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Retrieval-specific endocytosis of GluA2-AMPARs underlies adaptive reconsolidation of contextual fear

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Upon retrieval, fear memories are rendered labile and prone to modification, necessitating a restabilization process of reconsolidation to persist further. This process is also crucial for modulating both strength and content of an existing memory and forms a promising therapeutic target for fear-related disorders. However, the molecular and cellular mechanism of adaptive reconsolidation still remains obscure. Here we show that retrieval of fear memory induces a biphasic temporal change in GluA2-containing AMPA-type glutamate receptor (AMPAR) membrane expression and synaptic strength in the mouse dorsal hippocampus. Blockade of retrieval-induced, regulated, GluA2-dependent endocytosis enhanced subsequent expression of fear. In addition, this blockade prevented the loss of fear response after reconsolidation-update of fear memory content in the long-term. Thus, endocytosis of GluA2-containing AMPARs allows plastic changes at the synaptic level that exerts an inhibitory constraint on memory strengthening and underlies the loss of fear response by reinterpretation of memory content during adaptive reconsolidation.

Aversive associative memories formed by the association between a neutral conditioned stimulus and an aversive unconditioned stimulus are progressively made permanent by a process of consolidation[1]. However, upon retrieval, intervention by amnestic agents[2–7], either before or immediately after retrieval, results in disruption of the previously consolidated fear memory. This suggests that a consolidated memory returns to a transient destabilized state shortly after reactivation, necessitating a dynamic time-dependent process of reconsolidation to persist further. During this reconstruction, a memory is vulnerable to experimental intervention[8–10] leading to amnesia, but can also be enhanced[11–13] or modified in the long-term[14–16], thereby updating the previous memory with new information[14–17]. In clinical terms, the bidirectional and adaptive nature of reconsolidation is ideally placed to mediate the modification of both memory strength[12] and memory content[16,18], rendering this process a promising therapeutic target for counteracting the hyper-responsive fear system. To fully exploit reconsolidation-based therapies that adapt the content of fear memories, leading to a loss of fear response in the long term, it is crucial to elucidate the molecular underpinnings of reconsolidation, which remain obscure.

Long-lasting changes in synaptic efficacy brought about by gene transcription, protein synthesis and changes in strength of hippocampal glutamatergic synapses through AMPA receptor trafficking are believed to be the cellular substrates of learning and memory[19–21]. Although reconsolidation is not merely a recapitulation of the initial consolidation process[22], it has been shown that transcription, *de novo* protein synthesis and synaptic protein degradation in the hippocampus are necessary for memory remodeling after retrieval[4,7,17,23–25]. Here, we investigated whether the temporal profile of reconsolidation, which is hypothesized to be limited to a 6 h time window[5,8], reflects a sequential profile of defined dorsohippocampal AMPA receptor synaptic plasticity that is crucial to the synaptic remodeling that underlies subsequent fear expression (changes in memory strength) and reinterpretation of fear memory after retrieval (changes in memory content).

RESULTS

Memory recall induces acute hippocampal AMPAR endocytosis

To analyze whether glutamate receptors are regulated during reconsolidation in mice receiving the unconditioned stimulus and retrieval (US-R), we dissected the dorsal hippocampus at 1 and 4 h after retrieval and analyzed the synaptic membrane fraction, including membrane-bound proteins and associated proteins[26,27], by immunoblotting for subunits of AMPA receptors. A no-shock group experiencing retrieval (NS-R) was used to control for the specificity of an aversive associative memory (**Supplementary Fig. 1**). These two time points were chosen because they fall within the 6-h time window after retrieval during which the memory undergoes reconsolidation[5]. After retrieval, subsequent reconsolidation requires protein synthesis for the memory to persist further[7] (**Supplementary Fig. 1**). First, the 1 h time point was analyzed. All AMPAR subunits (GluA1–GluA3) were downregulated (31.4%, 16.4% and 50.20%, respectively; $P < 0.05$), indicating a weakened state of the synapse[28,29] (**Fig. 1a**,**b** and **Supplementary Fig. 2**). The observed downregulation was specific to retrieval of an associative contextual conditioned stimulus–unconditioned stimulus representation, with

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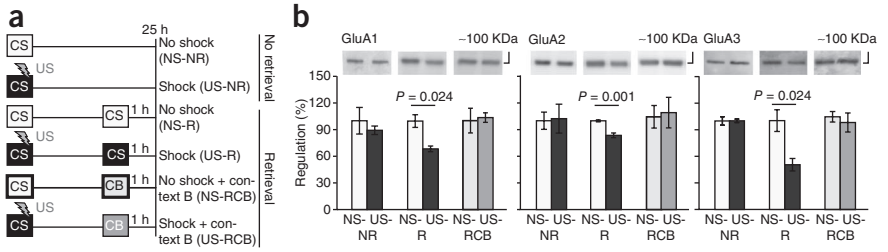

Figure 1 Retrieval after contextual fear consolidation leads to endocytosis of AMPARs. (**a**) Experimental design with six groups of mice that, 24 h before a retrieval session, were exposed to the conditioned stimulus (CS) context only (no shock: NS-R), or received a shock (unconditioned stimulus) in the same context (US-R) or in a different context (US-RCB), or did not experience retrieval (NS-NR and US-NR). Mice were then analyzed 1 h after the retrieval session. The timeline for collection of dorsal hippocampi for immunoblot analysis (NS-R, $n = 4$ samples; US-R, $n = 3$; US-RCB, $n = 4$) is indicated. (**b**) Quantification of synaptic membrane fraction AMPA receptor subunits, as a percentage of NS-R values. Representative blots with samples that were compared on the same gel are shown (approximate molecular weight indicated; for input material used for normalization, see **Supplementary Fig. 2**). Downregulation of subunits of AMPARs 1 h after retrieval was observed exclusively as result of retrieval in the conditioning context (GluA1, $F_{1,6} = 12.467$; GluA2, $F_{1,6} = 39.995$; GluA3, $F_{1,6} = 10.122$), but neither from consolidation alone nor from exposure to a novel context. All data points show mean ± s.e.m.; significant *P*-values are indicated.

no differences in GluA subunit expression observed in absence of a retrieval session or with retrieval in a novel context not associated with the unconditioned stimulus and hence not related to the fear memory (**Fig. 1b** and **Supplementary Fig. 1**). Furthermore, the downregulation was not due to nonspecific effects of the shock itself; no differences in GluA subunit expression were observed (**Fig. 2a**,**b**) when mice were shocked immediately upon placement in the conditioning context, a protocol in which mice do not learn to associate the conditioned stimulus with the shock[30] (**Supplementary Fig. 1**). To unequivocally demonstrate that changes in protein levels of AMPAR subunits measured in the synaptic membrane fraction represent differential surface expression, we performed a biotinylation experiment[31,32] and corroborated the downregulation of surface GluA2 receptor subunits 1 h after retrieval (**Fig. 2c**,**d**). Together these data point to a postsynaptic mechanism underlying reconsolidation of contextual memory rather than the initial consolidation of fear memory after conditioning.

Because regulated removal of AMPAR from postsynaptic membranes underlies alterations in synaptic strength[33], we recorded glutamatergic synaptic transmission onto CA1 pyramidal cells either in the absence of a retrieval session or 1 h after retrieval. The amplitude distribution and averages of pharmacologically isolated AMPAR-mediated miniature excitatory postsynaptic currents (mEPSCs) of conditioned mice were shifted to lower amplitudes (**Fig. 3**), an effect that was specific to the retrieval session. This depressed state continued over time, with GluA2 and GluA3 subunits robustly downregulated 4 h after retrieval (19.5% and 53.5%, respectively; $P < 0.05$), a time at which GluA1 subunit expression was normalized (**Fig. 4a**,**b**). Thus, memory retrieval resulted in a decreased strength of glutamatergic synapses onto CA1 pyramidal neurons, as predicted based on the observed reduction in synaptic AMPAR subunits (**Fig. 1b**) and decreased GluA2-containing surface receptors (**Figs. 2b**,**d** and **3**).

To test whether a specific increase in regulated endocytosis of GluA2-containing AMPARs[34,35] underlies reduced synaptic AMPAR protein levels, we examined whether blockade of regulated GluA2 endocytosis and synaptic strength by an HIV TAT-fused GluA2-derived C-terminal peptide (TAT-GluA2$_{3Y}$)[26,36] would interfere with retrieval-induced regulation of GluA1–GluA3. Conditioned mice and their NS-R controls received either TAT-GluA2$_{3Y}$ or TAT-GluA2$_{3A}$, a control containing a peptide (3A) in which the tyrosine residues were replaced by alanines, into the CA1 region of the dorsal hippocampus 1 h before retrieval (**Supplementary Fig. 3**). Preventing regulated endocytosis of GluA2-containing receptors indeed blocked the observed downregulation of GluA2 and GluA3, subunits. Hence, our data indicate that retrieval-induced downregulation of AMPARs and reduction of synaptic strength at these synapses during the reconsolidation time window could serve as a molecular process required for synaptic reorganization of the memory trace in the hippocampus.

Retrieval induces a second wave of AMPAR upregulation

Because a retrieved memory is reconsolidated approximately 6 h after retrieval, we hypothesized that the initial synaptic weakening at 1–4 h after retrieval would be followed by a stabilized state of previously induced

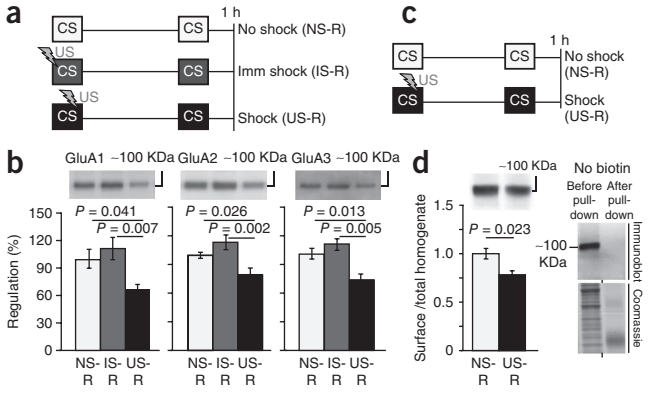

Figure 2 Endocytosis of AMPARs is specific to retrieval of a conditioned fear memory. (**a**–**d**) Experimental design with three or with two groups that, 24 h before retrieval, were exposed to context only (NS-R), or received a shock either immediately upon placing in the box (immediate (imm) shock; IS-R) or after a delay (US-R). All groups received a retrieval session the next day, and 1 h later the dorsal hippocampi were collected for immunoblot analysis ($n = 4$ samples per condition). (**b**,**d**) Quantification of AMPA receptor subunits, as a percentage of the NS-R value. Representative blots with samples that were compared on the same gel are shown (approximate molecular weight indicated; for input material used for normalization see **Supplementary Fig. 2**). (**b**) AMPAR subunits from the synaptic membrane fraction were downregulated 1 h after retrieval (GluA1, $F_{2,11} = 6.232$; GluA2, $F_{2,11} = 9.660$; GluA3, $F_{2,11} = 9.986$), and this effect was not due to unspecific effects of the shock (immediate shock). (**d**) Left: normalized ratio of AMPA receptor subunits present on the surface to those present in the total homogenate, determined using biotinylation. This corroborated the downregulation of surface GluA2 ($F_{1,7} = 10.441$; **Figs. 1b** and **2b**) compared with NS-R, and concomitant reduction in AMPAR currents (**Fig. 3**). Right: example of no-biotin control before and after addition of NeutrAvidin beads for immune precipitation. Top, GluA2 immunodetection; bottom, Coomassie stain to control for input differences. GluA2 cannot be detected after immune precipitation, indicating the specificity of the method. All data points show mean ± s.e.m.; significant *P*-values are indicated.

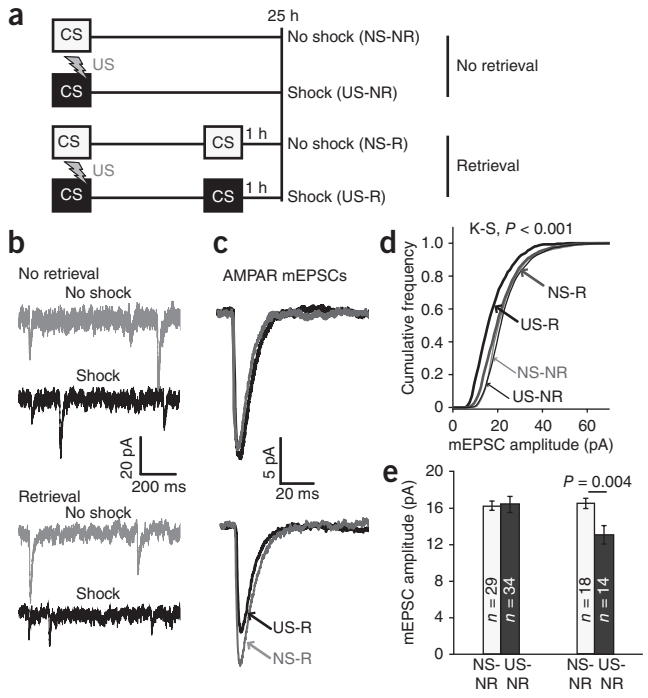

Figure 3 Fast retrieval-induced decrease in synaptic strength in dorsal hippocampus. (**a**) Experimental design with four groups, in which mice, 24 h before the presence or absence of a retrieval session, were exposed only to the conditioned stimulus (CS; groups NS-R or NS-NR) or received a shock (US-R or US-NR). The timeline is indicated for collection of brains for *in vitro* slice physiology ($n = 6$ for NS-NR; $n = 6$ for US-NR; $n = 4$ for NS-R; $n = 4$ for US-R). (**b**,**c**) Representative recordings of AMPAR mEPSCs (**b**) and resulting averages of events superimposed (**c**). (**d**) Cumulative frequency of mEPSC amplitudes, showing a significant ($P < 0.0001$) leftward shift in amplitude. (**e**) Bar graphs of AMPAR-mediated mEPSCs, showing decreased synaptic strength in shocked mice specifically 1 h after retrieval, without AMPAR current changes after conditioning. Number of individual cells measured are indicated. For cumulative frequencies, a Kolmogorov-Smirnov test (K-S) was performed. All data points show mean ± s.e.m.; significant *P*-values are indicated.

synaptic potentiation[2,5,7]. The first indication of this was the observed re-insertion of GluA1 into the membrane 4 h after retrieval, which could signify the start of a process that induces synaptic potentiation (**Fig. 4a**,**b**). This is in accordance with previous observations that LTP induction causes a transient increase in membrane GluA1-containing receptors, which are then gradually replaced by GluA2-containing receptors that stabilize synaptic strengthening[37,38]. At the maintenance phase of reconsolidation, 7 h after retrieval (**Fig. 4a**,**b**), GluA2 subunits were strongly upregulated (36.2%, $P < 0.05$). Moreover, a trend toward increase of the GluA3 subunit (11.7%, $P < 0.1$) was observed, along with the sustained presence of GluA1, indicating an LTP maintenance–like phase.

Next, to investigate whether the retrieval-induced increases in AMPAR subunits indeed translated into functional changes at glutamatergic synapses, we recorded pharmacologically isolated AMPAR-mediated mEPSCs 7 h after retrieval. We found that the decay of AMPAR-mediated mEPSCs was significantly faster in conditioned mice than in NS-R controls (**Fig. 4c**–**f**) without changes in mEPSC frequency (**Supplementary Fig. 4**). Changes in decay kinetics of AMPAR-mediated currents might result from differences in AMPAR subunit composition[39]. For example absence of synaptic GluA1-containing receptors leads to faster decay of

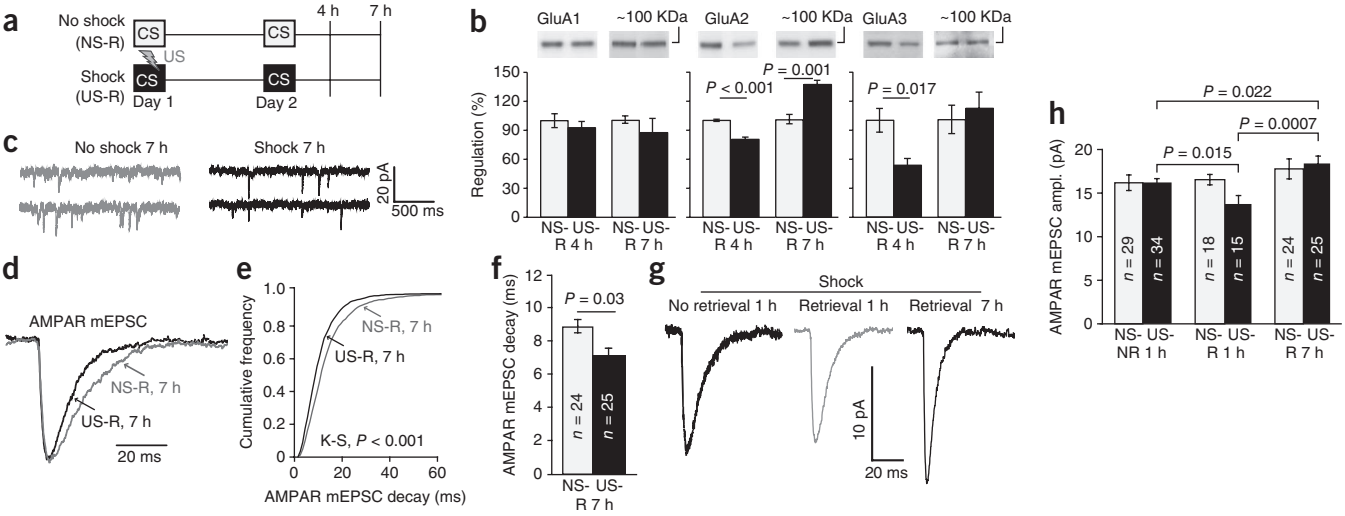

Figure 4 A biphasic wave of synaptic AMPAR levels after retrieval translates into functional synaptic changes in dorsal hippocampus. (**a**) Experimental design with two groups, in which mice, 24 h before retrieval, were exposed only to the conditioned stimulus (CS; group NS-R), or received a shock (US-R). Timeline is shown for collection of dorsal hippocampi for immunoblot analysis (4 and 7 h; $n = 4$ samples per condition) and of brains for *in vitro* slice physiology (7 h; $n = 10$ for NS-R, $n = 8$ for US-R). (**b**) Quantification, as a percentage of NS-R value, of AMPAR subunits in the synaptic membrane fraction. Representative blots with samples that were compared on the same gel are shown (approximate molecular weight indicated; for input material used for normalization, see **Supplementary Fig. 2**), showing a continued downregulation of GluA2 ($F_{1,7} = 60.951$) and GluA3 ($F_{1,7} = 10.824$) 4 h after retrieval, and an increase in GluA2 expression ($F_{1,7} = 36.65$) 7 h after retrieval. (**c**,**d**) Representative recordings of AMPAR mEPSCs (**c**) and resulting averages of events superimposed (**d**) 7 h after retrieval, showing a change in decay of AMPAR-mediated mEPSCs. (**e**) Cumulative frequency of mEPSC decay time, showing a significant ($P < 0.001$) leftward shift. For cumulative frequencies a Kolmogorov-Smirnov test (K-S) was performed. (**f**) Bar graphs of AMPAR-mediated mEPSCs, showing decreased decay time in shocked mice specifically 7 h after retrieval. Numbers of individual cells measured are indicated. (**g**,**h**) Temporal analysis of AMPAR-mediated mEPSCs, showing a biphasic wave of AMPAR regulation with decreased amplitudes 1 h after retrieval and increased amplitudes 7 h after retrieval, in the resulting averages of events (**g**) and in bar graphs representing AMPAR mEPSC amplitude (**h**). All data points show mean ± s.e.m.; significant *P*-values are indicated.

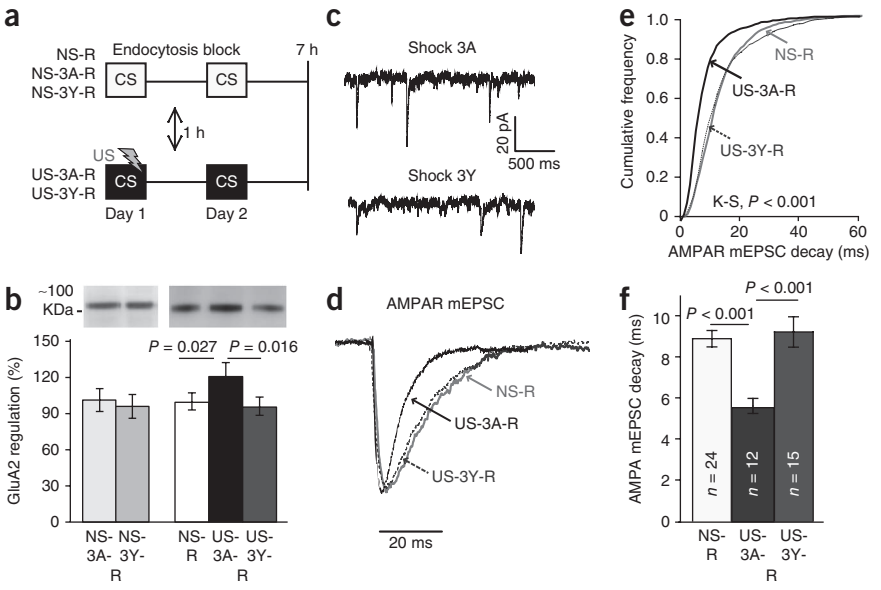

Figure 5 AMPAR endocytosis is crucial for subsequent AMPAR membrane insertion 7 h after retrieval. (**a**) Experimental design with two main groups, in which mice, 24 h before retrieval, were exposed only to the conditioned stimulus (CS; group NS-R), or received a shock (US-R), and in which regulated endocytosis of GluA2-AMPARs was blocked by the peptide $GluA2_{3Y}$ (3Y) or mice were treated with control peptide $GluA2_{3A}$ (3A). Timeline is indicated for intervention (1 h before retrieval) and for collection (7 h after retrieval) of dorsal hippocampi for immunoblot analysis ($n = 4$ samples per condition) and brains for *in vitro* slice physiology ($n = 8$ NS-R; $n = 4$ US-3A-R; $n = 5$ US-3Y-R). (**b**–**f**) Preventing retrieval-induced regulated endocytosis of AMPARs attenuated subsequent upregulation of GluA2 at the molecular level (**b**; $F_{2,11} = 8.096$; for input material, see **Supplementary Fig. 2**) and physiological level (**c**–**f**). (**c**) Representative recordings of AMPAR mEPSCs. (**d**,**e**) Scaled and superimposed resulting averages (**d**) and cumulative frequency of decays (**e**) of AMPAR-mediated mEPSCs in the presence of the $GluA2_{3Y}$ blocking peptide or the $GluA2_{3A}$ control peptide. For cumulative frequencies a Kolmogorov-Smirnov test (K-S) was performed. (**f**) Group data of AMPAR-mediated mEPSC decay time. Numbers of individual cells measured are indicated. All data points show mean ± s.e.m.; significant *P*-values are indicated.

AMPA currents[40]. Our results could thus reflect the relative increase in GluA2 and GluA3 observed. Although the amplitude of mEPSCs was similar to that in NS-R controls 7 h after retrieval (**Fig. 4c**), there was a significant ($P < 0.05$) time-dependent difference in amplitude, with increased amplitude 7 h after retrieval compared with that 25 h after conditioning or 1 h after retrieval (**Fig. 4g**,**h**).

Blocking initial AMPAR endocytosis by intrahippocampal TAT-$GluA2_{3Y}$ injection attenuated the subsequent retrieval-induced upregulation of AMPAR subunits (**Fig. 5a**,**b**). In addition, the decrease in decay time of AMPAR currents compared with NS-R controls was again observed using the TAT-$GluA2_{3A}$ control peptide (see **Fig. 4d**,**e**), a change that was completely reversed by blocking GluA2 endocytosis (**Fig. 5c**–**f**). This indicates that retrieval of contextual fear memory induces a second wave of glutamate receptor trafficking—dependent on the initial decrease in synaptic strength shortly after retrieval—and possibly relates to a subsequent increase in synaptic strength. Thus, this second wave of retrieval-induced trafficking of AMPARs is maintained after the reconsolidation window closes[5].

AMPAR endocytosis constrains memory strengthening

If this retrieval-induced wave of GluA2-containing AMPARs is a cellular correlate of reorganization at hippocampal memory storage sites, manipulating AMPAR endocytosis should affect synaptic reconsolidation and subsequent expression of fear over time. As reconsolidation can serve two purposes, maintaining memory strength and changing memory content[11,12,15,16], we attenuated regulated glutamate receptor endocytosis by injecting the TAT-$GluA2_{3Y}$ peptide into the dorsal hippocampus 1 h before retrieval.

We examined fear expression over multiple short conditioned stimulus–only presentations to analyze changes in memory strength (**Fig. 6a**,**b**). Blocking retrieval-induced regulated AMPAR endocytosis resulted in enhanced and stable fear expression. This effect was present acutely (retrieval test 2 (RT2), 2 h after retrieval RT1) indicative of the causal action of AMPAR endocytosis for the process of reconsolidation, and long term (RT3, 24 h after retrieval) as observed classically for reconsolidation experiments (treatment, $P < 0.01$; time × treatment, $P < 0.05$; treatment, RT2, $P < 0.05$; RT3, $P < 0.01$; **Fig. 6b**).

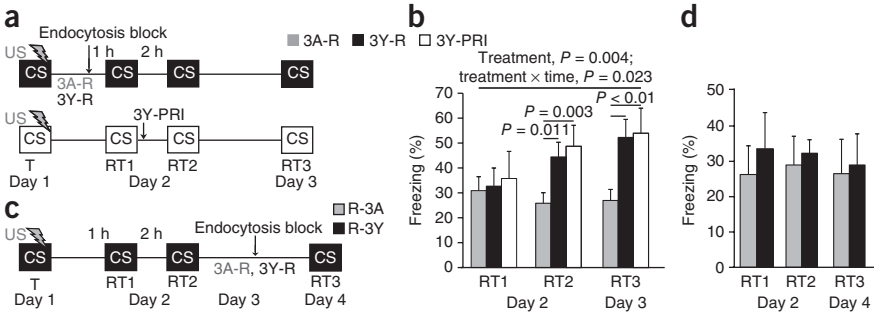

Figure 6 Retrieval-induced AMPAR endocytosis is crucial for modulating memory strength during reconsolidation. (**a**–**d**) Experimental design with two groups for the effect on reconsolidation of blocking regulated AMPAR endocytosis by dorsohippocampal injections of the $GluA2_{3Y}$ peptide (3Y) and control $GluA2_{3A}$ peptide (3A), showing timeline for training (T), testing using retrieval sessions (RT1–RT3), and dorsohippocampal injections, 1 h before retrieval (3Y-R, 3A-R, respectively) or 15 min after retrieval (3Y post-retrieval intervention, 3Y-PRI; **a**,**b**), or 24 h after retrieval (**c**,**d**). (**a**,**b**) 3A-R, $n = 10$; 3Y-R, $n = 11$; 3Y-PRI, $n = 6$; (**c**,**d**) R-3A, $n = 8$; R-3Y, $n = 8$. (**b**) On days 2 and 3, both a pre- or post-retrieval intervention resulted in a facilitated fear response (increased freezing) with a significant effect of treatment ($F_{2,24} = 6.980$) and interaction of time × treatment ($F_{2,24} = 4.178$) over all three retrieval sessions (RT1–RT3). Freezing was affected in both the short term (RT2; $F_{2,26} = 6.40$) and the long term (RT3; $F_{2,27} = 8.310$). (**d**) Blocking regulated AMPAR endocytosis outside the window of reconsolidation had no effect on freezing on the subsequent day (day 4), in contrast to blocking endocytosis within the reconsolidation window (see **a**,**b**). All data points show mean ± s.e.m.; significant *P*-values are indicated.

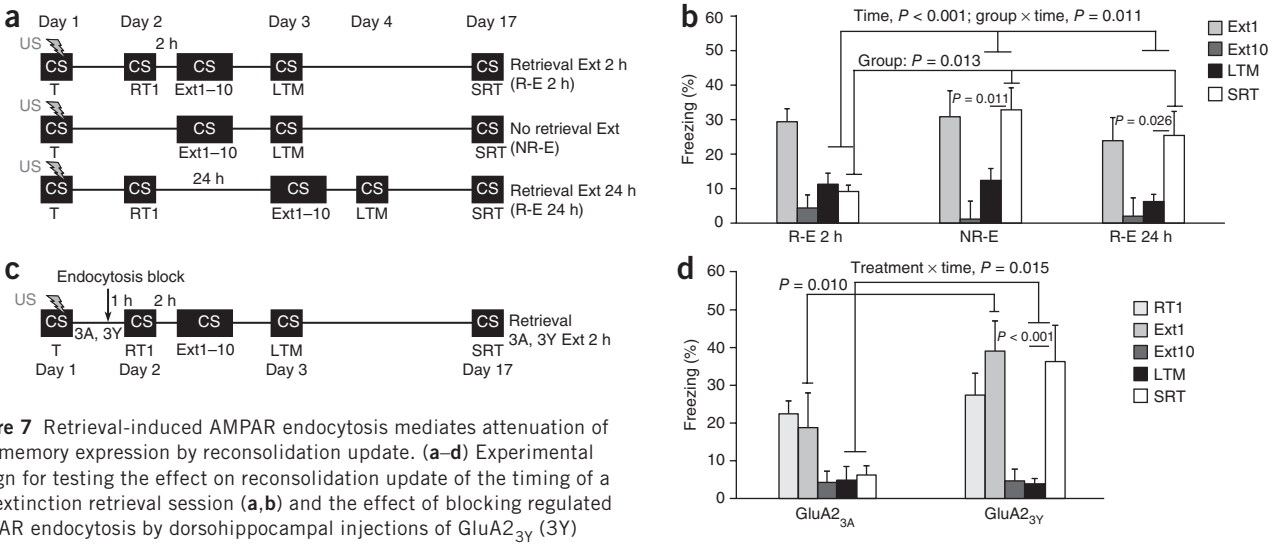

Figure 7 Retrieval-induced AMPAR endocytosis mediates attenuation of fear memory expression by reconsolidation update. (**a**–**d**) Experimental design for testing the effect on reconsolidation update of the timing of a pre-extinction retrieval session (**a**,**b**) and the effect of blocking regulated AMPAR endocytosis by dorsohippocampal injections of $GluA2_{3Y}$ (3Y) or control $GluA2_{3A}$ (3A) peptide (**c**,**d**), showing timeline for training (T), intervention and testing using retrieval sessions (RT1–RT3) or extinction (Ext1–10, a 30-min extinction session divided into 10 bins of 3 min each). LTM, long-term memory test of extinction; SRT, spontaneous recovery test. (**a**,**b**) R-E 2 h, $n = 10$; NR-E, $n = 10$; R-E 24 h, $n = 8$; (**c**,**d**) all $n = 5$. (**b**,**d**) All groups acquired extinction similarly (**Supplementary Fig. 6**), reached the same levels of freezing in the last 3 min (Ext10) of the 30-min session, and showed similar levels of freezing in the LTM test. (**b**) An effect of time ($F_{1,25} = 15.072$) and time × group ($F_{1,25} = 4.426$) was observed for groups between the LTM test on day 3 and SRT on day 17. A significant difference between groups was observed (SRT; $F_{2,27} = 5.175$), with the NR-E and R-E 24 h groups showing spontaneous recovery of fear, and R-E 2 h showing prevention of return of fear. (**d**) Treatment had an effect in the first 3 min (Ext1; $F_{1,9} = 10.01$) consistent with the acute effect on reconsolidation (**Fig. 6**). An effect of treatment ($F_{1,9} = 2.50$) and treatment × time ($F_{1,9} = 9.06$) was observed for groups between the LTM test on day 3 and SRT test on day 17. A significant difference between groups was observed (SRT; $F_{1,9} = 7.70$), with $GluA2_{3Y}$ groups showing spontaneous recovery of fear, whereas controls ($GluA2_{3A}$) showed a long-term loss of fear response. All data points show mean ± s.e.m.; significant *P*-values are indicated.

AMPAR endocytosis must not function in the initial retrieval of fear, as (i) treatment with the TAT-$GluA2_{3Y}$ peptide had no effect on fear expression in the first retrieval session (**Fig. 6b**), (ii) neither did it influence baseline activity (**Supplementary Fig. 5**), and (iii) treatment with the TAT-$GluA2_{3Y}$ peptide after retrieval yielded a similar behavioral profile, with increased expression of fear (**Fig. 6b**). In addition, the control peptide TAT-$GluA2_{3A}$ had no effect on baseline activity, with freezing behavior percentages comparable to those observed with a saline control (**Supplementary Fig. 5**).

To show that this blockade indeed coincides with the retrieval-induced GluA2 endocytosis and is specific for the time window of reconsolidation, we injected the TAT-$GluA2_{3Y}$ peptide 24 h after retrieval, a time at which there is no longer any regulation of the AMPAR subunits. Blockade of regulated endocytosis outside the retrieval-induced window of reconsolidation indeed had no effect on the expression of fear (**Fig. 6c**,**d**).

AMPAR endocytosis mediates modification of memory content

The first wave of AMPAR-mediated plasticity mirrors the time window of reconsolidation, and blocking this plasticity resulted in a more stable and enhanced fear memory. Reconsolidation by a short retrieval session represents a bidirectional modification of the original memory[3] that is time limited. Because retrieval-induced hippocampal synaptic depression seems to negatively regulate memory enhancement and memory strengthening during reconsolidation in a time-restricted manner (**Fig. 6**), we hypothesized that this molecular mechanism may also underlie the permanent attenuation of fear response by reconsolidation update; that is, modifying memory content[16,18]. Under this hypothesis, GluA2-containing AMPAR endocytosis would underlie the previously reported therapeutic effect that prevents the return of fear by the reinterpretation of emotional memories when a reconsolidation-inducing retrieval session is used before extinction[16,18].

Therefore, we first tested whether loss of fear response can be achieved for contextual memories in mice using a protocol similar to that used in rats and humans[16,18]. Mice received a retrieval session, or no retrieval, followed by a 30-min extinction session given within the reconsolidation window, 2 h after retrieval. These experiments showed that contextual memory was able to undergo reconsolidation-dependent attenuation of expression of fear memory, as only mice that received the extinction session within the time window of reconsolidation, 2 h after retrieval, showed a loss of fear response that was resistant to restoration in the long term when tested on day 17 in the spontaneous recovery test (SRT) (time, $P < 0.001$; time × group, $P < 0.05$; group: SRT, $P < 0.05$; **Fig. 7a**,**b**). Mice that did not receive a retrieval session before extinction, or that received extinction 24 h after the retrieval session, outside the reconsolidation window, spontaneously recovered fear with the passage of time, a well described passive re-emergence of fear associations[41]. A short conditioned stimulus presentation, as used in such a retrieval session, within the reconsolidation period does not result in long-term extinction (compare **Fig. 6b**, session R3). In all groups, acquisition of extinction was similar within the 30-min session (extinction session 10; Ext10), and no differences in consolidation of extinction were present as tested in a long-term extinction memory test 24 h after extinction (session LTM) (**Fig. 7b** and **Supplementary Fig. 6** (ref. 16)). This indicates that extinction leads to formation of a new memory that initially suppresses the fear memory trace, with the latter reemerging with the passage of time.

Next, to test the hypothesis that regulated AMPAR endocytosis is the mechanism that underlies this reconsolidation-dependent attenuation of expression of fear, mice that had been injected with the TAT-$GluA2_{3Y}$ blocking peptide or the TAT-$GluA2_{3A}$ control peptide into the dorsal hippocampus 1 h before retrieval were tested in the reconsolidation-update protocol (**Fig. 7c**). Blocking regulated endocytosis

of GluA2-containing AMPARs had a short-term effect in the first 3 min of the extinction session, which mimics the short-term effect on reconsolidation seen previously (treatment, $P < 0.01$; **Fig. 6b**). No effect of treatment was observed on the acquisition of the total extinction or in the last session (session Ext10), or on consolidation of extinction (session LTM) (**Fig. 7d** and **Supplementary Fig. 6**). Mice that were treated with the control peptide showed a long-term (~2.5 weeks) decrease of fear memory expression, similar to that in untreated controls (**Fig. 7b**,**d**). However, spontaneous recovery was observed in mice that received the GluA2 endocytosis blocking peptide ($P < 0.001$) (time × treatment, LTM versus SRT, $P < 0.05$), showing that the blockade of regulated AMPAR endocytosis is able to prevent an attenuation of fear memory expression. Hence, retrieval-induced regulated endocytosis of GluA2-AMPARs in the dorsal hippocampus is critical to the adaptive purpose of reconsolidation in modifying memory content, wherein extinction presented during reconsolidation leads to a persistent re-evaluation of the contextual conditioned stimulus, resulting in a long-term loss of fear response that is resistant to restoration.

DISCUSSION

Our data indicate a mechanism of biphasic GluA2-containing AMPAR plasticity in the dorsal hippocampus after retrieval that is required for adaptive reconsolidation of contextual fear memory. The hippocampus processes various properties of contextual stimuli and is thought to be crucial for reconsolidation of fear, when context is the main threatening conditioned stimulus[4,7]. We show that un-reinforced recall of contextual fear memory initially leads to regulated endocytosis of AMPARs and decrease in synaptic strength. The initial phase of synaptic depression (1–4 h), during which the memory returns to a labile state, is necessary for the subsequent increase in synaptic strength to be maintained (7 h) and is critical to the process of reconsolidation.

Initial consolidation of memory is known to depend on glutamate receptor plasticity[19,20]. Although previous studies have reported a synaptic insertion of AMPARs at hippocampal and amygdaloid synapses 24 h after auditory fear conditioning[19,42], there seems to be no increase in dorsohippocampal AMPAR surface expression 1 d after contextual foreground conditioning (without a tone) as measured here. This is in line with previous research that showed that disruption of GluA2 surface expression in the hippocampus 1 d after conditioning has no effect on maintenance of contextual fear memory[43,44], in contrast to disruption in the amygdala[43].

Reconsolidation has mostly been studied as the phenomenon that creates memory amnesia, owing to the well known effect of agents blocking the further expression of memory[3–8]. However, recent data indicates that reconsolidation is also adaptive in nature and has two main roles. The first one results in re-storage and strengthening of the memory, where the hippocampus is thought to have a putative inhibitory role[17,45]. The second one is the adaptive function of reconsolidation to incorporate new information and to update and modify previously established memories, thus altering the memory content[16,17,45]. Understanding the mechanisms occurring immediately after retrieval is instrumental in explaining how these two functions interact with each other and the effect it has on bidirectional behavioral plasticity. In line with this, the cellular mechanism identified here seems crucial to both aspects of reconsolidation. Hippocampal synaptic depression, which mirrors the period of memory malleability, seems to exert a gating, inhibitory constraint on re-storage and strengthening of memory during adaptive reconsolidation, because blocking synaptic depression leads to an enhanced expression of fear. Conversely, however, synaptic depression is critical to the adaptive reinterpretation and consequent long-term attenuation of the expression of fear memory by reconsolidation update, as blocking synaptic depression leads to the re-emergence of fear with passage of time.

Reducing or preventing the return of fear by extinction-based exposure therapies during the sensitive time window of reconsolidation could prove to be fundamental to intervention-based therapies for fear- and anxiety-related disorders. Here, we show for the first time that for contextual fear memories where context is the only threatening conditioned stimulus, therapy in the form of behavioral manipulation 2 h, but not 24 h, after an isolated retrieval trial results in persistent reevaluation of the conditioned stimulus and a long-term attenuation of the expression of fear. Most importantly, in the reconsolidation-update paradigm used by us, we measured spontaneous recovery of fear after reconsolidation update. This well described passive re-emergence of fear associations[41] only becomes apparent with passage of time (14 d in the present paradigm), as no difference between experimental groups was detected when it was assessed at shorter intervals, such as 24 h after extinction. These findings are similar to previous results[16,46]. Furthermore, we note that reconsolidation update was originally presented as a long-term loss of fear response[16,18], rather than an erasure of fear memory[42,47]. In the latter case, either the entire associative network containing the memory trace would have to be deleted, or the molecules responsible for maintaining long-term memories would have to be targeted[43,47,48]. It is more likely that expression of fear is reduced in the long term by modifying its content, with the aversive aspect of the memory being diminished[9]. Taken together, there seem to be certain conditions under which extinction training during reconsolidation yields long-term impairments of fear, which need to be further elucidated.

Extinction-induced loss of fear response has been attributed to an interference with reconsolidation of fear memory[16,18]. A recent report showed that GluA1-containing AMPARs in the lateral amygdala contribute to inhibition of expression of auditory conditioned fear[42], which fits into the conceptual framework of the results presented here, in which GluA2-containing AMPARs in the dorsal hippocampus contribute to expression of contextual fear. We show that retrieval-induced phased receptor trafficking facilitates synaptic reorganization and memory instability, allowing selective and robust manipulation of fear memory during a fixed time window to produce long-lasting effects. Indeed, blocking synaptic depression by blocking the retrieval-induced regulated endocytosis of GluA2-containing AMPARs resulted in an enhanced and stable expression of fear over time, and this fear memory imprint was rendered resistant to reinterpretation and loss of response when behavioral therapy was applied during the window of reconsolidation.

Of note, 7 h after retrieval we found reinsertion of GluA2-containing AMPARs into the synaptic membrane. This phase is dependent on the previous wave of AMPAR endocytosis and mimics the period during which a memory is fully reconsolidated, although retrieval-induced molecular and cellular changes might still be ongoing[5,49]. The results presented here show that interference with AMPAR endocytosis outside the window of reconsolidation has no effect on subsequent expression of fear. Furthermore, it has previously been shown that extinction therapy given outside the 6 h reconsolidation window does not permanently attenuate the expression of fear[16]. The first wave of AMPAR plasticity is necessary for adaptive reconsolidation to occur. This wave of synaptic weakening is pivotal for the observed behavioral effects both acutely (2 h after retrieval) and in the long term (24 h, 14 d) after interference. Although synaptic weakening is necessary for adaptive reconsolidation, we cannot rule out a contribution of the potentiated synapse 7 h after retrieval in the

modification of both memory strength and content. For the second wave of increased surface GluA2, one possibility is that it is involved in directing processes that interact with those triggered by the first wave, generating the long-term behavioral effects independent of GluA2 levels. Alternatively, the second wave, which is a consequence of the first phase (**Fig. 5**), may have no functional meaning. Further studies are required to elucidate the exact role this perpetuation of synaptic potentiation has in adaptive reconsolidation.

Taken together, this study demonstrates that adaptive reconsolidation in the hippocampus is characterized by a distinct plasticity response of hippocampal glutamatergic synapses governed by a GluA2-containing AMPAR expression profile having two discrete phases (**Supplementary Fig. 7**). The retrieval-induced AMPAR endocytosis is necessary for the time-limited synaptic remodeling that modulates the subsequent strength of expression and reinterpretation of a persistent fear memory imprint after retrieval.

METHODS

Methods and any associated references are available in the online version of the paper at http://www.nature.com/natureneuroscience/.

Note: Supplementary information is available on the Nature Neuroscience website.

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AUTHOR CONTRIBUTIONS

P.R.-R., A.B.S. and S.S. designed the molecular experiments. P.R.-R., D.C.R., H.D.M. and S.S. designed the physiological experiments. P.R.-R., O.S. and S.S. designed the behavioral experiments. P.R.-R. executed molecular experiments. D.C.R. executed physiological experiments. P.R.-R. and R.J.v.d.L. executed behavioral experiments. P.R.-R. and S.S. analyzed molecular experiments. D.C.R. and H.D.M. analyzed physiological experiments. P.R.-R. and S.S. analyzed behavioral experiments. P.R.-R., D.C.R., A.B.S. and S.S. wrote the manuscript.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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1. McGaugh, J.L. Memory–a century of consolidation. *Science* **287**, 248–251 (2000).
2. Frankland, P.W. *et al.* Stability of recent and remote contextual fear memory. *Learn. Mem.* **13**, 451–457 (2006).
3. Lee, J.L., Milton, A.L. & Everitt, B.J. Reconsolidation and extinction of conditioned fear: inhibition and potentiation. *J. Neurosci.* **26**, 10051–10056 (2006).
4. Mamiya, N. *et al.* Brain region-specific gene expression activation required for reconsolidation and extinction of contextual fear memory. *J. Neurosci.* **29**, 402–413 (2009).
5. Nader, K., Schafe, G.E. & Le Doux, J.E. Fear memories require protein synthesis in the amygdala for reconsolidation after retrieval. *Nature* **406**, 722–726 (2000).
6. Przybyslawski, J., Roullet, P. & Sara, S.J. Attenuation of emotional and nonemotional memories after their reactivation: role of beta adrenergic receptors. *J. Neurosci.* **19**, 6623–6628 (1999).
7. Suzuki, A. *et al.* Memory reconsolidation and extinction have distinct temporal and biochemical signatures. *J. Neurosci.* **24**, 4787–4795 (2004).
8. Nader, K. & Hardt, O. A single standard for memory: the case for reconsolidation. *Nat. Rev. Neurosci.* **10**, 224–234 (2009).
9. Kindt, M., Soeter, M. & Vervliet, B. Beyond extinction: erasing human fear responses and preventing the return of fear. *Nat. Neurosci.* **12**, 256–258 (2009).
10. Misanin, J.R., Miller, R.R. & Lewis, D.J. Retrograde amnesia produced by electroconvulsive shock after reactivation of a consolidated memory trace. *Science* **160**, 554–555 (1968).
11. Lee, J.L. Memory reconsolidation mediates the strengthening of memories by additional learning. *Nat. Neurosci.* **11**, 1264–1266 (2008).
12. Tronson, N.C., Wiseman, S.L., Olausson, P. & Taylor, J.R. Bidirectional behavioral plasticity of memory reconsolidation depends on amygdalar protein kinase A. *Nat. Neurosci.* **9**, 167–169 (2006).
13. Gordon, W.C. & Spear, N.E. The effects of strychnine on recently acquired and reactivated passive avoidance memories. *Physiol. Behav.* **10**, 1071–1075 (1973).
14. Lee, J.L. Reconsolidation: maintaining memory relevance. *Trends Neurosci.* **32**, 413–420 (2009).
15. Lee, J.L. Memory reconsolidation mediates the updating of hippocampal memory content. *Front. Behav. Neurosci.* **4**, 168 (2010).
16. Monfils, M.H., Cowansage, K.K., Klann, E. & LeDoux, J.E. Extinction-reconsolidation boundaries: key to persistent attenuation of fear memories. *Science* **324**, 951–955 (2009).
17. Tronson, N.C. & Taylor, J.R. Molecular mechanisms of memory reconsolidation. *Nat. Rev. Neurosci.* **8**, 262–275 (2007).
18. Schiller, D. *et al.* Preventing the return of fear in humans using reconsolidation update mechanisms. *Nature* **463**, 49–53 (2010).
19. Matsuo, N., Reijmers, L. & Mayford, M. Spine-type-specific recruitment of newly synthesized AMPA receptors with learning. *Science* **319**, 1104–1107 (2008).
20. Rumpel, S., LeDoux, J., Zador, A. & Malinow, R. Postsynaptic receptor trafficking underlying a form of associative learning. *Science* **308**, 83–88 (2005).
21. Schafe, G.E., Nader, K., Blair, H.T. & LeDoux, J.E. Memory consolidation of Pavlovian fear conditioning: a cellular and molecular perspective. *Trends Neurosci.* **24**, 540–546 (2001).
22. Alberini, C.M. Mechanisms of memory stabilization: are consolidation and reconsolidation similar or distinct processes? *Trends Neurosci.* **28**, 51–56 (2005).
23. Kida, S. *et al.* CREB required for the stability of new and reactivated fear memories. *Nat. Neurosci.* **5**, 348–355 (2002).
24. Lee, J.L., Everitt, B.J. & Thomas, K.L. Independent cellular processes for hippocampal memory consolidation and reconsolidation. *Science* **304**, 839–843 (2004).
25. Lee, S.H. *et al.* Synaptic protein degradation underlies destabilization of retrieved fear memory. *Science* **319**, 1253–1256 (2008).
26. Van den Oever, M.C. *et al.* Prefrontal cortex AMPA receptor plasticity is crucial for cue-induced relapse to heroin-seeking. *Nat. Neurosci.* **11**, 1053–1058 (2008).
27. Counotte, D.S. *et al.* Lasting synaptic changes underlie attention deficits caused by nicotine exposure during adolescence. *Nat. Neurosci.* **14**, 417–419 (2011).
28. Carroll, R.C., Lissin, D.V., von Zastrow, M., Nicoll, R.A. & Malenka, R.C. Rapid redistribution of glutamate receptors contributes to long-term depression in hippocampal cultures. *Nat. Neurosci.* **2**, 454–460 (1999).
29. Malenka, R.C. Synaptic plasticity and AMPA receptor trafficking. *Ann. NY Acad. Sci.* **1003**, 1–11 (2003).
30. Fanselow, M.S. Associative vs topographical accounts of the immediate shock-freezing deficit in rats: Implications for the response selection rules governing species-specific defensive reactions. *Learn. Motiv.* **17**, 16–39 (1986).
31. Robertson, S.D. *et al.* Insulin reveals Akt signaling as a novel regulator of norepinephrine transporter trafficking and norepinephrine homeostasis. *J. Neurosci.* **30**, 11305–11316 (2010).
32. Qiu, S., Zhao, L.F., Korwek, K.M. & Weeber, E.J. Differential reelin-induced enhancement of NMDA and AMPA receptor activity in the adult hippocampus. *J. Neurosci.* **26**, 12943–12955 (2006).
33. Malenka, R.C. Synaptic plasticity in the hippocampus: LTP and LTD. *Cell* **78**, 535–538 (1994).
34. Lee, S.H., Liu, L., Wang, Y.T. & Sheng, M. Clathrin adaptor AP2 and NSF interact with overlapping sites of GluR2 and play distinct roles in AMPA receptor trafficking and hippocampal LTD. *Neuron* **36**, 661–674 (2002).
35. Man, H.Y. *et al.* Regulation of AMPA receptor-mediated synaptic transmission by clathrin-dependent receptor internalization. *Neuron* **25**, 649–662 (2000).
36. Brebner, K. *et al.* Nucleus accumbens long-term depression and the expression of behavioral sensitization. *Science* **310**, 1340–1343 (2005).
37. Plant, K. *et al.* Transient incorporation of native GluR2-lacking AMPA receptors during hippocampal long-term potentiation. *Nat. Neurosci.* **9**, 602–604 (2006).
38. Kessels, H.W. & Malinow, R. Synaptic AMPA receptor plasticity and behavior. *Neuron* **61**, 340–350 (2009).
39. Jonas, P. The time course of signaling at central glutamatergic synapses. *News Physiol. Sci.* **15**, 83–89 (2000).
40. Lu, W. *et al.* Subunit composition of synaptic AMPA receptors revealed by a single-cell genetic approach. *Neuron* **62**, 254–268 (2009).
41. Rescorla, R.A. Spontaneous recovery. *Learn. Mem.* **11**, 501–509 (2004).
42. Clem, R.L. & Huganir, R.L. Calcium-permeable AMPA receptor dynamics mediate fear memory erasure. *Science* **330**, 1108–1112 (2010).
43. Migues, P.V. *et al.* PKMζ maintains memories by regulating GluR2-dependent AMPA receptor trafficking. *Nat. Neurosci.* **13**, 630–634 (2010).
44. Serrano, P. *et al.* PKMζ maintains spatial, instrumental, and classically conditioned long-term memories. *PLoS Biol.* **6**, 2698–2706 (2008).
45. Wang, S.H., de Oliveira Alvares, L. & Nader, K. Cellular and systems mechanisms of memory strength as a constraint on auditory fear reconsolidation. *Nat. Neurosci.* **12**, 905–912 (2009).
46. Chan, W.Y., Leung, H.T., Westbrook, R.F. & McNally, G.P. Effects of recent exposure to a conditioned stimulus on extinction of Pavlovian fear conditioning. *Learn. Mem.* **17**, 512–521 (2010).
47. Maren, S. Seeking a spotless mind: extinction, deconsolidation, and erasure of fear memory. *Neuron* **70**, 830–845 (2011).
48. Han, J.H. *et al.* Selective erasure of a fear memory. *Science* **323**, 1492–1496 (2009).
49. Nader, K. Memory traces unbound. *Trends Neurosci.* **26**, 65–72 (2003).

ONLINE METHODS

Mice. All experiments were carried out in accordance to the Animal User Care Committee of Vrije Universiteit. Adult male C57BL/6J mice (20–25 g, Charles River) were individually housed at a 12 h light/dark cycle with *ad libitum* access to food and water. Experiments were performed during the light phase. All mice were 9–10 weeks of age during testing. The number of mice used for testing is indicated in each figure. In all experiments, brains were removed after cervical dislocation.

Contextual fear conditioning. All experiments were carried out in a fear conditioning system (TSE Systems). Training and testing was performed in a Plexiglas chamber with a stainless steel grid floor with constant illumination (100–500 lx) and background sound (white noise, 68 dB sound pressure level), situated in a gray box to shield it from the outside. The chamber was cleaned with 70% ethanol before each session. Training consisted of placing mice in the chamber for a period of 180 s, after which a 2 s foot shock (0.7 mA) was delivered through the grid floor. Mice were returned to their home cage 30 s after the shock ended. For the immediate shock group, a 0.7 mA, 2 s foot shock was delivered immediately on placement in the conditioning chamber, after which the mice were allowed to explore the context for 210 s (180 s + 30 s). Baseline activity, exploration and freezing were assessed automatically. Freezing was defined as lack of any movement besides respiration and heart beat during 5 s intervals and is presented as a percentage of the total test time.

Contextual fear retrieval and spontaneous recovery test (SRT). Retrieval tests consisted of re-exposure (3 min) to context (conditioned stimulus), on day 2 (RT1 and RT2) and day 3 (RT3), 24 h after extinction training for the long-term memory test (LTM), and on day 17 to assess re-emergence of fear in the spontaneous recovery test (SRT). For retrieval in a novel context, mice were placed in an unfamiliar context (context B) 24 h after training. This context B was of the same shape and size as the conditioning context, but with a smooth floor (lacking a grid) with a white environment (380–480 lx) outside the gray box normally covering the conditioning chamber. This context was cleaned with 1% acetic acid and no background noise was provided.

Contextual fear extinction. Re-exposure (30 min) to context (conditioned stimulus) was done, 2 h or 24 h after retrieval test RT1, or 25 h after conditioning. Freezing measurements were binned per 3 min (Ext1–Ext10).

Tissue preparation and immunoblotting analysis. We dissected the dorsal half of the hippocampus at the desired time points from fresh brains and stored them at −80 °C. Synaptic membrane fractions were isolated (pooled from two or three mice, $n = 4$–6 pooled samples per group) on a discontinuous sucrose gradient, as described previously[26,27]. Protein concentration was measured by a Bradford assay (Bio-Rad). For all groups, 5 µg per sample were dissolved in SDS loading buffer and used for immunoblotting (Bio-Rad) using antibodies to GluA1 (Genscript, 1:1,000), GluA2 (Neuromab, 1:1,000), GluA3 (Abcam, 1:1,000), GluN1 (Millipore, 1:5,000), GluN2A (Abcam, 1:500) and GluN2B (Neuromab, 1:1,000). To correct for input differences, we compared the total protein amount from each sample[26], as this is a reliable method that is not dependent on a single protein for normalization. The gel was cut into two halves; the upper half, which contained the protein of interest, was used for quantitative immunoblotting analysis. The lower half was stained with Coomassie, quantified using the program Quantity One one-dimensional analysis software (Bio-Rad) and used for normalization of the input.

Slice surface biotinylation assay. These experiments were performed as described[31,32], with a few modifications. Briefly, the hippocampus was dissected from a minimum of three mice per condition, at the desired time points. Fresh slices of 300 µm containing the dorsal hippocampus were prepared in ice-cold modified artificial cerebrospinal fluid (ACSF; see the previously described procedures[26]. This was followed by incubation for 1 h in ACSF containing 1 mg ml^{-1} sulfo-NHS-SS-biotin (sulfosuccinimidyl-2-(biotinamido)ethyl-1,3-dithiopropionate; Pierce) at 4 °C with gentle shaking. One group of slices were not treated with biotin, to control for nonspecific binding to the beads. Unreacted reagent was removed by quenching with ice-cold ACSF containing 100 mM glycine. Homogenized tissue was resuspended in IP buffer (PBS containing 0.1% (wt/vol) SDS, 1% (vol/vol) Triton X-100 and protease inhibitors). An aliquot of the homogenate (200 µl) was kept aside for immunoblot analysis, and the remaining sample was incubated overnight with immobilized NeutrAvidin beads (Pierce Biotechnology). The beads were then washed in PBS buffer containing NP40 1% (vol/vol; Sigma) and treated with 2× Laemmli buffer (with 50 mM dithiothreitol) to elute the biotinylated proteins. The biotinylated proteins along with the whole homogenate were separated by SDS-PAGE and immunoblotted with an antibody to GluA2 (Neuromab, 1:1,000) as described above. After quantification, a ratio was determined for the surface biotinylated proteins to the total expression in the homogenate.

Systemic injection of protein synthesis inhibitor. To confirm that the behavioral protocols we used rendered the fear memory storage sites labile after retrieval, requiring new protein synthesis to persist further, we used systemic injections of a protein synthesis inhibitor before retrieval as described previously[7]. Briefly, anisomycin (150 mg per kilogram body weight, intraperitoneal, Sigma-Aldrich) was dissolved in PBS, pH 7.0–7.4, and injected 30 min before the first retrieval test on day 2 (**Supplementary Fig. 2**). No-shock controls received either anisomycin or an equivalent amount of PBS. At this dose, 95% of protein synthesis in the brain is blocked for the first 2 h (ref. 7).

Intrahippocampal injection of synthetic GluA2 derived peptide. Mice were anesthetized with avertin (1.2% (wt/vol), 0.02 ml g^{-1}, intraperitoneal) and chronically implanted with double guide cannulas (Plastics One) in the CA1 region of the dorsal hippocampus using a high-precision stereotaxic system, fixed to the skull using dental cement. Coordinates were based on the stereotaxic plates of the mouse brain atlas[50]. Anterior-posterior coordinates relative to bregma were 1.6 mm, and lateral coordinates relative to the midsagittal suture line were ± 1.03 mm. Buprenorphine was injected (0.1 mg kg^{-1}, subcutaneous) as an analgesic. Mice were allowed to recover for a period of at least 5 d before experimentation.

To block the regulated clathrin-coated endocytosis of AMPARs in the CA1 region of the dorsal hippocampus, we used a synthetic peptide derived from the GluA2 carboxyl terminal ($GluA2_{3Y}$: 869-YKEGYNVYG-877) and a control peptide in which the tyrosine residues were replaced by alanine ($GluA2_{3A}$: AKEGANVAG) (Genscript). Both peptides were fused to the cell membrane transduction domain of the HIV-TAT protein[26,36] and have an estimated half-life of 250–300 min after intravenous injection[36]. A dose of 15 pmol per side delivered in a volume of 0.25 µl artificial cerebrospinal fluid (ACSF) was bilaterally infused into the dorsal hippocampus using a microinjection pump (CMA/100, CMA/Microdialysis) at a flow rate of 0.33 µl min^{-1} 1 h before or 15 min after the first retrieval test (**Supplementary Figs. 2–6**) during a 90-s isoflurane (Forene; Abbott) inhalation anesthesia. The injector remained in place for 30 s after injections to prevent back flow into the double guide cannulas. To control for possible nonspecific effects of the control peptide, a saline injection was used as an extra control. For immunoblot experiments, we chose the time point 2 h after retrieval to allow optimal spread of the peptide in the dorsal hippocampus. At the end of experimentation, we verified the injection site by bilateral injection of 0.25 µl methylene blue solution, followed by histological analysis of coronal brain slices. Mice that did not receive symmetrical and bilateral injections in the CA1 region of the dorsal hippocampus were excluded from the study.

Electrophysiology. Horizontal slices of 400 µm containing the dorsal hippocampus were prepared in ice-cold modified ACSF (see previously described procedures[26]), followed by incubation in ACSF at room temperature (18–22 °C). All recording were performed at 32 °C.

Whole-cell recordings of AMPA mEPSCs from CA1 pyramidal cells were obtained in nominally Mg^{2+}-free conditions while voltage-clamping the cells at −70 mV in the presence of 100 µM DL-2-amino-5-phosphonovaleric acid. Firing-induced release of neurotransmitters and $GABA_A$-mediated currents were blocked (1 µM tetrodotoxin and 10 µM gabazine, respectively). The properties of mEPSCs were quantified using Mini Analysis software (Synaptosoft).

Statistics and analysis. Data from immunoblot experiments were analyzed using a univariate ANOVA, with unconditioned stimulus presentation or context presentation as a factor (significance set as $P < 0.05$). For multiple comparisons, significant effects were further analyzed using Fisher's least significant difference test.

Data from anisomycin experiments were analyzed using ANOVA for effects of anisomycin on the disruption of reactivated memory. Data from the fear conditioning were analyzed using a repeated-measures test for RT1 and RT2 on day 2, RT3 on day 3 and LTM and SRT to analyze the effects of all treatments on fear expression. A univariate ANOVA and *post hoc* least significant difference test were used to analyze significant effects of pharmacological treatment in specific tests (significance set as $P < 0.05$). For statistical comparisons of the electrophysiological experiments, we used two-tailed Student's *t*-tests or one-way ANOVA, Bonferroni matched.

50. Paxinos, G. & Franklin, K.B.J. *The Mouse Brain in Stereotaxic Coordinates* (Academic, San Diego, 1997).

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