Supporting Information

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SI Methods

Affinity Chromatography. GST-RIM1 351–429 or 351–596 (125,000 pmol) were phosphorylated *in vitro* for 3 h at room temperature in a standard buffer (10 mM Hepes, pH 7.4, 2 mM $MgCl_2$, 1 mM EGTA) in the presence of 100 μM ATP and 1000 U PKA. For nonphosphorylated negative controls, ATP was omitted from the reaction. GST-RIM fusion proteins were dialyzed into a coupling buffer (0.2 M $NaHCO_3$, 0.5 M NaCl, pH 8.2) and coupled to an Amino-link matrix (Pierce) according to the standard protocol provided with the matrix. Fresh rat brain extracts were homogenized at low speed with glass-Teflon homogenizers in a standard buffer (25 mM Hepes, pH 7.4, 150 mM NaCl, 2 mM EGTA, 1 mM dithiotreitol, 1 μg/ml pepstatin, 1 μg/ml leupeptin, 2 μg/ml aprotinin, 1 mM PMSF) in the presence of phosphatase inhibitors (phosphatase inhibitor mixture II; Sigma), and proteins were extracted in 1% Triton for 1 h at 4°C. The homogenate was spun twice (1 h, 164,000 × *g*, 4°C each spin), and supernatants were incubated with the affinity column overnight at 4°C. After extensive washing, the proteins were eluted from the column with increasing concentrations of NaCl and low pH (brain homogenate buffer with 500 mM, 750 mM, and 1 M NaCl, 100 mM glycine pH 3.2) and were then run on SDS/PAGE gels. Proteins were visualized with Coomassie and silver staining (1). The phospho-specific binding partner was identified by mass spectrometry. GST fusion protein purifications and GST-pulldown assays from native brain homogenates were performed as described (2), and *in vitro* phosphorylation was downscaled accordingly. Brain homogenates were prepared as described above.

Generation of RIM1 S413A-KI Mice. Mice were generated according to standard methods (3, 4).

We cloned the RIM1 gene containing exon 6 and flanking sequences from a genomic library. A targeting vector was constructed to replace the serine-413 residue with alanine; at the same time we introduced BglI and SphI restriction sites for genotyping. In addition, we flanked exon 6 with loxP recombination sites, a duplicated neomycin resistance gene (surrounded by flp recombination sites) was inserted in the intron 3′ of exon 6, and a diphtherotoxin-expressing cassette positioned at the 5′ end was used for negative selection. The linearized vector was transfected into R1 ES cells (5), and homologous recombination was identified by Southern blotting, and further characterized by PCR and sequencing. The positive ES cell clones were then injected into C57BL/6 blastocysts to produce chimeric offsprings carrying the knockin allele. Upon germline transmission of the mutant allele, we removed the neomycin resistance cassettes by crossing the mice with flp recombinase transgenic mice (6). This line was used for all experiments and will be referred to as RIM1 S413A-KI. The mouse line was submitted to the Jackson Laboratories and is freely available to the community.

Electrophysiology. Hippocampal and cerebellar slices (400 μm) were prepared from littermate wild-type control and RIM1 S413A-KI mice (3–6 weeks old). All animal procedures were performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Slices were prepared on a DTK-2000 vibratome (Dosaka) in ice-cold sucrose-containing cutting solution consisting of 215 mM sucrose, 2.5 mM KCl, 20 mM glucose, 26 mM $NaHCO_3$, 1.6 mM NaH_2PO_4, 1 mM $CaCl_2$, 4 mM $MgCl_2$, and 4 mM $MgSO_4$. The cutting solution was slowly exchanged (≈30 min) with artificial cerebrospinal fluid (ACSF) containing 124 mM NaCl, 2.5 mM KCl, 10 mM glucose, 26 mM $NaHCO_3$, 1.0 mM NaH_2PO_4, 2.5 mM $CaCl_2$, and 1.3 mM $MgCl_2$. Both cutting and ACSF solutions were saturated with 95% O_2 and 5% CO_2 (pH 7.4). The slices were incubated at room temperature for at least 1.5 h before recording. For whole-cell recording, the recording pipette solution was filled with internal solution consisting of 123 mM cesium gluconate, 8 mM NaCl, 1 mM $CaCl_2$, 10 mM EGTA, 10 mM Hepes, 10 mM glucose (pH 7.3, 290–295 mOsm). Mossy fiber LTP measurements were performed in the presence of 50 μM D-APV (D-2-amino-5-phosphonovalerate), and when LTP at parallel fiber to Purkinje cell synapses were examined, BAPTA [1,2-bis(*o*-aminophenoxy)ethane-*N,N,N′,N′*-tetraacetic acid] (20 mM) was added to the internal solution. In the latter case, $CaCl_2$ and EGTA were excluded, and the concentration of cesium gluconate was reduced to maintain optimal osmolality (290–295 mmol/kg). Field potentials were recorded extracellularly with patch-type pipettes filled with 1 M NaCl. Synaptic responses were evoked with patch pipettes filled with ACSF. Stimulating electrodes were placed in the molecular layer to activate cerebellar parallel fibers, in the dentate gyrus cell body layer to activate hippocampal mossy fibers, and in the CA1 stratum radiatum to activate Schaffer collateral fibers and interneuron axons. LTP at parallel fiber to Purkinje synapses were examined in the presence of 100 μM picrotoxin, and I-LTD was tested in the continuous presence of 25 μM D-APV and 10 μM NBQX (2,3-dihydroxy-6-nitro-7-sulfamoylbenzo[*f*]quinoxaline-2,3-dione). For mossy fiber LTP, 1 μM (2*S*,2′*R*,3′*R*)-2-(2′,3′-dicarboxycyclopropyl)glycine (DCG-IV) was applied at the end of every experiment, and the data were accepted only if synaptic responses were reduced by more than 90%. The synaptic response remaining in DCG-IV was then subtracted from all previous responses before further analysis to isolate mossy fiber-specific synaptic activity. All recordings were performed at 25.0°C ± 0.1°C using a 2-channel temperature controller (TC344B; Warner Instruments). Synaptic responses were acquired using a Multiclamp 700A amplifier (Axon Instruments). Data were digitized (3–5 kHz) and analyzed on line using custom software for IGOR PRO (Wavemetrics).

Behavioral Experiments. ***Locomotor activity and accelerating rotarod.*** To assess locomotor activity, mice were placed for 2 h in a fresh home cage with minimal bedding. Horizontal activity was monitored using photo beams linked to computer data acquisition software (San Diego Instruments) and averaged in 5-min bins. An accelerating rotarod designed for mice (IITC Life Sciences) was used essentially as described (7, 8) except that 9 trials were performed over 4 days (total of 36 trials). The rotarod was activated after placing mice on the motionless rod. The rod accelerated from 0 to 45 revolutions in 60 s, and the time to fall off the rod or to turn 1 full revolution was measured.

Tests of anxiety-like behaviors. The dark/light apparatus behavioral analysis was performed essentially as described (7, 8). Briefly, one side was kept dark (room light entry limited) while a light built into the top lit the other side (1700 lux, each chamber 25 cm × 26 cm). Mice were placed in the dark side and allowed to freely explore the light and dark sides for 10 min. Anxiety-like behavior was measured using latency to enter the light side. To measure locomotor habituation, mice were placed in the same apparatus 10 min/day for a total of 5 consecutive days, with locomotor activity measured each day. Student's *t* test was used to analyze anxiety-like behavior, whereas 2-way ANOVA was

used to analyze locomotor habituation data. In the elevated plus maze, mice were placed in the center of a black, Plexiglas elevated plus maze with white floors (each arm 33 cm long and 5 cm wide with 25-cm-high walls on closed arms) in a dimly lit room for 5 min, and video tracking software from Noldus (Ethovision 2.3.19) recorded time spent in the open and closed arms. Data were analyzed with Student's *t* test. The open field test was performed for 20 min in a brightly lit (≈800 lux), 48 × 48 × 48-cm white plastic arena using video tracking software from Noldus (Ethovision 2.3.19). Time spent in the center zone (15 × 15 cm) was recorded. Locomotor activity was also measured during the open field test.

Morris water maze. The Morris water maze and visible platform tests were performed as described (7, 8) except that the probe trial was performed on day 12. Briefly, a 4-foot-diameter, white, plastic, circular pool was filled to a depth of 13 in with 22°C ± 1°C water made opaque with gothic white, nontoxic, liquid tempera paint in a room with prominent extramaze cues. Mice were placed in 1 of 4 starting locations facing the pool wall and allowed to swim until they found a 10-inch-diameter, white platform submerged by 0.75 cm, or until a maximum of 60 s had elapsed. On finding the platform, mice remained on the platform for 15 s before being removed to the home cage. If mice did not find the platform within 60 s, they were guided by the experimenter to the platform, where they remained for 15 s before being removed to the home cage. Latency to reach the platform, distance traveled to reach the platform, thigmotaxis, and swim speed were measured using automated video tracking software from Noldus (Ethovision 2.3.19). Mice were trained with 4 trials per day with an intertrial interval of 1–1.5 min for 11 consecutive days between 8 a.m. and 1 p.m. A probe trial (free swim with the submerged platform removed) was performed as the first trial of the day on day 12. The percent time spent in the target quadrant was calculated using Ethovision 2.3.19. The time spent in the target quadrant was analyzed with a Student's *t* test, whereas latency to platform, distance to platform, thigmotaxis, and swim speed were analyzed with 2-way ANOVA. Two wild-type mice were objectively eliminated from analysis of the Morris water maze because they spent a significant amount of time floating across all days (outliers were identified using Grubb's test for outliers for swim speed).

Fear conditioning analysis. Fear conditioning was performed essentially as described (7). Briefly, mice were placed in the shock context for 2 min, then a 30-s, 90-dB tone coterminating in a 2-s, 0.5-mA foot shock was delivered twice with a 1-min interstimulus interval. Mice remained in the context for 2 min before returning to their home cage. Freezing behavior (motionless except respirations) was monitored at 10-s intervals by an observer blind to the genotype. To test for contextual learning 24 h after training, mice were placed into the same training context for 5 min and scored for freezing behavior every 10 s. To assess cue-dependent fear conditioning, mice were placed in a novel environment 3 h after the context test. Freezing behavior was assessed during a 3-min baseline, followed by a 3-min presentation of the tone. Cue-dependent fear conditioning was determined by subtracting baseline freezing from freezing during the tone. Student's *t* test was used to analyze the data.

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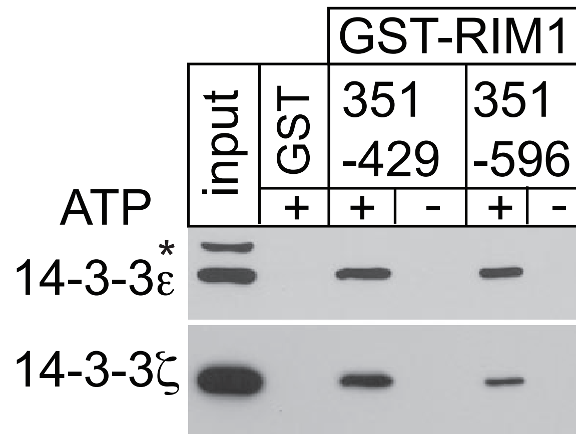

Fig. S1. GST-RIM1 proteins that contain a phosphorylated serine-413 bind to multiple 14-3-3 isoforms *in vitro*. GST fusion proteins of RIM1 were *in vitro* phosphorylated and used to pull down 14-3-3ε and 14-3-3ζ from fresh rat brain homogenates. ATP was omitted during the *in vitro* phosphorylation as a negative control. *, Unspecific band that sometimes appears with the 14-3-3ε antibody in brain homogenates.

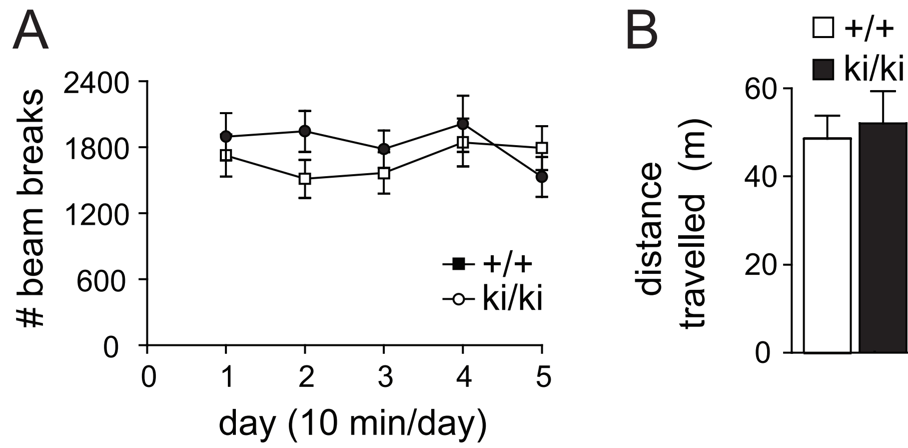

Fig. S2. RIM1 S413A-KI mice display normal locomotor response to novelty and normal locomotor activity. (*A*). RIM1 S413A mice displayed normal locomotor habituation as measured during a 10-min exposure to a novel chamber for 5 days. (*B*) RIM1 S413A-KI mice traveled the same distance as wild type during a 10-min test in the open field. Taken together, the KI mice have no abnormalities in locomotor activity.

Table S1. Numeric values for electrophysiologic recordings

Parameter	Mouse line	Genotype	Value, %	*n**	*P*	Statistical test	Figure
Parallel fiber LTP	S413A KI	+/+	131 ± 7	4/5	>0.5	2-way ANOVA, KI vs. wild-type	4*A*
		ki/ki	136 ± 10	4/6			
Mossy fiber LTP	S413A KI	+/+	176 ± 12	3/8	>0.9	2-way ANOVA, KI vs. wild-type	4*B*
		ki/ki	177 ± 12	3/6			
I-LTD	S413A KI	+/+	78 ± 6	4/4	>0.5	2-way ANOVA, KI vs. wild-type	4*C*
		ki/ki	80 ± 5	3/4			
Mossy fiber LTP	S413A KI/	1 ki/ki, 2α +/+	171 ± 6	2/5	>0.5	2-way ANOVA, KI 2α −/− vs. KI 2α +/+	4*D*
	2α KO	1 ki/ki, 2α −/−	171 ± 11	2/5			
Parallel fiber LTP	S413A KI/	1 ki/ki, 2α +/+	ND	ND	ND	ND	None
	2α KO	1 ki/ki, 2α −/−	138 ± 8	3/5			
PPF, 40 ms	S413A KI	+/+	1.8 ± 0.05	3/6	>0.5	2-way ANOVA, KI vs. wild-type	5*A*
		ki/ki	1.9 ± 0.03	3/5			
Train, 25th amplitude	S413A KI	+/+	1.2 ± 0.07	3/6	>0.3	2-way ANOVA, KI vs. wild-type	5*B*
		ki/ki	1.1 ± 0.06	3/5			

Values are given for all electrophysiologic experiments that were performed. PPF, paired pulse facilitation.
*Number of animals/slices per experiment.