The Ankyrin 3 Bipolar Disorder Gene Regulates Psychiatric-Related Behaviors that are Modulated by Lithium and Stress

Supplemental Information

Supplemental Methods and Materials

Animals

Male C57BL/6J mice for RNA interference studies were purchased from Jackson Laboratory (Bar Harbor, ME) at 7 weeks of age. Ankyrin 3 exon1b knockout mice (1) on a C57BL/6J background (backcrossed for >20 generations) were bred as *Ank3*+/- males crossed to C57BL/6J females to generate *Ank3*+/+ and *Ank3*+/- experimental progeny. Mice were 10-12 weeks of age at the start of behavior testing. Mice were housed 2-4/cage or, for chronic isolation stress, single housed for 6 weeks prior to and throughout testing, under a 12h:12h light-dark cycle (lights on at 7 am). All procedures were approved by the Massachusetts Institute of Technology Animal Care and Use Committee and followed the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals.

Lentivirus Preparation and Delivery

We performed lentiviral-mediated RNA interference to reduce Ank3 expression in mouse dentate gyrus. This region was specifically targeted due to its involvement in bipolar disorder (2), and response to mood stabilizers such as lithium (3, 4). Short hairpin RNA (shRNA) sequences were cloned into the pLKO.2 PU6-shRNA/PUbiC-eGFP vector (www.broadinstitute.org/rnai/trc): control non-targeting shCON (Sigma, SCH002); shRNA1 targeting *Ank3* exon 19, GGGATTGCGTCCGTCCATC (5); shRNA2 targeting exon 28, CCGCCTGGTAAAGAGACATAA (Sigma, NM_170730.1_302s1c1, TRCN0000090054). Exon 28 is present in the majority of *Ank3* splice isoforms, therefore shRNA2 targets a larger number of *Ank3* transcripts than shRNA1. *Ank3* knockdown efficiency was measured *in vitro* by lipofectamine transfection of mouse Neuro 2A cells (ATCC, Manassas, VA) and quantitative real time polymerase chain reaction of cell lysate using primers directed to exon 19 targeted by shRNA1 (Forward: AAAGAATGGCTATACACCACTGC; Reverse: AGGTTGACATTCGCGTTTCTACT) or exons 27-28 targeted by shRNA2 (Forward: ACTCCCTCAGACACTACAGTTGG; Reverse: TCCACCATAAAGCTAACCAGAAA). VSV-G pseudotype lentivirus was packaged in-house and by Systems Biosciences (Mountain View, CA) with final titers ranging from 2.6-4.9 x 10^9 IFU/ml. Virus (1 ul/side) was stereotaxically

infused (bilateral AP -2.0, L +/-1.5 relative to bregma, DV -1.8 mm relative to dura), and correct virus placement and expression confirmed by green fluorescent protein (GFP) localization. Any mice in which virus extended beyond dentate gyrus (including up the injection track) or that lacked virus expression either unilaterally or bilaterally, as confirmed by GFP expression, were excluded from all analyses (four mice excluded from the shRNA behavioral screen, seven mice excluded from the shRNA2 lithium experiment).

Primary Neuronal Cultures

Mixed cortical and hippocampal primary neuronal cultures established from embryonic day 18 (E18) C57BL/6 mouse embryos were plated at 15,000 cells/well in poly-d-lysine coated plates and grown at 37°C in Neurobasal medium supplemented with Penicillin/Streptomycin, B27 supplement, and Glutamax. At 7 days in vitro, cells were fixed with 4% paraformaldehyde and stained with antibodies against ankyrin G (Santa Cruz Biotechnology, #SC-28561), the neuronal marker MAP2 (Novus Biologicals, NB300-213), and the nuclear marker Hoechst 33342 (Invitrogen). Images were acquired using the Cellomics ArrayScan VTI fluorescence imaging system.

Behavioral Phenotyping

Behavior testing was performed using standard protocols and blinded to genotype and shRNA group. Mice received a battery of tests at least 24 hr apart with the most stressful tasks (e.g., forced swim test, fear conditioning) at the end of the battery. Assessment of RNA interference mice began 14 days after virus injection.

Open field. Locomotor activity in a novel environment was assessed for 60 min in an open field (40 x 40 cm Plexiglas, AccuScan Instruments, Columbus, OH) within a ventilated and lighted sound-attenuated chamber (55 cm x 55 cm). Horizontal activity (distance traveled) was measured by beam breaks using the Versamax analysis system (AccuScan Instruments, Columbus, OH) (6).

Elevated plus maze (EPM). The EPM is a task based on the conflicting tendencies of rodents to explore new environments while avoiding danger (7), and is sensitive to anxiolytic and anxiogenic drug (8). Mice were placed in the center of an EPM (45 x 10 cm arms and 30 cm high closed arm walls) in a moderately lit room and allowed to move freely for 5 min. Sessions were video recorded and scored for latency to enter the open arms, total time in the open or closed arms, total number of entries into the open or closed arms, and number of head dips and rears.

Light-dark transition (LD). The LD test, which is sensitive to anxiolytics, is based on the natural conflicting tendencies of mice to explore novel environments yet avoid bright light (7-9). Mice were placed in the dark side of a light-dark box (dark: 20 x 40 cm, light: 20 x 40 cm) for 10 min. Sessions were video recorded and scored for latency to enter the light side, total time in the light side, and number of transitions between light and dark sides.

Novelty-suppressed feeding (NSF). The NSF paradigm assesses the latency to approach and eat food in the center of a novel arena that mice innately avoid, and is considered an indicator of both anxiety- and depression-related behaviors (8). As previously described (10), mice were food restricted for 24 hours prior to testing with *ad libitum* access to water. A pellet of rodent chow was placed on a petri dish (diameter 10 cm) in the center of the novel arena (45W x 30.5L x 20H cm) under bright light (~850 lux) that decreased towards the edges of the arena (~550 lux). Mice were placed in a corner of the arena and the latencies to approach (defined as stepping onto the petri plate) and to bite the food pellet were recorded during the 10 min test. Mice that did not approach or eat during the test were assigned the maximum value (10 min). To control for differences in motivation or hunger, mice were screened for food consumption in their home cage immediately after NSF testing. One mouse did not eat within 5 min and was excluded from analyses.

Home cage activity. Mice were acclimated to single housing for 24 hours, followed by video recording in the home cage for 24 hours without disruption. For lithium studies, mice were administered lithium or vehicle 2 hours prior to video recording. Videos were analyzed using an automated mouse behavior detection system based on computational modeling (11) that detects walking, hanging, rearing, eating, drinking, grooming, micromovements, and resting. The time spent walking, hanging, rearing, grooming, or eating/drinking was summed to determine gross motor activity within the 12 hour light phase and the 12 hour dark phase.

Acoustic startle and prepulse inhibition (PPI). Sensorimotor gating was assessed by measuring PPI of acoustic startle as previously described (12). Briefly, mice were exposed to replicate trial blocks consisting of one pulse-alone trial (120 dB 40 ms white noise burst) and trials with a non-startling prepulse (70, 75, 80 or 85 dB for 20 ms) preceding the pulse by 100 ms. The acoustic startle response (ASR) was defined as the mean startle amplitude of the pulse-alone trials, and PPI as the percent reduction in ASR when preceded by a prepulse, calculated as 100% × (1–[mean startle from prepulse trial/ASR]).

Forced swim test (FST). As previously described (13), mice were placed in a transparent cylinder (15 cm diameter, 24 cm high) of water (27 ± 2°C, depth 12 cm) and video

recorded for 6 minutes. The total time spent immobile was determined using the EthoVision XT video tracking system (Noldus Information Technology, Leesburg, VA).

Fear conditioning. Cued and contextual associative learning (14) was assessed using a fear conditioning enclosure and FreezeFrame software (Colbourn Instruments, Whitehall, PA). Mice were exposed to a tone (85 dB, 30 s) paired with a footshock (0.9 mA) during the final 2 s of the tone presented twice during a 5 min training session. Twenty-four hours later, contextual learning was assessed using the same chamber by measuring the time exhibiting freezing behavior (an indicator of fear memory). Cued learning was assessed using an altered context (different chamber floor, walls, and scent) and measuring the freezing time during presentation of a tone (conditioned stimulus "CS", 85 dB 180 s), as well as prior to the tone (120 s) to confirm that the context was sufficiently altered and did not contribute to freezing behavior.

Visible platform water maze. To test for visual impairment (15), mice were placed in a water pool (1.8 m diameter, 24 ± 1°C) with a visible platform viewed by a "flag pole" in the platform center for 4 training trials (1 min/trial, 30 min inter-trial interval). Mice unable to locate the platform within 1 min were guided to it to facilitate task learning. The following day, latency to reach the visible platform was measured and averaged over 4 trials.

Sucrose preference. This task measures the preference mice exhibit for sweetened water over regular water and is frequently used to assess motivation for reward (16). Mice were acclimated to single housing for 48 hours, at which time two water bottles were placed in the home cage for an additional 48 hours of acclimation to the presence of two bottles. One water bottle and one bottle of 1% sucrose were placed in the cage for 5 days and daily consumption measured by weighing bottles. Location of the sucrose bottles (left or right) was alternated daily to reduce any bias. The percent preference for sucrose was calculated as 100% x (total consumption of sucrose / total consumption of water). Testing was carried out under single housing to determine the consumption of individual mice.

Drug Treatment

Lithium chloride (85 mg/kg i.p.; Sigma) or vehicle (saline with 0.2% acetic acid) was administered once daily for 14 days prior to and throughout the behavioral testing period one hour prior to testing. This dose and route of administration of lithium chloride was chosen based on experiments in C57BL/6J mice (n = 6) that detected blood serum levels of 1.0 ± 0.07 mEq/L one hour after administration, within the therapeutic range of 0.4-1.1 mmol/L (equivalent to 0.4-1.1 mEq/L for monovalent elements such as Li+) used for bipolar disorder treatment (17).

Lithium treatment was confirmed to not affect weight gain compared to vehicle treatment (Figure S6).

Social Isolation Stress

Social isolation was used as a stressor in *Ank3*$^{+/+}$ and *Ank3*$^{+/-}$ mice (18). Briefly, starting at 10 weeks of age, mice were housed individually in standard cages for 6 weeks prior to and throughout behavioral testing. Mice were tested in some behavioral paradigms prior to isolation, but were not repeatedly exposed to the same paradigm before and after isolation in order to avoid test-retest effects.

Corticosterone and Adrenal Gland Measurement

Whole blood samples were collected at baseline, as well as 30 and 180 minutes after the start of an acute restraint stress, which consisted of immobilization of the mice in immobilization bags for a period of 20 minutes. For group-housed mice (both *Ank3*+/+ and *Ank3*+/-), the disruption of consecutively removing mice from the home cage for blood sampling produced an increase in corticosterone levels with each mouse removed from the cage, thus accounting for the higher mean levels of corticosterone observed in group housed mice compared to isolated mice. Plasma samples were tested in duplicate for determination of corticosterone levels using a Corticosterone Enzyme ImmunoAssay Kit (Cat. No. ADI-900-097, Enzo Life Sciences). Wells were scanned using a Spectramax M5 plate reader. Corticosterone concentrations (ng/ml) were calculated from the average net optical density of samples plotted along a standard curve. Bilateral adrenal glands were collected at the same time as the blood collection and weighed wet (mg) to provide an additional measure of chronic stress load.

Immunohistochemistry

All experiments and analyses were performed blinded to genotype, shRNA, and/or drug treatment. Free-floating fixed brain sections (30 um) from transcardially perfused mice (4% paraformaldehyde) were analyzed using standard immunohistochemical techniques. Briefly, sections were incubated for 20 minute in 3% H_2O_2 in phosphate buffered saline (PBS), blocked in 5% bovine serum albumin, and then incubated overnight with the primary antibody (1:1000, anti-ankyrin G antibody, Santa Cruz Biotechnology, #SC-28561). Sections were washed with PBS and subsequently incubated with secondary antibody in blocking solution (biotinylated goat anti-rabbit) for 1 hour at room temperature, then incubated with a streptavidin conjugated fluorophore (Vector Labs #DL-1594). For ankyrin G quantification, an average of 5-7 axon initial

segments (AIS) selected from 2-3 different coronal brain sections for each of 4-7 mice per group were imaged using confocal microscopy and quantified using ImageJ software (NIH), with a free-hand region of quantification drawn around the AIS by a blinded investigator. The mean AIS area measured for each group did not statistically differ (Figure S2). Coronal sections chosen for imaging were within 1 mm of the injection site. Specificity of the ankyrin G antibody was confirmed in mouse primary neuronal cultures (Figure S1).

Nanostring Gene Expression

Probes specific to mouse *Ank3* exon 1b or exon 1e were designed by NanoString Technologies (Seattle, WA). mRNA was isolated from brain tissue harvested from eight C57BL/6J male mice, purified using the Qiagen RNeasy Kit (Valencia, CA), and quality assessed using an Agilent Bioanalyzer (Santa Clara, CA). An equal quantity of mRNA from each mouse was pooled and probe counts of the pool determined using the Nanostring nCounter Analysis System. Probe counts were normalized against the weighted average expression of 12 genes, including five reference genes (*Gapdh, Paxip1, Tbp, Zfp318, Wbp4*) selected for comparable expression across several human and mouse brain regions. Potential differences in hybridization affinities of the exon 1e and exon 1b probes were adjusted by including three concentrations of a synthetic transcript (BlueHeronBio, Bothell, WA) targeted by both probes in the Nanostring analysis, from which a binding affinity ratio was calculated and used to equalize raw expression counts for each probe in the experimental data set.

Statistical Analysis

All data are presented as means and standard errors of the mean (SEM). Where appropriate, one-way, two-way, and repeated measures analyses of variance were used, and group differences identified using Fisher's least significant difference post hoc tests. Statistical significance was accepted at the $p < 0.05$ level.

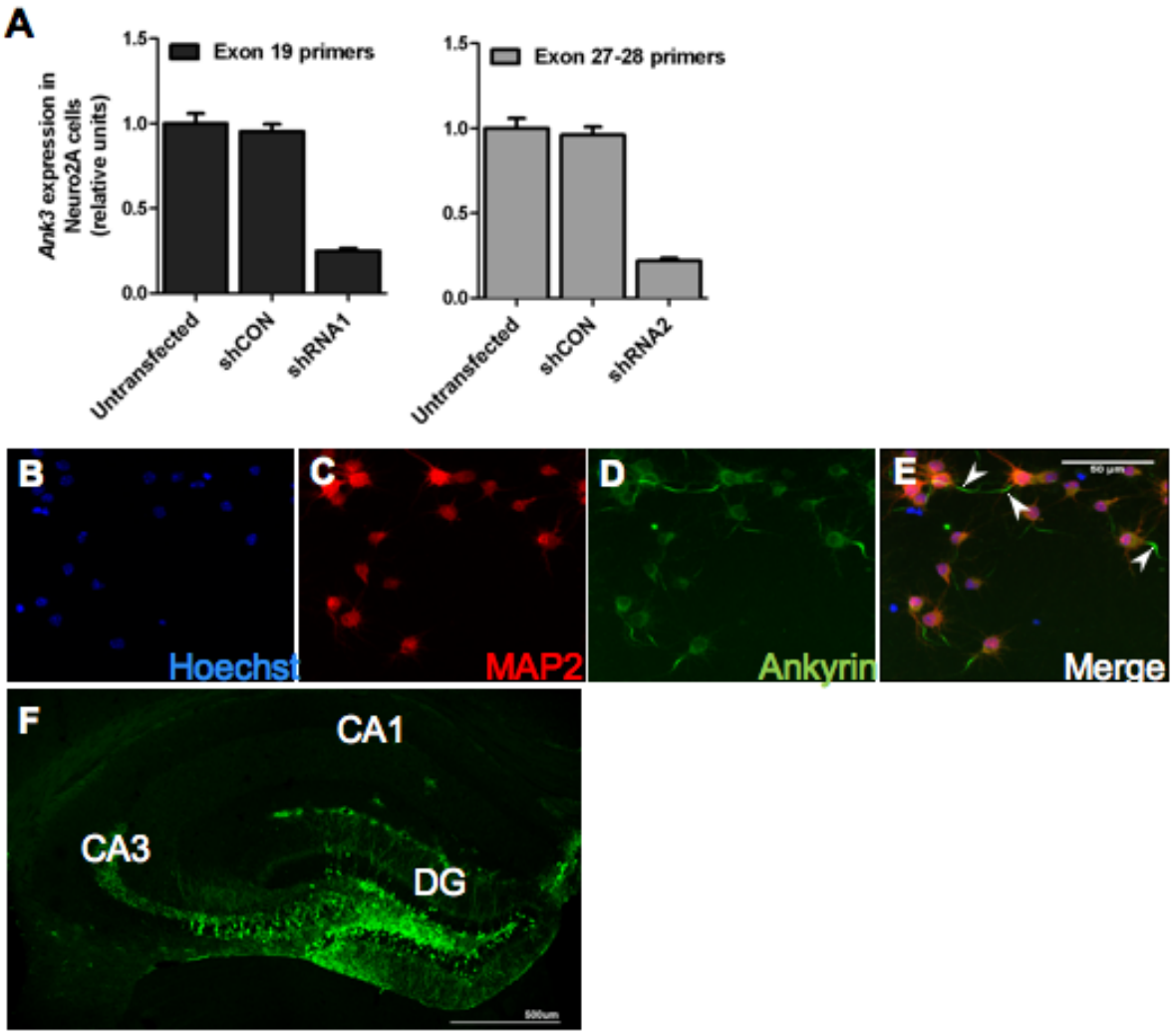

Figure S1. In vitro and in vivo validation of *Ank3* shRNA knockdown efficiency and ankyrin G antibody specificity. (**A**) Transfection of mouse Neuro-2a cells with shRNA1 or shRNA2 resulted in 75% to 80% decreased *Ank3* transcript levels compared to cells transfected with a non-targeting control shRNA (shCON), when measured using polymerase chain reaction primers located at *Ank3* exon 19 near the region targeted by shRNA1, or at exons 27-28 near the region targeted by shRNA2. Data are from three independent transfection experiments. Mean + SEM. (**B-E**) Immunofluorescent images of mouse primary neuronal cultures after 7 days in vitro (DIV7) documenting specificity of the ankyrin G antibody (Santa Cruz Biotechnology, #SC-28561) to detect ankyrin G at the axon initial segment (white arrowheads). (**F**) In mouse brain, representative image of hippocampus, demonstrating specificity of lentiviral infection to dentate gyrus (DG), including projection of DG neurons to CA3 (green, green fluorescent protein-positive infected cell).

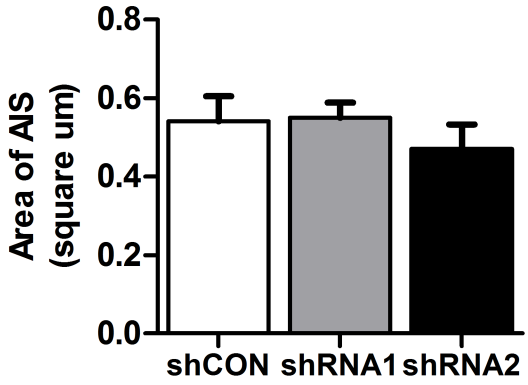

Figure S2. Relative areas quantified as neuronal axon initial segments (AIS) for each shRNA group. Ankyrin G expression was quantified at the AIS of dentate gyrus neurons by free-hand demarcation of the AIS structure and measuring immunofluorescence of ankyrin G within the demarcated area. There were no differences in the total area quantified as AIS between shCON and either of the *Ank3* shRNA groups. Mean + SEM; *n* = 4-7 mice/group, 5-7 AIS/mouse.

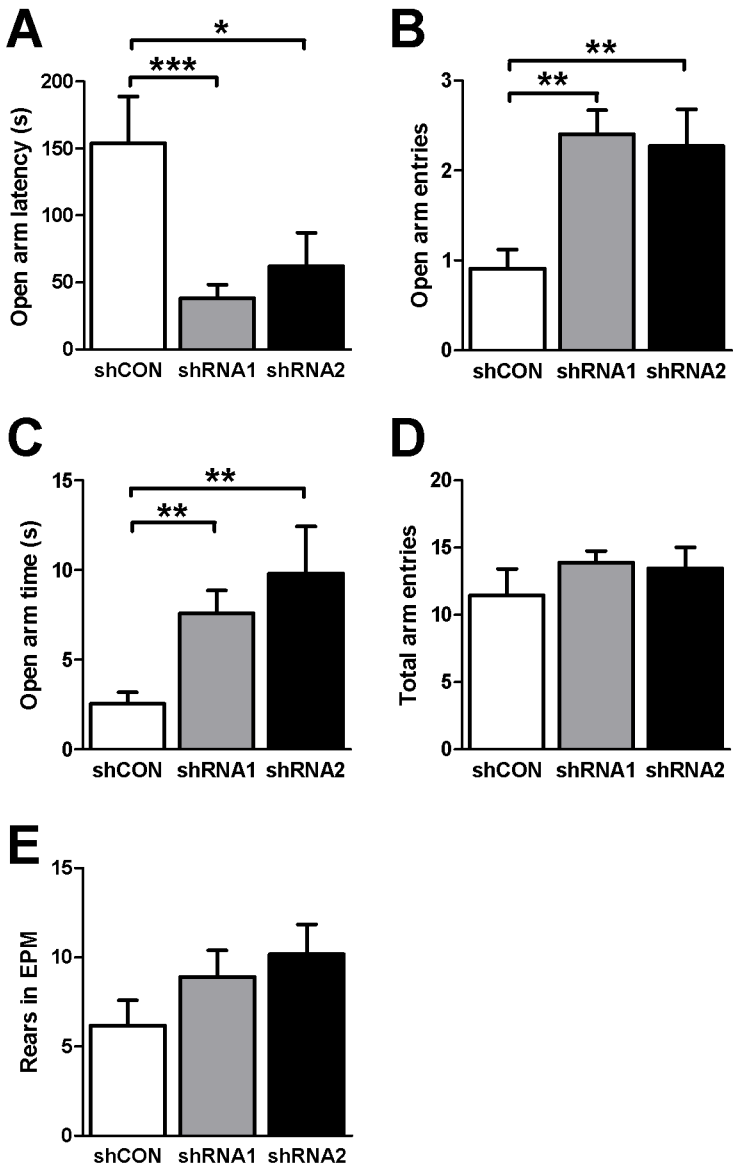

Figure S3. *Ank3* RNA interference behavioral screen highlights low anxiety-related behavior. Viral-mediated *Ank3* RNA interference in dentate gyrus significantly alters behavior in the elevated plus maze (EPM). Mice expressing shRNA1 or shRNA2 against *Ank3* (**A**) exhibit shorter latencies to enter the aversive open arms, (**B**) enter the open arms more frequently, and (**C**) spend more time in the open arms, compared to control mice expressing a non-targeting shRNA (shCON), indicative of decreased anxiety-related behavior. (**D**) Total arm entries and (**E**) rears in the EPM did not differ in shRNA1 and shRNA2 mice compared to shCON mice, indicating no change in general motor activity. Mean + SEM; n = 10-11 mice/group; *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

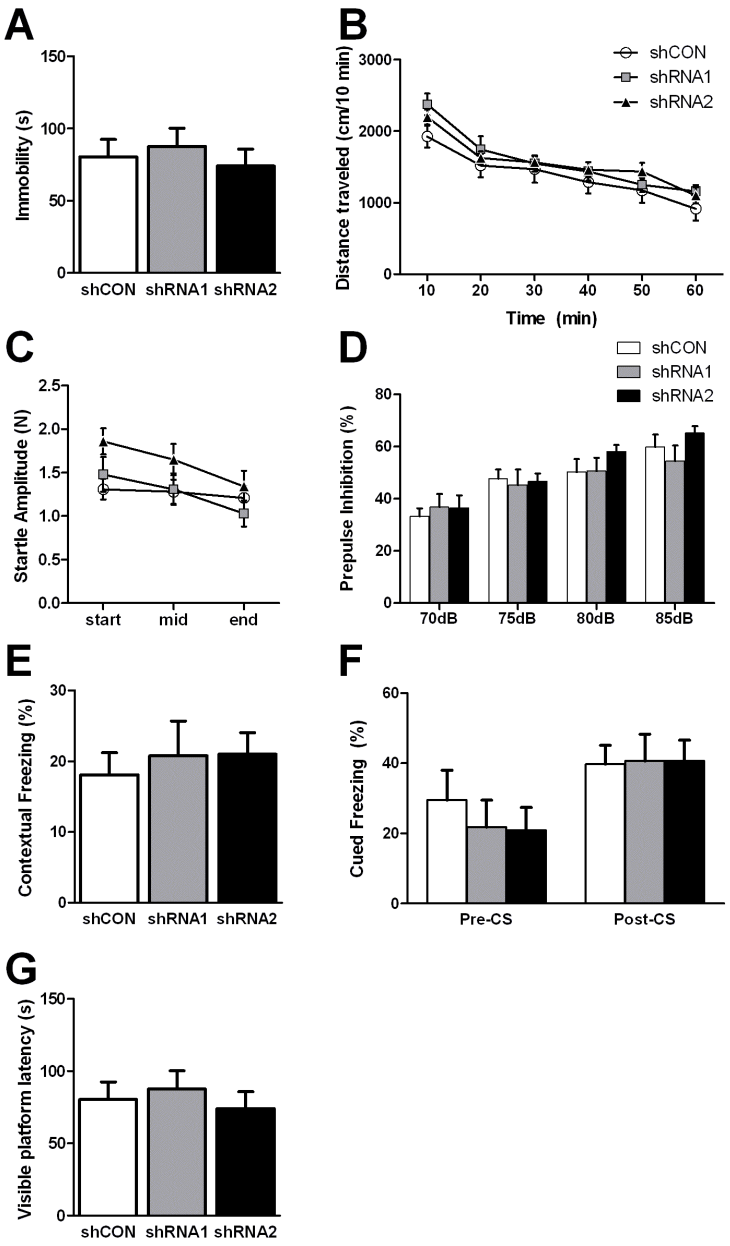

Figure S4. Behavioral screen indicates *Ank3* RNA interference in dentate gyrus (DG) does not globally alter brain function. Mice expressing shRNA1 or shRNA2 against *Ank3* in DG exhibit normal behavior compared to shCON mice in (**A**) forced swim test immobility time, (**B**) locomotor activity in a novel open field, (**C**) acoustic startle, (**D**) prepulse inhibition, (**E,F**) associative learning of a contextual or cued conditioned stimulus (CS), and (**G**) latency to find a visible platform in the water maze. These results suggest that the behavioral phenotype of mice expressing shRNA1 or shRNA2 is highly specific and not due to global changes in brain function. Mean + SEM; *n* = 10-11 mice/group.

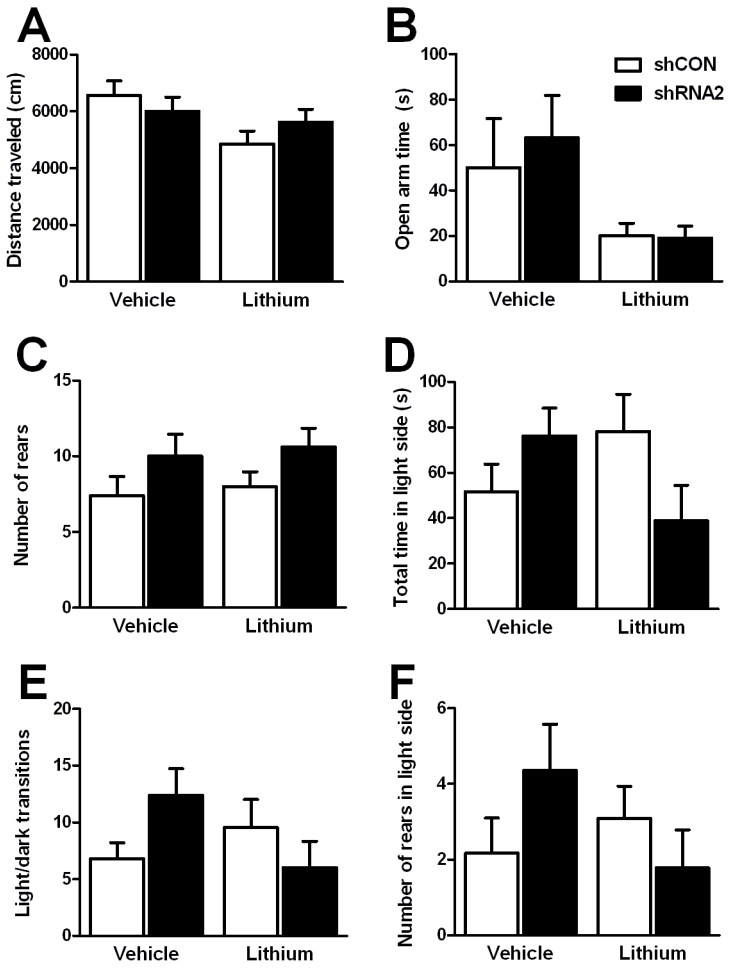

Figure S5. No changes in locomotor activity in *Ank3* shRNA2 mice treated with lithium or vehicle. (**A**) In the open field, total locomotor activity over a 60-minute period was not changed in shRNA2 mice compared to shCON mice treated with vehicle or with lithium (85 mg/kg i.p. daily, >14 days). There was no effect of *Ank3* knockdown by shRNA2 or of lithium treatment in the elevated plus maze task on (**B**) the total time in the open arms and (**C**) number of rears, or in the light-dark task on (**D**) time in the light side, (**E**) number of light-dark transitions, and (**F**) number of rears. Mean + SEM; *n* = 8-12 mice/group.

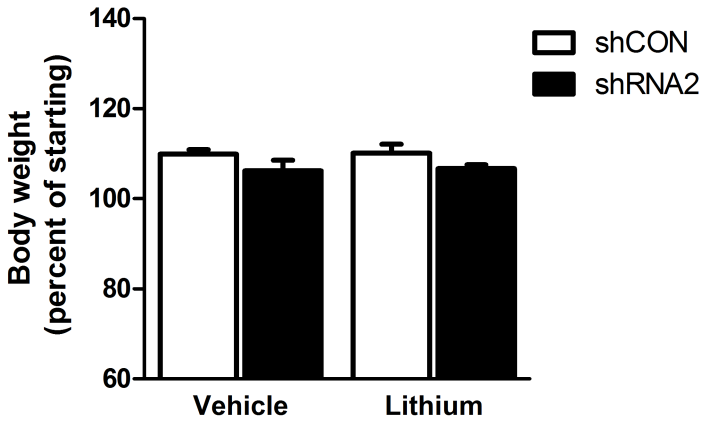

Figure S6. Lithium treatment does not affect weight in Ank3 shRNA mice. Mice treated with shCON or shRNA2 exhibit normal weight gain measured after three weeks of lithium treatment (85 mg/kg i.p. daily) when compared to vehicle-treated mice (all posthoc $p > 0.1$). Mean + SEM; n = 8-12 mice/group.

Table S1. Lack of broad behavioral changes in *Ank3+/-* mice highlights specificity of low anxiety/increased motivation phenotype. All data are group mean ± SEM.

	Ank3+/+	***Ank3+/-***	**Significance**
Open Field Test			
Distance traveled (cm)	7981 ± 336	7086 ± 667	ns
Elevated Plus Maze			
Open arm time (sec)	42.1 ± 8.3	70.5 ± 11.8	$p < 0.06$
Open arm entries (#)	5.3 ± 0.7	5.6 ± 0.7	ns
Total arm entries (#)	16.6 ± 1.3	15.5 ± 1.6	ns
Rears (#)	9.9 ± 0.9	11.6 ± 1.3	ns
Light Dark Test			
Light side time (sec)	155.2 ± 17.5	164.1 ± 26.9	ns
Transitions (#)	23.2 ± 2.1	26.2 ± 3.6	ns
Rears (#)	17.6 ± 3.1	19.6 ± 3.8	ns
Prepulse Inhibition (PPI)			
Acoustic Startle (N)	1.3 ± 0.1	1.6 ± 0.1	$p < 0.09$
Percent PPI at 70 dB	22.9 ± 3.4	18.2 ± 4.2	ns
Percent PPI at 75 dB	36.9 ± 6.0	38.1 ± 5.2	ns
Percent PPI at 80 dB	51.6 ± 3.9	48.2 ± 5.5	ns
Percent PPI at 85 dB	56.4 ± 4.9	54.7 ± 6.1	ns
Fear Conditioning			
Freezing to context (sec)	25.8 ± 3.8	25.8 ± 4.9	ns
Freezing to cue (sec)	52.8 ± 5.6	58.6 ± 5.1	ns

ns, not significant.

Table S2. Elevated plasma corticosterone levels (ng/ml) in *Ank3*+/- mice compared to *Ank3*+/+ mice under group or isolation housing conditions following exposure to acute restraint stress. All data are group mean ± SEM.

	Group		**Isolation**	
	***Ank3*+/+**	***Ank3*+/-**	***Ank3*+/+**	***Ank3*+/-**
Baseline	42.8 ± 14	137.6 ± 37	12.4 ± 5	24.0 ± 8
Acute – 30 min	887.5 ± 62	1392.2 ± 124	716.3 ± 56	1060.0 ± 111
Acute – 180 min	340.8 ± 114	925.0 ± 181	226.1 ± 35	641.1 ± 155

Table S3. Comparison of behavioral data from *Ank3* shRNA2 mice and *Ank3*+/- mice highlights consistency of phenotypes across these two models.

	***Ank3* shRNA2 (compared to shCON)**	***Ank3*+/- (compared to *Ank3*+/+)**	**Consistent between models?**
Open Field Test			
Activity (distance traveled)	NC	NC	Yes
Elevated Plus Maze			
Latency to enter open arm	¯*	¯#	Yes
Open arm time	- /NC	-	Yes
Open arm entries	- *	NC	No
Total arm entries	NC	NC	Yes
Rears	NC	NC	Yes
Light Dark Test			
Latency to enter light side	¯*	¯#	Yes
Light side time	NC	NC	Yes
Transitions	NC	NC	Yes
Rears	NC	NC	Yes
Novelty Suppressed Feeding			
Latency to approach	Not tested	¯#	?
Sucrose preference			
Preference for sucrose	Not tested	- #	?
Homecage activity			
Light phase	- *	Not tested	?
Dark phase	NC	Not tested	?
Prepulse Inhibition (PPI)			
Acoustic Startle	NC	NC	Yes
Percent PPI	NC	NC	Yes
Forced Swim Test			
Immobility	NC	NC#	Yes
Fear Conditioning			
Contextual	NC	NC	Yes
Cued	NC	NC	Yes

Studies in the *Ank3*+/- model were designed to complement and extend the findings from *Ank3* shRNA mice, thus *Ank3*+/- mice underwent additional characterization in the novelty suppressed feeding and sucrose preference tasks. The *Ank3*+/- model was not tested for homecage activity due to technical difficulties.

* Reversed by lithium treatment.

Altered by chronic isolation stress.

NC, No difference between *Ank3* shRNA mice or *Ank3*+/- mice and their respective controls.

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