The *ANK3* Bipolar Disorder Gene Regulates Psychiatric-Related Behaviors That Are Modulated by Lithium and Stress

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Background: Ankyrin 3 (*ANK3*) has been strongly implicated as a risk gene for bipolar disorder (BD) by recent genome-wide association studies of patient populations. However, the genetic variants of *ANK3* contributing to BD risk and their pathological function are unknown.

Methods: To gain insight into the potential disease relevance of *ANK3*, we examined the function of mouse *Ank3* in the regulation of psychiatric-related behaviors using genetic, neurobiological, pharmacological, and gene-environment interaction (G×E) approaches. *Ank3* expression was reduced in mouse brain either by viral-mediated RNA interference or through disruption of brain-specific *Ank3* in a heterozygous knockout mouse.

Results: RNA interference of *Ank3* in hippocampus dentate gyrus induced a highly specific and consistent phenotype marked by decreased anxiety-related behaviors and increased activity during the light phase, which were attenuated by chronic treatment with the mood stabilizer lithium. Similar behavioral alterations of reduced anxiety and increased motivation for reward were also exhibited by *Ank3*+/– heterozygous mice compared with wild-type *Ank3*+/+ mice. Remarkably, the behavioral traits of *Ank3*+/– mice transitioned to depression-related features after chronic stress, a trigger of mood episodes in BD. *Ank3*+/– mice also exhibited elevated serum corticosterone, suggesting that reduced *Ank3* expression is associated with elevated stress reactivity.

Conclusions: This study defines a new role for *Ank3* in the regulation of psychiatric-related behaviors and stress reactivity that lends support for its involvement in BD and establishes a general framework for determining the disease relevance of genes implicated by patient genome-wide association studies.

Key Words: Ankyrin G, dentate gyrus, GWAS, mouse, RNA interference, schizophrenia

Bipolar disorder (BD) is a severe psychiatric disorder for which the pathogenesis is poorly understood. BD is defined by alternating episodes of mania and depression, with manic symptoms including impulsivity, high-risk behavior, increased pleasure seeking (hedonia), and decreased sleep, whereas depressive symptoms include anhedonia, impaired cognition, and suicidality (1). Mood regulation in BD is unstable, and a manic or depressed episode can be triggered by many factors, including stress (2). Clinical studies highlight altered neurocircuit function in BD, notably increased limbic activity and decreased frontal cortical activation during emotional processing tasks (3).

It is well established that BD has a large genetic component (1). Patient genome-wide association studies (GWAS) have identified several genes associated with BD surpassing statistical correction for genome-wide testing (4). Of these, *ANK3* was among the most significant in a recent meta-analysis of BD GWAS data from nearly 17,000 subjects, although the results did not replicate in all samples (5). As reviewed elsewhere (6), *ANK3* also appears to be a shared risk factor between BD and schizophrenia based on a joint GWAS analysis (7) and has been implicated in anhedonia, stress signal processing, novelty seeking, and cognition in humans (8–10). *ANK3* expression is lower in schizophrenia postmortem brain (10), suggesting that downregulation may underlie psychopathology. However, like many genes implicated in multifactorial disorders, *ANK3* has a modest effect, with an odds ratio less than 1.35 for BD (5). The genetic variants associated with disease have no known function and may only be markers for the putative causal variant(s). It therefore remains unclear whether *ANK3* is indeed a psychiatric risk gene and, if so, how it contributes to psychopathology.

ANK3 encodes many diverse isoforms of the ankyrin G scaffold protein functioning in various biological processes (11). The most recognized function of ankyrin G in the brain is formation and maintenance of the axon initial segment (AIS) of neurons. In this subcellular domain and at nodes of Ranvier, ankyrin G links voltage-gated sodium and potassium channels to the cytoskeleton (11) and is required for the production and propagation of

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action potentials mediated by these channels (12). Ankyrin G is also necessary for localization of inhibitory gamma-aminobutyric acid (GABA) synapses at excitatory neuron AIS (13), subventricular zone neurogenesis (14), and potentially synaptic transmission (15,16), among other functions.

Increasingly, GWAS are identifying genetic loci associated with psychiatric disorders, but determining the basis for these associations remains a formidable challenge. Although current disease knowledge may suggest a compelling hypothesis for the influence of a particular gene, such information is typically too sparse to support a specific mechanism. In this study, we explored a new role of *ANK3* in neural circuits regulating mood using an integrative approach encompassing genetic, neurobiological, pharmacologic, and environmental components. We used two complementary genetic approaches to suppress the mouse *Ank3* gene in brain, RNA interference targeting specific circuits and whole brain transgenic knockout, that provide highly consistent and persuasive evidence for a novel function of *Ank3* in modulating psychiatric-related behaviors and stress reactivity. Our study provides compelling support for the human genetic data implicating *ANK3* in psychiatric disease. More broadly, this work establishes a general framework for validating newly implicated psychiatric GWAS risk genes and examining their putative disease-relevant functions.

Methods and Materials

Detailed methods can be found in Supplement 1.

Animals

Male 8-week-old C57BL/6J mice were used for RNA interference studies. Male 8- to 11- week old *Ank3*+/– and *Ank3*+/+ knockout mice (12) were generated from male *Ank3*+/– and female C57BL/6J crosses.

Lentiviral-Mediated RNA Interference

To minimize the possibility of off-target effects, two short hairpin RNA sequences (shRNA1 or shRNA2 targeting *Ank3* exons 19 or 28) that suppress mRNA expression in vitro by 75% to 80% (Figure S1A in Supplement 1) were compared with a control shRNA (shCON) that does not target any known mouse transcripts. Mouse hippocampal dentate gyrus was injected bilaterally with lentivirus expressing shRNA1, shRNA2, or shCON (n = 10–11 mice/group).

Behavior

Mice were evaluated using conventional, well-validated assays for measuring behaviors mediated by neural circuits implicated in psychiatric illness, as well as sensory and motor performance (17,18). These included locomotor activity in a novel open field, elevated plus maze (EPM), light-dark transition (LD), novelty-suppressed feeding (NSF), acoustic startle, prepulse inhibition, home cage activity, sucrose preference, forced swim test (FST), contextual and cued fear conditioning, and visual performance in a visible platform water maze.

Drug Treatment

Lithium chloride (85 mg/kg intraperitoneal; Sigma-Aldrich, St. Louis, Missouri) or vehicle (saline with .2% acetic acid) was administered once daily for 14 days before and throughout behavioral testing 1 hour before testing.

Immunohistochemistry

Coronal sections from 4% paraformaldehyde fixed brains were incubated with appropriate antibodies and processed using standard methods.

Corticosterone Measurements

Plasma corticosterone levels following acute restraint stress were measured using an enzyme immunoassay (EIA).

Statistical Analysis

All data are presented as means and standard errors of the mean (SEM). Where appropriate, one-way, two-way, and repeated-measures analysis of variance were used, and group differences identified using Fisher's least significant difference post hoc tests. Statistical significance was accepted at the $p < .05$ level.

Results

Ank3 RNA Interference in Dentate Gyrus Produces a Highly Specific Phenotype Marked by Lower Anxiety-Related Behavior

Ank3 expression was reduced by viral-mediated RNA interference in hippocampal dentate gyrus (DG), given its role in BD, mood and stress regulation, and mood stabilizer response (19–23). Across nine behavioral assays, *Ank3* suppression in the DG with either of two shRNA sequences targeting *Ank3*, shRNA1 or shRNA2, produced a highly specific and sizable reduction in anxiety-related behavior in the EPM. Mice expressing shRNA1 or shRNA2 exhibited 60% to 75% shorter latencies (i.e., less time) to enter the EPM open arms, 50% more open arm entries, and threefold more time in the open arms, compared with mice injected with a control sequence, shCON (Figure S3 in Supplement 1). These changes were not due to hyperactivity because the number of total arm entries or rears in the EPM (Figure S3 in Supplement 1) and open field activity (Figure S4 in Supplement 1) did not differ from shCON. *Ank3* knockdown mice did not differ from controls in conventional tasks assessing sensorimotor gating (prepulse inhibition), auditory and visual sensory performance (acoustic startle response and visible platform Morris water maze), associative learning (cued and contextual fear conditioning), or the FST (Figure S4 in Supplement 1). Overall, these data suggest that *Ank3* suppression in DG results in a specific reduction in anxiety-related behavior.

Following behavioral assessment and confirmation of correct virus placement, we measured knockdown of ankyrin G expression in DG granule cells infected with shRNA lentivirus by identifying cells expressing green fluorescent protein from the virus. We specifically examined neuronal AIS because ankyrin G is highly expressed and plays a critical role at this subcellular location. Ankyrin G AIS expression was significantly decreased by 45% and 62% in shRNA1 and shRNA2 mice, respectively, relative to shCON mice [n = 5/group; $F(2,8) = 8.8$, $p < .01$; Figure 1]. These results suggest that partial reduction of ankyrin G expression in DG granule cells is sufficient to cause marked behavioral changes.

Behavioral Changes Associated with *Ank3* Knockdown Are Reversed by Lithium Treatment

We performed an independent experiment to evaluate the effects of lithium in reversing the behavioral alterations induced by *Ank3* RNA interference. Given the consistent behavioral effects noted, we examined only shRNA2 because it targets more *Ank3* isoforms than shRNA1. Fourteen days after lentivirus injection into bilateral DG, mice were treated with lithium at a clinically

relevant dose (85 mg/kg intraperitoneal; see Supplement 1) or with vehicle for 14 days before and throughout behavioral testing (n = 8–12 mice/group). Lithium effects were assessed by comparing shRNA2 mice treated with lithium to shCON mice treated with vehicle that index the normal condition.

Replicating the previous result, *Ank3* RNA interference in DG substantially decreased anxiety-related behavior. Specifically, vehicle-treated shRNA2 mice exhibited 60% shorter latency and 40% increased frequency to enter the EPM open arms [$F(1,17) = 5.54$, $p = .03$; $F(1,17) = 4.61$, $p = .046$; Figure 2A,B] compared with vehicle-treated shCON mice. Notably, lithium treatment normalized the behavior, in that lithium-treated shRNA2 mice did not significantly differ from vehicle-treated shCON mice in EPM open arm latency or entries ($p > .1$ Figure 2A,B). These results were confirmed using the LD task, where there was a significant interaction between shRNA and lithium treatment in the latency to leave the dark and enter the light side [$F(1,33) = 4.78$, $p = .036$; Figure 2C]. Vehicle-treated shRNA2 mice exhibited 65% shorter latency to enter the light side compared with vehicle-treated shCON mice (post hoc $p = .02$), and lithium treatment increased this latency such that shRNA2 mice did not differ from vehicle-treated shCON mice (post hoc $p > .1$). We also tested NSF, which assesses the latency to approach and eat food in the center of a novel arena that mice innately avoid. There was a significant shRNA by drug interaction [$F(1,31) = 5.44$, $p = .026$; Figure 2D] in the NSF approach latency. Vehicle-treated shRNA2 mice exhibited 80% shorter latency to approach than vehicle-treated shCON mice (post hoc $p = .005$), whereas lithium treatment normalized the latency of shRNA2 mice such that there was no significant difference from vehicle-treated shCON mice (post hoc $p = .29$). As in the behavioral screen described earlier, mice expressing shRNA2 did not differ from shCON mice in motor activity in the open field, EPM, or LD tasks (Figure S5 in Supplement 1). Thus, across three paradigms, *Ank3* suppression in DG was associated with substantially decreased anxiety-related behaviors that were normalized by lithium treatment.

Because BD patients report sleep disruptions during both manic and depressive episodes (24), we assessed changes in motor activity across the light–dark cycle using an automated home-cage behavioral phenotyping system (25). Mice expressing shRNA2 showed no significant differences compared with shCON mice in the total time engaged in activity (walking, hanging, rearing, grooming, eating, drinking) during the dark phase [$F(1,33) = 1.9$, $p > .1$; Figure 2F] when mice, being nocturnal, are most active. However, during the light phase, shRNA2 mice were 53% more active than shCON mice [$F(1,33) = 4.61$, $p = .039$; Figure 2E]. Lithium treatment reversed the increased light phase activity of shRNA2 mice to levels similar to vehicle-treated shCON mice (post hoc $p = .6$; Figure 2E). These results indicate that in addition to decreased anxiety-related behaviors, *Ank3* suppression in DG is associated with elevated activity during the light phase of the light–dark cycle, which is normalized by lithium treatment.

Reduction of *Ank3* Brain-Specific Isoforms Induces Behavioral Changes Consistent with Dentate Gyrus Knockdown

To gather further support and extend our behavioral findings from *Ank3* RNA interference mice, we performed a

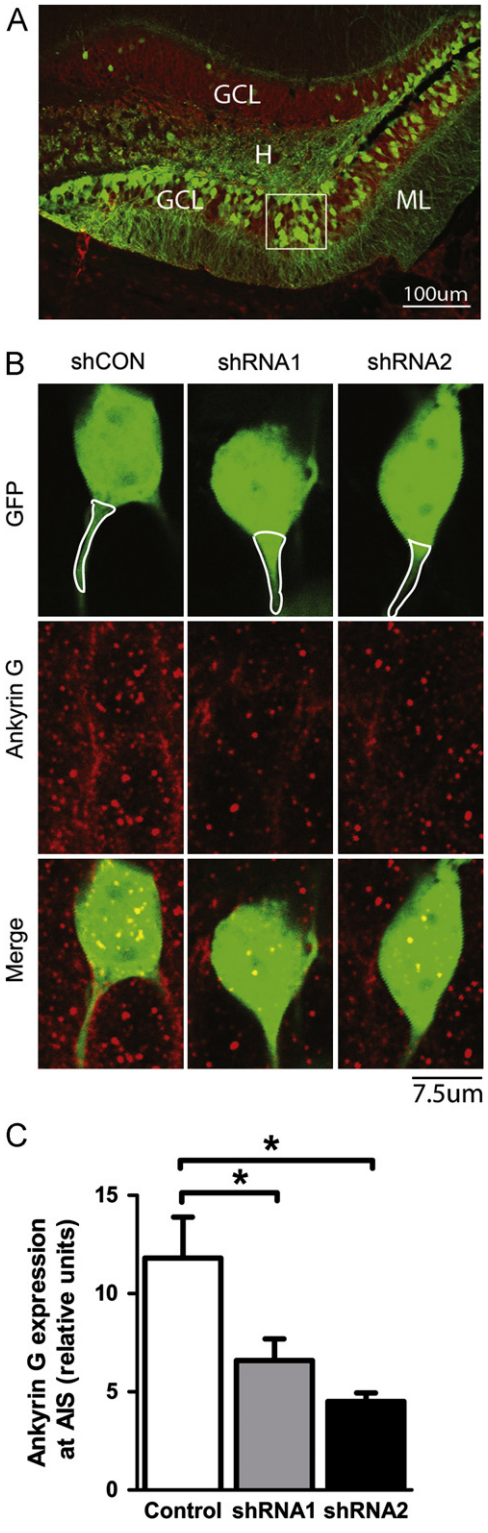

Figure 1. *Ank3* RNA interference markedly decreases ankyrin G expression at neuronal axon initial segments (AIS). **(A)** In mouse brain, dentate gyrus cells infected with lentivirus expressing shRNA (green, green fluorescent protein (GFP)-positive infected cells; red, ankyrin G). White box demarcates the region where ankyrin G expression was measured. **(B)** Representative images of neurons infected with lentiviral vectors expressing shCON, shRNA1, or shRNA2 targeting mouse *Ank3*. Ankyrin G (red) was measured at the AIS (demarcated by a white line) of neurons containing GFP (green) expressed from the lentiviral vector. **(C)** Quantification of ankyrin G expression showing decreases of 45% and 62% by shRNA1 and shRNA2, respectively, compared with shCON. Mean ± SEM; n = 5 mice/group, 6 AIS/mouse; *$p < .05$. GCL, granule cell layer; H, hilus; ML, molecular layer; shCON, control shRNA; shRNA, short hairpin RNA.

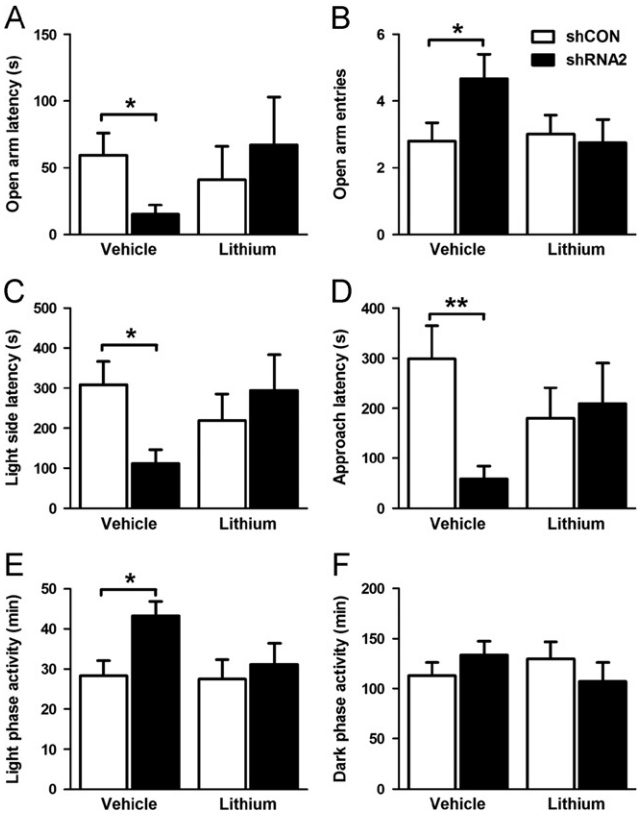

Figure 2. *Ank3* RNA interference in dentate gyrus (DG) is associated with lower anxiety-related behavior and increased light-phase activity that is normalized by lithium treatment. Vehicle-treated mice expressing shRNA2 against *Ank3* in the DG, when compared with shCON mice, exhibit **(A)** shorter latency to enter the open arms and **(B)** greater number of open-arm entries in the elevated plus maze, **(C)** shorter latency to enter the light–dark transition light side, **(D)** shorter latency to approach food in the novelty-suppressed feeding task, and **(E)** increased activity during the light phase but **(F)** no change in activity during the dark phase. **(A–E)** Lithium treatment (85 mg/kg intraperitoneal, >14 days) normalizes the behavioral alterations of shRNA2 mice to similar levels as vehicle-treated shCON mice. **(F)** Lithium has no impact on dark-phase activity in either shCON or shRNA2 mice. Mean ± SEM; n = 8–12 mice/group; *$p < .05$, **$p < .01$. shCON, control shRNA; shRNA, short hairpin RNA.

complementary study using a knockout mouse in which the *Ank3* exon 1b locus is disrupted (12). This disruption results in the exclusive loss of *Ank3* transcript variants containing exon 1b that are specifically expressed in brain but has no effect on transcripts containing alternative leading exons, which shRNA1 and shRNA2 target in addition to exon 1b transcripts. We confirmed in C57BL/6J mice that transcripts containing exon 1b are expressed in frontocortical and limbic regions relevant to mood-related behaviors (Figure 3). Exon 1b expression was highest in cerebellum, as previously reported (12), and was also substantial in DG and all other regions examined (hippocampus, amygdala, orbitofrontal cortex, prefrontal cortex, and striatum). Exon 1b transcripts were more highly expressed in all brain regions compared to transcripts containing an alternative leading exon 1e (Figure 3), which is found in brain and other tissues (12). These expression data suggest that the brain-specific exon 1b isoforms function in many brain regions, including several implicated in mood regulation.

Because *Ank3*–/– mutant mice exhibit early-onset ataxia (12) that interferes with general locomotion, only *Ank3*+/– and *Ank3*+/+ mice were examined (n = 7–18/group). Compared with wild-type *Ank3*+/+ mice, heterozygous *Ank3*+/– mice are reported to have reduced forebrain and cerebellar expression of the 270- and 480-kD ankyrin G isoforms that localize to the AIS (12,26). We verified that *Ank3*+/– mice have a significant 39% reduction in ankyrin G expression at neuronal AIS in the DG granule cell layer compared with *Ank3*+/+ littermates [t(6 *df*) = 4.6, $p < .01$; Figure 4A], similar to the RNA interference mice (Figure 1). Furthermore, ankyrin G expression at the AIS of cortical neurons was decreased by 41% in *Ank3*+/– mice compared with *Ank3*+/+ mice [$t(8\ df) = 6.2$, $p < .001$; Figure 4B]. Together with the published data, these results suggest that *Ank3*+/– mice with a single functional copy of brain-specific isoforms have reduced ankyrin G expression at neuronal AIS throughout the brain.

We found that male *Ank3*+/– mice exhibited behavioral alterations that were highly consistent with the decreased anxiety-related phenotype of RNA interference mice (Table S3 in Supplement 1) and also displayed other relevant behaviors. Compared to *Ank3*+/+ littermates, *Ank3*+/– mice exhibited substantially shorter latencies to enter the EPM open arms (57% reduced, post hoc $p < .05$, Figure 5A) and LD light side (59% reduced, post hoc $p = .07$, Figure 5B), and to approach food in the NSF task (83% reduced, post hoc $p < .05$, Figure 5C). To consider other attributes relevant to mood, we measured preference to drink a 1% sucrose solution over water. *Ank3*+/– mice exhibited 53% greater sucrose preference compared to *Ank3*+/+ littermates (post hoc $p < .05$, Figure 5D), suggesting heightened motivation to obtain a reward. As with *Ank3* RNA interference mice, *Ank3*+/– mice were normal in all other behavioral tests including open field activity, acoustic startle, prepulse inhibition, contextual and cued fear conditioning, and motor activity in the EPM and LD tasks (Figure 5E; Tables S1 and S3 in Supplement 1). The highly consistent behavioral changes in *Ank3*+/– mice further demonstrate a previously unknown function of *Ank3* brain-specific isoforms in regulating psychiatric-related behaviors.

Chronic Stress Induces a Shift to Depressive-like Features in *Ank3*+/− Mice

Because stress is a major risk factor for BD episode recurrence (2) and the hypothalamic-pituitary-adrenal (HPA) axis is implicated in BD (27), we examined the effect of chronic stress on *Ank3*+/– mice.

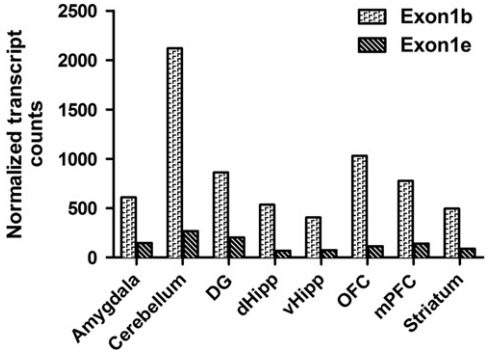

Figure 3. *Ank3* exon 1b transcripts are expressed in multiple brain regions. Normalized expression of *Ank3* transcripts containing the brain-specific exon 1b or widely expressed exon 1e across brain regions, as measured by Nanostring gene expression analysis. For each brain region, mRNA from eight C57BL/6J mice was pooled before measurement. Exon 1b transcripts are more highly expressed than exon 1e transcripts across all brain regions examined. DG, dentate gyrus; dHipp, dorsal hippocampus; mPFC, medial prefrontal cortex; OFC, orbitofrontal cortex; vHipp, ventral hippocampus.

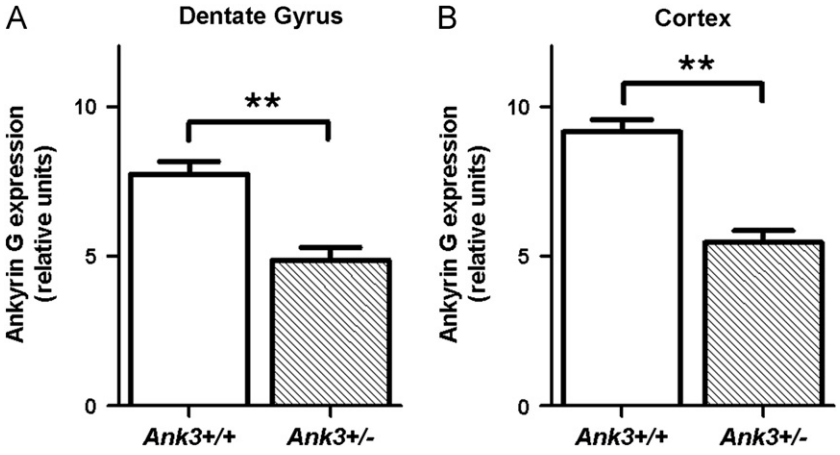

Figure 4. *Ank3*+/– mice have substantially reduced ankyrin G expression at the axon initial segments (AIS) of dentate gyrus and cortical neurons. Compared with *Ank3*+/+ littermates, heterozygous *Ank3*+/– mice exhibit an approximately 40% reduction in ankyrin G at the AIS of neurons in the **(A)** dentate gyrus and **(B)** cortex, as measured by immunohistochemistry. Mean ± SEM; $n = 5$–7 mice/group, 6–7 AIS/mouse; $^{**}p < .01$.

Compared with *Ank3*+/+ littermates, *Ank3*+/– mice singly housed for 6 weeks did not exhibit increased motivation for reward or decreased anxiety-related behaviors (Figure 5 isolated condition), as found under standard group-housed conditions described above (Figure 5, group condition). Specifically, isolated *Ank3*+/– mice did not display significantly shorter latencies to enter the EPM open arms or LD light side or to approach food in the NSF task when compared with either group-housed or isolated *Ank3*+/+ mice (all post hoc p values $> .09$, Figure 5A–C). There was also a change in motivation (Figure 5D): isolated *Ank3*+/– mice did not display increased sucrose preference compared with isolated *Ank3*+/+ mice ($p > .05$), as was observed under group-housed conditions, and had substantially lower sucrose preference, relative to group-housed *Ank3*+/– mice (post hoc $p < .05$). Chronic isolation also resulted in 50% greater FST immobility in *Ank3*+/– mice versus *Ank3*+/+ mice ($p < .05$, Figure 5E), whereas group-housed *Ank3*+/– and *Ank3*+/+ mice did not differ ($p > .05$, Figure 5E). The latter results are suggestive of a depression-related phenotype in *Ank3*+/– mice exposed to stress, because the increase in FST immobility is opposite to the reduced immobility induced by antidepressant treatment (28). Thus, chronic stress induced a transition in *Ank3*+/– mice from decreased anxiety-related behaviors and increased motivation to depression-related features, highlighting an environmental component in mood regulation. Interestingly, the isolation stress had no effect on *Ank3*+/+ mice (all p values $> .09$; Figure 5, group vs. isolation housed), suggesting a putative gene-environment (G×E) interaction in which diminished *Ank3* is associated with elevated stress sensitivity.

Evidence for Altered Stress Hormone Reactivity in *Ank3*+/– Mice

To investigate the mechanism through which chronic stress modifies the phenotype of *Ank3*+/– mice, we measured plasma levels of corticosterone, the predominant stress hormone in rodents, and weight of the adrenal gland that produces corticosterone, as a measure of chronic stress load (29). Under group housing, the ratio of adrenal weight to body weight ratio did not differ between *Ank3*+/+ and *Ank3*+/– mice, nor did isolation have any effect on the adrenal:body weight ratio of wild-type *Ank3*+/+ mice (all post hoc p values $> .1$; Figure 6A). In contrast, isolated *Ank3*+/– mice exhibited a 27% increase in adrenal:body weight ratio compared to group-housed *Ank3*+/– mice ($p < .01$; Figure 6A), suggesting that *Ank3*+/– mice are more reactive to chronic stress than wild-type mice. *Ank3*+/– mice exhibited higher basal corticosterone levels than *Ank3*+/+ littermates, regardless of group or isolation housing [$F(1,10) = 9.03$ and $F(1,11) = 9.04$, both p values $< .05$; Figure 6C,D; Table S2 in

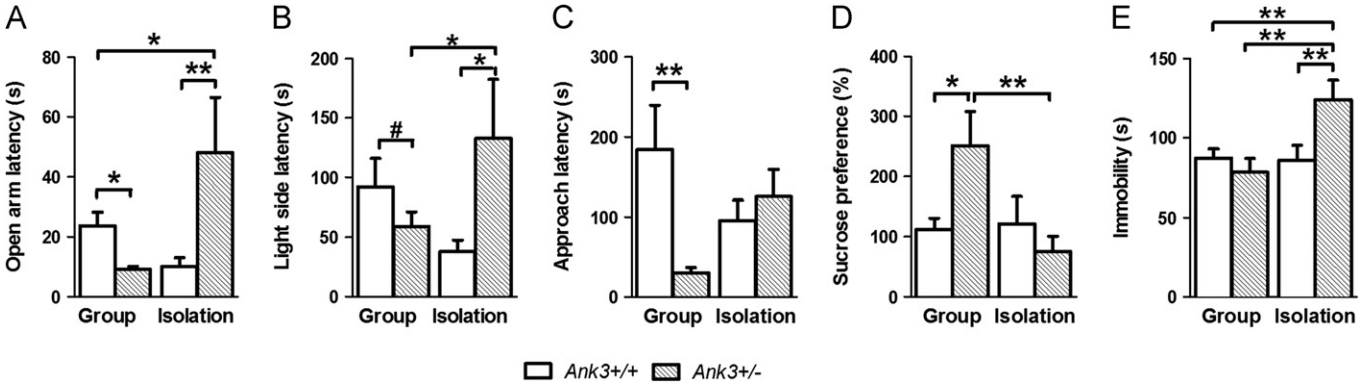

Figure 5. *Ank3*+/– mice exhibit distinctive behavioral alterations that are modified by chronic stress. Under standard group-housed conditions compared with *Ank3*+/+ littermates, *Ank3*+/– mice display shorter latencies **(A)** to enter the elevated plus maze (EPM) open arms, **(B)** to enter the light–dark (LD) transition light side, and **(C)** to approach food in the novelty-suppressed feeding (NSF) task; **(D)** they also exhibit greater preference for sucrose over water. In contrast, after prolonged isolation housing, *Ank3*+/– mice no longer exhibit shorter latencies to enter **(A)** the EPM open arms or **(B)** the LD light side, **(C)** or to approach food in the NSF task, **(D)** nor do they display greater sucrose preference, compared with isolation-housed *Ank3*+/+ mice. **(E)** Group-housed *Ank3*+/– mice do not differ from *Ank3*+/+ mice in FST immobility time, whereas isolation housing significantly increased *Ank3*+/– immobility time. Mean ± SEM; $n = 7$–18 mice/genotype; $^{**}p < .01$, $^{*}p < .05$, $^{\#}p < .07$, unpaired t test.

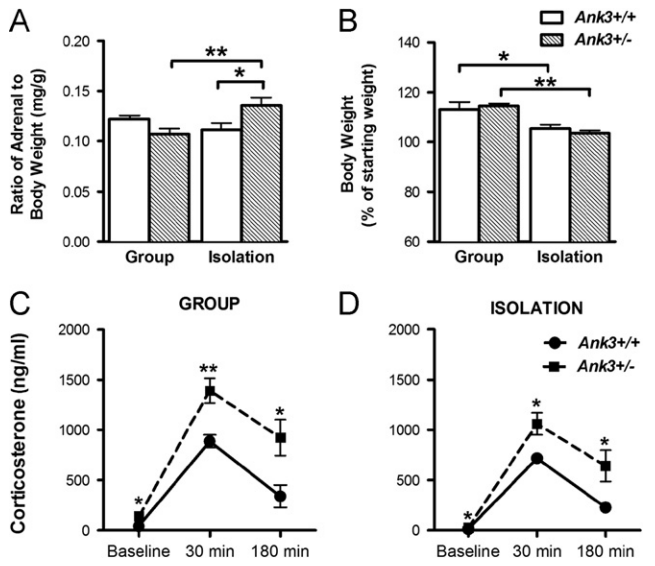

Figure 6. *Ank3*+/– mice exhibit heightened physiologic stress reactivity. Following chronic social isolation, **(A)** adrenal gland weight (milligrams) relative to body weight (grams) is higher in *Ank3*+/– mice compared with isolation-housed *Ank3*+/+ mice or to group-housed mice, suggesting a higher sustained response to chronic stress in *Ank3*+/– mice. **(B)** Isolation-housed *Ank3*+/+ and *Ank3*+/– mice exhibit a smaller increase in body weight than group-housed mice. Corticosterone levels (ng/mL) are elevated in *Ank3*+/– mice compared to *Ank3*+/+ mice whether **(C)** group-housed or **(D)** isolated for 6 weeks. In particular, *Ank3*+/– mice exhibit a higher corticosterone peak 30 min after acute restraint stress as well as a slower return to baseline after 180 min. Mean ± SEM; $n = 6$–8 mice/genotype; $^{**}p < .01$, $^{*}p < .05$.

Supplement 1). Following acute stress (20-min restraint), corticosterone levels were more elevated and returned to baseline more slowly in *Ank3*+/– mice compared with *Ank3*+/+ mice (Figure 6C,D). These results demonstrate a persistent elevation in basal corticosterone levels in *Ank3*+/– mice that is further exaggerated by acute stress, suggesting impaired HPA axis regulation.

Discussion

This study uncovered a novel function of *Ank3* in the regulation of psychiatric-related traits that implicates a central role in neural circuits underlying psychopathology. *Ank3* suppression in mouse brain, either by viral delivery of shRNAs to the DG or disruption of brain-specific *Ank3* in a heterozygous knockout mouse, resulted in lower anxiety-related behavior denoted by rapid entry into aversive novel environments in three tasks. Notably, these changes were reversed by chronic lithium, a first-line treatment for BD mania. Decreased anxiety-like behaviors in the absence of anxiolytic drugs can be interpreted as increased risk taking (30,31), a classic feature of BD mania (1). Risk taking can be considered on the same spectrum but at the opposite end from anxiety, thus the rapid entry into aversive environments of mice with diminished *Ank3* could reflect increased risk taking. We also detected increased activity during the light phase in the *Ank3* RNA interference mice, and characterization of *Ank3*+/– mice identified heightened motivation to obtain a reward, which are both features of BD mania (1). Thus, converging evidence from two genetic models and several behavioral paradigms (Table S3 in Supplement 1) suggests that ankyrin G functions in neural circuits relevant to BD, given that the behavioral changes were reversed by lithium, a clinically effective prophylactic for manic episodes.

Another striking finding of this study was the observation that chronic isolation stress reversed the lower anxiety-related behavior and elevated motivation of *Ank3*+/– mice and induced depression-related behavior (increased FST immobility). The stress-induced transition is particularly interesting because stress predicts relapse of both depressive and manic episodes in BD (2). The elevated stress reactivity of *Ank3*+/- mice compared to *Ank3*+/+ mice suggests a G×E interaction in which the level of *Ank3* expression modulates the impact of stress on brain function. One putative mechanism through which *Ank3* may mediate stress reactivity is through dysregulated HPA axis activity, as suggested by elevated corticosterone levels and adrenal gland weight following chronic isolation stress in *Ank3*+/– mice compared with *Ank3*+/+ mice. Notably, mice with reduced neural expression of the glucocorticoid receptor exhibit a phenotype remarkably similar to *Ank3*+/– mice, including decreased anxiety-related behavior, impaired behavioral response to stress, and altered HPA axis regulation (32), supporting a link between HPA axis function and the behavioral changes observed in *Ank3*+/– mice. Future experiments to examine HPA axis integrity in *Ank3*+/– mice, as well as ankyrin G function following chronic stress, will be important to elucidate the role of ankyrin G in stress reactivity.

Our data suggest that *Ank3* may influence a broader repertoire of emotional behaviors beyond those associated mainly with BD. Further insight into its nuanced role could be obtained through human genetic association studies of other psychiatric disorders and psychological traits, as well as by evaluating defined behaviors and related neural circuitry in mice with perturbed *Ank3* expression (18). For example, tasks that specifically assess impulsivity and risk-taking may clarify the observed decrease in anxiety-related behaviors (30,31). This is an important distinction given that low anxiety is not typically associated with BD, whereas impulsive and risk-taking behaviors are key characteristics. It will also be important to assess sleep and circadian rhythm, which are implicated in BD (24), to follow-up our finding of increased light phase activity in *Ank3* RNA interference mice.

There are several potential reasons why the *ANK3* genetic variants identified by human GWAS have small effects, whereas robust changes in mood states are observed in *Ank3*+/– mice. It is possible that the human variants produce a more subtle change in ankyrin G function than that caused by the approximately 40% reduced expression in *Ank3*+/– mice. Alternatively, the human *ANK3* genetic changes may have larger effects in some individuals than others because of variation in genetic background or other factors, but the effect is diluted across heterogeneous human populations. In contrast, robust effects may be more easily revealed in precisely controlled experiments using genetically homogeneous mouse lines.

Although our data provide compelling evidence that *Ank3* functions in neural processes regulating psychiatric-related behaviors, more research is clearly required to determine whether *Ank3* suppression in mice is a valid model of BD. We are aware of only two other genetic manipulations in mice that induce features of both decreased anxiety and increased depression. These are knockout of the D-box binding protein gene (*Dbp*) and RNA suppression of *Clock,* which, interestingly, reduces anxiety-related behaviors similar to that observed in this study (33,34).

A contrasting phenotype of increased anxiety (decreased exploration) and antidepressant-like behavior is found in mice haploinsufficient for another BD GWAS-implicated gene, the voltage-gated calcium channel alpha subunit *Cacna1c* (35). A similar mood-stabilized phenotype also occurs in mice haploinsufficient for the lithium target glycogen synthase kinase 3 beta (GSK-3β) or overexpressing its substrate β-catenin (36–38), which is notable given the reversal of behavioral changes by lithium in this study and that GSK-3 and β-catenin have been implicated in ankyrin G tethering at the AIS (39).

The overwhelming consistency between both *Ank3* mouse models has several implications. First, it indicates that the *Ank3* shRNA sequences were "on target" because two distinct shRNAs are unlikely to produce the same off-target effects. Second, the role of *Ank3* in behavioral regulation is likely attributable to one or more brain-specific transcripts that are disrupted in *Ank3*+/– mice and suppressed by both shRNA sequences. However, pinpointing the specific transcript(s) is impeded by the large number of *Ank3* splice variants (>10 reported to date) that necessitates in-depth molecular studies, for example, reversal studies using viral-mediated gene transfer of individual complementary DNA (cDNA) sequences into brain. The unwieldy cDNA size of the 270- and 480-kD ankyrin G isoforms located at the AIS, however, precludes packaging into conventional viruses with persistent in vivo expression. Lastly, we can speculate that the DG is critical for the observed phenotypes because it was targeted by our RNA interference and *Ank3* exon 1b transcripts disrupted in *Ank3*+/– mice are expressed in this region. The DG is indeed implicated in exploration (40) and untrained anxiety-related responses such as those elicited in the EPM, LD, and NSF tasks (41). Altered DG function may also perturb hippocampal and downstream circuits that could directly regulate behavior, such as the septohippocampal circuit that mediates inhibition of behavioral responses(42,43).

There are several mechanisms through which lithium may exert the observed behavioral effects. Known targets include GSK-3, AKT kinase, the phosphoinositol pathway, and the extracellular regulated kinase/mitogen activated protein kinase cascade (44). Alternatively, other known properties of lithium may be involved, such as increasing hippocampal volume (21), DG synaptic plasticity and granule cell firing (23), or adult DG neurogenesis (22). Interestingly, *Ank3* loss in the subventricular zone disrupts the production of adult newborn neurons in mice (14). *Ank3* may have a similar role in the DG, such that *Ank3* downregulation could impair adult neurogenesis, leading to behavioral alterations that are restored by lithium.

Our study provides a framework for addressing the daunting challenge in medical genetics research of elucidating the functional relevance of genes implicated by patient GWAS. We used two complementary genetic approaches to manipulate gene expression, assessed several biological processes related to the disease, established pharmacologic validity using a clinical medication, and demonstrated a G×E interaction with an environmental trigger of disease symptoms. Furthermore, our study followed recent recommendations for animal studies of candidate psychiatric risk genes (18) because it evaluated changes in behavior using a wide range of paradigms and focused on chronic manipulations of environment (e.g., stress). Applying a similar integrative approach to other risk genes may also provide insight into their possible pathological functions.

Overall, this study provides compelling evidence that reduced *Ank3* expression is associated with altered psychiatric-related behaviors and stress reactivity. Our discovery of a previously unknown role of *Ank3* in behavioral regulation highlights avenues for future investigation of the potential functional relevance of *ANK3* to psychopathology.

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Supplementary material cited in this article is available online.

1. Barnett JH, Smoller JW (2009): The genetics of bipolar disorder. *Neuroscience* 164:331–343.
2. Bender RE, Alloy LB (2011): Life stress and kindling in bipolar disorder: Review of the evidence and integration with emerging biopsychosocial theories. *Clin Psychol Rev* 31:383–398.
3. Langan C, McDonald C (2009): Neurobiological trait abnormalities in bipolar disorder. *Mol Psychiatry* 14:833–846.
4. Lee KW, Woon PS, Teo YY, Sim K (2011): Genome wide association studies (GWAS) and copy number variation (CNV) studies of the major psychoses: What have we learnt? *Neurosci Biobehav Rev* 36:556–571.
5. Sklar P, Ripke S, Scott LJ, Andreassen OA, Cichon S, Craddock N, *et al.* (2011): Large-scale genome-wide association analysis of bipolar disorder identifies a new susceptibility locus near ODZ4. *Nat Genet* 43: 977–983.
6. Leussis M, Madison JM, Petryshen TL (2012): Ankyrin 3: Genetic association with bipolar disorder and relevance to disease pathophysiology. *Biol Mood Anxiety Disord* 2:18.
7. Ripke S, Sanders AR, Kendler KS, Levinson DF, Sklar P, Holmans PA, *et al.* (2011): Genome-wide association study identifies five new schizophrenia loci. *Nat Genet* 43:969–976.
8. Ruberto G, Vassos E, Lewis CM, Tatarelli R, Girardi P, Collier D, *et al.* (2011): The cognitive impact of the ANK3 risk variant for bipolar disorder: Initial evidence of selectivity to signal detection during sustained attention. *PloS One* 6:e16671.
9. Roussos P, Giakoumaki SG, Georgakopoulos A, Robakis NK, Bitsios P (2011): The CACNA1C and ANK3 risk alleles impact on affective personality traits and startle reactivity but not on cognition or gating in healthy males. *Bipolar Disord* 13:250–259.
10. Roussos P, Katsel P, Davis KL, Bitsios P, Giakoumaki SG, Jogia J, *et al.* (2011): Molecular and genetic evidence for abnormalities in the nodes of Ranvier in schizophrenia. *Arch Gen Psychiatry* 69:7–15.
11. Bennett V, Lambert S (1999): Physiological roles of axonal ankyrins in survival of premyelinated axons and localization of voltage-gated sodium channels. *J Neurocytol* 28:303–318.
12. Zhou D, Lambert S, Malen PL, Carpenter S, Boland LM, Bennett V (1998): AnkyrinG is required for clustering of voltage-gated Na channels at axon initial segments and for normal action potential firing. *J Cell Biol* 143:1295–1304.
13. Ango F, di Cristo G, Higashiyama H, Bennett V, Wu P, Huang ZJ (2004): Ankyrin-based subcellular gradient of neurofascin, an immunoglobulin family protein, directs GABAergic innervation at Purkinje axon initial segment. *Cell* 119:257–272.
14. Paez-Gonzalez P, Abdi K, Luciano D, Liu Y, Soriano-Navarro M, Rawlins E, *et al.* (2011): Ank3-dependent SVZ niche assembly is required for the continued production of new neurons. *Neuron* 71:61–75.

15. Koch I, Schwarz H, Beuchle D, Goellner B, Langegger M, Aberle H (2008): Drosophila ankyrin 2 is required for synaptic stability. *Neuron* 58:210–222.
16. Pielage J, Cheng L, Fetter RD, Carlton PM, Sedat JW, Davis GW (2008): A presynaptic giant ankyrin stabilizes the NMJ through regulation of presynaptic microtubules and transsynaptic cell adhesion. *Neuron* 58: 195–209.
17. Crawley JN (2008): Behavioral phenotyping strategies for mutant mice. *Neuron* 57:809–818.
18. Nestler EJ, Hyman SE (2010): Animal models of neuropsychiatric disorders. *Nat Neurosci* 13:1161–1169.
19. Joels M, Karst H, Alfarez D, Heine VM, Qin Y, van Riel E, *et al.* (2004): Effects of chronic stress on structure and cell function in rat hippocampus and hypothalamus. *Stress* 7:221–231.
20. Sahay A, Drew MR, Hen R (2007): Dentate gyrus neurogenesis and depression. *Prog Brain Res* 163:697–722.
21. Ng WX, Lau IY, Graham S, Sim K (2009): Neurobiological evidence for thalamic, hippocampal and related glutamatergic abnormalities in bipolar disorder: A review and synthesis. *Neurosci Biobehav Rev* 33:336–354.
22. Boku S, Nakagawa S, Masuda T, Nishikawa H, Kato A, Toda H, *et al.* (2011): Effects of mood stabilizers on adult dentate gyrus-derived neural precursor cells. *Prog Neuropsychopharmacol Biol Psychiatry* 35: 111–117.
23. Shim SS, Hammonds MD, Ganocy SJ, Calabrese JR (2007): Effects of sub-chronic lithium treatment on synaptic plasticity in the dentate gyrus of rat hippocampal slices. *Prog Neuropsychopharmacol Biol Psychiatry* 31:343–347.
24. Murray G, Harvey A (2010): Circadian rhythms and sleep in bipolar disorder. *Bipolar Disord* 12:459–472.
25. Jhuang H, Garrote E, Yu X, Khilnani V, Poggio T, Steele AD, *et al.* (2010): Automated home-cage behavioural phenotyping of mice. *Nat Commun* 1:68.
26. Zhang X, Bennett V (1998): Restriction of 480/270-kD ankyrin G to axon proximal segments requires multiple ankyrin G-specific domains. *J Cell Biol* 142:1571–1581.
27. Daban C, Vieta E, Mackin P, Young AH (2005): Hypothalamic-pituitary-adrenal axis and bipolar disorder. *Psychiatr Clin North Am* 28:469–480.
28. Cryan JF, Holmes A (2005): The ascent of mouse: Advances in modelling human depression and anxiety. *Nat Rev Drug Discov* 4:775–790.
29. Blanchard RJ, Nikulina JN, Sakai RR, McKittrick C, McEwen B, Blanchard DC (1998): Behavioral and endocrine change following chronic predatory stress. *Physiol Behav* 63:561–569.
30. Flaisher-Grinberg S, Einat H (2010): Strain-specific battery of tests for domains of mania: Effects of valproate, lithium and imipramine. *Front Psychiatry* 1:10.
31. Einat H (2007): Different behaviors and different strains: Potential new ways to model bipolar disorder. *Neurosci Biobehav Rev* 31:850–857.
32. Tronche F, Kellendonk C, Kretz O, Gass P, Anlag K, Orban PC, *et al.* (1999): Disruption of the glucocorticoid receptor gene in the nervous system results in reduced anxiety. *Nat Genet* 23:99–103.
33. Le-Niculescu H, McFarland MJ, Ogden CA, Balaraman Y, Patel S, Tan J, *et al.* (2008): Phenomic, convergent functional genomic, and biomarker studies in a stress-reactive genetic animal model of bipolar disorder and co-morbid alcoholism. *Am J Med Genet B Neuropsychiatr Genet* 147B:134–166.
34. Mukherjee S, Coque L, Cao JL, Kumar J, Chakravarty S, Asaithamby A, *et al.* (2010): Knockdown of *Clock* in the ventral tegmental area through RNA interference results in a mixed state of mania and depression-like behavior. *Biol Psychiatry* 68:503–511.
35. Dao DT, Mahon PB, Cai X, Kovacsics CE, Blackwell RA, Arad M, *et al.* (2010): Mood disorder susceptibility gene CACNA1C modifies mood-related behaviors in mice and interacts with sex to influence behavior in mice and diagnosis in humans. *Biol Psychiatry* 68:801–810.
36. Gould TD, Einat H, O'Donnell KC, Picchini AM, Schloesser RJ, Manji HK (2007): Beta-catenin overexpression in the mouse brain phenocopies lithium-sensitive behaviors. *Neuropsychopharmacology* 32:2173–2183.
37. O'Brien WT, Harper AD, Jove F, Woodgett JR, Maretto S, Piccolo S, *et al.* (2004): Glycogen synthase kinase-3beta haploinsufficiency mimics the behavioral and molecular effects of lithium. *J Neurosci* 24: 6791–6798.
38. Beaulieu JM, Marion S, Rodriguiz RM, Medvedev IO, Sotnikova TD, Ghisi V, *et al.* (2008): A beta-arrestin 2 signaling complex mediates lithium action on behavior. *Cell* 132:125–136.
39. Tapia M, Del Puerto A, Puime A, Sanchez-Ponce D, Fronzaroli-Molinieres L, Pallas-Bazarra N, *et al.* (2012): GSK3 and beta-catenin determines functional expression of sodium channels at the axon initial segment [published online ahead of print July 5]. *Cell Mol Life Sci.*
40. Saab BJ, Georgiou J, Nath A, Lee FJ, Wang M, Michalon A, *et al.* (2009): NCS-1 in the dentate gyrus promotes exploration, synaptic plasticity, and rapid acquisition of spatial memory. *Neuron* 63:643–656.
41. Engin E, Treit D (2007): The role of hippocampus in anxiety: Intracerebral infusion studies. *Behav Pharmacol* 18:365–374.
42. Gray JA, McNaughton N (1996): The neuropsychology of anxiety: Reprise. *Nebr Symp Motiv* 43:61–134.
43. Hirshfeld-Becker DR, Biederman J, Faraone SV, Segool N, Buchwald J, Rosenbaum JF (2004): Lack of association between behavioral inhibition and psychosocial adversity factors in children at risk for anxiety disorders. *Am J Psychiatry* 161:547–555.
44. Machado-Vieira R, Manji HK, Zarate CA Jr (2009): The role of lithium in the treatment of bipolar disorder: convergent evidence for neurotrophic effects as a unifying hypothesis. *Bipolar Disord* 11(2 suppl): 92–109.