

Mania-like behavior induced by genetic dysfunction of the neuron-specific Na⁺,K⁺-ATPase α 3 sodium pump

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Bipolar disorder is a debilitating psychopathology with unknown etiology. Accumulating evidence suggests the possible involvement of Na⁺,K⁺-ATPase dysfunction in the pathophysiology of bipolar disorder. Here we show that *Myshkin* mice carrying an inactivating mutation in the neuron-specific Na⁺,K⁺-ATPase α 3 subunit display a behavioral profile remarkably similar to bipolar patients in the manic state. *Myshkin* mice show increased Ca²⁺ signaling in cultured cortical neurons and phospho-activation of extracellular signal regulated kinase (ERK) and Akt in the hippocampus. The mood-stabilizing drugs lithium and valproic acid, specific ERK inhibitor SL327, roflumilast, and transgenic expression of a functional Na⁺,K⁺-ATPase α 3 protein rescue the mania-like phenotype of *Myshkin* mice. These findings establish *Myshkin* mice as a unique model of mania, reveal an important role for Na⁺,K⁺-ATPase α 3 in the control of mania-like behavior, and identify Na⁺,K⁺-ATPase α 3, its physiological regulators and downstream signal transduction pathways as putative targets for the design of new antimanic therapies.

Atp1a3 | mouse model | sodium potassium adenosine triphosphate alpha 3

Bipolar disorder is a genetically heterogeneous, heritable, and highly debilitating mood disorder defined by the presence of one or more manic episodes of abnormally elevated mood, arousal, or energy levels, with or without one or more depressive episodes. Numerous genes have been linked to bipolar disorder, including *ATPLA3* that encodes the Na⁺,K⁺-ATPase α 3 sodium pump, but no clear causal relationships have been established for any genetic factor (1). To enhance understanding of the neurobiology of the disorder and aid the development of novel therapies, fully validated and appropriate animal models are urgently needed.

The Na⁺,K⁺-ATPase (NKA) is a membrane-bound enzyme abundant in brain tissue and comprised of a catalytic α -subunit and regulatory β -subunit. Three α -isoforms are present in the brain: α 1 and α 2 are expressed in neurons and glia, and α 3 is expressed exclusively in neurons. NKA activity maintains and restores electrochemical gradients necessary for neuronal function by active exchange of Na⁺ and K⁺, which consumes 40% to 50% of total brain ATP. In addition, the NKA is linked to intracellular signal transduction pathways in the brain (2).

Cardiotonic steroids, commonly referred to as ouabain-like compounds (OLC), selectively bind and inhibit NKA α -isoforms (3). Endogenous OLCs include the structurally similar molecules ouabain, digoxin, marinobufagenin, and proscillaridin A (4). All human α -isoforms and rodent α 2 and α 3 isoforms, have a high binding affinity for endogenous OLCs, which regulate Na⁺,K⁺-ATPase activity and initiate signaling (4–6). Intracerebroventricular (ICV) administration of ouabain to rats stimulates locomotor hyperactivity and NKA signaling and is considered as a model of mania (7–9), but the similar affinities of α 2 and α 3 for ouabain have precluded conclusive pharmacological studies of isoform specificity.

ICV ouabain also excites the sympathetic nervous system and elevates blood pressure and heart rate in mice (10); however, these effects are mediated by the α 2 isoform (11, 12). The binding of OLC to neuronal NKA initiates intracellular Ca²⁺ signaling, and the phospho-activation of extracellular signal regulated kinase (ERK) and Akt (8, 9, 13). Therefore, genetic changes that decrease NKA activity could alter neuronal signaling, both directly and through pleiotropic effects on downstream pathways.

Postmortem gene-expression analysis of bipolar disorder patients has revealed lower expression of NKA α 2 in the temporal cortex (14) and α 3 in the prefrontal cortex (15). Genetic studies have reported an association between bipolar disorder and variants of the genes encoding α 1, α 2, and α 3 (1, 16), but the functional effects of these genetic changes remain unknown. There is also evidence that abnormal regulation of endogenous OLC may influence NKA activity in bipolar disorder. Relative to healthy controls, bipolar individuals show lower ouabain levels in serum (17, 18) but higher ouabain levels and binding in the parietal cortex (19). Finally, digitalis toxicity can be accompanied by manic and depressive symptoms in healthy humans (20).

By dint of the links between NKA and bipolar disorder, we assessed whether heterozygous *Myshkin* (*Atp1a3*^{Myk/+}; *Myk*^{+/+}) mice that carry a missense mutation in the neuron-specific NKA α 3 isoform exhibit mood-related behavioral abnormalities. Briefly, the *Myk*^{+/+} mutation was created through *N*-nitroso-*N*-ethylurea mutagenesis and results in a normally expressed but inactive enzyme, leading to a 36% to 42% reduction in total NKA activity in the brain (21). Mutations in the *ATPLA3* gene have been identified in rapid-onset dystonia parkinsonism; however, a known rapid-onset dystonia parkinsonism mutation reduces Na⁺ binding, whereas the *Myk*^{+/+} mutation is inactivating (22). Because abnormal behaviors are the primary diagnostic indicators of bipolar disorder, we undertook a detailed analysis of the behavioral phenotype of *Myk*^{+/+} mice in assays that model its fundamental symptoms. Herein, we report that *Myk*^{+/+} mice display behavioral, pharmacological, and biochemical phenotypes associated with mania observed in bipolar patients.

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Results

Absence of Stress-Induced Seizures in *Myshkin* Mice Backcrossed 20 Generations to C57BL/6Ncr Strain. Previously, we reported that *Myk*^{+/+} mice backcrossed 12 generations (N12) to the C57BL/6Ncr strain display increased susceptibility to stress-induced seizures (21). In the current study, we used *Myk*^{+/+} mice that were backcrossed to the seizure-resistant C57BL/6Ncr strain (23) for 20 generations (N20). *Myk*^{+/+} mice with this genetic background have increased total brain NKA activity (Fig. S1) and do not exhibit stress-induced seizure activity in electrocorticography (ECoG) recordings.

***Myshkin* Mice Display Increased Exploratory Locomotion and Sensitivity to Amphetamine.** Within a novel environment, manic humans explore novel objects more frequently, travel longer distances (hyperambulation), and show a chaotic path of exploration compared with healthy individuals (24). We observed similar behavior in *Myk*^{+/+} mice. In a novel-object test and a hole-board test, *Myk*^{+/+} mice explored objects and nose-poked more frequently than wild-type (+/+) mice (Fig. 1*A* and *B*). In contrast to +/+ mice, *Myk*^{+/+} mice did not habituate hole-board exploration (Fig. 1*B*). In a novel open field, *Myk*^{+/+} mice exhibited hyperambulation, faster walking speed, and decreased freezing than +/+ mice (Fig. 1*C* and Fig. S2). Hyperambulation in *Myk*^{+/+} mice was not greater in response to light; instead, they were more hyperactive in the dark (Fig. S2). Although both genotypes had similar total rearing activity, the amount of rearing decreased over time in +/+ mice but increased over time in *Myk*^{+/+} mice, suggesting a deficiency in habituation (Fig. S2). Finally, the walking path of *Myk*^{+/+} mice was chaotic and they had greater locomotor activity in the center compared with +/+ mice (Fig. 1*D* and *E*), suggesting decreased anxiety-like behavior.

Bipolar patients exhibit a greater response to amphetamine (25). Amphetamine exacerbates hyperactivity in bipolar disorder, but decreases locomotor activity in attention-deficit hyperactivity disorder (26). Mice were treated with an acute dose of D-amphetamine (0.5 mg/kg) and locomotor activity was assessed in an open field. As expected (27), the behavior of +/+ mice was unchanged by a low dose of D-amphetamine, but *Myk*^{+/+} mice showed increased ambulation (Fig. 1*F*), rearing, stereotypy, and circling behavior (Fig. S2), suggestive of an increase in dopamine signaling. This enhanced sensitivity of *Myk*^{+/+} mice to D-amphetamine is consistent with mania, rather than attention-deficit hyperactivity disorder.

***Myshkin* Mice Display Sleep and Circadian Rhythm Abnormalities.** A decreased need for sleep while maintaining energy is the most common symptom of mania (28). Incidentally, we found that *Myk*^{+/+} mice have more wake time than +/+ mice across 24 h, at the expense of non-rapid eye movement (non-REM) and REM sleep (Fig. 2*A*). *Myk*^{+/+} mice showed a deficit in the amount of sleep only during the light phase (Fig. 2*B* and *C*). In the light phase, *Myk*^{+/+} mice exhibited a reduced number of REM sleep bouts and shorter non-REM sleep bout length (Fig. 2*D* and *E*). Furthermore, similar to humans, REM sleep latency, as measured by the average duration of non-REM sleep that precedes entrance into REM sleep, was significantly reduced in *Myk*^{+/+} mice (Fig. 2*F*).

The majority of bipolar individuals have altered circadian functions (29). *Myk*^{+/+} mice successfully entrain to light and show normal circadian periods in a 12-h light:12-h dark environment. However, when external zeitgebers are removed, +/+ mice show an expected endogenous circadian period of 23.5 h (30) but *Myk*^{+/+} mice show an extended endogenous circadian period of 25 h because of an increase in activity (Fig. 2*G–I*).

***Myshkin* Mice Display Lowered Anxiety and a Greater Preference for Reward.** Low levels of anxiety, greater risk-taking, and greater impulsivity are core symptoms of mania (31). To assess levels of anxiety-like behavior, we used the elevated plus maze (EPM) and light-dark box (LDB). In the EPM, general locomotor activity did not differ between *Myk*^{+/+} and +/+ mice (Fig. S3); however, *Myk*^{+/+} mice made more open-arm entries and exploratory head dips (Fig. S3) and demonstrated a preference for the open arms (Fig. 3*A*). In the LDB, *Myk*^{+/+} mice spent a higher percentage of time in the light (Fig. 3*B*), and did not show a preference for the dark compartment. However, +/+ mice appeared to be driven by anxiety, but *Myk*^{+/+} mice were focused on exploration in unprotected spaces. Because NKA $\alpha 3$ is expressed in all neuronal-type cells of the retina (32) and anxiety-related behaviors are affected by visual impairment (33), we assessed the head-tracking response of *Myk*^{+/+} mice in an optokinetic drum (34), but found no difference between genotypes in this test of visual acuity (Fig. S4).

Excessive motivation, such as increased reward-seeking behavior or drive to perform or to achieve goals, is common during mania (35). To assess preference for a natural reward, we tested sucrose preference. We found that *Myk*^{+/+} mice consumed more sucrose solution relative to water than +/+ mice (Fig. 3*C*). In addition, *Myk*^{+/+} mice initially consumed more sucrose solution before the choice test (Fig. 3*D*). The increased preference for

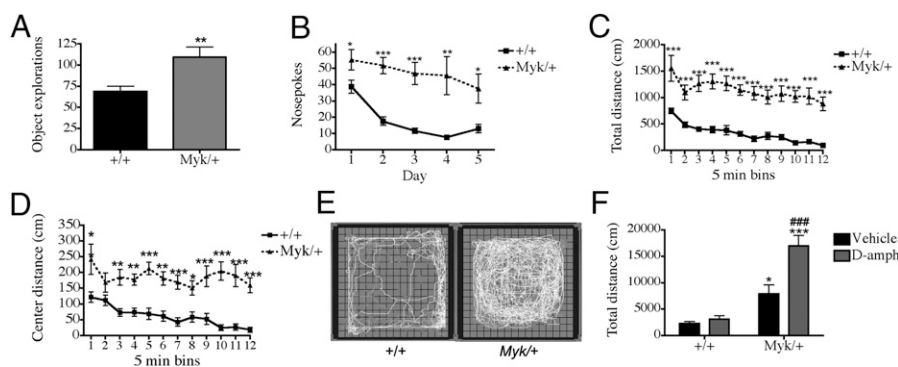


Fig. 1. Exploration and ambulation in *Myk*^{+/+} mice. *Myk*^{+/+} show an increased number of explorations of (A) novel objects (+/+ mice $n = 10$, *Myk*^{+/+} $n = 11$) and (B) nosepokes compared with +/+ mice and do not habituate (+/+ mice $n = 8$, *Myk*^{+/+} $n = 7$). (C) In an open field *Myk*^{+/+} travel a further distance and (D) spend more time in the center (+/+ mice $n = 27$, *Myk*^{+/+} $n = 22$) in 5-min bins compared with +/+ mice. (E) *Myk*^{+/+} exhibit unusual walking patterns compared with +/+ mice. (F) In 60 min in the open field, *Myk*^{+/+} increase locomotor activity in response to D-amph, but +/+ mice do not (+/+ vehicle $n = 9$, +/+ D-amph $n = 9$; *Myk*^{+/+} vehicle $n = 8$, *Myk*^{+/+} D-amph $n = 9$). All data are presented as mean SEM, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ compared with +/+ mice, #### $P < 0.001$ compared with *Myk*^{+/+} vehicle mice.

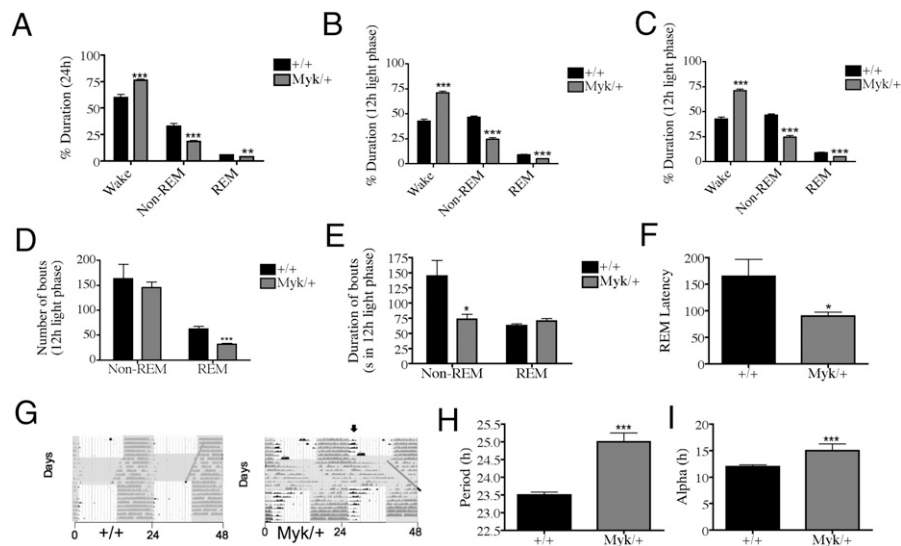


Fig. 2. Sleep and endogenous circadian period in *Myk1⁺* mice. (A) *Myk1⁺* ($n = 6$) experience more wake time than +/+ mice ($n = 6$) across 24 h with a reduction of both non-REM and REM sleep, as assessed by EEG and EMG. (B and C) *Myk1⁺* show deficits in sleep duration only during the light phase; (D) *Myk1⁺* have fewer REM sleep bouts but no change in non-REM bouts. (E) Non-REM bout length is reduced and REM bout length was unchanged in *Myk1⁺*. (F) REM sleep latency is reduced in *Myk1⁺*. (G) Wheel running actograms from +/+ and *Myk1⁺* mice. Animals were held on a light-dark (LD) cycle for 14+ d, released into constant dark for 7 d to assess free running period, and reentrained to a LD12:12 cycle. Shaded area represents dark portion of LD cycle. Vertical arrows indicate continuation of nocturnal activity into light. (H) Endogenous period is extended in *Myk1⁺* because of longer periods of (I) activity (α). All data are presented as means \pm SEM, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ compared with +/+ mice.

sucrose is indicative of a hyperhedonic state common to mania. To assess drive and motivation, we used the Porsolt forced swim test, which involves measurement of escape-directed behavior. In this test, *Myk1⁺* mice spent a longer time active than +/+ mice (Fig. 3E). Antidepressant drugs have been shown to increase the duration of mobility in the forced swim test (36) and the increased escape-directed behavior of *Myk1⁺* mice suggests a lower level of depressive-like behavior, which correlates with their increase in preference for rewarding stimuli.

Manic individuals and their unaffected siblings show abnormal deficits in prepulse inhibition (PPI) and habituation of startle (37, 38). We found that *Myk1⁺* mice demonstrate deficits in both PPI and startle habituation (Fig. 3F–H), suggesting that they share the abnormal sensorimotor gating observed in bipolar patients. The behavioral profile of *Myk1⁺* mice is remarkably similar to that of bipolar patients in the manic state (Table S1).

Manic-Like Behavior of *Myshkin* Mice Can Be Attenuated with Mood Stabilizers and Transgenic Restoration of NKA $\alpha 3$.

Lithium and valproic acid (VPA) are mood stabilizers that are effective in treating mania (39). We found the behavioral abnormalities of *Myk1⁺* mice were reduced by chronic lithium carbonate and VPA treatment, but the behavior of +/+ mice was unaffected. In the open field, lithium and VPA reduced the total distance traveled by *Myk1⁺* mice (Fig. 4A and B). Lithium and VPA also reduced duration on the open arms (Fig. 4C and D), entries to the open arms, and exploratory head dips (Fig. S3) by *Myk1⁺* mice in the EPM. In the LDB, lithium reduced the time spent in the light by *Myk1⁺* mice (Fig. S3).

To verify a causal link between the *Atp1a3^{Myk}* mutation—and its reduction in NKA activity—and the observed phenotype, we attempted to rescue the mania-like behavioral phenotype of *Myk1⁺* mice by transgenic restoration of functional NKA $\alpha 3$. To achieve this verification, we crossed *Myk1⁺* mice with Tg-

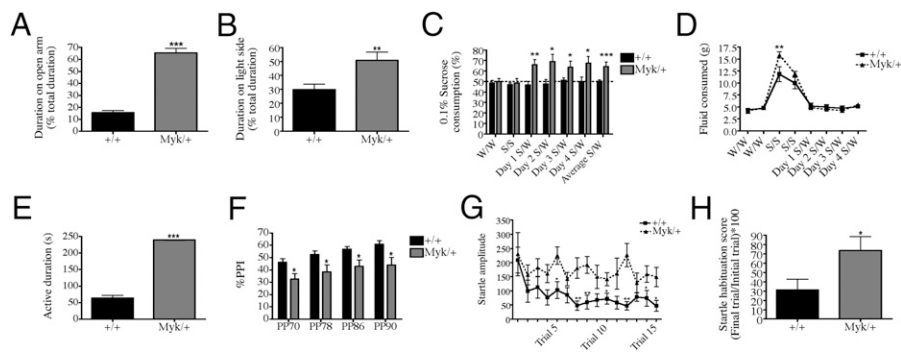


Fig. 3. Mania-like behavior in *Myk1⁺* mice. (A) *Myk1⁺* prefer to explore the open arm of the EPM (+/+ mice $n = 29$, *Myk1⁺* $n = 19$) and (B) the light side of the LDB (+/+ mice $n = 27$, *Myk1⁺* $n = 18$) for longer durations than +/+ mice. (C) *Myk1⁺* ($n = 8$) show a higher preference for 0.1% sucrose than +/+ mice ($n = 16$) over 4 d and (D) consume more sucrose on the first presentation of sucrose. W, water; S, sucrose. (E) *Myk1⁺* ($n = 14$) are active for a longer duration than +/+ mice ($n = 15$) in the Porsolt forced swim test. (F) PPI scores are impaired in *Myk1⁺* ($n = 20$) compared with +/+ mice ($n = 33$) at all prepulse intensities tested. (G and H) *Myk1⁺* ($n = 16$) had a startle habituation deficit compared with +/+ when presented with a repeated auditory stimulus ($n = 12$). All data are presented as means \pm SEM, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ compared with +/+ mice.

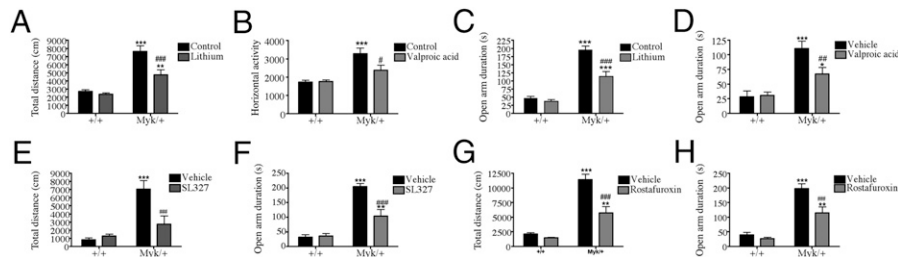


Fig. 4. Attenuation of mania-like behavior by lithium and VPA in *Myk1+* mice. (A) Chronic treatment with lithium reduces total distance traveled by *Myk1+* ($n = 20$) compared to untreated *Myk1+* ($n = 22$) over 30 min in the open field and had no effect in *+/+* mice ($n = 28$ control, $n = 27$ lithium). (B) VPA reduced total distance traveled by *Myk1+* ($n = 20$) compared to vehicle-treated *Myk1+* ($n = 22$), and had no effect on *+/+* mice ($n = 28$ vehicle, $n = 27$ VPA) over 30 min. (C) Lithium reduces open-arm duration in *Myk1+* ($n = 18$) compared with control *Myk1+* ($n = 19$), but had no effect on *+/+* mice ($n = 29$ control, $n = 29$ lithium) in the EPM. (D) VPA reduces open-arm duration in *Myk1+* ($n = 12$) compared with vehicle-treated *Myk1+* ($n = 10$) but had no effect on *+/+* mice ($n = 12$ control, $n = 12$ VPA) in the EPM. (E) ERK inhibitor SL327 decreased distance traveled in *Myk1+* ($n = 12$) compared with vehicle-treated *Myk1+* ($n = 9$), and had no effect in *+/+* mice ($n = 12$ vehicle, $n = 12$ SL327) in the open field. (F) SL327 reduced open-arm duration in *Myk1+* ($n = 6$) compared with *Myk1+* vehicle ($n = 6$) but had no effect on *+/+* mice ($n = 6$ control, $n = 6$ SL327) in the EPM. (G) Rostafuroxin decreased distance traveled in *Myk1+* ($n = 12$) compared with vehicle treatment ($n = 12$) and had no effect in *+/+* mice ($n = 13$ vehicle, $n = 15$ rostauroxin) in the open field. (H) In the EPM rostauroxin reduced open arm duration in *Myk1+* ($n = 12$) compared with vehicle treated *Myk1+* ($n = 12$) and had no effect in *+/+* mice ($n = 12$ vehicle, $n = 15$ rostauroxin). All data are presented as means \pm SEM, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ compared with *+/+* mice, # $P < 0.05$, ### $P < 0.01$, #### $P < 0.001$ compared with *Myk1+* vehicle mice.

Atp1a3^{1Stel} transgenic (Tg) mice to yield *+/+*, *Tg*⁺, *Myk1+*, and *Myk1+/Tg* littermates. Inheritance of the *Tg-Atp1a3*^{1Stel} transgene by *Myk1+/Tg* mice was previously shown to increase brain NKA activity to 74% of wild-type levels (21). We found that transgenic *Myk1+/Tg* mice perform at wild-type levels in the open field, EPM, and LDB (Fig. S5). This normalization of the behavioral phenotype shows that NKA $\alpha 3$ function is important in the regulation of mania-like behavior. We deduce the *Tg-Atp1a3*^{1Stel} transgene is located on the X chromosome (Table S2).

Signal-Transduction Pathways Downstream of NKA $\alpha 3$ Are Up-Regulated in *Myshkin* Mice. The binding of ouabain to NKA induces calcium (Ca^{2+}) release from intracellular stores via the activation of the inositol 1,4,5-trisphosphate receptor (40, 41). We used fura-2 microfluorometry to compare intracellular free Ca^{2+} ($[Ca^{2+}]_i$) in cortical neurons cultured from *Myk1+* and *+/+* mice. We found that *Myk1+* neurons exhibit higher resting $[Ca^{2+}]_i$, as measured by the fura-2 fluorescence emission ratio F340/F380 (Fig. 5A). Application of 10 μ M glutamate evoked transient $[Ca^{2+}]_i$ increases that were qualitatively similar in neurons from both genotypes. However, neurons from *Myk1+* mice demonstrated markedly prolonged glutamate-evoked $[Ca^{2+}]_i$ transients, as revealed by comparing the normalized fura-2 ratio over time (Fig. 5B and C).

ICV administration of 1 mM ouabain to rats induces locomotor hyperactivity and phosphorylation of ERK and Akt in the hippocampus (7–9). We expected ouabain-treated rats and *Myk1+* mice to show similarities. To determine the phosphorylation level of ERK and Akt, hippocampal extracts were subjected to Western blot analysis. We found that the immunoreactivity of p-ERK1/2 and p-Akt1/2/3 normalized to the corresponding total protein was elevated in *Myk1+* mouse hippocampus (Fig. 5D), although the degree of phospho-activation of ERK in *Myk1+* samples was variable (Fig. S6). Transgenic overexpression of NKA $\alpha 3$ in *Myk1+/Tg* mice, with 26% lower brain NKA activity than *+/+* mice, showed normalized hippocampal levels of p-Akt but not p-ERK (Fig. 5D). The persistent reduction in NKA activity may explain why the increase in ERK activation is maintained in the *Myk1+/Tg* mice.

Given the increased p-ERK in *Myk1+* mice, we investigated the behavioral effects of acute SL327, an inhibitor of ERK, at a dose shown to reduce ERK activity in *+/+* mice and have no effect on locomotion (42). SL327 reduced total distance traveled in the open field, duration on the open arm, and the number of exploratory head dips in the EPM in *Myk1+* mice (Fig. 4E and F and Fig. S7). We also investigated the behavioral effects of

rostauroxin (PST-2238; Sigma-Tau/Rostaquo), a compound that selectively displaces ouabain from the NKA in a rat model of hypertension (43). We expected that a reduction in endogenous ouabain binding would increase NKA activity or reduce NKA signaling in *Myk1+* mice and restore behavior. Chronic rostauroxin reduced total distance traveled in the open field, duration on the open arms, and exploratory head dips in the EPM, and minimized light side duration in the LDB in *Myk1+* mice (Fig. 4G and H and Fig. S7). These findings suggest a possible relationship between the mania-like behavioral phenotype and NKA signaling pathways in the *Myk1+* brain (Fig. 5E).

Discussion

The behavioral profile of *Myk1+* mice carrying an inactivating mutation in the neuron-specific $\alpha 3$ isoform of the NKA is remarkably similar to bipolar patients during the manic state, including their treatment by lithium and VPA. In light of emerging evidence implicating abnormal NKA function in mania, *Myk1+* mice represent a convincing model of human mania, with construct validity and significant face and predictive validity. *Myk1+* mice are behaviorally similar to other genetic models of mania, including reduction of Clock, ERK, and GluR2, and overexpression of glycogen synthase kinase-3 β (GSK3 β) (44–47). These genes may be interconnected in a pathway regulating mania-like behaviors.

Increasing the contribution of the seizure-resistant C57BL/6Ncr strain (23) to the genetic background of *Myk1+* mice had a significant phenotypic impact. In contrast to N12 C57BL/6Ncr *Myk1+* mice, *Myk1+* mice at N20 C57BL/6Ncr did not show stress-induced seizure activity in ECoG recordings and had increased total brain NKA activity. These results support our previous finding that an increase in NKA activity contributes to seizure resistance (21). Nonetheless, the possibility remains that unobserved subcortical epileptiform discharges contribute to mania-like behavior in *Myk1+* mice. Interestingly, epilepsy and bipolar disorder can be comorbid in humans (48) and they share a common pathophysiology (49). Given that *Myk1+* mice and ICV ouabain-treated rats exhibit mania-like behavior and increased susceptibility to seizures (50, 51), these models may help to explain why these debilitating conditions can be comorbid and suggest that increasing NKA activity may serve as therapy for mania and epilepsy.

Thus far, there have been no indications that *Myk1+* mice cycle between mania and depression, and future studies may determine whether depression-like symptoms occur after stress, sleep deprivation, or administration of antidepressants. However, we have shown that mice heterozygous for a point mutation in

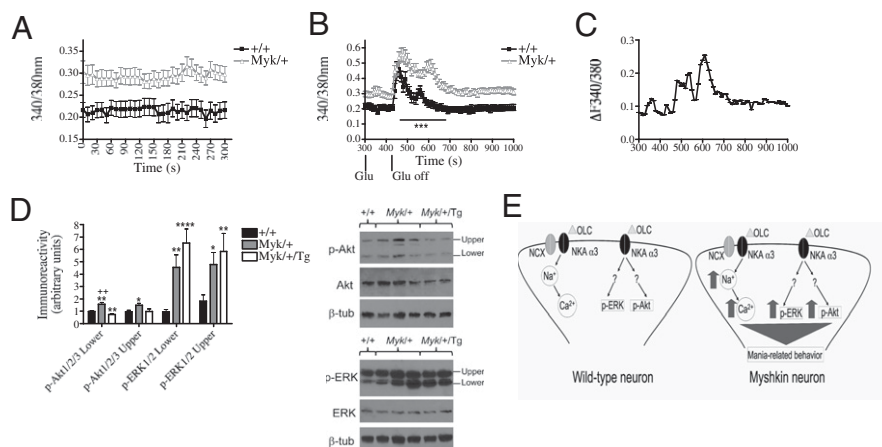


Fig. 5. Free intracellular Ca^{2+} and Ca^{2+} -dependent signaling in *Myk1⁺* mice. (A) Mean resting intracellular ($[\text{Ca}^{2+}]_i$) is stably elevated in cortical cells cultured from *Myk1⁺* ($n = 47$) than *+/+* mice ($n = 19$), as measured by the ratio of fura-2 fluorescence emission upon 340-nm and 380-nm excitation ($P < 0.01$). (B) *Myk1⁺* cortical neurons show a prolonged peak in $[\text{Ca}^{2+}]_i$ compared with *+/+* in response to bath superfusion of 10 μM glutamate (Glu). (C) When normalized to baseline $[\text{Ca}^{2+}]_i$, glutamate-evoked $[\text{Ca}^{2+}]_i$ transients were prolonged in neurons from *Myk1⁺* compared with neurons from *+/+* (D) Immunoreactivity of p-Akt1/2/3 and p-ERK1/2 was elevated in *Myk1⁺* compared with *+/+* hippocampus. Transgenic overexpression of NKA $\alpha 3$ in *Myk1⁺/Tg* mice did not alter hippocampal levels of p-ERK1/2 but reduced p-Akt1/2/3. *+/+*, $n = 8$ (Akt, ERK); *Myk1⁺*, $n = 11$ (Akt), $n = 10$ (ERK); *Myk1⁺/Tg*, $n = 5$ (Akt, ERK). (E) Model of NKA $\alpha 3$ signaling at the synapse in *+/+* and *Myk1⁺* mice. *Myk1⁺* mice have reduced NKA activity that augments $[\text{Ca}^{2+}]_i$ and activation of p-ERK and p-Akt. These intracellular signals may independently, additively or synergistically contribute to behavioral phenotypes of mania. All data are presented as means \pm SEM, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$ compared with *+/+* mice, ** $P < 0.01$ compared with *Myk1⁺/Tg* mice.

Atp1a3 intron 4 (*Atp1a3^{tm1Ling}*) exhibit increased susceptibility to depression-related behavior and a 33% reduction of brain NKA activity following chronic variable stress (52). Taking these data together, our work suggests that mood is significantly correlated with reductions in brain NKA activity. Theoretically, as previously hypothesized (53, 54), modulation of NKA activity could account for changes in mood in bipolar disorder.

Myk1⁺ mice may be a valuable tool for the development of novel mood stabilizers. We expected that the reduction in neuronal NKA activity in *Myk1⁺* brain would increase NKA signal transduction given that NKA-dependent signal transduction pathways are Ca^{2+} -dependent (13). Cortical neurons cultured from *Myk1⁺* mice demonstrated higher resting and glutamate-evoked $[\text{Ca}^{2+}]_i$ signals. Similarly, NKA activity is reduced and $[\text{Ca}^{2+}]_i$ is elevated in erythrocytes during manic and depressed states (55, 56). Elevated $[\text{Ca}^{2+}]_i$ may be studied as a drug target in *Myk1⁺* mice. Calcium channel blockers, such as nimodipine, are prescribed as treatments for bipolar disorder (57), and variation in calcium channel genes, such as *CACNA1C* and *TRPM2*, has shown strong association with bipolar disorder (58, 59), underscoring a possible role for dysregulation of the influx and efflux of calcium in mood disorders.

Similar to an ouabain model of mania (8), phospho-activation of the ERK signaling cascade was enhanced in *Myk1⁺* hippocampus, possibly by increasing transmitter-evoked Ca^{2+} signaling. However, p-ERK levels were not restored in *Myk1⁺/Tg* mice, suggesting that multiple signals contribute to mania-like behavior. In parallel with previous findings (42), the dose of SL327 that we used had no effect on locomotor activity in *+/+* mice. In contrast, a higher dose of SL327 (60) or deletion of the *Mapk3* (ERK1) gene (44, 61) have been shown to increase locomotor activity in rats and mice, respectively. These results suggest that the degree of ERK activation is correlated with mania-like behavior in animal models, and support the ERK pathway as a promising target for mood stabilizers (62). Also in accordance with an ouabain model of mania (9), the *Myk1⁺* hippocampus showed elevated phospho-activation of Akt. p-Akt was reduced in *Myk1⁺/Tg* mice, suggesting it may contribute to the regulation of mania-like behavior. Activated Akt phosphorylates and inhibits the activity of GSK-3 β , a well-known molecular target of

lithium and VPA (63), mood stabilizing drugs that diminished many of the behavioral abnormalities of *Myk1⁺* mice. Because Akt/GSK-3 β signaling is regulated by dopamine (64), our results suggest that Akt may also be a promising target for mood stabilizers. *Myk1⁺* mice could be used as a tool to investigate the potential of ERK and Akt modulators as antimanic therapies and to assess the prophylactic effect of novel mood stabilizers. Finally, because *Myk1⁺* mice and ouabain-treated rats show similar p-ERK and p-Akt increases in the hippocampus, the genetic *Myk1⁺* model of mania may replace the pharmacological ouabain model of mania, thus providing a less laborious model for exploring potential therapeutic approaches.

Rostafuroxin is a digitoxigenin derivative that antagonizes the signaling action of endogenous ouabain on the NKA that acts upstream of ERK to reduce ouabain-mediated NKA signaling (43). Its effective reduction of mania-like behavior in *Myk1⁺* mice supports the notion that the phenotype of *Myk1⁺* mice is caused by increased NKA downstream signaling.

Our results highlight the potential involvement of genes regulating NKA activity or downstream signaling pathways that are engaged by this transporter in bipolar etiology, and suggest that at least some manic individuals possess hypofunctional NKA sodium pumps and hyperfunctional NKA signal transduction.

Materials and Methods

All procedures were approved by the Animal Care Committee of the Toronto Centre for Phenogenomics and followed the Province of Ontario Animals for Research Act 1971 and requirements of the Canadian Council on Animal Care. The *Myshkin* and *Tg-Atp1a3^{15Tcl}* mouse lines have been described previously (21). Lithium carbonate was administered in chow (Harlan Teklad) at 0.4% for 28 d. VPA (Sigma-Aldrich) was administered at 150 mg/kg intraperitoneally for 28 d. The ERK inhibitor SL327 (Enzo Life Sciences) was acutely administered intraperitoneally at 30 mg/kg. Rostafuroxin (Sigma-Tau/Rostaquo) was administered for 21 d by oral gavage at 100 $\mu\text{g}/\text{kg}$. See *SI Materials and Methods* for more detailed discussion.

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