Supporting Information

Radwanska et al. 10.1073/pnas.1109680108

SI Materials and Methods

Drugs. Anisomycin (Sigma-Aldrich) and actinomycin D (Sigma-Aldrich) were dissolved in PBS. Rapamycin was dissolved in PBS and DMSO (1:5).

Cannulation of the Dorsal Hippocampus. Mice were anesthetized by inhalation of 3–5% isoflurane (IsoFlo; Abbott Animal Health) in oxygen and positioned in a stereotaxic frame (Kopf Instruments). Two holes were drilled in the skull, and a double guide cannulae (2 mm apart and 2 mm long; Plastics One) was lowered into the holes such that the cannula tip was 1.3 mm posterior to bregma, ±1 mm lateral, and −2 mm vertical, thus placing them 0.5 mm above the target area according to the mouse brain atlas (1). Cannulae were kept patent by using 33-gauge internal dummy cannulae (Plastics One). The animals were used in contextual fear conditioning not earlier than 5 d and not later than 10 d after the cannulation.

Foreground Fear Conditioning. The animals were trained in a conditioning chamber (Med Associates) in a sound-attenuating box with background noise supplied to the chamber by a white noise generator. The chamber floor had a stainless steel grid for shock delivery. Mice were placed in the chamber, and after a 148-s introductory period, a foot shock (2 s, 0.7 mA) was presented. The shock was repeated five times, with an intertrial interval of 90 s. Thirty seconds after the last shock, the mouse was returned to its home cage. Contextual fear memory was tested 24 h after training by reexposing the mouse to the conditioning chamber for 5 min. A video camera was fixed inside the door of the sound-attenuating box, which allowed the behavior to be observed and scored. Freezing behavior (defined as complete lack of movement, except for respiration) was scored for 2 s in every 5 s. All of the procedures were done blind to genotype and experimental treatment.

Fear Memory Extinction and Reconsolidation. Contextual fear memory was tested and extinguished 24 h after training by reexposing the mouse to the conditioning chamber for 5 min, followed by two 15-min testing sessions during two consecutive days. Contextual fear memory was reactivated 24 h after training by reexposing the mouse to the conditioning chamber for 5 min. Next, the animals received bilateral drug injections (0.5 μL per side for 1 min) immediately after memory reactivation. LTM after reconsolidation was reassessed 1 or 6 d later in the conditioning chamber during 5 min (Fig. 5).

Slice Electrophysiology. Mice were anesthetized and decapitated. The hippocampus was dissected and cut in 450-μm-thick slices with a tissue chopper. The slices were transferred into the recording chamber and kept in interface at 28 °C for 1.5 h. Hippocampal slices were perfused with artificial cerebrospinal fluid (ACSF) with the following composition: 124 mM NaCl, 5 mM KCl, 26 mM $NaHCO_3$, 1.24 mM NaH_2PO_4, 2.5 mM $CaCl_2$, 1.3 mM $MgSO_4$, 10 mM glucose, and bubbled with a mixture of 95% O_2 and 5% CO_2. The perfusion rate of ACSF was 1 mL/min. Two bipolar twisted nickel-chrome electrodes (50 μm each) were used to stimulate two different sets of Schaffer's collaterals. One pathway was used for baseline recording and the other one for LTP. Extracellular field excitatory postsynaptic potentials (fEPSP) were recorded in the stratum radiatum of the CA1 region with low resistance (2–5 MΩ) glass microelectrodes filled with ACSF. Test stimuli were biphasic (0.08 ms for each pulse) constant-voltage pulses delivered every minute with an intensity adjusted to evoke an ≈40% maximal response. The slope of the fEPSP was measured on the average of four consecutive responses. LTP was induced by applying two series (45-min interval) of four trains (50-Hz, 1-s, 5-min interval). For each slice, the fEPSP slopes were normalized with respect to the mean slope of the fEPSPs recorded during the 30-min period the preceding induction of LTP.

Immunohistochemistry. After perfusion and fixation, coronal brain sections (40 μm thick) were prepared (Microm HM560) and stored at –20 °C in PBSAF [PBS, 20% sucrose (Sigma-Aldrich), 15% ethylene glycol (Sigma-Aldrich), and 0.05% NaN_3 (Sigma-Aldrich)]. The sections were washed with PBS before being placed in hydrogen peroxide (3% H_2O_2/PBS) for 6 min. The sections were washed in PBS/0.3% Triton X-100 (Sigma-Aldrich) followed by 2-h incubation in a blocking solution (3% normal goat serum in PBS/0.3% Triton X-100) and incubation with the selective antibodies directed against c-Fos (sc-52; Santa Cruz Biotechnology), Nur77 (sc-5569; Santa Cruz Biotechnology), Zif268 (sc-110; Santa Cruz Biotechnology), and PSD95 (ab18258; Abcam). Next, the sections were washed in PBS with 0.3% Triton X-100 and incubated with the secondary antibody (biotinylated; Vector Laboratories). After incubation with the secondary antibody, the sections were washed again in PBS/0.3% Triton X-100 and incubated with avidin-biotin horseradish peroxidase complex (Vector Laboratories). The reaction was developed with 1 mg/mL diaminobenzidine/0.005% H_2O_2 in PBS or incubated with streptavidin-bound Alexa Fluor 647 (Invitrogen) for 1 h (PSD95 staining). The sections were mounted on poly-lysine–coated slides (Sigma-Aldrich), air-dried, dehydrated in ethanol solutions and xylene, and embedded in Entellan (Merck). Sections stained with Alexa Fluor 647 were covered with hard-set medium for fluorescence (Vector Laboratories).

Analysis of immunostaining is described in detail in *Materials and Methods*.

Phalloidin Staining. Brain sections were prepared as for immunostaining and washed three times in PBS before being placed for 1 h in buffer containing phalloidin bound with TRITC (1:500; Sigma-Aldrich) in PBS with 0.3% Triton X-100. After washing with PBS, the sections were mounted on poly-lysine–coated slides (Sigma-Aldrich) and covered with hard-set medium for fluorescence (Vector Laboratories).

Western Blots. The hippocampal tissue was homogenized in lysis buffer [0.2 M NaCl, 0.1 M Hepes, 10% glycerol, 1% Triton X-100, 10 mM sodium pyrophosphate, 2 mM EDTA, 2 mM EGTA, 0.2 mM phenylarsine oxide, 0.1 mM molybdate, 10 mM NaF, 27 mM sodium pervanadate, and Complete EDTA-free Protease Inhibitor mixture tablet (Roche Diagnostics)], incubated on ice for 10 min, and then centrifuged (10,000 × g, 15 min) at 4 °C. Equal protein amounts were run in precast gels (Bio-Rad; 4–15% Tris·HCl) and transferred to PVDF membranes. The blots were incubated in blocking buffer [TBS (10 mM Tris at pH 7.6 and 150 mM NaCl) with 3% BSA and 0.05% Tween 20] and overnight at 4 °C with anti-PSD95 primary antibody in blocking buffer. After washing in TBS/0.05% Tween 20 (TBST), the blots were incubated with a horseradish peroxidase-conjugated secondary antibody in blocking buffer and washed again in TBST, and signals were visualized with ECL system (Pierce Super Signal). Band intensities from X-ray film (Amersham

Biosciences) were quantified with ImageJ software. The blots were stripped with stripping buffer (Perbio) and reprobed with anti-NSE antibodies (Chemicon) to normalize for the amount of loaded protein.

1. Paxinos G, Franklin KJ (2001) *The Mouse Brain in Stereotaxic Coordinates* (Academic Press, San Diego, CA).

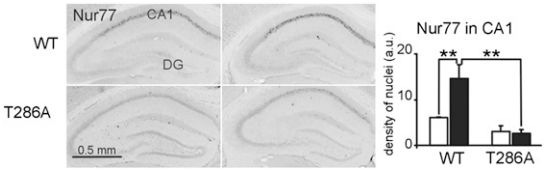

Fig. S1. Impaired Nur77 protein expression in the dorsal hippocampus in T286A mutants despite contextual LTM formation. WT mice and T286A mutants were trained with massed foreground conditioning. Nur77 immunostaining (density of stained nuclei) was analyzed in the area CA1 of the dorsal hippocampus 75 min after training. $n = 6$; $^{**}P < 0.01$.

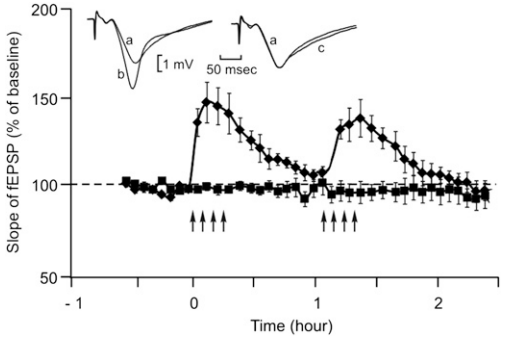

Fig. S2. Strong stimulation cannot induce late CA1-LTP in T286A mutants. LTP was induced by two series of four trains (arrows, 50 Hz, 5 min apart). Evolution of fEPSP slope (mean ± SEM) in a tetanized pathway (diamonds) and in a control pathway (squares) is shown. (*Inset*) Representative fEPSP traces before (*a*), just after (*b*), and 1 h after the last train (*c*). $n = 4$.

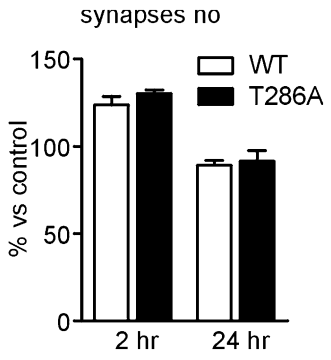

Fig. S3. Training-induced increase in total synapse number in T286A mutants and WT mice is short-lasting. Synapse density was analyzed in stratum radiatum of hippocampal area CA1 in wild-type and T286A mutant mice, 2 and 24 h after training by using 3D or 2D electron microscopy, respectively. The data are presented as percentage of changes vs. naïve animals.

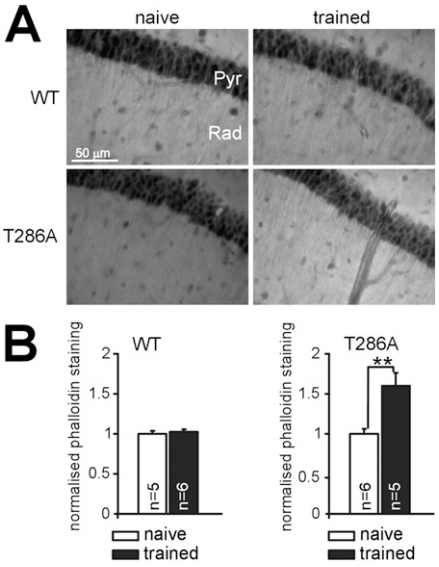

Fig. S4. Training induced up-regulation of F-actin level in T286A mutants but not in WT mice. WT mice and T286A mutants were trained with massed foreground conditioning. (*A*) The amount of F-actin was analyzed in the stratum radiatum of CA1 area of the dorsal hippocampus by detection with TRITC-bound phalloidin 2 h after conditioning. Pyr, pyramidal layer of CA1 hippocampal area; Rad, stratum radiatum of CA1. (*B*) Quantification revealed a significant up-regulation in trained T286A mutants, but not in WT mice (two-way ANOVA: genotype x training interaction: $F_{(1,21)} = 9.21$, $P < 0.01$; post hoc analysis: T286A mutants trained vs. naïve: $P < 0.01$, WT mice trained vs. naïve: $P > 0.05$).

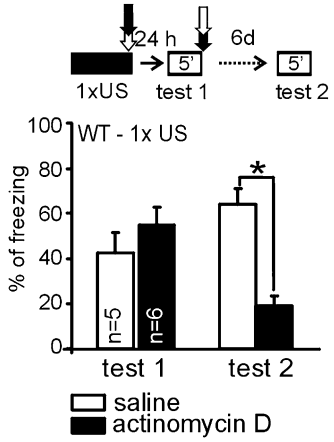

Fig. S5. Hippocampal transcription is necessary for memory reconsolidation but not consolidation. WT mice were trained with foreground conditioning. Actinomycin D (10 ng/side) was bilaterally injected into dorsal hippocampus immediately after training and 24 h after conditioning the animals were tested for contextual LTM (test 1). Next, the animals were once again bilaterally injected with actinomycin D (10 ng per side) or saline (the animals previously injected with saline now received actinomycin D, and the actinomycin D group received saline injection) immediately after memory reactivation (test 1). LTM after reactivation was tested 6 d later (test 2).

Table S1. Contextual fear conditioning up-related Zif268 expression in hippocampus and cortex in WT mice, but not in αCaMKII autophosphorylation-deficient mutants

	WT		T286A	
Brain region	Naive	Trained	Naive	Trained
Hippocampus				
CA1	7.29 ± 1.49	**17.84 ± 4.17***	7.92 ± 3.34	6.52 ± 1.48†
DG	0.01 ± 0.01	0.12 ± 0.05	0.03 ± 0.03	0.03 ± 0.03
Amygdala				
LA	1.41 ± 0.40	1.63 ± 0.31	0.64 ± 0.41	0.27 ± 0.06†
BL	0.19 ± 0.06	0.17 ± 0.05	0.04 ± 0.03	0.08 ± 0.04
Ce	0.08 ± 0.04	0.18 ± 0.07	0.20 ± 0.18	0.11 ± 0.06
Striatum				
CaPu	1.33 ± 0.85	3.74 ± 1.50	1.47 ± 0.52	1.38 ± 0.35
AcbSh	1.29 ± 0.46	2.32 ± 0.60	1.06 ± 0.64	1.01 ± 0.33
AcbC	1.21 ± 0.41	2.04 ± 0.54	0.72 ± 0.46	0.63 ± 0.15
Cortex				
Cg1	3.03 ± 1.06	5.00 ± 1.52	1.92 ± 0.53	1.33 ± 0.57
PrL	2.06 ± 0.94	4.17 ± 0.74	1.59 ± 0.46	1.20 ± 0.44†
IL	0.82 ± 0.34	**2.67 ± 0.47***	0.96 ± 0.27	0.80 ± 0.34†

Quantification of Zif268 immunostaining in the hippocampus, amygdala, striatum, and neocortex in WT mice and T286A mutants before and after five foreground contextual fear conditioning trials. Data represent mean ± SEM, $n = 6$ per group. For the ease of presentation only, results from the post hoc analyses are shown, which revealed significant training-induced up-regulation in WT mice (*$P < 0.05$) and significant difference between expression in trained WT mice versus trained T286A mutants (†$P < 0.05$). The training-induced up-regulations are shown in bold; there were no such up-regulations in T286A mutants. AcbC, accumbens nucleus, core; AcbSh, accumbens nucleus, shell; BL, basolateral amygdaloid nucleus; CA1, field CA1 of hippocampus; Ce, central amygdaloid nucleus; CPu, caudate putamen; Cg1, cingulate cortex, area 1; DG, dentate gyrus; IL, infralimbic cortex; LA, lateral amygdaloid nucleus; PrL, prelimbic cortex.

Table S2. Contextual fear conditioning up-related c-Fos expression in hippocampus, amygdala, and striatum in WT mice, but not in αCaMKII autophosphorylation-deficient mutants

	WT		T286A	
Brain region	Naive	Trained	Naive	Trained
Hippocampus				
CA1	0.64 ± 0.15	**4.70 ± 0.94***	0.45 ± 0.17	0.66 ± 0.43†
DG	0.93 ± 0.23	**1.84 ± 0.29***	0.10 ± 0.04	0.19 ± 0.12†
Amygdala				
LA	0.54 ± 0.09	**2.02 ± 0.40***	0.53 ± 0.18	0.46 ± 0.15†
BL	1.03 ± 0.29	**2.86 ± 0.48***	0.65 ± 0.19	0.64 ± 0.18†
Ce	0.29 ± 0.08	0.86 ± 0.21	0.28 ± 0.18	0.36 ± 0.12
Striatum				
CaPu	0.12 ± 0.04	**0.59 ± 0.10***	0.16 ± 0.06	0.27 ± 0.13
AcbSh	0.78 ± 0.15	**2.23 ± 0.38***	0.54 ± 0.17	0.99 ± 0.30†
AcbC	0.31 ± 0.08	**0.82 ± 0.16***	0.25 ± 0.09	0.50 ± 0.11
Cortex				
Cg1	4.14 ± 1.29	6.28 ± 0.91	2.19 ± 0.91	3.53 ± 1.12
PrL	4.60 ± 1.01	6.36 ± 1.71	1.72 ± 071	4.53 ± 1.87
IL	2.39 ± 0.94	5.44 ± 1.66	1.49 ± 0.66	2.21 ± 0.81

Quantification of c-Fos immunostaining in the hippocampus, amygdala, striatum, and neocortex in WT mice and T286A mutants before and after five foreground contextual fear conditioning trials. Data represent mean ± SEM, $n = 6$ per group. For the ease of presentation only results from the post hoc analyses are shown, which revealed significant training-induced up-regulation in WT mice (*$P < 0.05$) and significant difference between expression in trained WT mice versus trained T286A mutants (†$P < 0.05$). The training-induced up-regulations are shown in bold; there were no such up-regulations in T286A mutants. AcbC, accumbens nucleus, core; AcbSh, accumbens nucleus, shell; BL, basolateral amygdaloid nucleus; CA1, field CA1 of hippocampus; Ce, central amygdaloid nucleus; CPu, caudate putamen; Cg1, cingulate cortex, area 1; DG, dentate gyrus; IL, infralimbic cortex; LA, lateral amygdaloid nucleus; PrL, prelimbic cortex.

Table S3. Contextual fear conditioning up-related Nur77 expression in hippocampus, and amygdala in WT mice, but not in αCaMKII autophosphorylation-deficient mutants

	WT		T286A	
Brain region	Naive	Trained	Naive	Trained
Hippocampus				
CA1	6.03 ± 1.42	**14.57 ± 3.11***	3.01 ± 1.29	2.64 ± 0.93†
DG	0.07 ± 0.03	**0.32 ± 0.14**	0.12 ± 0.05	0.04 ± 0.02
Amygdala				
LA	0.73 ± 0.19	**1.38 ± 0.39***	0.24 ± 0.12	0.17 ± 0.09†
BL	0.60 ± 0.18	0.69 ± 0.17	0.08 ± 0.04	0.10 ± 0.05
Ce	1.07 ± 0.62	0.23 ± 0.05	0.07 ± 0.05	0.06 ± 0.05
Striatum				
CaPu	0.25 ± 0.08	1.28 ± 0.83	0.25 ± 0.05	0.64 ± 0.19
AcbSh	1.36 ± 0.19	1.85 ± 0.43	0.43 ± 0.20	0.81 ± 0.45†
AcbC	1.03 ± 0.15	1.53 ± 0.27	0.45 ± 0.11	0.71 ± 0.66
Cortex				
Cg1	0.35 ± 0.16	1.92 ± 0.83	0.21 ± 0.08	0.37 ± 0.21
PrL	1.35 ± 0.14	1.86 ± 0.34	0.43 ± 0.18	0.81 ± 0.31
IL	1.03 ± 0.15	1.52 ± 0.39	0.46 ± 0.17	0.77 ± 0.31

Quantification of Nur77 immunostaining in the hippocampus, amygdala, striatum, and neocortex in WT mice and T286A mutants before and after five foreground contextual fear conditioning trials. Data represent mean ± SEM, $n = 6$ per group. For the ease of presentation only results from the post hoc analyses are shown, which revealed significant training-induced up-regulation in WT mice (*$P < 0.05$) and significant difference between expression in trained WT mice versus trained T286A mutants (†$P < 0.05$). The training-induced up-regulations are shown in bold; there were no such up-regulations in T286A mutants. AcbC, accumbens nucleus, core; AcbSh, accumbens nucleus, shell; BL, basolateral amygdaloid nucleus; CA1, field CA1 of hippocampus; Ce, central amygdaloid nucleus; CPu, caudate putamen; Cg1, cingulate cortex, area 1; DG, dentate gyrus; IL, infralimbic cortex; LA, lateral amygdaloid nucleus; PrL, prelimbic cortex.