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Supporting Online Material for

Calcium-Permeable AMPA Receptor Dynamics Mediate Fear Memory Erasure

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Supporting Online Material

Materials and Methods

Subjects

GluA1 serine-831 and serine-845 phosphorylation site mutants (S831A and S845A, respectively) were generated using polymerase chain reaction mutagenesis. Mutation sites were previously verified using phosphorylation-selective antibodies against GluA1 (*S1*). S831A and S845A founders were backcrossed 14 and 9 times, respectively, to C57BL6/J6 and bred as heterozygotes to generate wildtype and homozygous mutant littermates. All experiments were performed on male mice aged postnatal day 30 to 50 (P30-50), which had been weaned on P18 and maintained on a 12h light/ 12h dark schedule. In experiments in which no mutant mice were used, wildtype subjects originated from C57BL6/J6 matings. All manipulations were approved in advance by the Johns Hopkins University Animal Care and Use Committee.

Fear conditioning

For each of two days prior to conditioning, subjects were acclimated to experimenter by 10 minutes of light handling. The conditioning arena was located inside of a custom-built sound isolation box. Each box contained a modular test cage with an electrifiable floor grid and an ambient light supply. On the day of conditioning (Day 0), unpaired and paired groups received training that was divided into two sessions, separated by 30 minutes. Unpaired animals were presented with 6 unpaired tones (conditioned stimuli, CS) in session 1, and 6 unpaired foot shocks (unconditioned stimuli, US) in session 2. Paired animals were presented with no stimuli in session 1, and 6 paired CS and US during session 2. During each session, a period of acclimation lasting 200 seconds preceded the presentation of cues. The CS consisted of an 80-dB 2 KHz pure tone lasting 20 seconds. The US consisted of a 0.5-mA current lasting 2 seconds. During paired conditioning, CS and US were co-terminating. Cue presentations were separated

by 100 seconds. Following conditioning, mice were returned to their homecages until preparation of brain slices or further behavioral examination.

Reconsolidation-update training

To develop a protocol for reconsolidation-update in mice, we relied on the initial descriptions of this technique using rats (*S2*) and humans (*S3*) as subjects. In pilot experiments we determined that adolescent mice required more CS trials to undergo robust extinction than were employed during the reconsolidation period for rats (*S2*). Consequently, we modified the protocol of Monfils et al. (*S2*) by increasing the number of CS during extinction, while maintaining the defining feature of reconsolidation-update, the presentation of an isolated CS prior to extinction. Extinction was divided into 2 blocks to minimize the confounding effect of increasing subject inactivity on measurement of freezing after long periods of exposure to the extinction arena.

Reconsolidation-update was performed in a context distinct from that in which conditioning took place. This arena consisted of a textured polymer box scented with acetic acid, vanilla or pomegranate extract. A period of 100 seconds preceded the presentation of any CS. The Retrieval group (those animals undergoing reconsolidation-update) were presented with 1 CS, and then returned to their homecage. 30 minutes after the isolated CS, Retrieval animals underwent extinction training divided into 2 blocks, which were separated by 30 minutes. Three separate controls were used for physiological experiments. Unpaired controls were subjected to unpaired fear conditioning and received no further manipulation until behavioral testing or brain slice preparation. No-retrieval controls were treated identically to Retrieval animals, except that the CS was omitted from the retrieval session and 1 additional CS was added to extinction block 1 to balance the total number of CS presented. Context-only controls received paired fear conditioning and were exposed to the extinction context for an amount of time equivalent to Retrieval and No-retrieval animals, but no CS were presented. CS consisted of 80-dB 2 KHz pure tones lasting 20 seconds, separated by a 50-s interval during extinction. A total of 19 CS were delivered during each extinction block, except in the case of block 1 for No-retrieval controls, as stated above. After reconsolidation-update, mice were returned to their homecages

until sacrificed for preparation of brain slices, or until further behavioral examination was conducted.

Spontaneous recovery and renewal tests

After reconsolidation-update training, mice were returned to the extinction arena and presented with 4 CS to measure spontaneous recovery of fear. 30 minutes later, the same animals were placed in the conditioning arena and presented with 4 CS to measure renewal. While spontaneous recovery can in principle manifest at any point after extinction, our analysis indicated that 6 days (but not 1 day) was sufficient time for freezing in No-retrieval controls to recover to levels comparable to pre-extinction and to renewal. Furthermore, the level of freezing observed on Day 7 in No-retrieval controls was not significantly different than Context-only controls, suggesting 6 days is sufficient time for substantial spontaneous recovery.

Measurement and analysis of fear behavior

Percentage time freezing during CS presentation was quantified using automated motion sensitive software. A single set of algorithmic parameters optimized to the age range of our subjects were used for every experiment. For longitudinal studies of fear relapse, statistical analysis of mean freezing values was conducted using two-way repeated measures ANOVA (group x test interactions). Tukey's post-hoc comparison was then used to detect significant differences between groups. Non-longitudinal comparisons were conducted using 1-way ANOVA, and significant group differences were established with Tukey's post-hoc comparison. When neither of these tests was appropriate, a Student's 2-tailed t-test was used.

Brain slice electrophysiology

After sacrificing anesthetized mice, brains were dissected into ice-cold buffer containing the following (in mM): 210.3 sucrose, 11 glucose, 2.5 KCl, 1 NaH_2PO_4, 26.2 $NaHCO_3$, 0.5 ascorbate, 0.5 $CaCl_2$, 4 $MgCl_2$. Acute coronal slices were obtained at 350 μm thickness on a vibratome and transferred to normal artificial cerebrospinal fluid (ACSF) composed of the following (in mM): 119 NaCl, 2.5 KCl, 1 NaH_2PO_4, 26.2 $NaHCO_3$, 11 glucose, 2 $CaCl_2$, and 2 $MgCl_2$. Following recovery at 35°C for 40 minutes, slices were maintained at 22–25°C. Whole-cell recordings were performed using borosilicate electrodes (3–5 MΩ) on submerged slices containing lateral amygdala (LA). All recordings were targeted to the upper portion of the lateral nucleus, because this region receives the densest input from the auditory thalamus, which conveys CS information to the LA. Presynaptic stimulation was delivered to thalamic fiber bundles traversing the amygdalo-striatal transition area with a bipolar microelectrode. Monosynaptic responses were judged on their latency, which was 3-4 ms on average and constant across stimuli. In all recordings, fast GABAergic inhibition was blocked with picrotoxin (100 μM) to yield pure excitatory responses. Because we found that in a small number of cases this resulted in polysynaptic bursting, all recordings were performed in solution containing elevated divalent cations (4 mM Ca^{2+} and Mg^{2+}) to reduce network excitability, except when action potentials were blocked with TTX. Recording electrode internal solution contained (in mM): 130 cesium-methanesulfonate, 10 HEPES, 0.5 EGTA, 8 NaCl, 1 TEA, 4 Mg-ATP, 1 QX-314, 10 Na-phosphocreatine, and 0.4 Na-GTP. All measurements of AMPAR currents, including rectification, AMPAR-mEPSC amplitude, and NASPM sensitivity, were performed in ACSF containing D,L-APV (100 μM). To quantify rectification, internal solution was supplemented with 100 μM spermine to compensate for the dialysis of endogenous polyamines by the patch pipette. Spontaneous mEPSCs were collected in the presence of 1 μM tetrodotoxin (TTX). Data were acquired at 10 KHz using Multiclamp 700B and pCLAMP 10 and analyzed offline in pCLAMP. AMPA:NMDA ratios were calculated as the ratio of peak current at –70 mV to the current at 100 ms after stimulus onset at +40 mV (since we determined from synaptic responses in D,L-APV that AMPAR currents make a negligible contribution at this interval). There were no effects of conditioning or extinction on the kinetics of NMDAR currents (data not shown). Rectification index was calculated as a ratio of slopes of a linear fit of

I–V points, in which the index = slope at negative holding potentials (–70 to 0 mV) divided by the slope at positive holding potentials (0 to +50 mV). Accordingly, an index of 1 represents perfect linearity, while values >1 indicate inward rectification. Naphthylacetyl spermine (NASPM, 50 μM) was used to block CP-AMPARs in the presence of D,L-APV (100 μM). To determine the contribution of CP-AMPAR trafficking to LTD, NASPM was applied 20 minutes after LTD induction for a duration (>20 minutes) sufficient for saturation of NASPM block. In these experiments, comparisons of EPSC amplitude were performed prior to (15-20 minutes after LTD) and after the application of NASPM (45-50 minutes after LTD) to determine both the amount of LTD (1st timepoint) as well as the residual contribution of CP-AMPARs (2nd timepoint). AMPAR-mEPSCs were detected at 5 pA threshold (>2x RMS noise) and analyzed using MiniAnalysis. To obtain decay times, mean mEPSCs were fitted with a single exponential. Significance for all group comparisons was assessed by one-way ANOVA followed by a Tukey's *post-hoc* comparison or, when appropriate, a Student's 2-tailed t-test.

References

S1. H. S. Crombag *et al.*, *Behav Brain Res* **191**, 178 (Aug 22, 2008).

S2. M. H. Monfils, K. K. Cowansage, E. Klann, J. E. LeDoux, *Science* **324**, 951 (May 15, 2009).

S3. D. Schiller *et al.*, *Nature* **463**, 49 (Jan 7).

Supplemental Figure Legends

Fig. S1. Pathway-specific analysis of AMPAR transmission at thalamo-amygdala synapses. EPSCs were evoked by stimulation of separate thalamic axon bundles terminating onto the same postsynaptic cell. (A) Representative EPSCs at membrane holding potential (V_h) = -70, 0 and +40 mV during stimulation of separate pathways (A and B) from mice that were subjected to unpaired (N = 9) or paired (N = 11) fear conditioning 24 hrs prior to slice preparation. Scale bars = unpaired A 200 pA, B 200 pA, paired cell 1 A 200 pA, cell 1 B 200 pA, cell 2 A 100 pA, cell 2 B 100 pA x 200 ms. (B) Mean AMPA:NMDA ratio. * P < 0.00001 Student's t-test. (C) AMPA:NMDA ratio as a function of pathway within each cell. Blue line indicates group mean. (D) Distribution of individual AMPA:NMDA ratios from all pathways. Dotted line indicates 2 standard deviations above unpaired mean. 50% of recorded pathways, and 63.6% of recorded cells in paired slices had AMPA:NMDA ratios more than 2 standard deviations above the unpaired mean.

Fig. S2. Fear conditioning potentiates AMPAR-mEPSCs. (A) Cumulative histogram of AMPAR-mEPSC amplitudes. * P < 0.0001 ANOVA followed by Tukey's post-hoc comparison with unpaired. (B) Cumulative histogram of AMPAR-mEPSC interevent intervals. * P < 0.001 ANOVA followed by Tukey's post-hoc comparison with unpaired. A significant increase in frequency was present at 24 hours after fear conditioning in paired animals. (C) Mean AMPAR-mEPSC rise time (20-80%). (D) Mean AMPAR-mEPSC decay time (τ decay). No significant differences in the kinetics of mean AMPAR-mEPSCs were detected. Paired, N = 9-22 cells; unpaired, N = 9-18 cells per timepoint.

Fig. S3. Expression of CP-AMPARs at thalamic inputs to LA in naïve mice. (A) Inhibition of AMPAR-EPSCs by the CP-AMPAR antagonist NASPM (50 μM, N = 6). (B) Rectification of AMPAR-EPSCs in naïve mice (baseline, N = 17), and elimination by pre-incubation in NASPM (post-NASPM, N = 6). Representative AMPAR-EPSCs at V_h = -70, -60, -40, -20, 0, +20, +40, and +50 mV. Scale bar = 50 pA x 30 ms. * P < 0.01 Student's 2-tailed t-test.

Fig. S4. Analysis of AMPAR-EPSC rectification after fear conditioning. Normalized current-voltage (I-V) plots were constructed from peak amplitude of AMPAR-EPSCs at varying

intervals after fear conditioning. Paired, N = 6-9; unpaired, N = 5-7 per timepoint. * $P < 0.01$ Student's 2-tailed t-test.

Fig. S5. Pathway-specific analysis of AMPAR-EPSC rectification at thalamo-amygdala synapses. AMPAR-EPSCs were evoked by stimulation of separate thalamic axon bundles terminating onto the same postsynaptic cell. (A) Representative AMPAR-EPSCs at membrane holding potential (V_h) = -70, -60, -40, -20, 0, +20, +40 and +50 mV during stimulation of separate pathways (A and B) from mice that were subjected to unpaired (N = 9) or paired (N = 14) fear conditioning 24 hrs prior to slice preparation. Scale bars = unpaired A 100 pA, B 100 pA, paired cell 1 A 200 pA, cell 1 B 100 pA, cell 2 A 100 pA, cell 2 B 100 pA x 50 ms. (B) Mean rectification index. * $P < 0.001$ Student's t-test. (C) Rectification index as a function of pathway within each cell. Blue line indicates group mean. (D) Distribution of individual rectification indices from all pathways. Dotted line indicates 2 standard deviations above unpaired mean. 67.8% of recorded pathways, and 78.6% of recorded cells in paired slices had rectification indices more than 2 standard deviations above the unpaired mean.

Fig. S6. Inhibitory effect of NASPM on AMPAR-EPSCs as a function of time since training. NASPM (50 μM) was applied to acute slices from unpaired (N = 4-5) and paired (N = 5 each) subjects following fear conditioning, in the continuous presence of APV (50 μM). * $P < 0.001$ Student's t-test for the final 5 min of recording.

Fig. S7. Pharmacological requirements for LTD resulting from ppLFS-pairing. ppLFS-pairing was delivered at thalamo-LA inputs in slices from naïve animals, in the absence of any drug (N = 10), or in the continuous presence of DL-APV (100 μM, N =6), LY367385 (100 μM, N = 5), or MPEP (10 μM, N =6). * $P < 0.01$ Student's t-test versus no drug.

Fig. S8. Reversal of increased AMPAR rectification after fear conditioning by LTD or NASPM. EPSCs were evoked at thalamo-amygdala synapses in slices prepared 24 hrs after fear conditioning. (A) Induction of LTD by ppLFS-pairing (3 Hz stimulation, 50-ms interpulse interval, for 3 min at -50 mV), followed by blockade of NMDARs (100 μM DL-APV) for construction of AMPAR-EPSC current-voltage plots (N = 6). (B) Timecourse of effect of NASPM (50 μM) on AMPAR-EPSCs. (C) Representative AMPAR-EPSCs following LTD (N =

6) or NASPM (N = 4) alongside interleaved control experiments in unpaired (N = 6) or paired (N = 6) cells. Scale bars = unpaired 100 pA, paired 150 pA, paired LTD 100 pA and paired NASPM 50 pA x 50 ms. (D) Mean rectification index. * $P < 0.01$ ANOVA, Tukey's post-hoc.

Fig. S9. Long-term attenuation of fear relapse and AMPA transmission by reconsolidation-update. Reconsolidation-update was performed on Day 1 after conditioning on Day 0, as described in Fig. 3. (A) Comparison of freezing in spontaneous recovery and renewal tests on Day 7. * $P < 0.01$ ANOVA, Tukey's post-hoc (N = 6-8). (B) AMPA:NMDA ratio at thalamo-LA synapses on Day 7 after reconsolidation-update on Day 1. Scale bars = No-retrieval 150 pA, Retrieval 100 pA x 50 ms. * $P < 0.01$ ANOVA, Tukey's post-hoc (N = 7-10).

Fig. S10. Unaltered fear behavior during conditioning and extinction in S831A and S845A mutants. (A) Timeline for fear conditioning and reconsolidation-update training in GluA1 S831A (N = 7) and S845A mutants (N = 8), examined alongside their wildtype littermates (N = 7-8) in interleaved experiments. CS-evoked freezing was measured during fear conditioning and subsequent reconsolidation-update training in S831A (B) and S845A mutants (C).

Fig. S11. S831A knockin mutation does not disrupt fear erasure. S831A knockins and their wildtype littermates were subjected to reconsolidation-update, as described in Fig. 2 and fig. S10. Freezing was averaged for the first and last 4 trials of extinction, and relapse was measured by comparison to the last 4 trials. Spontaneous recovery and renewal tests were performed on Day 7 after reconsolidation-update on Day 1. Repeated measures ANOVA, group x test: wildtype $F(3,30) = 4.16$, $P < 0.05$; S831A knockins $F(3,36) = 3.48$, $P < 0.05$. * $P < 0.01$ Tukey's post-hoc comparison with last 4 trials. # $P < 0.01$.

Figure S1

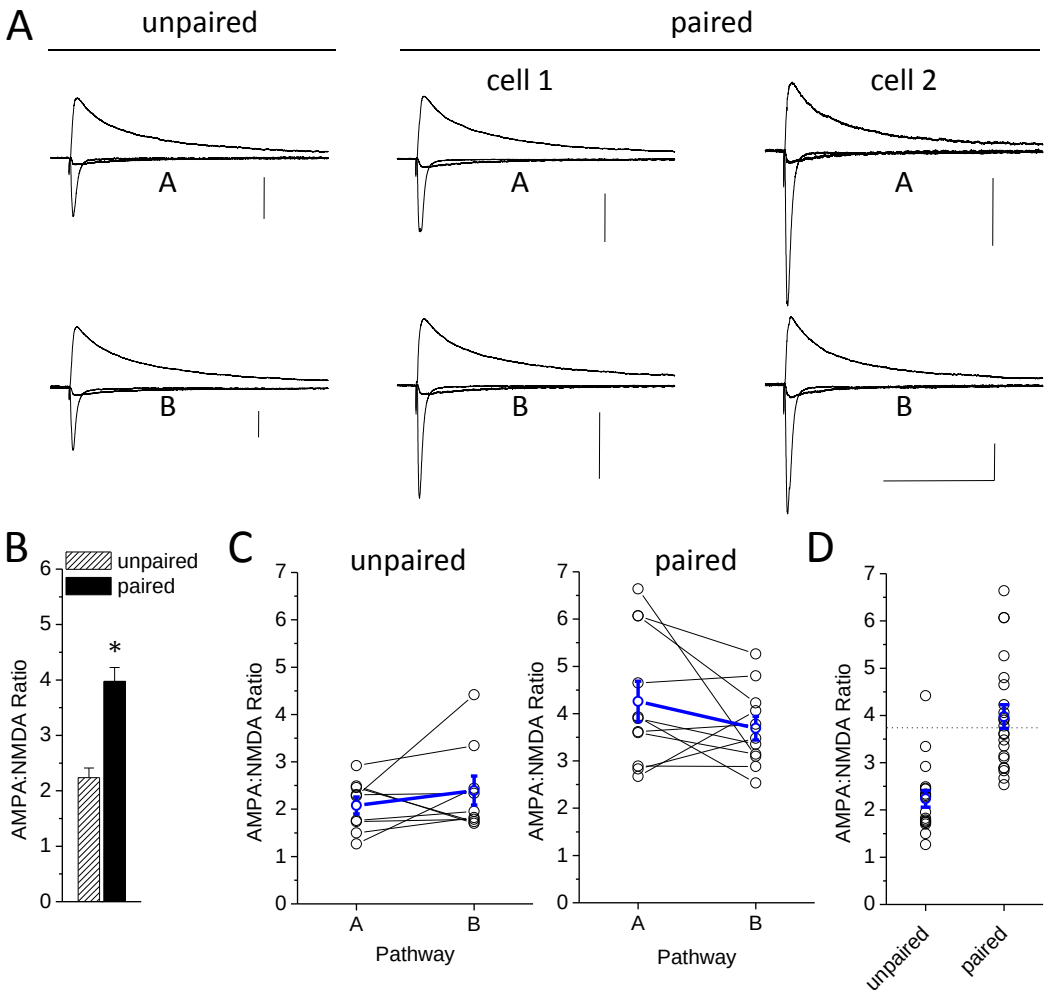
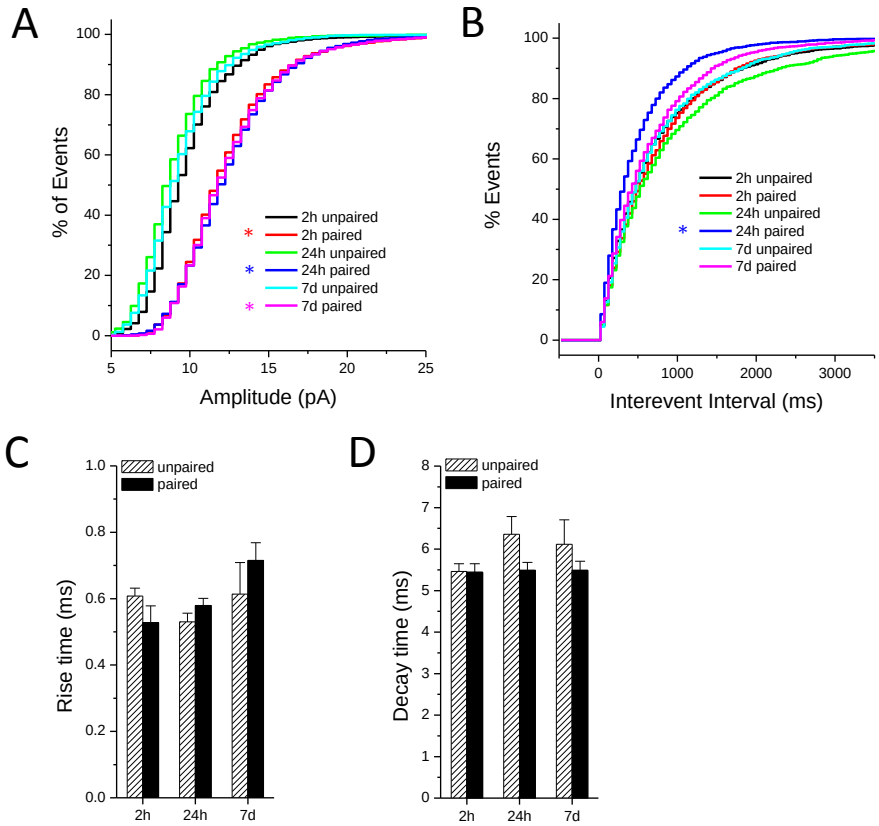

A
unpaired
paired
cell 1
cell 2
A
B
B
unpaired
paired
*
AMPA:NMDA Ratio
C
unpaired
paired
Pathway
D
unpaired
paired

Figure S2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% of Events
Amplitude (pA)
2h unpaired
2h paired
24h unpaired
24h paired
7d unpaired
7d paired
B
% Events
Interevent Interval (ms)
2h unpaired
2h paired
24h unpaired
24h paired
7d unpaired
7d paired
C
Rise time (ms)
unpaired
paired
2h
24h
7d
D
Decay time (ms)
unpaired
paired
2h
24h
7d

Figure S3

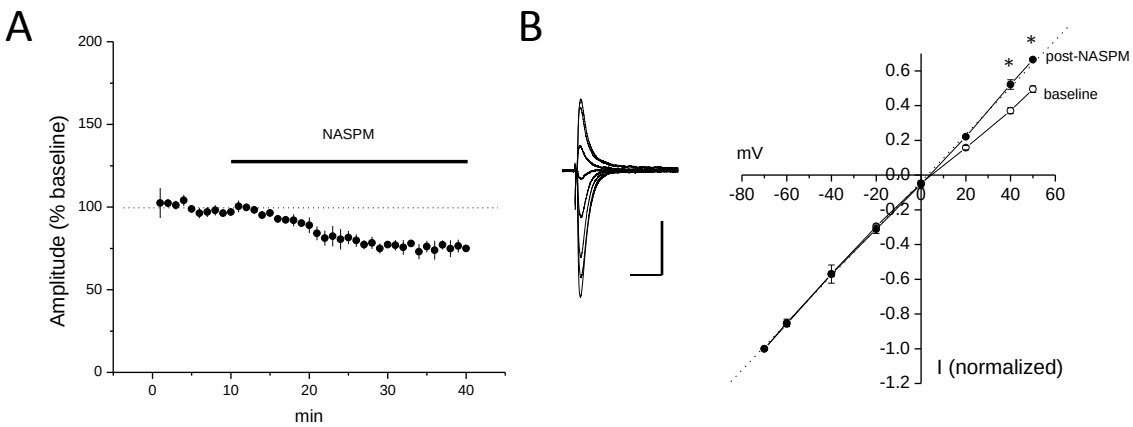

A
B
NASPM
Amplitude (% baseline)
min
post-NASPM
baseline
mV
I (normalized)

Figure S4

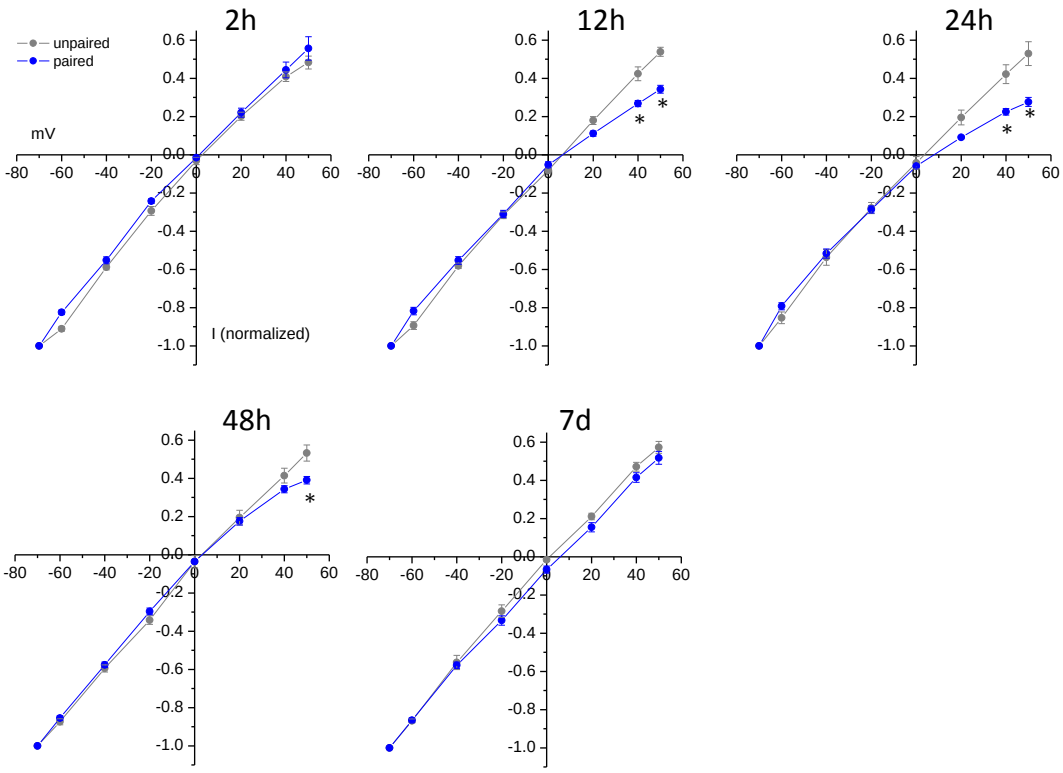

2h
12h
24h
48h
7d
unpaired
paired
mV
I (normalized)
0.6
0.4
0.2
0.0
-0.2
-0.4
-0.6
-0.8
-1.0
-80
-60
-40
-20
0
20
40
60
*

Figure S5

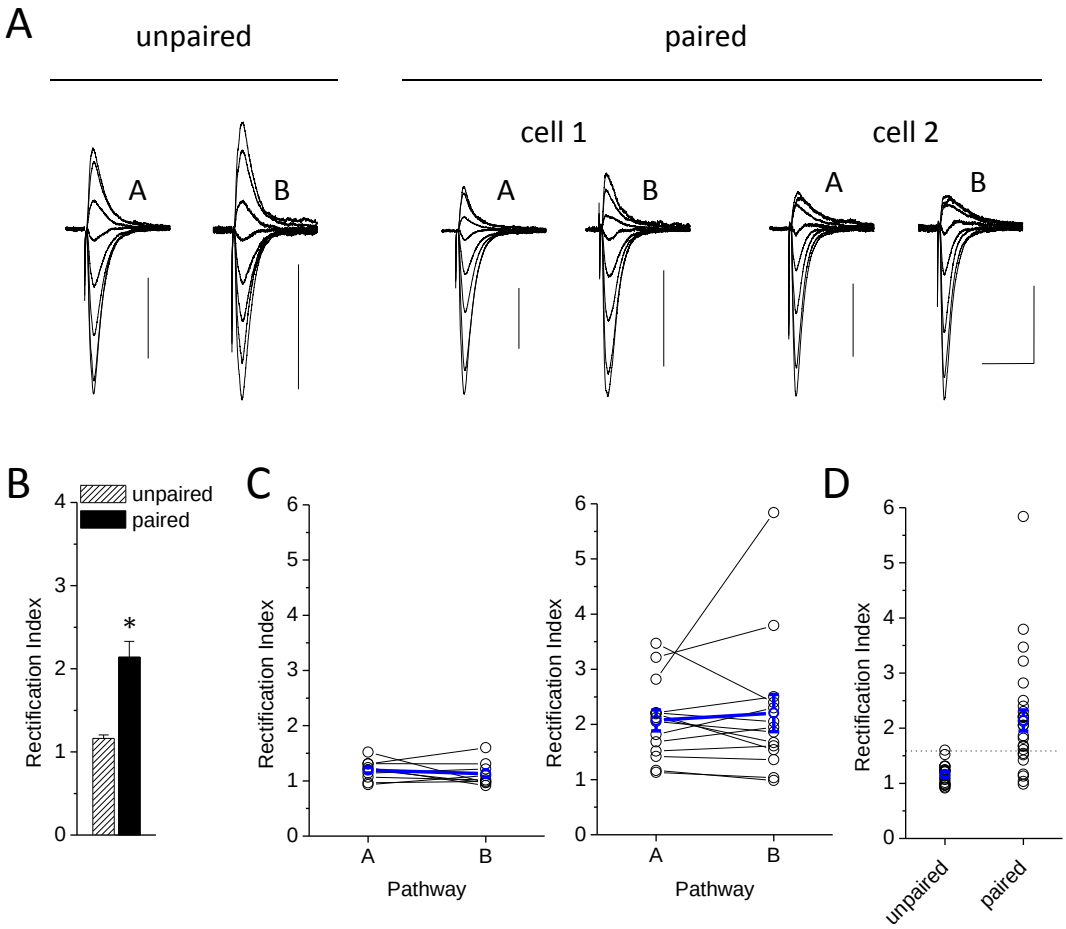
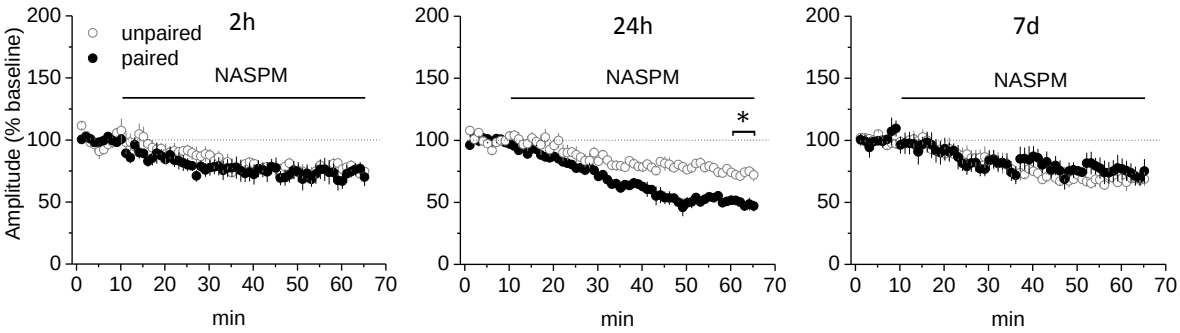

A
unpaired
paired
cell 1
cell 2
A
B
A
B
A
B
B
unpaired
paired
Rectification Index
*
C
Rectification Index
Pathway
A
B
D
Rectification Index
unpaired
paired

Figure S6


Amplitude (% baseline)
unpaired
paired
2h
24h
7d
NASPM
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min
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100
150
200
0
10
20
30
40
50
60
70

Figure S7

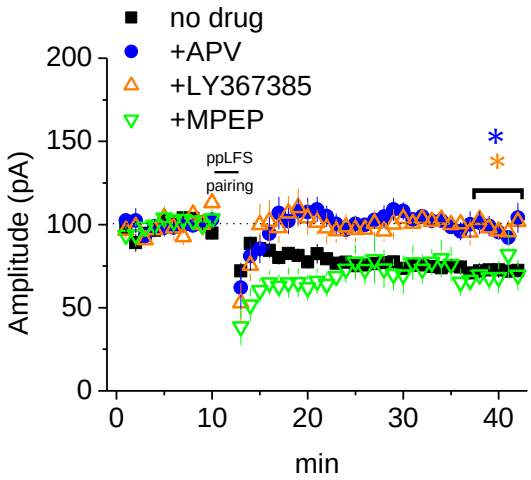

no drug
+APV
+LY367385
+MPEP
ppLFS
pairing
Amplitude (pA)
200
150
100
50
0
0
10
20
30
40
min
*
*

Figure S8

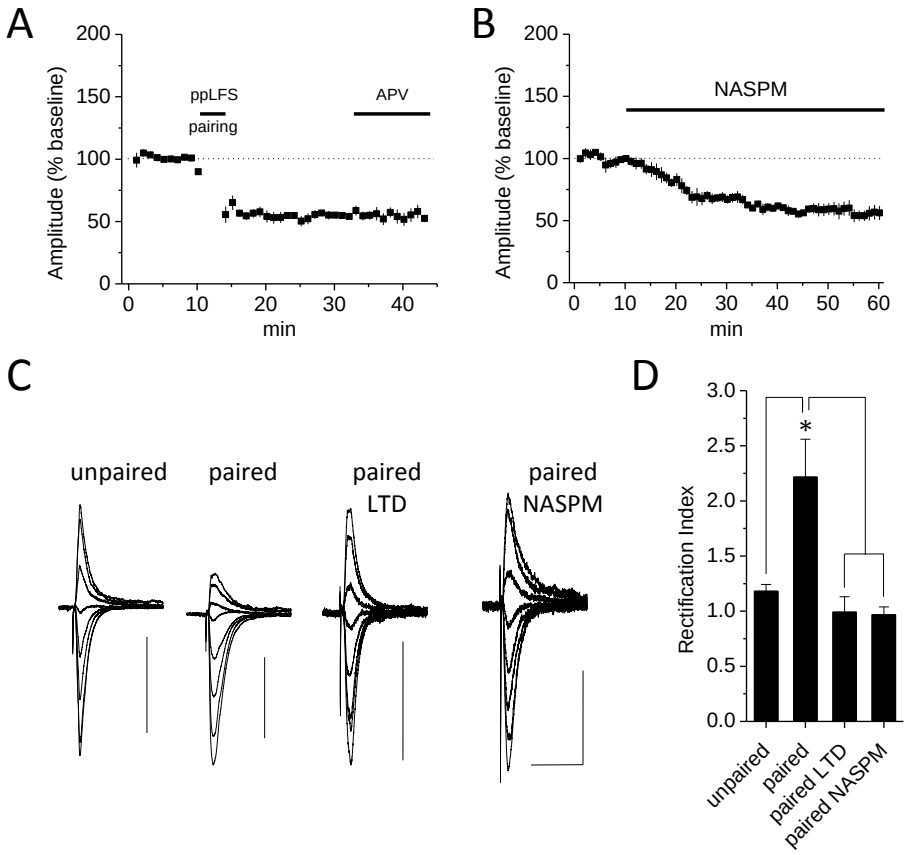
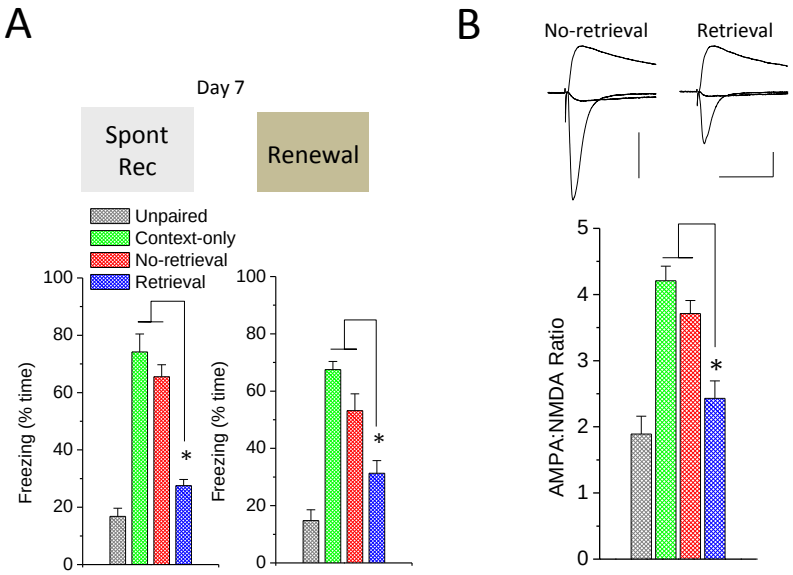

A
200
150
100
50
0
Amplitude (% baseline)
ppLFS
pairing
APV
0
10
20
30
40
min
B
200
150
100
50
0
Amplitude (% baseline)
NASPM
0
10
20
30
40
50
60
min
C
unpaired
paired
paired
LTD
paired
NASPM
D
3.0
2.5
2.0
1.5
1.0
0.5
0.0
Rectification Index
*
unpaired
paired
paired LTD
paired NASPM

Figure S9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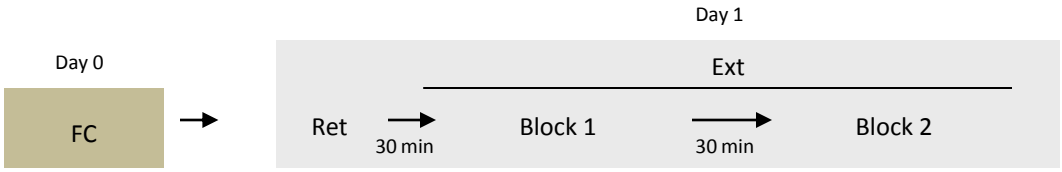
Day 7
Spont Rec
Renewal
Unpaired
Context-only
No-retrieval
Retrieval
Freezing (% time)
100
80
60
40
20
0
*
B
No-retrieval
Retrieval
AMPA:NMDA Ratio
5
4
3
2
1
0
*

Figure S10

A



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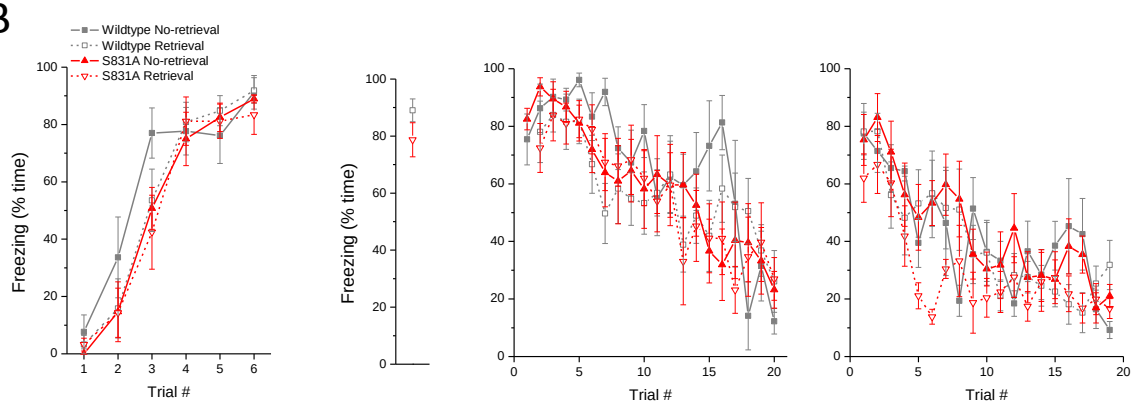


C

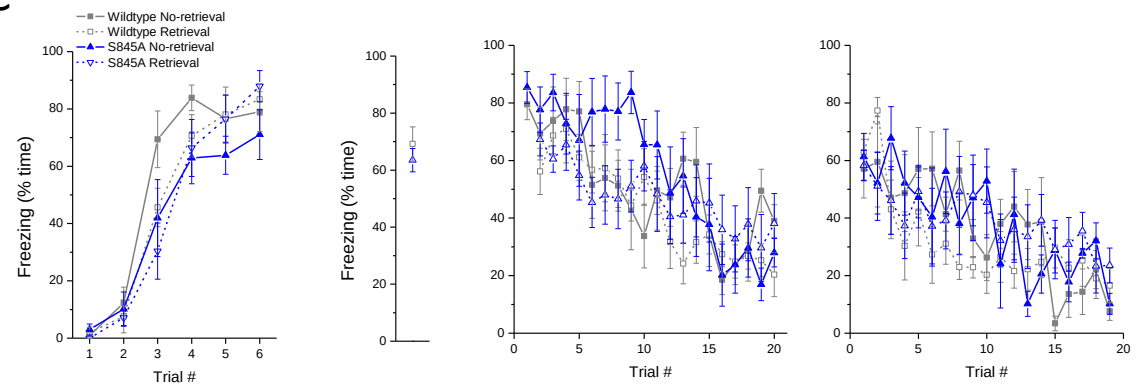
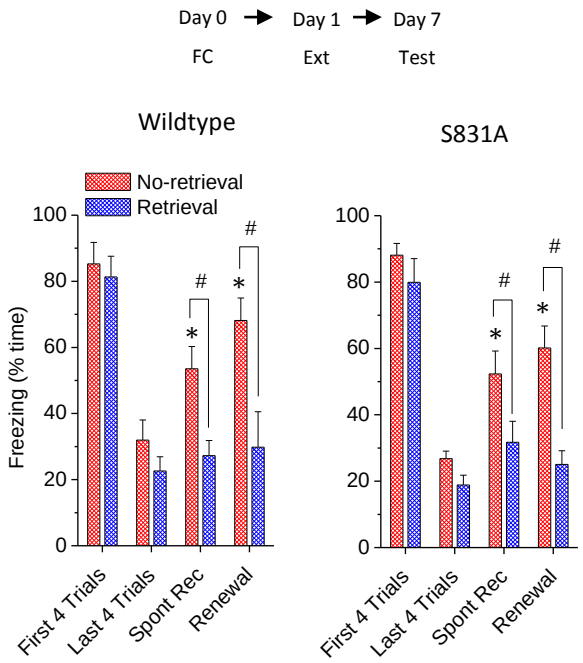


Figure S11


Day 0
Day 1
Day 7
FC
Ext
Test
Wildtype
S831A
No-retrieval
Retrieval
Freezing (% time)
100
80
60
40
20
0
#
*
First 4 Trials
Last 4 Trials
Spont Rec
Renewal