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Upon retrieval, fear memories are rendered labile and prone to modifications, necessitating a restabilization process of reconsolidation to persist further. This process is also crucial to modulate 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 α-amino-3-hydroxyl-5-methyl-4-isoxazole-propionate receptor (AMPAR) membrane expression and synaptic strength in the mouse dorsal hippocampus. Blocking 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 on 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 (CS+) and an aversive unconditioned stimulus (US+) are progressively made permanent by a process of consolidation[1]. However upon retrieval, intervention by amnestic agents[2-7], either prior to 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 in order 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 on 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 both the modification of memory strength[12], as well as memory content[16,18], rendering this process a promising therapeutical target to counteract the hyper-responsive fear system. In order to fully exploit reconsolidation-based therapies that adapt the content of fear memories, leading to a loss of fear response on the long term, it is crucial to elucidate the molecular underpinnings of reconsolidation, which to this date remain obscure.

Long-lasting changes in synaptic efficacy brought about by gene transcription, protein synthesis and changes in strength of hippocampal glutamatergic synapses via 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 also necessary for memory remodeling after retrieval[4,7,17,23-25]. Here, we investigated whether the temporal profile of reconsolidation that is hypothesized to be limited to a 6 h time window[5,8] actuates 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

In order to analyze whether glutamate receptors are regulated during reconsolidation in animals receiving the US+ and retrieval (US-R), we dissected the dorsal hippocampus at 1 and 4 h post-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. S1**). These two time points were chosen as they fall within the 6-h time window after retrieval during which the memory undergoes reconsolidation[5]. After retrieval and subsequent reconsolidation the memory requires protein synthesis to persist further[7] (**Supplementary Fig. S1**). All AMPA receptor (AMPAR) subunits (GluA1-3) exhibited a down-regulation 1 h post-retrieval (31.4, 16.4, 50.20%, respectively, $p<0.05$), indicative of a depotentiated state of the synapse[28,29] (**Fig. 1a**,**b, Supplementary Fig. S2**). The observed down-regulation was specific to retrieval of an associative-contextual CS+–US+ representation, with no differences in GluA subunit expression observed in absence of a retrieval session or with retrieval in a novel context, not associated with the US+ and hence not related to the fear memory (**Fig. 1b, Supplementary Fig. S1**). Furthermore, the down-regulation was not due to aspecific 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 animals do not learn to associate the CS+ with the shock[30] (**Supplementary Fig. S1**). Together these data point to a post-synaptic 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[31], we recorded glutamatergic synaptic transmission onto CA1 pyramidal cells in the absence of a retrieval session, or 1 h after retrieval. The amplitude distribution and averages of pharmacologically isolated AMPAR-mediated miniature EPSCs (mEPSCs) of conditioned animals 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 exhibiting robust down-regulation 4 h post-retrieval (19.5, 53.5%, $p<0.05$), at a moment in which GluA1 subunits were 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**). 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[32,33]. Indeed, we corroborated the down-regulation of GluA2 receptor subunits 1 h after retrieval (**Fig. 2c**,**d**), in agreement with the observed lower amplitude of AMPAR currents (**Fig. 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 a TAT-fused GluA2-derived C-terminal peptide (TAT-GluA2$_{3Y}$)[26,36] would interfere with retrieval-induced regulation of GluA1–3. Conditioned mice and their NS-R controls received either TAT-GluA2$_{3Y}$ or its control TAT-GluA2$_{3A}$ into the CA1 region of the dorsal hippocampus 1 h prior to retrieval (**Supplementary Fig. S3**). Indeed, preventing regulated endocytosis of GluA2-containing receptors blocked the observed down-regulation of GluA2 and GluA3, but not of GluA1 subunits. Hence, our data indicate that retrieval-induced down-regulation 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 up-regulation

Because a retrieved memory is reconsolidated approximately within 6 h post-retrieval, we hypothesized that the initial depotentiation at 1-4 h post-retrieval would be followed by a stabilized state of previously induced synaptic potentiation[2,5,7]. The first indication for this was the observed re-insertion of GluA1 into the membrane 4 h post-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 that are then gradually replaced by GluA2-containing receptors that stabilize synaptic strengthening[37,38]. At the maintenance phase of reconsolidation, i.e., 7 h post-retrieval (**Fig. 4a**,**b**), GluA2 exhibited a strong up-regulation (36.2%, $p<0.05$). Moreover, a trend towards increased levels 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 increased levels of AMPAR subunits indeed translated into functional changes at glutamatergic synapses, we recorded pharmacologically isolated AMPAR-mediated mEPSCs 7 h post-retrieval. We found that the decay of AMPAR-mediated mEPSCs was significantly faster in conditioned mice than in NS-R controls (**Fig. 4c**–**f**). Changes in decay kinetics of AMPAR-mediated currents might result from differences in AMPAR subunit composition[39]. For example absence of synaptic GluA1-lacking receptors leads to faster decay of AMPA currents[40]. Our results could thus reflect the relative increase in GluA2 and GluA3 levels observed. Although the amplitude of mEPSC was similar to NS-R control levels 7 h post-retrieval (**Fig. 4c**), a significant ($p<0.05$) time-dependent difference in amplitude was measured with increased levels 7 h post-retrieval compared with those 25 h after conditioning or at 1 h post-retrieval (**Fig. 4g**,**h**).

Blocking initial AMPAR-endocytosis by intrahippocampal TAT-GluA2$_{3Y}$ injection attenuated the subsequent retrieval-induced up-regulation of AMPAR subunits (**Fig. 5a**,**b**). In addition, the decrease in decay time of AMPAR currents was again observed using the TAT-GluA2$_{3A}$ control peptide, 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 impact synaptic reconsolidation and subsequent expression of fear over time. As reconsolidation can serve two purposes, i.e., 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 prior to retrieval.

We examined fear expression over multiple short CS+-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 on the long term (RT3, 24 h after retrieval) as observed classically for reconsolidation experiments (Treatment: $p<0.01$, time x treatment: $p<0.05$; treatment: RT2, $p<0.05$; R3, $p<0.01$; **Fig. 6b**). AMPAR-endocytosis does not play a role in the initial retrieval of fear as 1) treatment with the TAT-GluA2$_{3Y}$ peptide had no effect on fear expression in the first retrieval session (**Fig. 6b**), 2) neither did it influence baseline activity (**Supplementary Fig. S4**), 3) treatment with the TAT-GluA2$_{3Y}$ peptide after retrieval showed a similar behavioral profile with increased expression of fear (**Fig. 6b**). In addition, the control peptide TAT-GluA2$_{3A}$ had no effect on base-line activity and similar levels of freezing were observed when compared to a saline control (**Supplementary Fig. S4**).

In order to show that this block 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, at a moment that there is no regulation of the AMPAR

subunits anymore. Indeed, a block of regulated endocytosis outside the retrieval-induced window of reconsolidation 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 bi-directional modification of the original memory[3] that is time-limited. Since retrieval-induced hippocampal synaptic depression appears to negatively regulate memory enhancement and memory strengthening during reconsolidation in a time-controlled manner (**Fig. 6**), we hypothesized that this molecular mechanism may also underlie the permanent attenuation of fear response by reconsolidation-update, i.e., modifying memory content[16,18]. Hence, 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 prior to extinction[16,18].

Thereto, 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]. Animals received a retrieval session – or no retrieval – followed by a 30 min extinction session given within the reconsolidation window, i.e., 2 h after retrieval. Here, we could show that contextual memory was able to undergo reconsolidation-dependent attenuation of expression of fear memory, as only animals that received the extinction session within the time window of reconsolidation, i.e., 2 h after retrieval, exhibited a loss of fear response at the spontaneous recovery test (SRT) that was invulnerable to restoration on the long term (time: $p<0.001$, time x group: $p<0.05$, group: SRT, $p<0.05$, **Fig. 7a**,**b**). Animals that did not receive a pre-extinction retrieval session, or received extinction outside the reconsolidation window, i.e., 24 h after the retrieval session, exhibited spontaneous recovery with the passage of time, a well described passive re-emergence of fear associations[41]. A short CS+ presentation, as used in such a retrieval session, within the reconsolidation period does not result in long-term extinction (*c.f.* **Fig. 6b**: session R3). In all groups, acquisition of extinction was similar within the 30 min session (session 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. S5)[16]. This indicates that extinction leads to formation of a new memory that initially suppresses the fear memory trace, with the latter re-emerging with the passage of time.

Next, in order to test the hypothesis that regulated AMPAR-endocytosis is the mechanism that underlies this reconsolidation-dependent attenuation of expression of fear, animals – injected with the TAT-GluA2$_{3Y}$ blocking peptide or the TAT-GluA2$_{3A}$ control peptide into the dorsal hippocampus 1 h prior to retrieval– were tested in the reconsolidation-update protocol (**Fig. 7c**). Blocking regulated GluA2-containing AMPAR-endocytosis 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 the last session (session Ext10), or consolidation of extinction (session LTM) (**Fig. 7d** and **Supplementary Fig. S5**). Animals that were treated with the control peptide (3A) showed long-term (~2.5 weeks) decrease of fear memory expression, similar to non-treated controls (**Fig. 7b**,**d**). However, spontaneous recovery was observed in animals that received the GluA2-endocytosis block (GluA2$_{3Y}$; $p<0.001$) (time x treatment: LTM *vs.* SRT: $p<0.05$), showing that the block 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 reevaluation of the contextual CS+, resulting in a long-term loss of fear response that was invulnerable 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 plays a major role in processing of various properties of contextual stimuli, and is thought to be crucial for reconsolidation of fear, when context is the main threatening CS+[4,7]. We showed that non-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 (**Fig. 7**).

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 appears to be no increase in dorsohippocampal AMPAR surface expression 1 day after contextual foreground conditioning (without tone) as measured here. This is in line with previous research that showed that disruption of GluA2 surface expression in the hippocampus 1 day after conditioning has no effect on maintenance of contextual fear memory[43,44], in contrast to the amygdala[43].

Reconsolidation has mostly been studied as the phenomenon that creates memory amnesia, due to the well-known effect of agents to block 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 post retrieval is instrumental in elucidating 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, appears to exert a gating, inhibitory constraint on re-storage and strengthening of memory during adaptive reconsolidation as blocking synaptic depression leads to an enhanced expression of fear (*c.f.* **Fig. 6**). On the other hand, synaptic depression is critical to the adaptive re-interpretation and consequent long-term attenuation of the expression of fear memory by reconsolidation-update (**Fig. 7**, and **Supplementary Fig. S7**), 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 CS+, therapy in the form of behavioral manipulation 2 h, but not 24 h after an isolated retrieval trial resulted in persistent reevaluation of the CS+ 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 days in the present paradigm), as no difference between experimental groups is detected when assessed at shorter intervals, e.g., 24 h after extinction (**Fig. 7**, LTM), similar as shown by both Chan et al.[46], and Monfils et al.[16]. Furthermore, it is good to note that reconsolidation-update has originally been 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 on the long-term by modifying its content, with the aversive aspect of the memory being diminished[9]. Taken together, there appear 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 play a role in the mechanism of inhibition of expression of auditory conditioned fear[42], which fits into the conceptual framework of the results presented here. We show that retrieval-induced phased-receptor trafficking facilitates synaptic re-organization and memory instability allowing for long-lasting effects of selective and robust manipulation of fear memory during a fixed time window. 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 invulnerable to reinterpretation and loss of response when behavioral therapy was employed during window of reconsolidation (**Fig. 4**).

Interestingly, 7 h after retrieval we find a reinsertion of GluA2-containing AMPARs into the synaptic membrane (**Supplementary Fig. S7**). It is important to note that 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 of AMPAR-endocytosis outside the window of reconsolidation has no effect on subsequent expression of fear (*c.f.* **Fig. 7**). Furthermore, it has previously been shown that extinction therapy given outside the 6 h reconsolidation window does not permanently attenuate the expression of fear. The first wave of AMPAR plasticity is necessary for adaptive reconsolidation to occur. This wave of depotentiation is pivotal for the observed behavioral effects both acutely (2 h post retrieval) and on the long term (24 h, 14 days) 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 GluA2 levels, one option 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**), has 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 uniquely demonstrates that adaptive reconsolidation in the hippocampus is characterized by a distinct plasticity response of hippocampal glutamatergic synapses governed by a biphasic temporal GluA2-containing AMPAR expression profile. 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.

Author contributions

PR-R, ABS, SS designed the molecular experiments

PR-R, DCR, HDM, SS designed the physiological experiments

PR-R, OS, SS designed the behavioral experiments

PR-R executed molecular experiments

DCR executed physiological experiments

PR-R, RvdL executed behavioral experiments

PR-R, SS analyzed molecular experiments

DCR, HDM analyzed physiological experiments

PR-R, SS analyzed behavioral experiments

PR-R, DCR, ABS, SS wrote the manuscript

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Figure legends

Fig. 1 **Retrieval after contextual fear consolidation leads to endocytosis of AMPARs.** (**a**) Experimental design with 5 groups, in which mice –24 h prior to a retrieval session– were exposed to the context only (no shock: NS-R), or received a shock in the same context (US-R) or in a different context (US-RCB), or did not experience retrieval (NS-NR and US-NR), were analyzed 1 h after this 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 (% *vs.* NS-R values). Representative blots with samples that were compared on the same gel are shown (approximate MW indicated; for input material, see **Supplementary Fig. S2**). Down-regulation of subunits of AMPARs 1 h post-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 or exposure to a novel context. All data points show mean±SEM, significant p-values are indicated.

Fig. 2 **Endocytosis of AMPARs is specific to retrieval of a conditioned fear memory.** (**a**,**c**) Experimental design with 3 or 2 groups that –24 h prior to retrieval– were exposed to context only (NS-R), or received a shock either immediately upon placing in the box (immediate shock: IS-R), or a delayed shock (US-R). All groups received a retrieval session the following 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 (% to NS-R). Representative blots with samples that were compared on the same gel are shown (approximate MW indicated; for input material, see **Supplementary Fig. S2**). (**b**) AMPAR subunits from the synaptic membrane fraction were downregulated 1 h post-retrieval (GluA1: $F_{(2,11)}$=6.232, GluA2: $F_{(2,11)}$=9.660, GluA3: $F_{(2,11)}$=9.986), and were not due to unspecific effects of the shock (immediate shock). (**d**) (*Left*) Ratio of AMPA receptor subunits present on the surface *vs.* present in the total homogenate using a biotinylation experiment, with ratios ≤ 1 signify decreased surface expression when compared with total homogenate. This corroborated the down-regulation of GluA2 ($F_{(1,7)}$=10.441; **Fig. 1b** and **Fig. 2b**), and concomitant reduction in AMPAR currents (**Fig. 3**). (*Right*) Example of no-biotin control before and after addition of NeutrAvidin beads for immune precipitation (upper panel: GluA2 immuno-detection; lower panel: Coomassie stain to control for input differences) shows that GluA2 cannot be detected

anymore after immune precipitation, indicative of the specificity of the method. All data points show mean±SEM, significant p-values are indicated.

Fig. 3 **Fast retrieval-induced decrease in synaptic strength in dorsal hippocampus.** (**a**) Experimental design with 4 groups, in which mice –24 h prior to the presence or absence of a retrieval session– were exposed only to the CS+ (NS-R or NS-NR), or received a shock (US-R or US-NR). The timeline 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; number of cells are indicated) is indicated. Representative recordings (**b**) of AMPAR mEPSC and resulting averages of events superimposed (**c**). (**d**) Cumulative frequency of mEPSC amplitudes shows a significant ($p<0.0001$) leftward shift in amplitude. (**d**) Bar graphs of AMPAR-mediated mEPSCs showing decreased synaptic strength in shocked mice specifically 1 h after retrieval, without AMPAR current changes after conditioning. All data points show mean±SEM, significant p-values are indicated.

Fig. 4 **Biphasic wave of synaptic AMPAR levels post-retrieval translate into functional synaptic changes in dorsal hippocampus.** (**a**) Experimental design with 2 groups, in which mice –24 h prior to retrieval– were exposed only to the CS+ (NS-R), or received a shock (US-R), and timeline for collection of dorsal hippocampi for immunoblot analysis (n=4 samples per condition), and brains for in vitro slice physiology (n=10 for 7 h NS-R, n=8 for 7 h US-R; number of cells is indicated). (**b**) Quantification (% to NS-R) of synaptic membrane fraction AMPAR subunits. Representative blots with samples that were compared on the same gel are shown (approximate MW indicated; for input material see **Supplementary Fig. S2**), showing a continued down-regulation 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. Representative recordings (**c**) of AMPAR mEPSC and resulting averages of events superimposed (**d**) 7 h after retrieval showed a change in decay of AMPAR-mediated mEPSCs. (**e**) Cumulative frequency of mEPSC decay time indicated a significant ($p<0.001$) leftward shift. (**f**) Bar graphs of AMPAR-mediated mEPSCs revealed decreased decay time in shocked mice specifically 7 h after retrieval. (**g**,**h**) Temporal analysis of AMPAR-mediated mEPSCs showed 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 bar graphs representing AMPAR mEPSC amplitude (**h**). All data points show mean±SEM, significant p-values are indicated.

Fig. 5 **AMPAR-endocytosis is crucial for subsequent AMPAR membrane insertion 7 h after retrieval.** (**a**) Experimental design with 2 main groups, in which mice –24 h prior to

retrieval– were exposed only to the CS+ (NS-R), or received a shock (US-R), and in which regulated endocytosis of GluA2-AMPARs was blocked by the peptide $GluA2_{3Y}$ vs. its control peptide $GluA2_{3A}$. Timeline for intervention (1 h before retrieval) and collection 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; number of cells is indicated) are indicated (7 h after retrieval). Preventing retrieval-induced regulated endocytosis of AMPARs attenuated subsequent up-regulation of GluA2 at the molecular (**b**; $F_{(2,11)}$=8.096; for input material, see **Supplementary Fig. S2**) and physiological level (**d–f**). Scaled and superimposed resulting averages (**d**) and cumulative frequency of decays (**e**) of AMPAR-mediated mEPSC in the presence of the $GluA2_{3Y}$ blocking peptide or the $GluA2_{3A}$ control peptide. (**f**) Group data of AMPAR-mediated mEPSC decay time. All data points shown are mean±SEM, significant p-values are indicated.

Fig. 6 **Retrieval-induced AMPAR-endocytosis is crucial to modulate memory strength during reconsolidation. (a**,**c)** Experimental design with 2 groups for the effect of blocking regulated AMPAR-endocytosis by dorsohippocampal injections for the $GluA2_{3Y}$ peptide and control ($GluA2_{3A}$) on reconsolidation and timeline for dorsohippocampal injections (1 h pre-retrieval (3Y-R, 3A-R, respectively) or 15 min post-retrieval intervention (3Y-PRI) and testing (**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 day 2 and 3, both a pre- or post-retrieval intervention resulted in a facilitated fear response with a significant effect of treatment ($F_{(2,24)}$=6.980, and interaction of time x treatment ($F_{(2,24)}$=4.178) over all 3 retrieval sessions (RT1–RT3). Freezing was affected both on 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 shown are mean±SEM, significant p-values are indicated.

Fig. 7 **Retrieval-induced AMPAR-endocytosis mediates attenuation of fear memory expression by reconsolidation-update. (a**,**c)** Experimental design for effect and timing of a pre-extinction retrieval session, and blocking regulated AMPAR-endocytosis by dorsohippocampal injections of $GluA2_{3Y}$ or control $GluA2_{3A}$ peptide, on reconsolidation-update, and timeline for intervention and testing (**a**,**b**: R-E2 h: n=10, NR-E: n=10, R-E24 h: n=8; **c**,**d**: all n=5). Ext1–10 indicates a 30-min extinction session divided into 10 bins of 3 min measurements. (**b**,**d**) All groups acquired extinction similarly (**Supplementary Fig. S6**), reached the same levels of freezing in the last 3 min (Ext10) of the 30-min session, and exhibited similar levels of freezing in the long-term memory test (LTM) of extinction. (**b**) An

effect of time ($F_{(1,25)}$=15.072) and time x group ($F_{(1,25)}$=4.426) was observed for groups between LTM and spontaneous recovery test (SRT, day 17). A significant difference between groups was observed (SRT; $F_{(2,27)}$=5.175), with the NR-E and R-E24 h groups exhibiting spontaneous recovery of fear, and prevention of return of fear in R-E2 h. (**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 x time ($F_{(1,9)}$=9.06) was observed for groups between the LTM on day 3 and spontaneous recovery at day 17. A significant difference between groups was observed (SRT; $F_{(1,9)}$=7.70), with $GluA2_{3Y}$ groups exhibiting spontaneous recovery of fear, while controls ($GluA2_{3A}$) showed a long-term loss of fear response. All data points shown are mean±SEM, significant p-values are indicated.

Methods online

Animals and fear conditioning

All experiments were carried out in accordance to the Animal User Care Committee of the VU University. 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.

Contextual fear conditioning - All experiments were carried out in a fear conditioning system (TSE-Systems, Bad Homburg, Germany). 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). The chamber was cleaned with 70% ethanol prior to 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 shock termination. 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). Base-line 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 (CS+), 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; spontaneous recovery test (SRT). For retrieval in a novel context, animals were placed in a novel, 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 (without grid) floor with a white surrounding environment (380 – 480 lx) outside of the fear conditioning box. This context was cleaned with 1% acetic acid and no background noise was provided.

Contextual fear extinction – Re-exposure (30 min) to context (CS+) was done, 2 h or 24 h after retrieval test RT1, or 25 h after conditioning. Freezing measurements was 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/group) on a discontinuous sucrose gradient, as described previously[26,27]. Protein concentration was measured by a Bradford assay (Biorad). For all groups 5 µg/sample was dissolved in SDS loading buffer and used for immunoblotting (Biorad) using antibodies against 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 that contained the protein of interest was used for quantitative immunoblotting analysis. The lower half was stained with Coomassie, quantified using the program Quantity One® 1-D analysis software (Biorad), and used for normalization of the input.

Slice surface biotinylation assay - These experiments were performed as described before[32,33], with a few modifications. Briefly, the hippocampus was dissected from a minimum of three animals 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 previously described procedures[26]. This was followed by incubation for 1 h in aCSF containing 1 mg/ml sulfo-NHS-SS-biotin (Pierce) at 4 °C with gentle shaking. One group of slices were not treated with biotin, to control for unspecific 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 (1X phosphate buffered saline (PBS) containing 0.1% SDS, 1% Triton X-100 and protease inhibitors). An aliquot of the homogenate (200 µl) was kept aside for immunoblot analysis while the remaining sample was incubated overnight with immobilized NeutrAvidin beads (Pierce Biotechnology). The beads were then washed in 1X

PBS-NP40 (Sigma) buffer and treated with 2X Laemmli buffer (with 50 mM DTT) to elute the biotinylated proteins. The biotinylated proteins along with the whole homogenate were separated by SDS-PAGE, and immunoblotted with an antibody against 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

In order to confirm that the behavioural protocols we use render the fear memory storage sites labile after retrieval, requiring new protein synthesis to persist further, we used systemic injections of a protein synthesis inhibitor prior to retrieval as described previously[7]. Briefly, anisomycin (Ani, 150 mg/kg, i.p., Sigma-Aldrich, St. Louis, MO, USA) was dissolved in phosphate buffered saline (Sal) (pH adjusted to 7–7.4), and injected 30 min prior to the first retrieval test on day 2 (**Supplementary Fig. 2**). No shock controls received either anisomycin or an equivalent amount of phosphate buffered saline. At this dose 95% of protein synthesis in the brain is blocked for the first 2 h[7].

Intra-hippocampal injection of synthetic GluA2 derived peptide

Mice were anaesthetized with avertin (1.2%, 0.02 ml/g, i.p.) and chronically implanted with double guide cannulas (Plastics One, Roanoke, VA, USA) in the CA1 region of the dorsal hippocampus using a high precision stereotaxic system, and 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 mid sagittal suture line were ± 1.03 mm. Buprenorphine was injected (0.1 mg/kg, s.c.) as analgesic. Animals were allowed to recover for a period of 5 days prior to experimentation.

To block the regulated clathrin-coated endocytosis of AMPARs in the CA1 region of the dorsal hippocampus we made use of a synthetic peptide derived from the GluA2 carboxyl terminal ($GluA2_{3Y}$: ^{869}YKEGYNVYG^{877}) and a control scrambled peptide, in which the tyrosine residues are replaced by alanine ($GluA2_{3A}$: AKEGANVAG) (Genscript, USA). Both peptides are 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 i.v. injection[36]. A dose of 15 pmol/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, Solna, Sweden) at a flow rate of 0.33 µl/min 1 h prior to or 15 min after the first retrieval test (**Supplementary Fig. 2–6**) during a 90 s isoflurane (Forene, Abbott, Kent, UK) 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 unspecific effects of the control peptide, a saline injection was used as additional control. For immunoblot experiments, the time point of 2 h post-retrieval was chosen, to allow for optimal spread of the peptide within the dorsal hippocampus. At the end of experimentation, verification of injection site was carried 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

Mice were decapitated either 1, 2 or 7 h after exposure to the CS+ and 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. All recording were performed at 32 °C.

Whole cell recordings of AMPA miniature synaptic currents (mEPSC) from in 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 APV. Firing-induced release of neurotransmitters and GABAA mediated currents were blocked (1 µM TTX and 10 µM gabazine, respectively). The properties of mEPSCs were quantified using Mini Analysis software (Synaptosoft, Decatur, GA, USA).

Statistics and Analysis

Data from immunoblot experiments were analysed using a univariate ANOVA, with US^{+} presentation or context presentation as a factor (significance set as $p<0.05$). For multiple comparisons significant effects were further analyzed using a Fisher's least significant difference (LSD) 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 LSD test was used to analyze significant effects of pharmacological treatment in specific tests (significance set as $p<0.05$). For statistical comparisons of the electrophysiological experiments, the two tailed Student's t-tests were used or the one-way ANOVA, Bonferroni matched.

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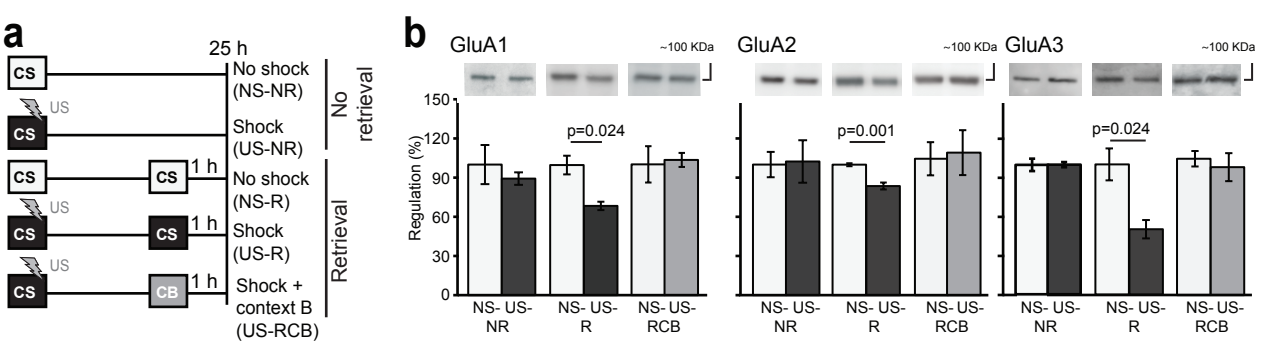
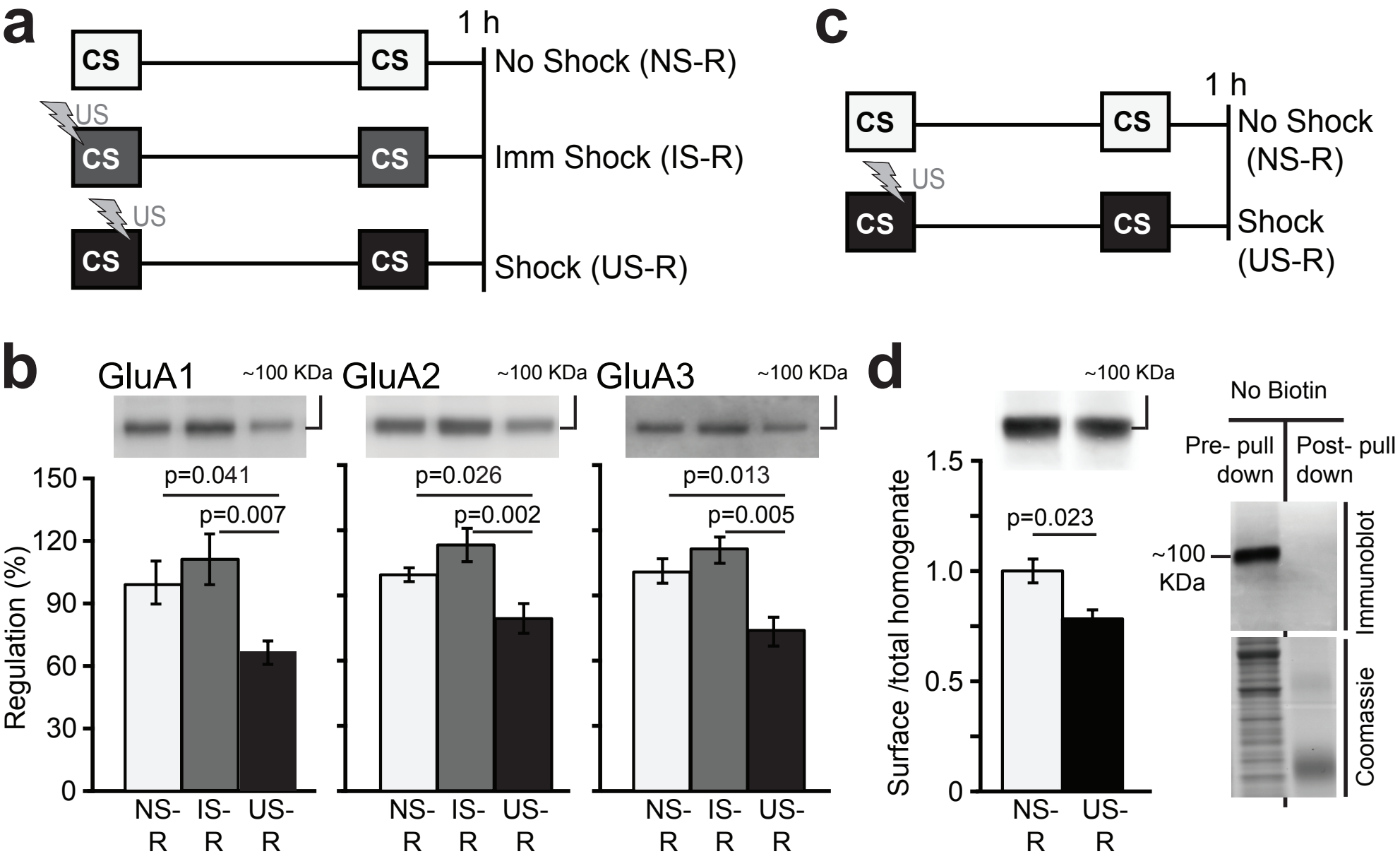
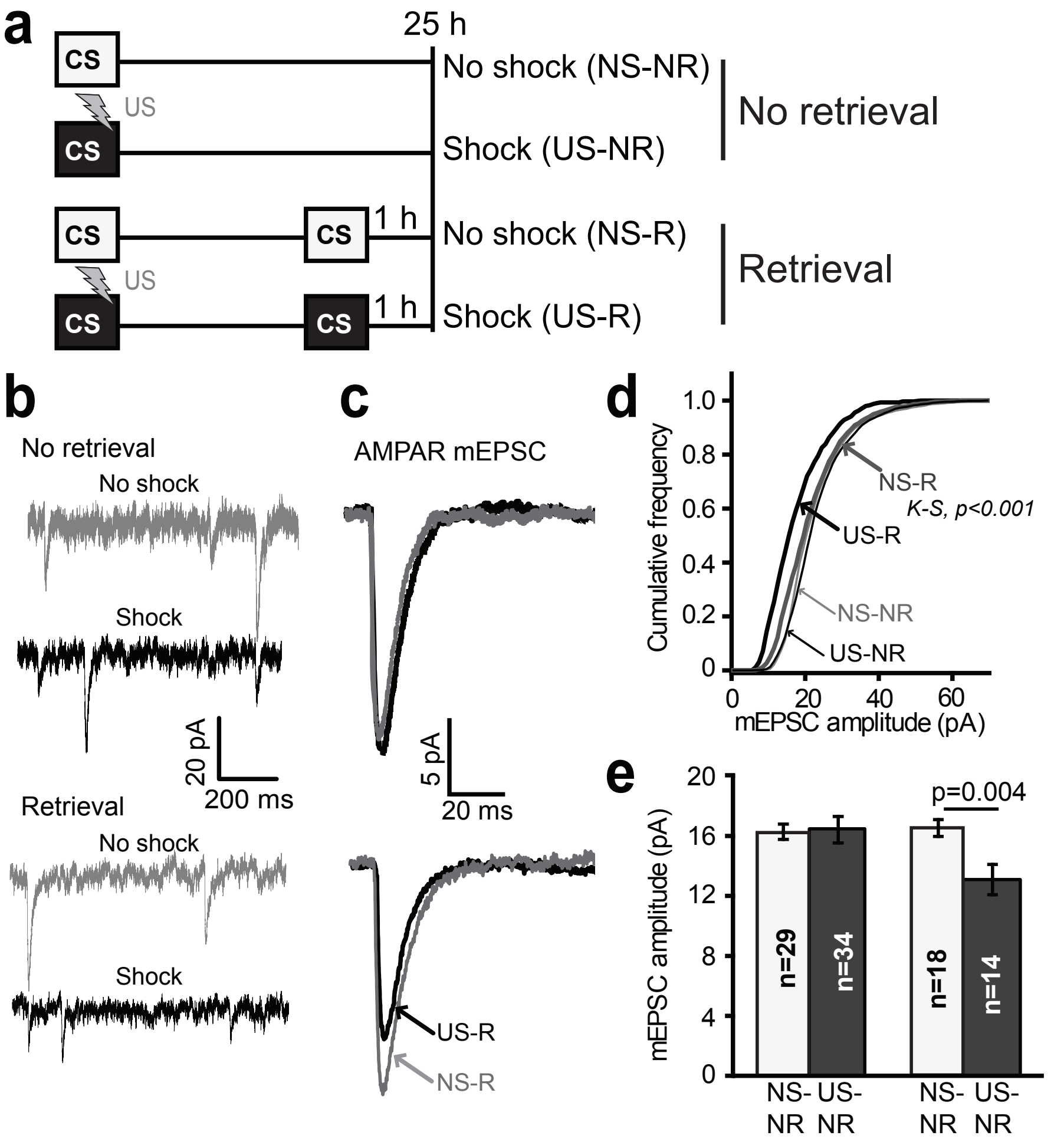
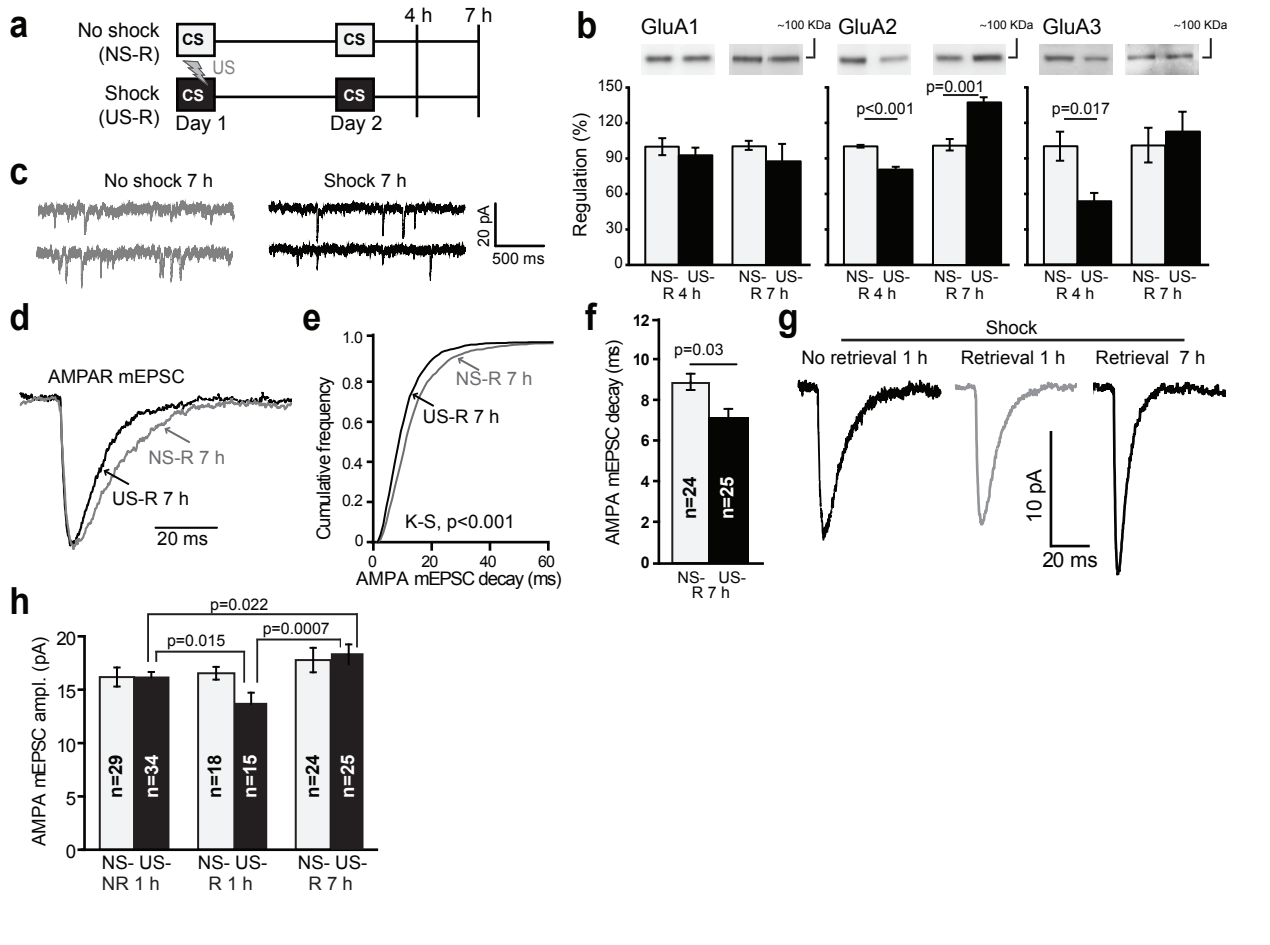
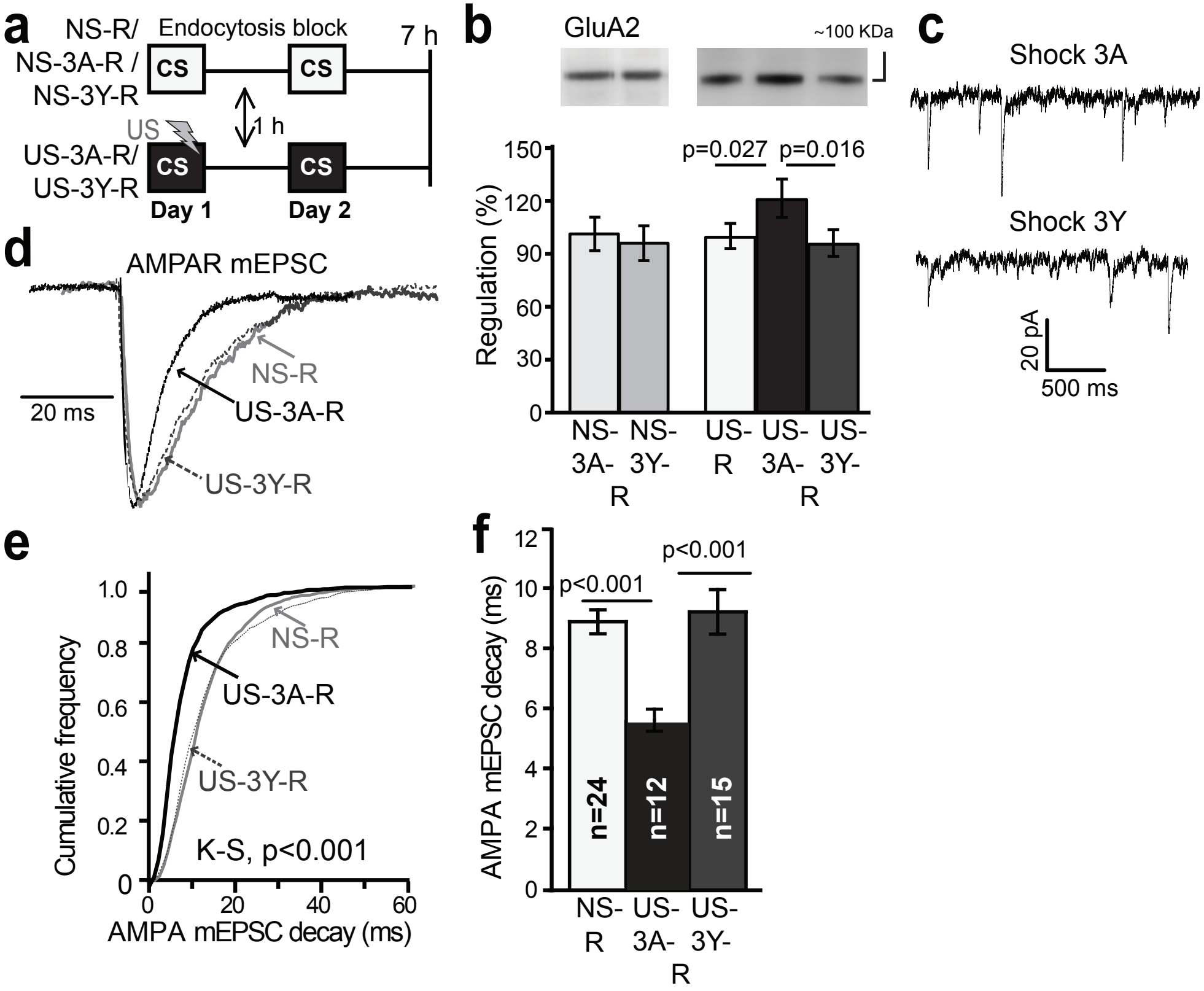
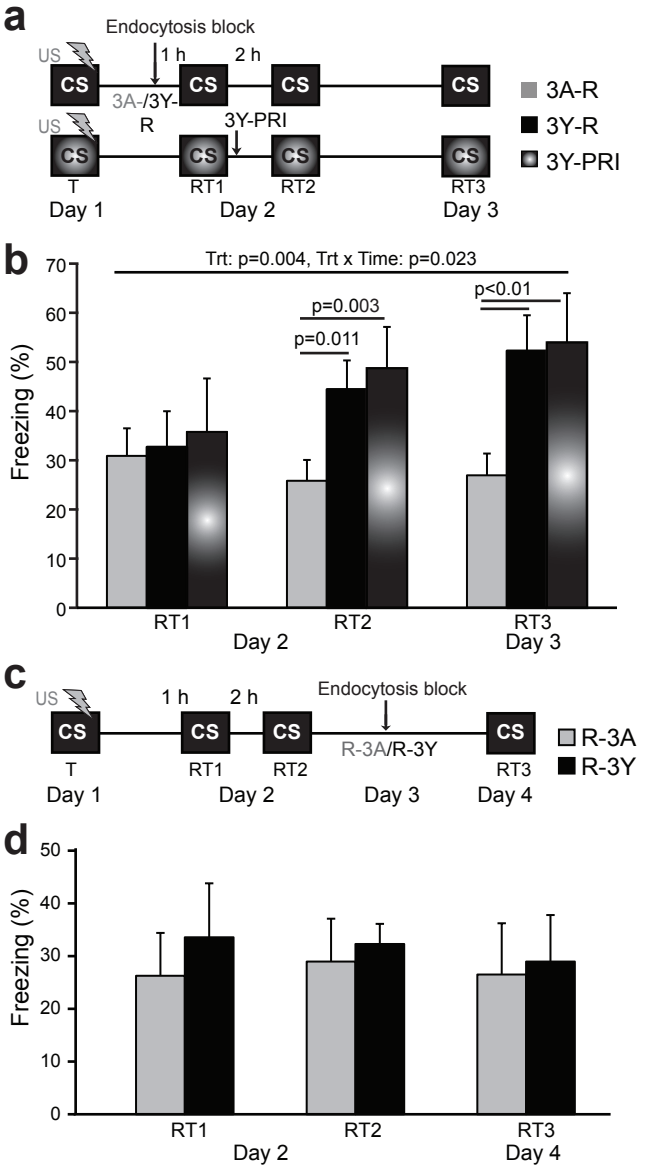

a
25 h
CS
No shock
(NS-NR)
No retrieval
US
CS
Shock
(US-NR)
CS
CS
1 h
No shock
(NS-R)
US
CS
CS
1 h
Shock
(US-R)
US
CS
CB
1 h
Shock +
context B
(US-RCB)
Retrieval
b
GluA1
~100 KDa
GluA2
~100 KDa
GluA3
~100 KDa
Regulation (%)
150
120
90
60
30
0
p=0.024
p=0.001
p=0.024
NS- US-
NR
NS- US-
R
NS- US-
RCB

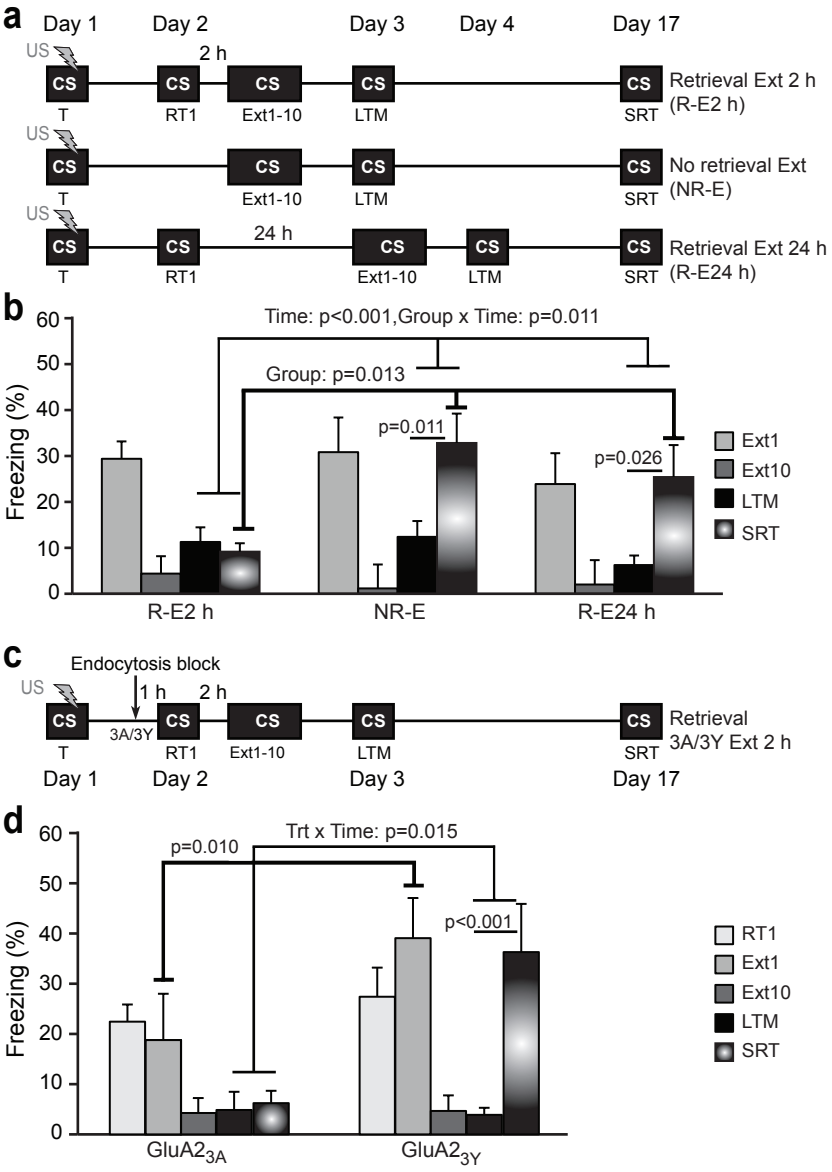

a
1 h
CS
CS
No Shock (NS-R)
US
CS
CS
Imm Shock (IS-R)
US
CS
CS
Shock (US-R)
c
1 h
CS
CS
No Shock
(NS-R)
US
CS
CS
Shock
(US-R)
b
GluA1
~100 KDa
GluA2
~100 KDa
GluA3
~100 KDa
p=0.041
p=0.007
p=0.026
p=0.002
p=0.013
p=0.005
Regulation (%)
150
120
90
60
30
0
NS-R
IS-R
US-R
d
~100 KDa
No Biotin
Pre- pull down
Post- pull down
~100 KDa
Immunoblot
Coomassie
Surface /total homogenate
1.5
1.0
0.5
0
p=0.023
NS-R
US-R


a
25 h
CS
US
No shock (NS-NR)
Shock (US-NR)
No retrieval
1 h
No shock (NS-R)
Shock (US-R)
Retrieval
b
No retrieval
No shock
Shock
20 pA
200 ms
Retrieval
c
AMPAR mEPSC
5 pA
20 ms
US-R
NS-R
d
Cumulative frequency
NS-R
K-S, p<0.001
US-R
NS-NR
US-NR
mEPSC amplitude (pA)
e
mEPSC amplitude (pA)
p=0.004
n=29
n=34
n=18
n=14
NS-NR
US-NR


a
No shock (NS-R)
Shock (US-R)
CS
US
4 h
7 h
Day 1
Day 2
b
GluA1
GluA2
GluA3
~100 KDa
Regulation (%)
p<0.001
p=0.001
p=0.017
NS- R 4 h
US- R 4 h
NS- R 7 h
US- R 7 h
c
No shock 7 h
Shock 7 h
20 pA
500 ms
d
AMPAR mEPSC
NS-R 7 h
US-R 7 h
20 ms
e
Cumulative frequency
K-S, p<0.001
AMPA mEPSC decay (ms)
f
AMPA mEPSC decay (ms)
p=0.03
n=24
n=25
g
Shock
No retrieval 1 h
Retrieval 1 h
Retrieval 7 h
10 pA
20 ms
h
AMPA mEPSC ampl. (pA)
p=0.022
p=0.015
p=0.0007
n=29
n=34
n=18
n=15
n=24
n=25
NS- NR 1 h
US- NR 1 h
NS- R 1 h
US- R 1 h
NS- R 7 h
US- R 7 h


a
NS-R/
NS-3A-R /
NS-3Y-R
Endocytosis block
7 h
CS
CS
1 h
US
US-3A-R/
US-3Y-R
CS
CS
Day 1
Day 2
b
GluA2
~100 KDa
p=0.027
p=0.016
Regulation (%)
150
120
90
60
30
0
NS-3A-R
NS-3Y-R
US-R
US-3A-R
US-3Y-R
c
Shock 3A
Shock 3Y
20 pA
500 ms
d
AMPAR mEPSC
20 ms
NS-R
US-3A-R
US-3Y-R
e
Cumulative frequency
1.0
0.8
0.6
0.4
0.2
0
NS-R
US-3A-R
US-3Y-R
K-S, p<0.001
0
20
40
60
AMPA mEPSC decay (ms)
f
AMPA mEPSC decay (ms)
12
10
8
6
4
2
0
p<0.001
p<0.001
n=24
n=12
n=15
NS-R
US-3A-R
US-3Y-R


a
Endocytosis block
US
1 h
2 h
CS
3A-/3Y-R
3Y-PRI
3A-R
3Y-R
3Y-PRI
T
RT1
RT2
RT3
Day 1
Day 2
Day 3
b
Trt: p=0.004, Trt x Time: p=0.023
p=0.003
p=0.011
p<0.01
Freezing (%)
RT1
RT2
RT3
Day 2
Day 3
c
R-3A/R-3Y
R-3A
R-3Y
Day 4
d


a
Day 1
Day 2
Day 3
Day 4
Day 17
US
2 h
CS
T
RT1
Ext1-10
LTM
SRT
Retrieval Ext 2 h
(R-E2 h)
No retrieval Ext
(NR-E)
24 h
Retrieval Ext 24 h
(R-E24 h)
b
Time: p<0.001,Group x Time: p=0.011
Group: p=0.013
p=0.011
p=0.026
Freezing (%)
Ext1
Ext10
LTM
SRT
R-E2 h
NR-E
R-E24 h
c
Endocytosis block
1 h
2 h
3A/3Y
Retrieval
3A/3Y Ext 2 h
d
Trt x Time: p=0.015
p=0.010
p<0.001
RT1
Ext1
Ext10
LTM
SRT
GluA2$_{3A}$
GluA2$_{3Y}$