Supporting Information

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SI Methods

Materials. MAPK inhibitors and kainic acid were from Tocris, His-MAPKs were from Calbiochem. Antibodies from Cell Signaling Technologies were rabbit anti-phosphorylated Erk1/2, rabbit anti-phosphorylated MAPK kinase (anti–phospho-MEK1/2), rabbit anti-MEK1/2, rabbit anti-phosphorylated JNK, rabbit anti-JNK, rabbit anti-phosphorylated tyrosine kinase receptor A/B (anti–phospho-TrkA/B), rabbit anti- phosphorylated Akt, mouse anti-Akt, rabbit anti-phosphorylated PKC, rabbit anti-phosphorylated PKA substrates, rabbit anti-PKC, rabbit anti-phosphorylated Src, rabbit anti-phosphorylated calmodulin-dependent protein kinase II (CaMKII). Antibodies from Millipore were mouse anti-CamKII, mouse anti-actin, mouse anti-phosphorylated tyrosine, mouse anti–synapsin-I, rabbit anti-NMDA receptor 1 (anti-NR1), -NR2A, and -NR2B, rabbit anti-phosphorylated glutamate receptor type 1 (anti–phospho-GluR1), rabbit anti-phosphorylated NR1, and mouse anti-PSD95. Mouse anti-phosphorylated neurofilament (anti-pNF) was from Covance, mouse anti-Erk and rabbit anti-tyrosine kinase receptor B (anti-TrkB) were from BD Transduction Labs, mouse anti-Arc antibody was from Santa Cruz Biotech, and rabbit anti-phosphorylated synapsin I was from Phosphosolutions, rabbit anti-phosphorylated TrkB was a kind gift from Moses Chao (New York University, New York). Rabbit anti-ZnT3 and guinea pig anti-vesicular glutamate transporter 1 (anti-VGLUT1) antibodies were from Synaptic Systems. Agatoxin-IVA was from Alomone Laboratories. Zinpyr-1 was from Toronto Research Chemicals. Ni-NTA agarose beads were from Qiagen. All other reagents were from Sigma.

Tissue Lysates, Immunoblotting, and Subcellular Fractionation. Whole-cell fractions from naïve or treated mice were obtained by Dounce-homogenizing hippocampi in a buffer made of (in mM): 10 Hepes, 5 EDTA, 1.5 $MgCl_2$, 150 NaCl, and 1% Nonidet P-40, 0.5% deoxycholate, 10% glycerol, with protease/phosphatase inhibitors. Lysates were centrifuged for 15 min at 14,000 × *g*, and supernatant was mixed with loading buffer before separation by SDS/PAGE using Criterion Tris-HCl gels (Bio-Rad). Proteins were transferred to Immobilon-P membranes (Millipore), blocked in 5% BSA in phosphate-buffered saline polysorbate 20 (PBST), and incubated with primary antibodies at 4 °C followed by HRP- or alkaline phosphatase-conjugated secondary antibodies. Immunoreactions were detected with ECL Plus or CDP-Star systems.

For subcellular fractions, fresh hippocampi from four or five mice were pooled for each experiment and Dounce-homogenized in 2 mL of buffer made of (in mM): 4 Hepes (pH 7.4) and 320 sucrose with protease/phosphatase inhibitors. Lysates were centrifuged for 10 min at 800 × *g* at 4 °C. Supernatant S1 was centrifuged for 10 min at 9,200 × *g*; the pellet was resuspended and centrifuged again for 15 min at 10,200 × *g*. Pellet P2 was resuspended in sucrose buffer and lysed by adding nine volumes of ice-cold water with three vigorous strokes. After adding Hepes to 7.5 mM final concentration, the lysate was rocked for 30 min and centrifuged for 20 min at 25,000 × *g*. LP1 was resuspended in Hepes buffer containing 1% Nonidet P-40, was layered onto a discontinuous gradient of 0.8/1.0/1.2 M sucrose, and was centrifuged for 2 h at 80,000 × *g*. The synaptosomal membrane fraction was obtained from the 1.0/1.2 M sucrose interface. Supernatants S2 and LS1 were centrifuged for 2 h at 165,000 × *g* to obtain S3, P3, and LP2. Final pellets were resuspended in 1% Nonidet P-40 and 0.3% SDS in 40 mM Tris, pH 8.

To obtain hippocampal synaptic membrane fractions from individual mice, tissue was homogenized in 500 μL of buffer made of (in mM): 10 Tris (pH 7.4), 2 EDTA, 2 EGTA, and 320 sucrose with protease/phosphatase inhibitors. Lysates were centrifuged 10 min at 800 × *g*, supernatant S1 was centrifuged for 15 min at 10,200 × *g*, and P2 was resuspended in 36 mM sucrose buffer. 0.5% Triton X-100 was added, and lysates were rocked for 30 min before centrifugation for 20 min at 25,000 × *g*.

MAPK Phosphorylation and Dephosphorylation Assays. The Erk2 kinase assay was performed with 30 ng of purified phosphorylated Erk2 (pErk2) protein per sample and using myelin basic protein as the substrate following the manufacturer's instructions (Millipore). Control zinc-free solutions consisted of 10 mM Tricine in the absence of added zinc. For 1 μM free zinc, 200 μM $ZnCl_2$ was added to 10 mM Tricine. For 10–100 μM free zinc, the concentration of added zinc was increased by 100 μM to a solution containing 100 μM CaEDTA. For dephosphorylation assays, hippocampi were lysed in 25 mM Hepes (pH 7.5), 150 mM NaCl, 0.5% Nonidet P-40, and protease inhibitors. Lysates were spun for 10 min at 14,000 × *g*, and the supernatant was mixed 1:5 in assay buffer made of 10 mM Hepes, 10 mM $MgCl_2$, and 10 μM U0126. Phosphorylated His_6-Erk2 was added at 40 ng per sample and incubated for up to 30 min at RT. Reactions were stopped by adding 8 M urea buffer containing 10 mM imidazole (pH 8.6), and His-Erk2 was precipitated by incubation with 20 μL of Ni-agarose beads per sample for 90 min at 4 °C. Samples were washed twice in urea buffer, pH 6.8, and once in 300 mM NaCl, 25 mM Tris, pH 7.5.

Acute Hippocampal Slices. Mice lacking zinc transporter ZnT3 (ZnT3KO mice) and control mice (3 mo old) were decapitated, and their brains were submerged quickly in ice-cold oxygenated sucrose-based solution containing (in mM) 280 sucrose, 26 $NaHCO_3$, 1.3 KCl, 1 $CaCl_2$, 10 $MgCl_2$, 10 D-glucose, and 2 kynurenate. Transverse hippocampal slices (400 μm) were cut with a vibratome in a 2% agarose block and recovered for 60 min in standard artificial cerebrospinal fluid (CSF) containing (in mM) 124 NaCl, 4 KCl, 1 $MgCl_2$, 2.5 $CaCl_2$, 1.25 NaH_2PO_4, 26 $NaHCO_3$, and 10 D-glucose. For chemical depolarization, slices were perfused with 5 mM 4-aminopyridine for 15 min in the presence of DL-2-amino-5-phosphonopentanoic acid (100 μM) to minimize dendritic pErk activation overlapping with MFs. Drug incubations were performed in 10-mL wells for 60 min in the presence of 10 μM U0126 to isolate the effect of phosphatases. Slices then were fixed in 4% formaldehyde, cryoprotected, and recut to 40-μm thickness in a cryostat before immunostaining.

Stereotaxic Injections. Coordinates from bregma were (in mm) −2 AP, −2 L, −2 V for CA3 and −1.9 AP, −1.5 L, −1.9 V for dentate gyrus. Infusion needles (30-G) were connected to a syringe pump (World Precision Instruments, Inc.) via polyethylene tubing. *N,N,N′,N′*-tetrakis(2-pyridylmethyl)ethylenediamine (TPEN) (2 mM) was dissolved in 10% DMSO in PBS. Agatoxin (50 nmol) was dissolved in PBS. Tetanus toxin (0.2 μg) was dissolved in 0.15% BSA in PBS. Solutions were infused at 0.1 μL/min for a total 0.5 μL per side. Lentivirus expressing the double-negative form of MEK (dnMEK) under the control of a CMV promoter or a control internal ribosome entry site (IRES)-GFP (1) was prepared by standard transfection of 293T cells, and viral particles were concentrated by centrifugation to 3 × 10^8 IU/mL in a solu-

tion containing 10 mM PBS, 1 mg/mL BSA, and 4 μg/mL protamine. Lentivirus-injected mice were trained 3–4 wk later.

Tissue Preparation, Immunohistochemistry, and Zinc Staining. Anesthetized mice were transcardially perfused with 4% formaldehyde in phosphate buffer 0.1M and processed for immunostaining as previously reported (2). Confocal images used for group comparisons were taken under identical settings using a Leica SP1 confocal microscope and were analyzed off-line with Metamorph software. Image J was used for quantification of grayscale intensities in sections stained for pErk and developed with 3,3′-diaminobenzidine. Background staining obtained in the absence of primary antibody was subtracted from grayscale values. Mean mossy fiber (MF) pErk intensity in control mice was used to calculate changes in pErk in treated mice. Synaptic vesicle zinc was detected by the Neo-Timm method (3) or by incubating tissue slices in 10 μM zinpyr-1 in PBS for 5 min.

Behavior. Mice were individually housed and handled daily for 1 wk before behavioral training. Mice were coded so all experiments and data collection were carried out blind to the genotype.

Object recognition. During each phase, mice freely explored the chamber under dim light conditions before being returned to their home cages. Proximal spatial cues were placed outside the chamber. Each phase was separated by a 10-min interval. Three objects (plastic blue cylinder, green cube, or red pyramid) were always present within 2 cm from the corners of a rectangular chamber. After 15 min of initial exploration, one object was moved to the opposite corner, the other two remained fixed, and mice explored the new arrangement for 5 min. In the third phase, the displaced object was replaced by a novel object, and object preference was measured over 5 min again. Displaced/replaced objects were counterbalanced among mice to rule out preference for an object regardless of novelty.

Passive avoidance. Mice were habituated to the lit side of the chamber for 1 min and then allowed to cross over the dark side, in which they remained for 10 min before delivery of a 0.6-mA, 2-s foot shock. Mice were returned to their home cages 20 s after the shock. This procedure was repeated for six consecutive days or until cross-over latency was greater than 5 min. Cutoff time was 10 min. Latencies were recorded again for each mouse 1 wk after the last shock.

Contextual fear conditioning and extinction. Mice were placed in a square chamber with a metal grid floor (Coulbourn Instruments) and allowed to explore it for 2 min before delivery of a single 0.7-mA, 2-s foot shock. After 1 min, mice were returned to their home cages. For unpaired controls, mice were shocked immediately and removed 1 min later. For gradual fear conditioning, preexposure time was reduced to 40 s, shock intensity to 0.4 mA, and training was repeated for 5 d. Freezing scores were obtained by sampling once every 10 s for the entire 3 min. Freezing was defined as complete absence of movement except for respiration for at least 2 s.

Contextual discrimination. Mice were preexposed to two chambers (A and B) for 10 min each the day before conditioning. Mice received 3 d of conditioning in chamber A to learn to discriminate between chambers A and B. The order of daily exposure to chambers was counterbalanced, and at least 4 h were allowed between each exposure. The chambers were located in the same room and had identical grid floors. However, they were different in shape (square versus triangle), scent (70% ethanol versus 1% acetic acid), and background noise. Conditioning took place 150 s after the mouse was placed in chamber A with a 0.75-mA, 2-s foot shock. Mice were returned to their home cages 28 s later. Mice remained in chamber B for 3 min without a shock. Freezing scores were calculated for the first 150 s during each exposure. For chamber C, a flat white plastic surface replaced the grid floor in chamber A.

Elevated T-maze. Mice were kept at 85% of their free-feeding body weight and given water ad libitum. The T-maze was elevated 1.5 m from the floor in a well-lit room and was surrounded by fixed external cues. Mice were habituated to the maze and sugar pellets (20 mg) during three 10-min sessions before training. Each daily session consisted of five trials, and each trial consisted of two runs separated by 20 s. On the first, sample run, mice were forced to go left or right (pseudorandom sequence, with no more than two runs in the same direction), and a food pellet was available at the end of the goal arm. On the second run, both arms were open, but only the previously unvisited arm contained a food pellet (rewarded alternation, or nonmatching-to-place test).

Open field activity. Unsupervised spontaneous activity in Plexiglas boxes was recorded automatically by Truscan software under dim light conditions.

Statistical Analysis. Data sets were subjected to parametric analysis (two-way t test or ANOVA followed by Bonferroni pairwise comparisons). Unless otherwise stated, n refers to the number of mice, and measures are expressed as mean ± SD. Significance was set at $P = 0.05$.

1. Mansour SJ, et al. (1994) Transformation of mammalian cells by constitutively active MAP kinase kinase. *Science* 265:966–970.
2. Sindreu CB, Scheiner ZS, Storm DR (2007) Ca2+ -stimulated adenylyl cyclases regulate ERK-dependent activation of MSK1 during fear conditioning. *Neuron* 53:79–89.
3. Danscher G (1981) Histochemical demonstration of heavy metals. A revised version of the sulphide silver method suitable for both light and electronmicroscopy. *Histochemistry* 71:1–16.

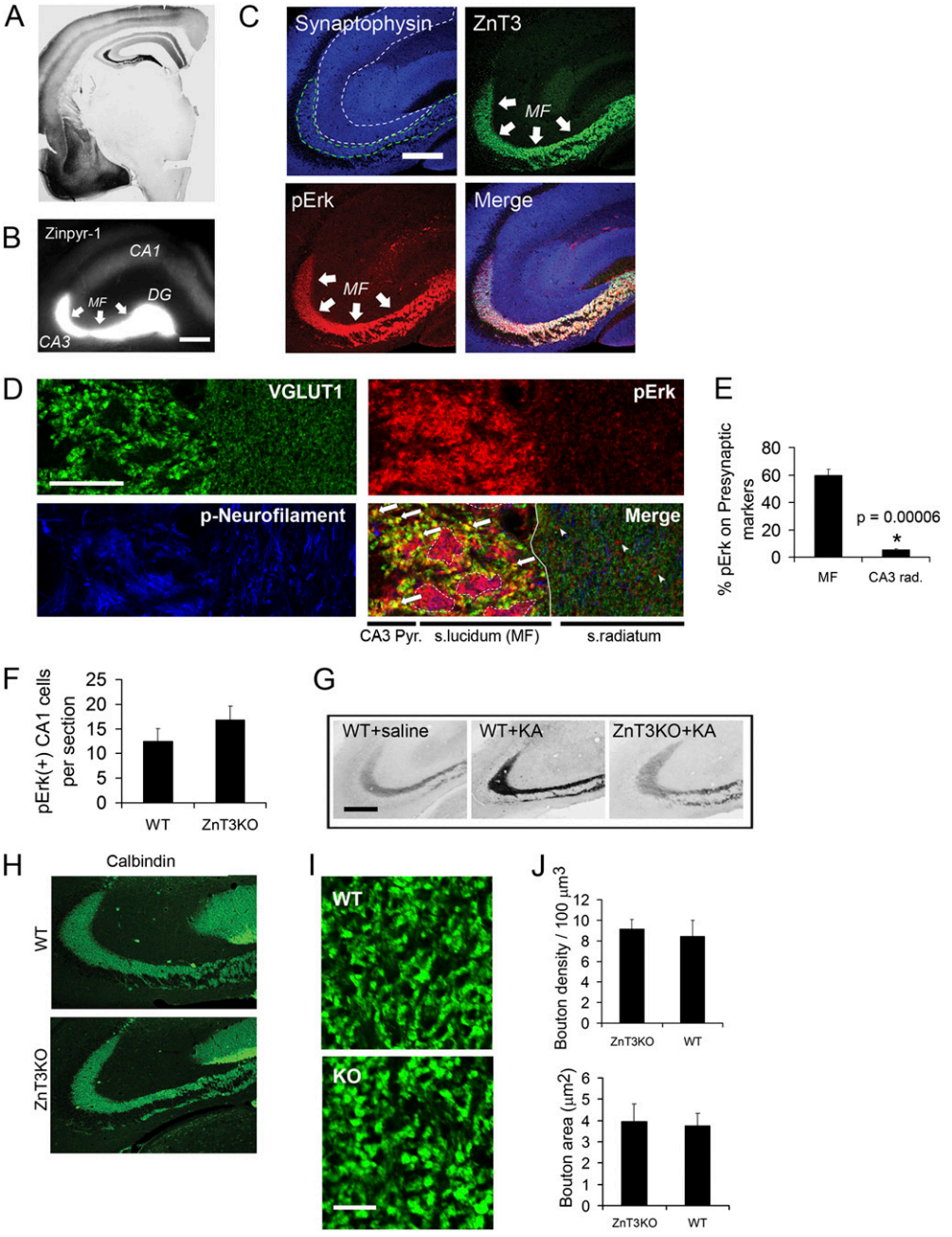

Fig. S1. pErk is selectively targeted to presynaptic sites in MF. (*A*) Timm's staining of a coronal mouse brain section revealing vesicular zinc. (*B*) Zinpyr-1 fluorescence in hippocampus. (*C*) Immunofluorescence staining for synaptophysin, ZnT3, and pErk. Boundaries of the stratum lucidum (i.e., MF terminal region) are indicated by the dashed green line. The stratum radiatum is indicated with a dashed white line. (Scale bar: 100 μm.) Ratio of ZnT3 to synaptophysin signal in MFs, 1.4 ± 0.3; ratio in CA3 radiatum, 0.6 ± 0.1; $P < 0.001$; n = 8. Ratio of pErk to synaptophysin in MFs, 2.2 ± 0.5; ratio in CA3 radiatum, 1 ± 0.4; $P < 0.0001$; n = 8. (*D*) Single optical sections from hippocampus at the level of CA3a/b, stained for VGLUT1, pErk, and pNF. Arrows in merged image indicate colocalized pErk and VGLUT1. Discontinuous outlines show regions of significant pErk and pNF colocalization. Arrowheads indicate segregated pErk and VGLUT1 or pNF. The solid line marks the limit between MF terminals and stratum radiatum. CA3 Pyr, pyramidal cell layer in CA3. (Scale bar: 20 μm.) (*E*) Quantification of pErk colocalization with presynaptic markers. (*F*) Number of pErk-immunopositive cells in the pyramidal layer of region CA1. WT vs. KO, P = 0.27; n = 17–18 per group. (*G*) pErk staining in MF of mice injected with kainate (KA) 90 min earlier. (Scale bar: 100 μm.) (*H*) Calbindin staining in MF of WT and ZnT3KO mice. (*I*) VGLUT1 staining of presynaptic terminals in the stratum lucidum. (Scale bar: 10 μm.) (*J*) Summary data of density and size of MF boutons. WT vs. KO density, P = 0.29; WT vs. KO area, P = 0.53; n = 493–455 boutons per genotype.

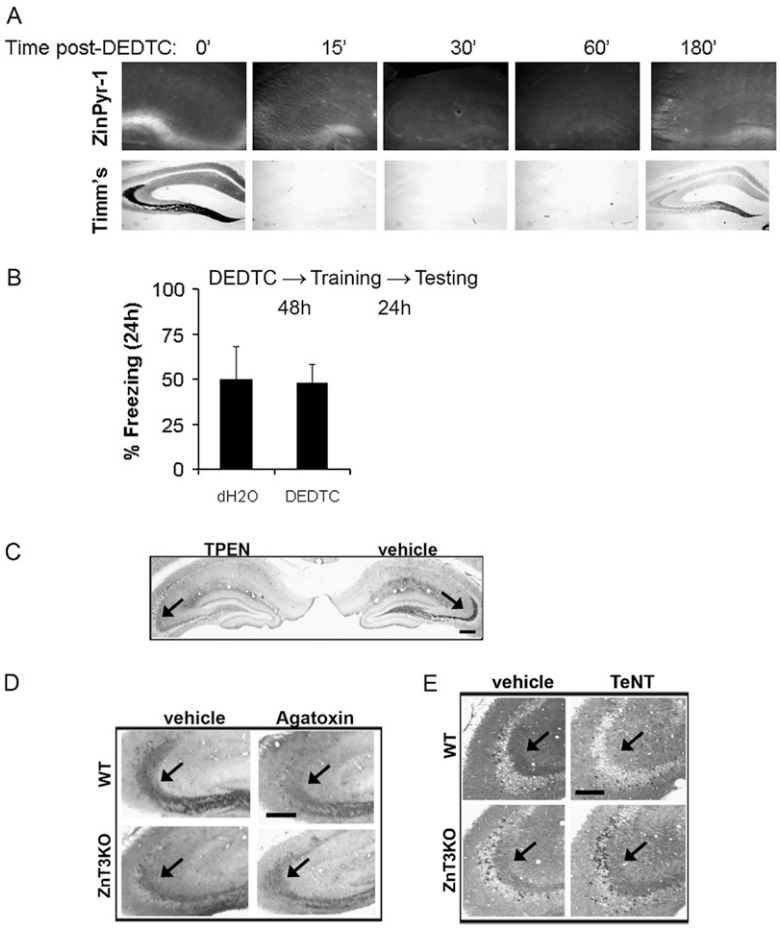

Fig. S2. Effect of zinc chelators and exocytosis blockade. (*A*) Changes in levels of synaptic zinc at 0, 15, 30, 60, and 180 min after diethyldithiocarbamate (DEDTC) injection, as visualized with Timm's staining or zinpyr-1 fluorescence. (*B*) Mice were trained for contextual fear conditioning 2 d after DEDTC or vehicle (dH_2O) injection and were tested for memory 24 h later. There was no difference between groups ($P > 0.05$; $n = 5/5$). (*C*) Example of hippocampus from a mouse infused with TPEN (2 mM) or vehicle (10% DMSO in PBS) on opposite sides of the hippocampus and killed 20 min later. (Scale bar: 250 μm.) (*D*) Examples of MF pErk staining in mice that received hippocampal infusions of agatoxin IVA or vehicle (PBS) and were killed 30 min later. Effect of treatment, $F_{(1,8)} = 10.34$, $P = 0.018$. (Scale bar: 100 μm.) (*E*) Examples of MF pErk staining in mice that received hippocampal infusions of tetanus toxin or vehicle (0.15% BSA) and were killed 14 h later. Interaction between treatment and genotype, $F_{(1,10)} = 7.03$, $P = 0.024$. (Scale bar: 100 μm.)

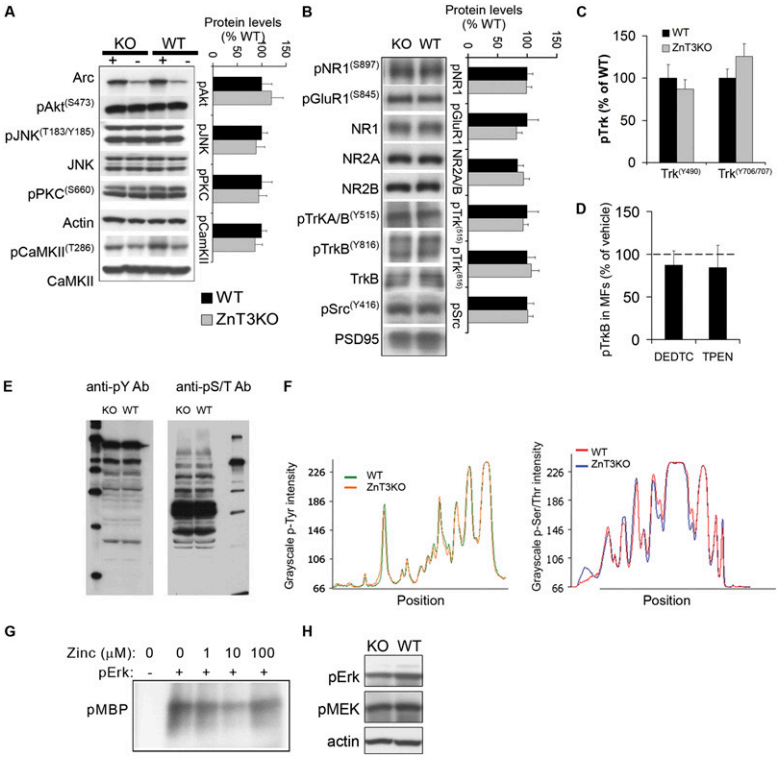

Fig. S3. Protein phosphorylation in ZnT3KO mice. (*A*) Immunoblots of pooled hippocampal lysates were probed for various signaling proteins. Lysates were obtained from mice 90 min after injection with 10 mg/kg kainate (+) or saline (–). Bar graphs illustrate normalized data from mice analyzed separately and expressed relative to WT in basal conditions ($P > 0.05$ for all comparisons; $n = 10$ per group). (*B*) Immunoblots of synaptic membrane fractions (Triton-insoluble) from pooled hippocampi. Bar graphs were obtained from analysis of individual mice and normalized to WT ($P > 0.05$ for all comparisons; $n = 6$ per group). (*C*) Levels of phosphorylated TrkB (pTrkB) at Y490 and pTrkA/B at Y706/707, relative to TrkB, in soluble fractions of hippocampus. $n = 5$ per group; $P > 0.05$. (*D*) Summary data of pTrkB levels in MFs following injection of zinc chelators in WT mice, normalized to vehicle controls ($n = 9$ per group; $P > 0.05$). (*E*) Pooled hippocampal samples from ZnT3KO or WT mice probed with phosphotyrosine (p-*Y*) or phosphoserine/threonine (p-S/*T*) antibodies. (*F*) Quantification of the grayscale intensity of protein bands obtained from line scans of immunoreacted membranes. (*G*) His-pErk2 and myelin basic protein (MBP) were coincubated for 30 min in increasing zinc concentrations, and phosphorylation of MBP was resolved by Western blotting. Phospho-MBP (p-MBP) was not affected by the addition of zinc; $P > 0.05$. (*H*) Immunoblot of hippocampal lysates showing normal phosphorylated MAP kinase (pMEK) levels in ZnT3KO mice. Blots were stripped and reprobed sequentially for pErk and actin. pErk/pMEK ratio in ZnT3KO, 73 ± 8% of WT; $n = 13$–14 per group; $P = 0.0053$.

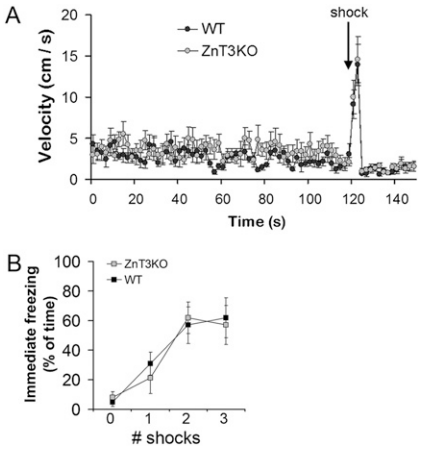

Fig. S4. Normal immediate responses to foot shock in ZnT3KO mice. (*A*) Velocity of movements before, during, and after foot shock delivery ($n = 8$ per group). (*B*) Freezing Immediately after shock was scored during 1-min intervals following delivery of three consecutive shocks. $P = 0.67$; $n = 7$ mice per group.

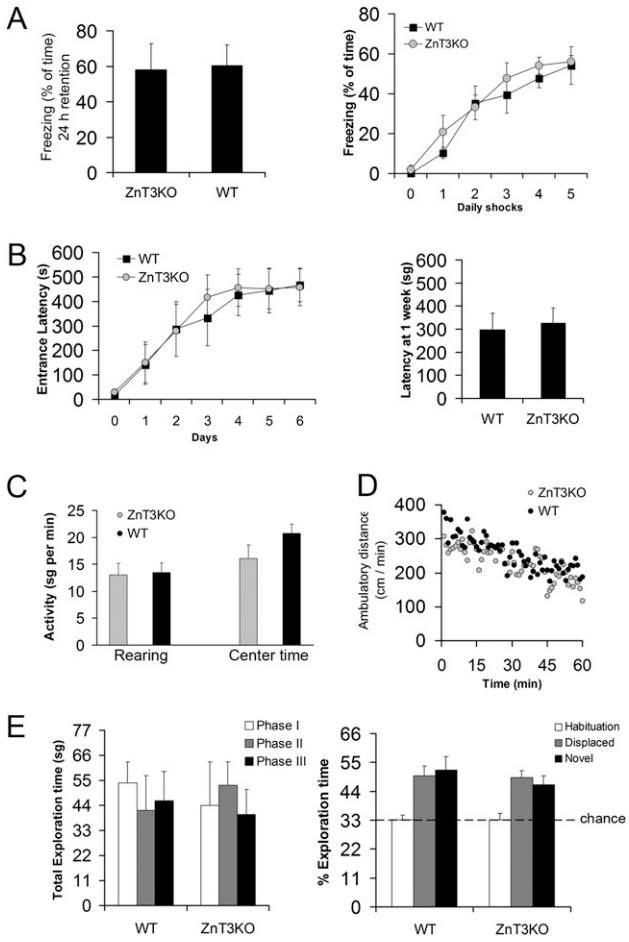

Fig. S5. Normal behaviors in ZnT3KO mice. (*A*) (*Left*) Conditioned freezing 24 h after a single foot shock (0.7 mA, 2 s) delivered after the mouse explored the novel chamber for 2 min ($P = 0.9$; $n = 12–14$ per group). (*Right*) Gradual acquisition of contextual fear conditioning. Mice received a milder foot shock (0.4 mA, 2 s) after spending only 40 s in the novel chamber and then were removed immediately. The procedure was repeated for 5 d. $P = 0.86$; $n = 8$ per group. (*B*) (*Left*) Gradual passive avoidance. Mice were allowed to cross over to the dark side of the chamber, and a foot shock was delivered 10 min later. The procedure was repeated for 6 d. $n = 14–15$ per group. (*Right*) Cross-over latency was retested 1 wk after the last shock. $P = 0.75$. (*C*) Measures of spontaneous rearing activity and center time in an open field ($n = 8$ per group). (*D*) Mean ambulatory distances recorded over 60 min in dim light conditions. (*E*) (*Left*) Total time spent exploring objects during the initial phase (phase I, habituation), after relocation in the open field of one of three objects (phase II, displaced), and finally after replacement of one of three objects with a novel object (phase III, novel). (*Right*) Percentage time mice spent exploring the displaced or novel objects relative to the nonaltered objects. Novelty preference, WT vs. KO, $P = 0.39$; $n = 8$ per group.

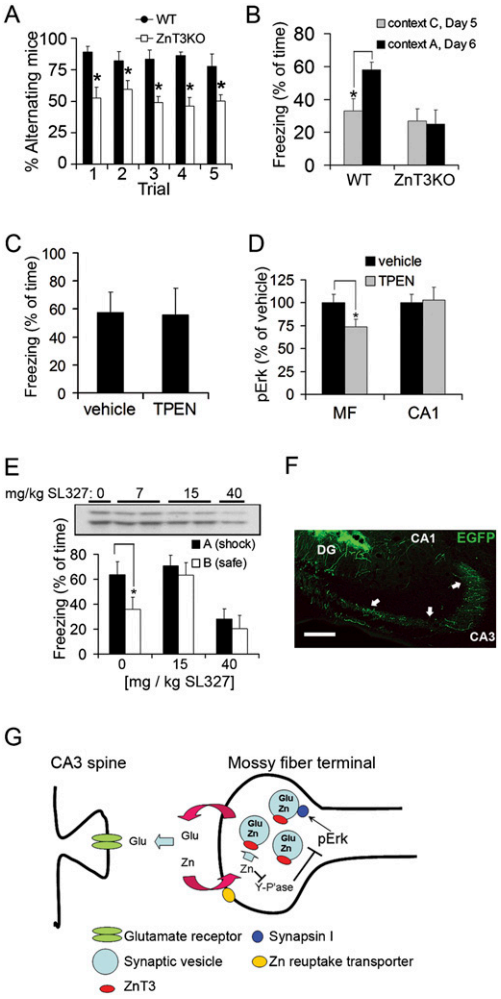

Fig. S6. Cognitive deficits in ZnT3KO and drug-injected mice. (*A*) Fraction of mice that alternated correctly for each of the five trials performed each day between days 3 and 6. Effect of genotype, $F_{(1,30)} = 14.12$; $P = 0.0007$. (*B*) WT mice ($P = 0.037$) but not ZnT3KO mice ($P = 0.8$) continued to show increased freezing in the shock chamber after exposure to a (third) novel chamber. (*C*) TPEN infusion in the CA3 spared contextual fear conditioning in WT mice ($P = 0.88$; $n = 9$–10 per group). (*D*) TPEN infusion in the CA3 decreased pErk in MFs ($P = 0.007$; $n = 6$ per group) but not in CA1 ($P = 0.51$). (*E*) The MEK1/2 inhibitor SL327 blocked contextual discrimination at low doses and conditioned freezing at higher doses. Effect of dose, $F_{(1,15)} = 7.38$; $P = 0.0348$. (*F*) Lentivirus injection in the dentate gyrus resulted in EGFP expression in MFs (arrows). (Scale bar: 100 μm.) (*G*) Schematic model. MF activity releases both glutamate and zinc from synaptic vesicles. Glutamate acts on postsynaptic receptors on CA3 spines and is cleared by glial cells, whereas zinc is recycled by action of transporters of the Slc39 family. Cytoplasmic zinc inhibits a tyrosine phosphatase (Y-P'ase), thereby preventing inactivation of pErk and enhancing Erk-dependent activities, such as phosphorylation of synapsin I. Absence of ZnT3 (Slc30a3) prevents zinc recycling, leading to disinhibition of Y-P'ase and reduced pErk.