Inhibition of 14-3-3 Proteins Leads to Schizophrenia-Related Behavioral Phenotypes and Synaptic Defects in Mice

Supplemental Information

Supplementary Methods

Open Field Test

General activity was assessed using an open field testing apparatus (open field arena, 43.2 cm x 43.2 cm x 30.5 cm, with IR photobeam sensors, Med Associates). Activity was recorded for 30 min and total distance traveled was determined using Med Associates Activity Monitor software.

Rotarod

Balance and motor coordination was assessed using the rotarod (Med Associates) test apparatus. Up to five mice were placed on the rotarod with a baseline speed of 2 RPM. Latency to fall was measured as the rotation speed increased from 2 to 20 RPM over a 2 min period (Med Associates).

Light/Dark Box Test

Anxiety behaviors were assessed using the light/dark box testing paradigm. The testing apparatus is made up of two chambers, one dark chamber with a black Plexiglas floor and one illuminated chamber with white Plexiglas floor (Shuttle Box Avoidance Chamber for Mouse, Med Associates), separated by an automated guillotine door. After a 5 min habituation period in the dark chamber, the automated guillotine door opened and the test mouse was allowed to freely

explore both chambers for 10 min. During testing, the time spent in each chamber was recorded using IR photobeam sensors.

Passive Avoidance

Passive avoidance was performed as previously described (1). The passive avoidance task was performed over 2 consecutive days using the Shuttle Box Avoidance Chamber for Mouse (Med Associates). For training on day 1, the test mouse was placed in the light chamber and their latency to enter the dark chamber was recorded. Upon entering the dark chamber, the automatic guillotine door was closed and mice were given a brief 2 sec footshock (0.70 pA) and remained in the dark chamber for 1 min. For testing on day 2, mice were placed back into the light chamber and their latency to enter the dark chamber was recorded.

Contextual Fear Conditioning

Contextual fear conditioning was performed as previously described (1). The contextual fear conditioning test was performed over 2 consecutive days using the Contextual NIR Video Fear Conditioning System for Mouse and Video Freeze Software (Med Associates). For training on day 1, mice were placed in the testing chamber and their freezing behavior was recorded before (3 min), during, and after (2 min) 3 footshocks (2 sec, 0.50 pA, separated by a 2 min intershock interval). For testing on day 2, mice were placed back into the same chamber and their freezing behavior was recorded for 3 min.

Social Interaction Test

Social interaction testing was performed as previously described (2). Sociability and social novelty preference tests were performed using a Plexiglas testing arena (24” x 24” x 12”) divided equally into 3 chambers by two walls with 3” diameter cut-outs. For sociability, the test mouse was habituated to the middle chamber for 5 min. Then the test mouse was allowed to freely explore all chambers for 10 min, and the time spent in each chamber (empty, middle, containing an unfamiliar mouse) was recorded. For social novelty, the test mouse was subsequently rehabituated to the middle chamber for 5 min, and then allowed to freely explore all three chambers for 10 min, in which the previously empty chamber contains a new unfamiliar mouse. Time spent in each chamber (middle, containing either unfamiliar or familiar mouse) was recorded.

Y-Maze Test

Analysis of continuous spontaneous alternation behavior in the Y-maze, as a measure of working memory, was assessed as previously described (3). Briefly, the testing apparatus was constructed from clear Plexiglas consisting of 3 arms (24” x 6” x 12”) angled 120° apart in the shape of a ‘Y’ with removable walls at the center end and joined by a central triangular platform. Each arm had distinct markings on the walls (labeled A, B, C) and visual cues were placed around the testing room. During testing, the test mouse was placed on the center platform, with all arms blocked off, for a 5 min habituation period. The arms were then unblocked and the test mouse was allowed to freely explore the maze for 10 min while activity was recorded with a video camera. Video files were analyzed to determine the order of arm choices, total number of arm choices (T), and total number of complete alternations (Alt) made which was scored when

the mouse consecutively entered all 3 different arms (i.e., B-A-C). Alternation percentage was calculated as: [(Alt/(T-2))*100%].

Sensorimotor Gating

Startle response and prepulse inhibition (PPI) of the acoustic startle response were assessed using Acoustic Startle Reflex package (Med Associates). The test mouse was placed in a clear Plexiglas cylinder holder within a sound attenuating cubicle, and their responses to acoustic stimuli were recorded with 60 dB background noise. Baseline startle amplitude responses were first measured during a block of 10 trials consisting only of startle tone (90 dB). PPI was determined in the subsequent block which consisted of pseudo-randomized delivery of 8 trials each of startle tone only, no stimuli, pulse tone only (67, 70, 73, or 76 dB), and pulse paired with startle tone. PPI percentage was calculated as: [1- ((Mean of Startle with Prepulse – Mean of Startle Only)/Mean of Startle Only)*100%].

Fluorescent Microscopy

Mice were anesthetized and transcardially perfused with 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4 (PBS). After an overnight postfixation in the same fixative at 4°C, the brains were then cut into 40 μm sections on a Vibratome (Leica Microsystems). Brain sections were mounted with Vectashield to retard fluorescence fading, and imaged on a fluorescence microscope (Eclipse FN1, Nikon, Japan) using a 4X objective. The pictures of complete sagittal brain sections were acquired as stitched images using the NIS-Elements AR software (Nikon). Transgene expression patterns were determined by scoring fluorescent signal intensity levels in different brain regions as background or no expression, low expression, or high expression (4).

To evaluate the transgene expression in cortical and hippocampal regions, brain sections were immunostained with a monoclonal anti-NeuN antibody (Millipore), followed by incubation with Alexa Fluor® 647 donkey anti-mouse secondary antibodies (Invitrogen). The images were attained on a Leica TCS SP2 SE laser scanning confocal microscope (Leica Microsystems) using a 20 X objective.

Electrophysiology

Slice preparation. Mice (4-6 weeks old, males) were sacrificed after being deeply anesthetized with ketamine (100 mg/kg) and xylazine (10 mg/kg). The whole brain was removed and placed in ice-cold and oxygenated (95% O_2/5% CO_2) dissecting buffer including (in mM): 230 sucrose, 2.5 KCl, 10 $MgSO_4$, 1.25 Na_2HPO_4, 26 $NaHCO_3$, 0.5 $CaCl_2$, 10 D-glucose. The coronal slices comprising medial prefrontal cortex were cut with VT1200S vibratome (Leica) and moved to the artificial cerebrospinal fluid (ACSF) saturated with 95% O_2/5% CO_2. The ACSF contained (in mM): 124 NaCl, 5 KCl, 2.5 $CaCl_2$, 1.3 $MgSO_4$, 1.2 KH_2PO_4, 26 $NaHCO_3$, and 10 glucose. The slices were then incubated at 37°C for at least 45 min in oxygenated ACSF prior to use.

Whole-cell recordings. Whole-cell recordings of spontaneous excitatory and inhibitory postsynaptic currents (sEPSC and sIPSC) and miniature excitatory and inhibitory postsynaptic currents (mEPSC and mIPSC) from medial prefrontal cortex layer V pyramidal neurons were carried out as previously described (5, 6). For recordings, a single slice was placed in the submersion recording chamber (Warner Instruments) under a fluorescence microscope (ECLIPSE FN1, Nikon) equipped with infrared differential interference contrast and water immersion-objective for visualization of neurons in live tissues. During recordings, the chamber

was continuously superfused with ACSF (2 ml/min) saturated with 95% O_2/ 5% CO_2 at room temperature (22 ± 0.5^{o}C). The layer V pyramidal neurons were recorded in the voltage-clamp mode using a Multiclamp 700A amplifier (Molecular Devices). The recording electrodes (DC resistance: 7-9 MΩ) were filled with a Cs^{+}-based intracellular solution containing (in mM): 120 Cs-gluconate, 6 $CsCl_2$, 0.2 EGTA, 1 ATP-Mg, 0.2 Na_2GTP, 10 Hepes, 5 QX-314, (290 - 300 mOsm, pH 7.4 with CsOH). As described previously (5, 6), sEPSCs were recorded at the reversal potential of GABAA receptors (-65 mV) in the presence of picrotoxin (50 μM), while sIPSCs were recorded at the reversal potential of ionotropic glutamate receptors (0 mV) in the presence of both D-AP5 (50 μM) and CNQX (20 μM). For mEPSCs and mIPSCs recordings, tetrodotoxin (1 μM) was included in the perfusion solution. Currents were filtered at 2 kHz with a low-pass filter, and data were digitized at 10 kHz and acquired using the pCLAMP 10 software (Molecular Devices). Series resistance was continuously monitored for each neuron. The neuron was rejected from statistical analysis if the series resistance increased more than 20%. Data analyses were conducted using the Mini Analysis (Synaptosoft, USA) program.

Stereology

Estimations of the number of parvalbumin (PV) containing interneurons were performed as previous described (7). Briefly, sequential sections (6 slices) of the prefrontal region (Bregma +5.0 to +2.0) were used in fluorescence immunohistochemistry to detect parvalbumin-containing interneurons with primary antibody anti-parvalbumin 1:1000 (Sigma-Aldrich), followed by incubation with Alexa Fluor® 647 donkey anti-mouse secondary antibody (Invitrogen). The tiled, z-stack images at 40x magnification were attained on a Leica TCS SP2 SE laser scanning confocal microscope (Leica Microsystems). The settings of the confocal microscope were kept

constant throughout the imaging such that fluorescence intensities could be compared between animals and imaging days. Stereological estimation of the number of PV-containing interneurons was determined using StereoInvestigator system (Microbrightfield Europe).

Golgi Stain

Golgi staining was performed using the FD Rapid GolgiStain^{TM} Kit (FD NeuroTechnologies Inc., Columbia MD) according to the manufacturer's instructions. Briefly, mice were anesthetized, decapitated, and brains were removed. Tissue was placed in impregnation solution containing equal volumes of Solutions A and B, and stored at room temperature for 2 weeks in the dark. Brains were then placed in Solution C for 48 h at 4°C in the dark. Coronal sections were cut to 200 µm using a vibratome (Leica Microsystems), mounted, and air dried overnight. Sections were then rinsed in double distilled water, placed in Solution D+E, and dehydrated in 50%, 75%, 95%, and absolute ethanol. Lastly, sections were cleared with xylene and coverslipped with Permount mounting medium. All neurons selected for analysis satisfied the following criteria: (i) the cell body and processes were completely impregnated; (ii) the cell and processes were isolated from surrounding impregnated cells; (iii) the cell body was located in the hippocampus CA1 cell layer, cortex layer III, or cortex layer V (8).

Western Blot

Mouse hippocampal lysates were prepared as previously described (1, 9). Briefly, dissected hippocampi (100 mg) were homogenized in TEVP buffer (10 mM Tris base, 5 mM NaF, 1 mM Na3VO4, 1 mM EDTA, 1 mM EGTA, pH 7.4) + 320 mM sucrose solution. Each

sample was a combined pool of tissue from 2-3 animals. The lysates were then sonicated and separated by SDS-PAGE and probed with specific antibodies. The relative amount of GAPDH (probed with anti-GAPDH, EnCor Biotechnology) was used as a loading control for quantification. Other primary antibodies used in this experiment include polyclonal antibodies (Cell Signaling) against cofilin-1, phospho-cofilin (Ser3), and (Santa-Cruz) d-catenin. The western blot signals were generated by incubating the membranes with fluorescently-labeled secondary antibodies (LI-COR Biosciences), and acquired using the LI-COR Odyssey Infrared Fluorescent scanner. Protein densities on western blots were then analyzed and quantified with ImageJ software.

Table S1. Transgene expression pattern in different 14-3-3 FKO founder lines

	Founder Line			
	132	**142**	**153**	**177**
Cortex Layer:				
I	-	-	-	-
II/III	-	+	+	-
IV	+	+	-	-
V	++	+	+	+
VI	++	-	-	-
Hippocampus:				
CA1	++	-	-	-
CA3	++	+	-	-
Dentate Gyrus	++	+	-	-
Olfactory Bulb	+	+	+	+
Striatum	+	+	-	+
Thalamus	+	+	+	+
Midbrain	+	+	+	+
Pons	++	+	+	+
Medulla	+	+	+	+
Cerebellum Layer:				
Molecular	-	-	+	-
Granule cells	++	+	-	+
Purkinje cells	-	-	-	-

Transgene expression scored as: ++, high level expression; +, low level expression; and -, denotes no detectable signal.

Table S2. Behavior characterization of different 14-3-3 FKO founder lines

Test *Measurement (units)*	14-3-3 Functional Knockout Founder Lines				Wildtype
	132	**142**	**153**	**177**	
Open Field *Distance (cm)*	18980.5 ± 1289.9*** *(n = 34)*	9499.0 ± 666.5*** *(n = 26)*	7090.0 ± 740.3 *(n = 21)*	7616.8 ± 668.2** *(n = 21)*	5839.6 ± 353.21 *(n = 22)*
Rotarod *Latency to fall (s)*	110.5 ± 16.07* *(n = 46)*	180.3 ± 19.58 *(n = 25)*	158.1 ± 16.41 *(n = 14)*	178.9 ± 27.73 *(n = 11)*	190.9 ± 19.42 *(n = 29)*
Light/Dark Box *Time spent in light chamber (s)*	244.9 ± 31.65 *(n = 23)*	315.4 ± 47.99 *(n = 6)*	248.1 ± 15.65 *(n = 7)*	N/A	259 ± 30.96 *(n = 17)*
Startle Response *Startle Amplitude (unit)*	1407.3 ± 120.4 *(n = 27)*	1786.6 ± 307.3 *(n = 8)*	1661.2 ± 426.3 *(n = 4)*	2808.9 ± 319.8*** *(n = 10)*	1448.9 ± 112.04 *(n = 22)*
Prepulse Inhibition (PPI) *PPI (73dB) (%)*	32.53 ± 5.91*** *(n = 27)*	52.77 ± 9.57* *(n = 17)*	61.51 ± 6.31 *(n = 10)*	40.50 ± 12.53** *(n = 8)*	67.17 ± 4.28 *(n = 23)*
Contextual Fear Conditioning *Freezing (%)*	35.40 ± 5.36** *(n = 29)*	58.76 ± 12.55 *(n = 5)*	26.28 ± 4.65*** *(n = 12)*	31.68 ± 6.66** *(n = 16)*	50.33 ± 4.88 *(n = 21)*
Passive Avoidance *Latency to dark chamber (s)*	76.6 ± 14.93** *(n = 19)*	149.0 ± 20.66 *(n = 22)*	N/A	N/A	125.52 ± 17.22 *(n = 15)*

All values are represented as mean ± S.E.M. Statistical significance compared with that of wildtype is represented as: *$p < 0.05$; **$p < 0.01$; and ***$p < 0.0001$.
"N/A", denotes the behavior test was not assessed.

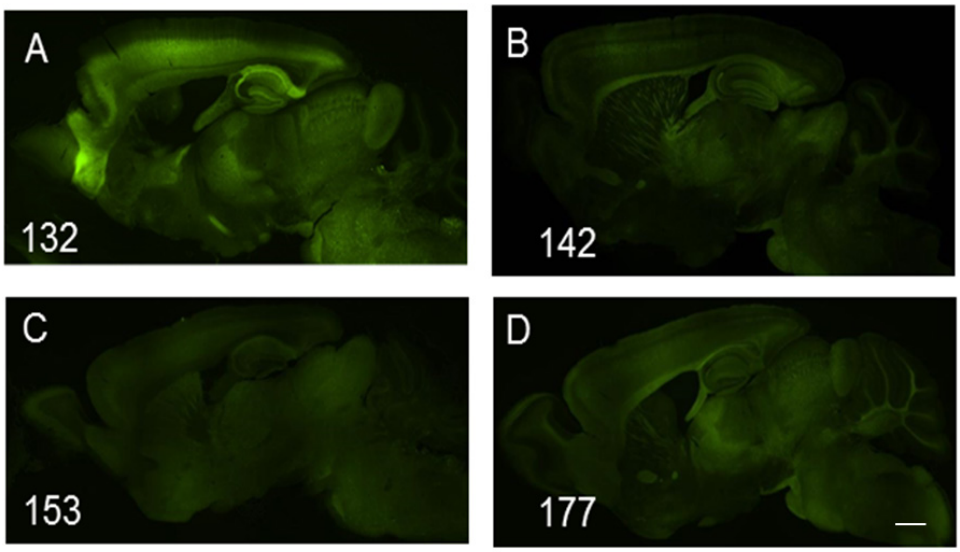

Figure S1. Transgene expressions of different 14-3-3 FKO founder lines. Representative images of sagittal brain sections from various 14-3-3 FKO founder lines (A, line 132; B, line 142; C, line 153; D, line 177). Transgene expression (green) was detected by fluorescent microscopy. Scale bar, 1 mm.

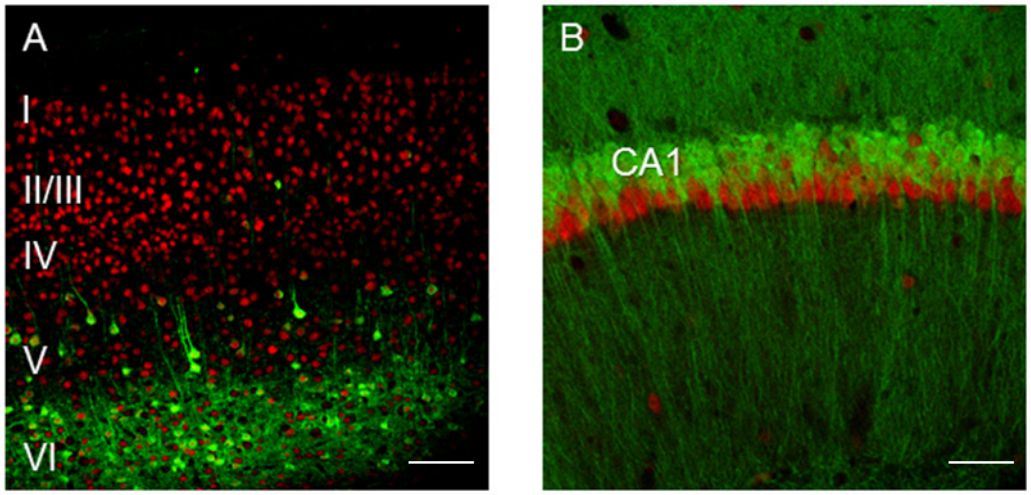

Figure S2. Transgene expression in cortex and hippocampus of the 14-3-3 FKO mice. In the 14-3-3 FKO mice (132 founder line), the transgenes are highly expressed in pyramidal neurons of the deep layers of the cortex (layer V/VI) (**A**) and hippocampus CA1 (**B**). Scale bars, 100 μm (A) and 50 ṁm (B). Transgene, green; NeuN staining, red.

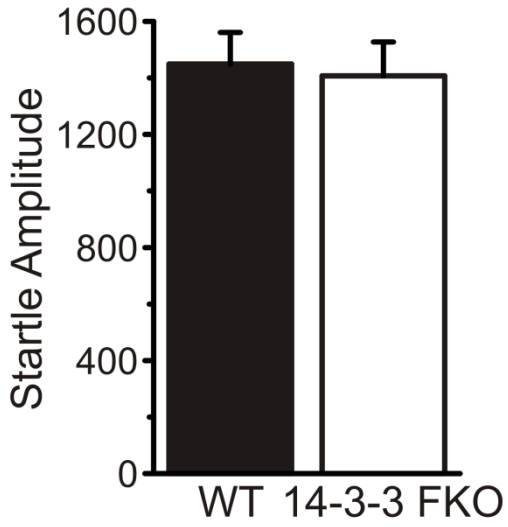

Figure S3. Baseline startle amplitude response in the 14-3-3 FKO mice. Baseline startle response (arbitrary units) is similar in both WT (*n* = 22) and 14-3-3 FKO mice (132 line; *n* = 29). Data are presented as mean ± S.E.M., one-way ANOVA.

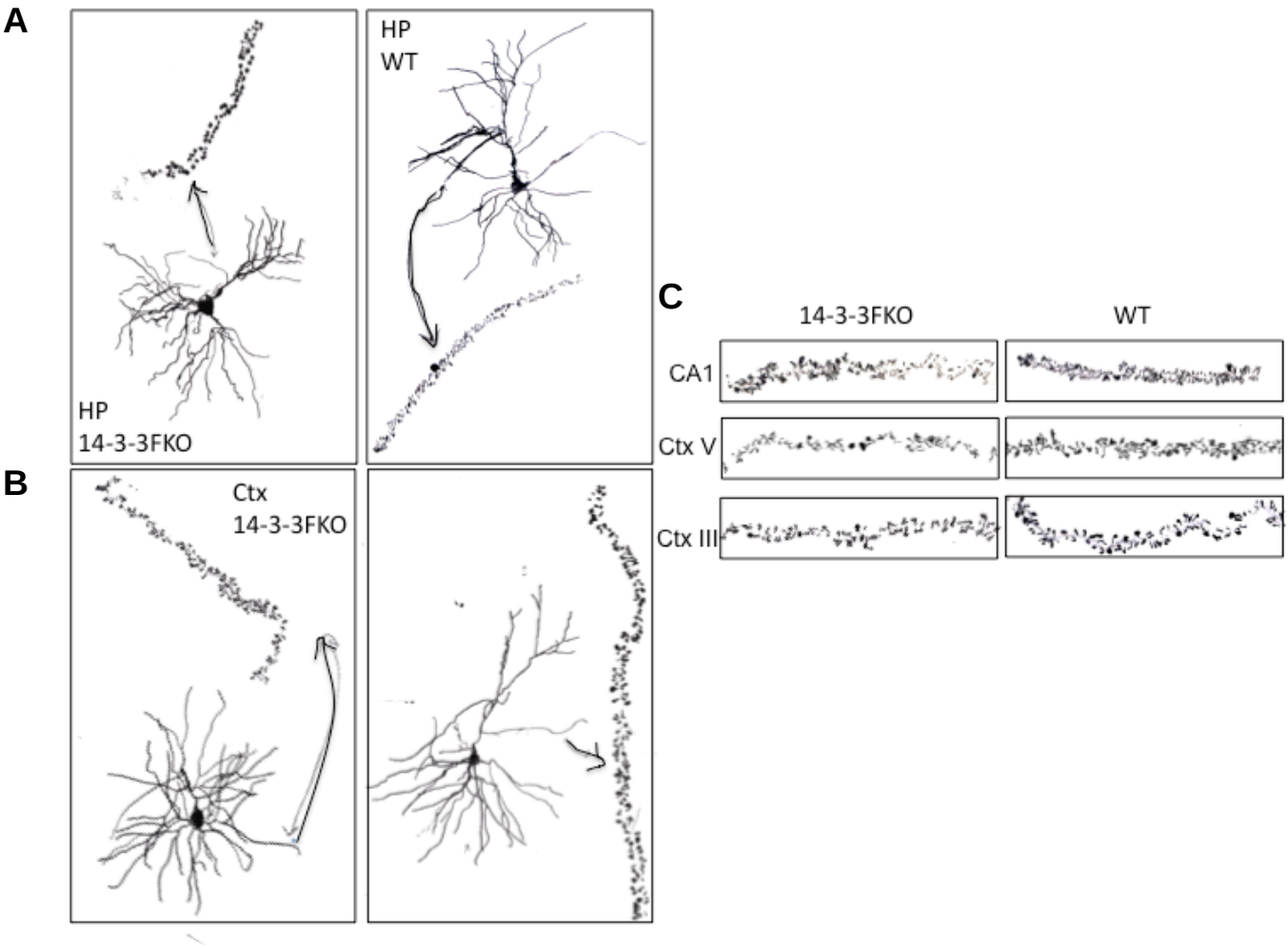

Figure S4. Hippocampal and cortical neurons and dendrites in the 14-3-3 FKO and wildtype mice. Representative tracings of (**A**) hippocampal neurons, (**B**) cortical neurons and (**C**) apical dendrites from hippocampus CA1, cortex layer V and cortex layer III. Images were traced at 25x and 100x magnification for neurons and dendrites respectively.

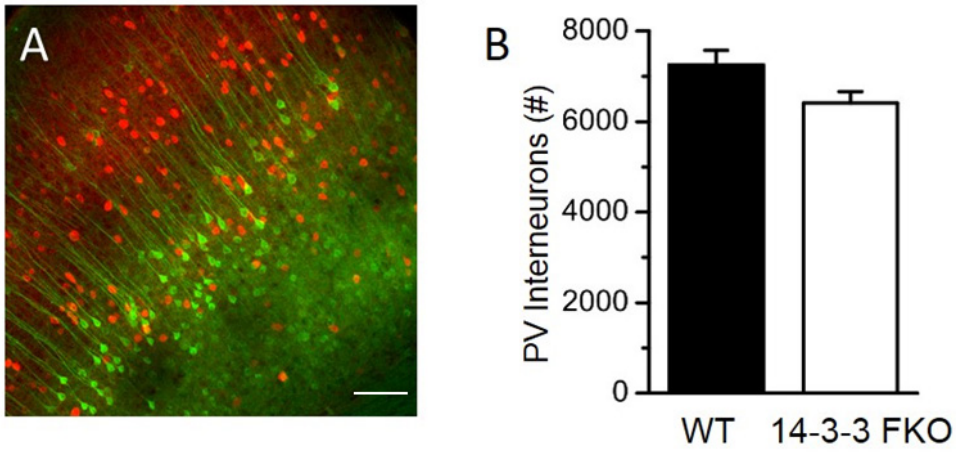

Figure S5. Transgene expression does not alter PV-containing interneurons. (**A**) Transgene (green) is minimally expressed in parvalbumin (PV) containing interneurons (red) in the cortex of the 14-3-3 FKO mice. Scale bar, 100 µm. (**B**) No significant difference in the number of PV-containing interneurons in the medial PFC between WT and 14-3-3 FKO mice. Data are presented as mean ± S.E.M.; one-way ANOVA.

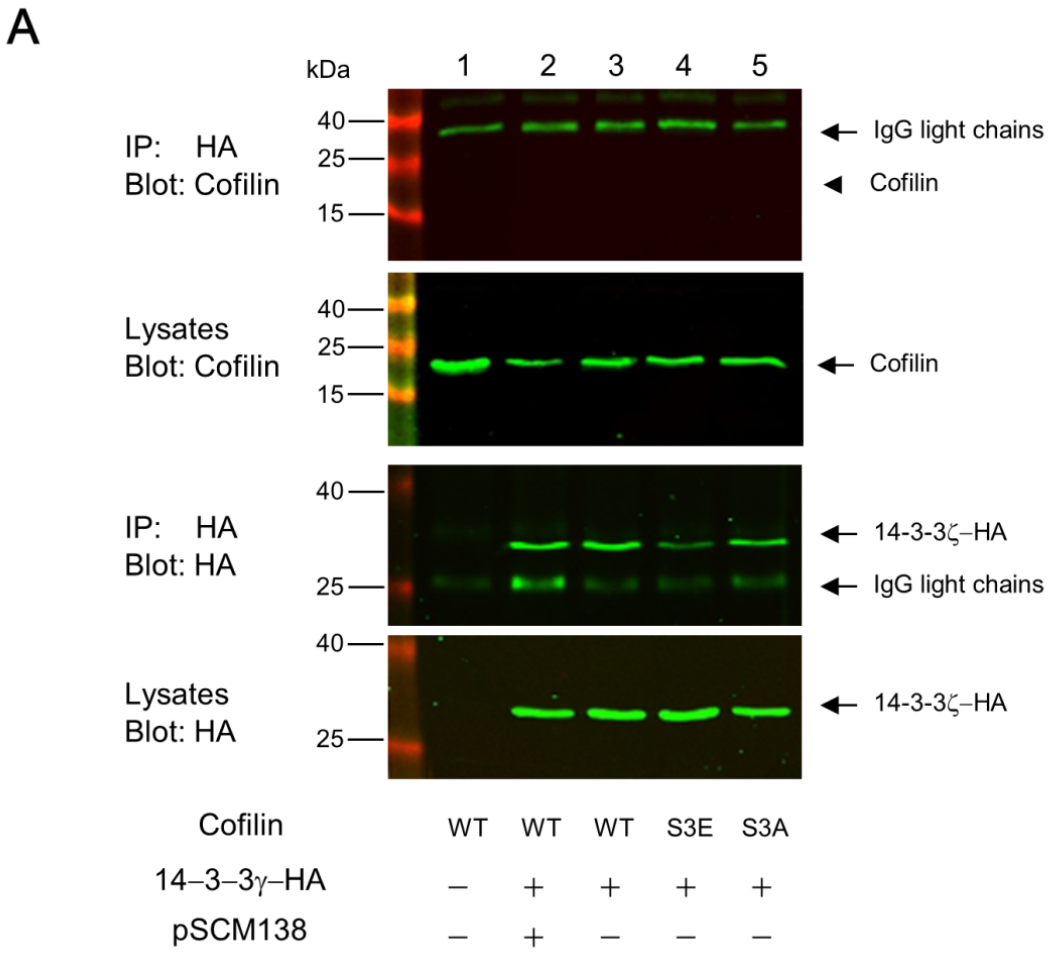

Figure S6. 14-3-3 does not interact with cofilin in co-transfected cells. Western blot analyses of HA immunoprecipitates and cell lysates from tsA201 cells in in which 14-3-3z-HA was co-transfected with either wildtype (lanes 2, 3), S3E (lane 4) or S3A mutant cofilin (lane 5). Cofilin (*position indicated by arrowhead*) was not detected in 14-3-3z-HA immunoprecipitates under any of these conditions. Lane 1 depicts a negative control without co-transfected 14-3-3.

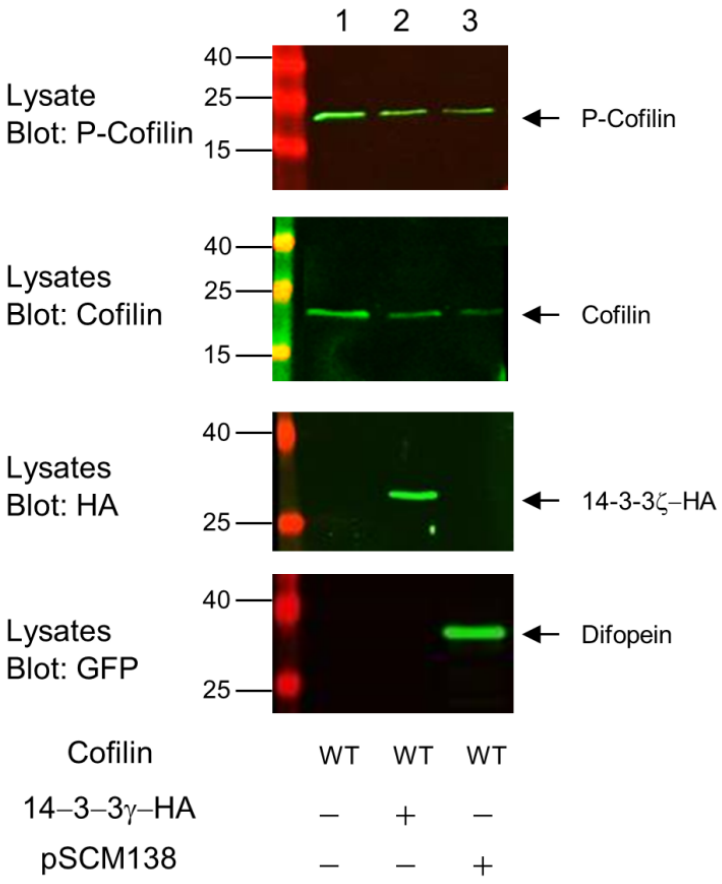

Figure S7. 14-3-3 does not affect the level of phospho-cofilin in tsA201 cells. Western blot analyses of cell lysates from tsA201 cells in in which cofilin was transfected either alone (lane 1), or together with exogenous 14-3-3z-HA (lane 2) or pSCM138 that encodes the 14-3-3 inhibitor difopein (lane 3). The ratio of phosphorylated and total cofilin was similar under these different transfection conditions. Note: the level of cofilin is lower in co-transfected conditions for this particular experiment.

Supplemental References

1. Qiao H, Foote M, Graham K, Wu Y, Zhou Y (2014): 14-3-3 proteins are required for hippocampal long-term potentiation and associative learning and memory. *J Neurosci*. 34:4801-4808.

2. Moy SS, Nadler JJ, Perez A, Barbaro RP, Johns JM, Magnuson TR, et al. (2004): Sociability and preference for social novelty in five inbred strains: an approach to assess autistic-like behavior in mice. *Genes Brain Behav*. England, pp 287-302.

3. Deacon RM, Rawlins JN (2006): T-maze alternation in the rodent. *Nat Protoc*. England, pp 7-12.

4. Caroni P (1997): Overexpression of growth-associated proteins in the neurons of adult transgenic mice. *J Neurosci Methods*. Netherlands, pp 3-9.

5. Verret L, Mann EO, Hang GB, Barth AM, Cobos I, Ho K, et al. (2012): Inhibitory interneuron deficit links altered network activity and cognitive dysfunction in Alzheimer model. *Cell*. 149:708-721.

6. Li YC, Kellendonk C, Simpson EH, Kandel ER, Gao WJ (2011): D2 receptor overexpression in the striatum leads to a deficit in inhibitory transmission and dopamine sensitivity in mouse prefrontal cortex. *Proc Natl Acad Sci U S A*. 108:12107-12112.

7. Lodge DJ, Behrens MM, Grace AA (2009): A loss of parvalbumin-containing interneurons is associated with diminished oscillatory activity in an animal model of schizophrenia. *J Neurosci*. 29:2344-2354.

8. Koyama Y, Hattori T, Shimizu S, Taniguchi M, Yamada K, Takamura H, et al. (2013): DBZ (DISC1-binding zinc finger protein)-deficient mice display abnormalities in basket cells in the somatosensory cortices. *J Chem Neuroanat*. 53:1-10.

9. Hallett PJ, Collins TL, Standaert DG, Dunah AW (2008): Biochemical fractionation of brain tissue for studies of receptor distribution and trafficking. *Curr Protoc Neurosci*. Chapter 1:Unit 1.16.