Neurobiology of Learning and Memory 139 (2017) 98–108

Contents lists available at ScienceDirect

Neurobiology of Learning and Memory

journal homepage: www.elsevier.com/locate/ynlme

Altered AMPA receptor expression plays an important role in inducing bidirectional synaptic plasticity during contextual fear memory reconsolidation

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ARTICLE INFO

Article history:
Received 1 June 2016
Revised 19 December 2016
Accepted 23 December 2016
Available online 27 December 2016

Keywords:
Reconsolidation
Memory
Potentiation
Depression
Glutamate
Learning
Plasticity

ABSTRACT

Retrieval of a memory appears to render it unstable until the memory is once again re-stabilized or *reconsolidated*. Although the occurrence and consequences of reconsolidation have received much attention in recent years, the specific mechanisms that underlie the process of reconsolidation have not been fully described. Here, we present the first electrophysiological model of the synaptic plasticity changes underlying the different stages of reconsolidation of a conditioned fear memory. In this model, retrieval of a fear memory results in immediate but transient alterations in synaptic plasticity, mediated by modified expression of the glutamate receptor subunits GluA1 and GluA2 in the hippocampus of rodents. Retrieval of a memory results in an immediate impairment in LTP, which is enhanced 6 h following memory retrieval. Conversely, memory retrieval results in an immediate enhancement of LTD, which decreases with time. These changes in plasticity are accompanied by decreased expression of GluA2 receptor subunits. Recovery of LTP and LTD correlates with progressive overexpression of GluA2 receptor subunits. The contribution of the GluA2 receptor was confirmed by interfering with receptor expression at the postsynaptic sites. Blocking GluA2 endocytosis restored LTP and attenuated LTD during the initial portion of the reconsolidation period. These findings suggest that altered GluA2 receptor expression is one of the mechanisms that controls different forms of synaptic plasticity during reconsolidation.

1. Introduction

Memory formation and retrieval constitute two important aspects of cognition. Failures of memory are critical markers of a variety of neurodegenerative conditions. However, in recent years it has become clear that even when pathology is not an issue, memory reliability (by forgetting, persistence, or distortion) can be altered by multiple external manipulations. Although many cognitive (i.e., top-down) processes have been used to identify and explain decreased memory reliability (Loftus, 2003), the physiological process of storing and retrieving memories may provide cues for some of its failures. For example, it is well known that new memories are in a labile state until they are *consolidated* (i.e., stabilized), a process that takes place over a period of time ranging from minutes to hours (Dudai, 1996; McGaugh, 2000). Stabilization through consolidation, however, does not make these memories permanent. Instead, recent evidence suggests consolidated memories can return to a transient labile state each time they are retrieved, and these labile traces must undergo a process similar to consolidation; that is, they must be *reconsolidated* (Kida, 2014; Kim, Moki, & Kida, 2011; Misanin, Miller, & Lewis, 1968; Nader & Einarsson, 2010; Nader & Hardt, 2010; Nader, Schafe, & Le Doux, 2000). This reconsolidation process occurs over a period of approximately 6 h in rodents (Krawczyk et al., 2015; Nader & Hardt, 2010; Nader et al., 2000; Przybyslawski, Roullet, & Sara, 1999; Suzuki et al., 2004), and it appears to be essential for

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http://dx.doi.org/10.1016/j.nlm.2016.12.013
1074-7427/

memory maintenance. For example, procedures that disrupt the reconsolidation process (e.g., protein synthesis inhibitors, such as anisomycin) can result in amnesia of recently-retrieved information (Rodriguez-Ortiz, Garcia-DeLaTorre, Benavidez, Ballesteros, & Bermudez-Rattoni, 2008). Furthermore, new information acquired during the reconsolidation period can permanently alter retrieved memories (Monfils, Cowansage, Klann, & LeDoux, 2009; Rose & McGlynn, 1997; Zelikowsky, Bissiere, et al., 2013; Zelikowsky, Hast, et al., 2013).

Characterizing the processes that underlie memory reconsolidation is fundamental to our understanding of memory formation, maintenance, modification, and disruption. Over the last decade, there has been mounting experimental support for the critical involvement of de novo protein synthesis and early gene expression in memory reconsolidation in the hippocampus (Matsuo, Reijmers, & Mayford, 2008; Rumpel, LeDoux, Zador, & Malinow, 2005). Furthermore, there also seems to be a critical role for glutamate receptor expression and activity on reconsolidation. Alterations in α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid receptors (AMPA receptor) trafficking and function have been proposed as mediators of these downstream cellular processes in the hippocampus (Rao-Ruiz et al., 2011). AMPA receptors also play a crucial role in synaptic efficacy, being critical for both synaptic strengthening (long-term potentiation [LTP]; Bear & Abraham, 1996; Fonseca, Nägerl, & Bonhoeffer, 2006) and weakening (long-term depression [LTD]; Bear & Abraham, 1996). The balance between LTP and LTD (i.e., metaplasticity) is important for new memory traces to be processed for long-term storage. However, there is limited information on how basal synaptic communication and plasticity change through the reconsolidation period. Synaptic plasticity may be impaired after retrieval, since LTP appears to be insensitive to protein synthesis inhibitors unless re-stimulation occurs during the maintenance phase (Fonseca et al., 2006), and it is also known that hippocampal miniature excitatory post-synaptic currents (mEPSCs) shift to lower amplitudes (Rao-Ruiz et al., 2011). There is little to no information on how retrieval affects LTD, but manipulations that result in reduced memory after retrieval also result in decreased AMPA receptor activity, a characteristic of LTD (Clem & Huganir, 2010). Importantly, these previous studies take a snapshot of synaptic plasticity at a single point after retrieval, undermining the possible changes that may take as time progresses. Starting at the point of memory retrieval, expression of the GluA1 and GluA2 subunits of AMPA receptor is reportedly initially reduced (1 h), normalizes midway through the reconsolidation period (4 h), and GluA2 expression is increased after reconsolidation (7 h; Rao-Ruiz et al., 2011). However, how LTP and LTD are expressed during the reconsolidation period, and whether this expression is consistent with changes in AMPA receptor expression at different time points after memory retrieval is not known. To assess this question, the present studies investigate the physiological relations among alterations in AMPA receptor trafficking, synaptic plasticity, and protein synthesis during memory reconsolidation in the hippocampus. Furthermore, we also investigate if interfering with AMPA receptor expression during the initial period of reconsolidation impacts the pattern of plasticity occurring post retrieval. Finally, our studies investigate whether *de novo* protein synthesis has to occur in conjunction with AMPA receptor expression for maintenance of a fear memory after reconsolidation. This strategy should advance our overall and basic understanding of the physiological mechanisms underlying synaptic plasticity changes during memory reconsolidation, and provide a comprehensive model of the pattern of plasticity induced by memory retrieval that can be used for future manipulations of memory stability and reliability under various conditions.

2. Methods

2.1. Fear conditioning and behavioral assessment of fear memory

The subjects were outbred, male, Sprague-Dawley rats (2–4 months of age, Charles River Laboratories, Wilmington, MA). Animals were placed in a standard rat operant chamber (housed in a sound-isolation cubicle). The chamber's (hereon, the *context*) grid floors could be electrified to deliver a foot-shock. Animals were trained with a *conditioned freezing* protocol. During fear conditioning, animals in the Retrieval (Rtv) group were placed in context A for 180 s, at which time a 2-s, 0.75-mA foot-shock was delivered. Control animals received the same treatment, but experienced no shock in context A; thus, although a memory of having been in the context should be activated, this memory should not be associative. A further subset of subjects received shock in a different context, B (NoRtv); thus, these animals had experience with shock but not associated to the target context. The data from this latter group of subjects were contrasted against those of the Control group to determine the validity of the Control. Twenty-four h later, all animals were returned to context A for 180 s (retrieval manipulation). No shocks were delivered during retrieval. Memory of the conditioning manipulation was expected to result in freezing behavior (absence of all movements except for those related to respiration). Subjects were randomly divided into 3 groups and euthanized after 1, 4, or 6 h after retrieval (see Fig. 1A for a summary and timeline of the interventions). Control subjects were also euthanized at 1, 4, or 6 h after retrieval. There were no differences between control subjects based on time to euthanasia, and the data from all control subjects were pooled together for data analysis. Animals that received conditioning in a second context were euthanized at the same time points as the previous two groups. Once again, differences were not observed based on time to euthanasia, and the data from these subjects was pooled together for data analyses yielding the NoRtv condition. All live animal procedures were approved by the Auburn University Animal Care and Use Committee (IACUC), and animals were euthanized (in a CO_2 chamber) in accordance with the American Veterinary Medical Association (AVMA) Panel on Euthanasia regulations.

2.2. PSD-95 pull down

Rat brains were dissected, and hippocampi and cerebella were separated with continuous washing in PBS for protein extraction. PSD-95 fractions were pulled down using Magnetic Beads (Millipore, Billerica, MA). Briefly, 40 µl magnetic beads were washed with 500 µl of 1X IMP buffer (pH = 7.4) and incubated with PSD-95 antibody (1:10, Santa-Cruz, Dallas, TX). The immunoprecipitated fraction was purified by washing it several times with 1X IMP buffer (pH = 7.4). Equal amounts of samples were loaded on to an SDS PAGE gel to probe for the presence of PSD-95, as well as its interaction with GluA1/2 receptor subunits (1:1000, Cell Signaling, Danvers, MA). All blots were probed with Dy-Light conjugated 550 anti-rabbit secondary (1:10,000, Thermo Scientific, Waltham, MA).

2.3. Systemic injection of drugs and behavioral assessment

The GluA2 endocytosis blocker TAT-GluA2-3Y [1.5 nM/gm, Ana Spec, Freemont, CA, (Dias, Wang, & Phillips, 2012)] was used to control GluA2 levels and determine their role on observed plasticity patterns recorded during the reconsolidation period. Control subjects received the scrambled peptide TAT-GluA2-3A or equivalent volume of vehicle; all compounds were administered intravenously [i.v.] through the lateral tail vein 30 min prior to the

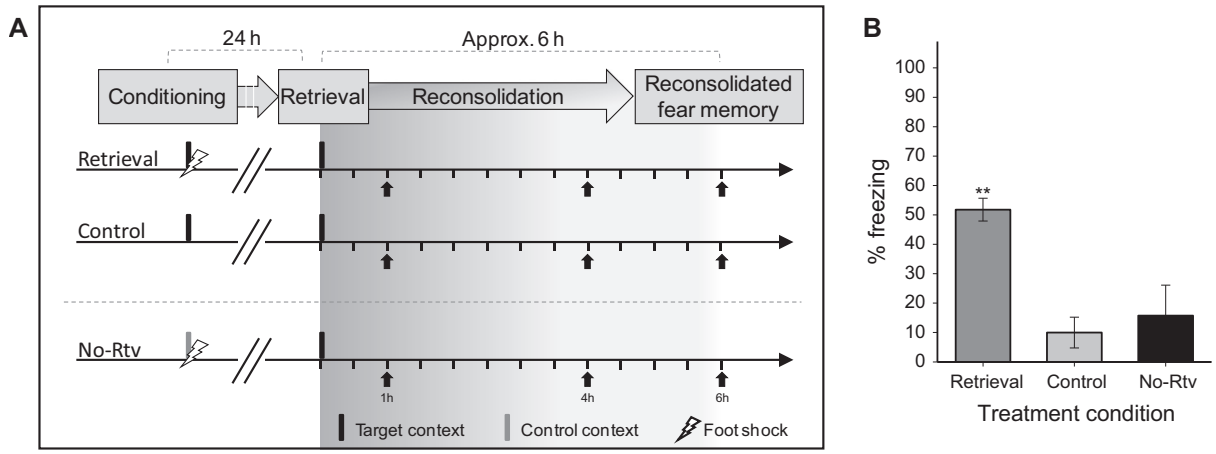

Fig. 1. A. Schematic design and timing of events for the basic retrieval manipulation. During conditioning, a 3-min context exposure (represented by small rectangles above the timeline) was (Retrieval and NoRetrieval [No-Rtv]) or was not (Control) paired with delivery of electric footshock (lightning symbol). Memory of the conditioning trial was assessed as percent time freezing in the conditioning context (Retrieval and Control) or a novel context (NoRetrieval) 24 h later in a second 3-min session. Animals were then euthanized for biochemical and electrophysiological experiments 1, 4, or 6 h after retrieval (upward arrows). Tick marks represent 30-min intervals. The shaded area represents the progression of reconsolidation, from highest destabilization of memory (darker portion) to memory restabilization (lighter portion). B. Freezing scores expressed as percent freezing during retrieval of memory of the conditioning experience. The figure presents freezing after pairings of the context with footshock (Retrieval, Rtv, *ns* = 14–18), exposure to the context (Control, *ns* = 9), or conditioning in a context different from the context of Retrieval (NoRtv, *ns* = 9). Freezing was observed only in the Rtv condition, $F_{1,\ 74} = 41.08$ ($^{**} = p < 0.01$ in post hoc comparisons [Tukey's test] against control).

retrieval manipulation. The effects of blocking GluA2 endocytosis on memory retrieval were assessed behaviorally as follows. On the retrieval day, animals received their scheduled drug or vehicle 30 min prior to retrieval and, 5 min following retrieval, they received a single intraperitoneal [i.p.] dose of anisomycin [50 mg/kg, s.c., dissolved in 99.5% pure DMSO at 20 mg/ml, Sigma Aldrich, St. Louis, MO; (Kwapis, Jarome, Schiff, & Helmstetter, 2011; Lee, 2008; Mac Callum, Hebert, Adamec, & Blundell, 2014; Rodriguez-Ortiz et al., 2008; Schiller et al., 2010)] or vehicle (DMSO alone, equal volume needed to dissolve amount equivalent to 50 mg/kg anisomycin). Animals were then returned to their home cages and memory of the conditioning experience was again assessed after a 3d retention interval (see Fig. 3 for details).

2.4. Long term potentiation and depression

Transverse hippocampal slices (350 μM) were sectioned using a Leica VT-1200S (Parameshwaran et al., 2013) 1, 4 and 6 h after memory retrieval following the same time point design as in the PSD pull down experiments. Briefly, slices were sectioned while submerged in high sucrose cutting solution (in mM: 85 NaCl, 2.5KCl, 4 MgSO4, 0.5 CaCl2, 1.25 NaH2PO4, 25 NaHCO3, 25 glucose, 75 sucrose, 0.5 ascorbate, and 2 kynurenic acid) maintained at 0-4 °C. After sectioning was completed, the slices were incubated for 1 h in artificial Cerebrospinal Fluid (aCSF in mM: 119 NaCl, 2.5 KCl, 1.3 MgSO4, 2.5 CaCl2, 1 NaH2PO4, 26 NaHCO3 and 11 dextrose). Kynurenic acid was used in cutting solution to reduce excitotoxicity of tissue during slicing. Slice incubation and recording solutions were devoid of any kynurenic acid. Note that kynurenic acid might potentially affect the glutamatergic neurotransmission necessary for LTP/LTD. Thus, the effect of kynurenic acid on LTP/LTD was controlled for by comparing all retrieval groups against the control groups (noShock and NoRtv), in which normal LTP was expressed at all time points assessed (time of slicing in cutting solution with kynurenic acid to field recording for each group remained same; for further details on the method of slicing, see Parameshwaran et al., 2013). All solutions were bubbled with 95%O2/5%CO2. Following incubation, electrophysiological recordings were performed in a submerged chamber with continuous aCSF perfusion (2–3 ml/min) at room temperature (25 °C). Field excitatory postsynaptic potentials (fEPSPs) from Schaffer Collateral pathway SC-CA1 synapses were measured with a glass pipette filled with aCSF (2–4 MΩ). Stimulating pulses were applied at Schaffer collaterals via a bipolar stimulating electrode positioned 300 μm closer to CA3 subfield than recording electrode. Frequency of test stimulation was 0.33 Hz (every 20 s). For stimulus response curves current intensity was altered from 0, 5, 10, and then 20–100 μA at steps of 20μA. For LTP and LTD experiments, baseline was recorded at 50% of amplitude at which initial population spike appeared. LTP was induced after 10 min of stable baseline recording using Theta Burst Stimulation protocol (10 bursts of stimuli, each of four pulses at 100 Hz, 200 ms, and 20 s intervals between individual TBS), and recording was continued till 50–60 min post TBS. Separately, fEPSP was recorded for 50–60 min without inducing any protocol to verify that baseline was stable (for both LTP and LTD recordings). LTD was induced using two low frequency stimuli (LFS: 900 pulses at 1 Hz) delivered at an interval of 10 min and preceded by 15 min of stable baseline. Stimulation intensity while recording baseline, in between two trains of LFS, and immediately after induction were set to 30% of maximal fEPSPs. The stimulation intensity was set to 50% when 1 Hz LFS trains were delivered (Hrabetova et al., 2000; Kochlamazashvili et al., 2010; Parameshwaran et al., 2013).

2.5. Statistical analysis

All data were analyzed using analyses of variance (ANOVA), either as one-way analyses or factorial analyses as detailed in the results section. All significant omnibus tests were followed by Tukey post hoc comparisons, except where expected effects were assessed with planned pairwise comparisons derived from the error term of the main ANOVA. Nonsignificant effects are reported where appropriate.

3. Results

3.1. Fear conditioning

Percent time freezing during the 3-min retrieval session was used as an index of memory of the conditioning experience.

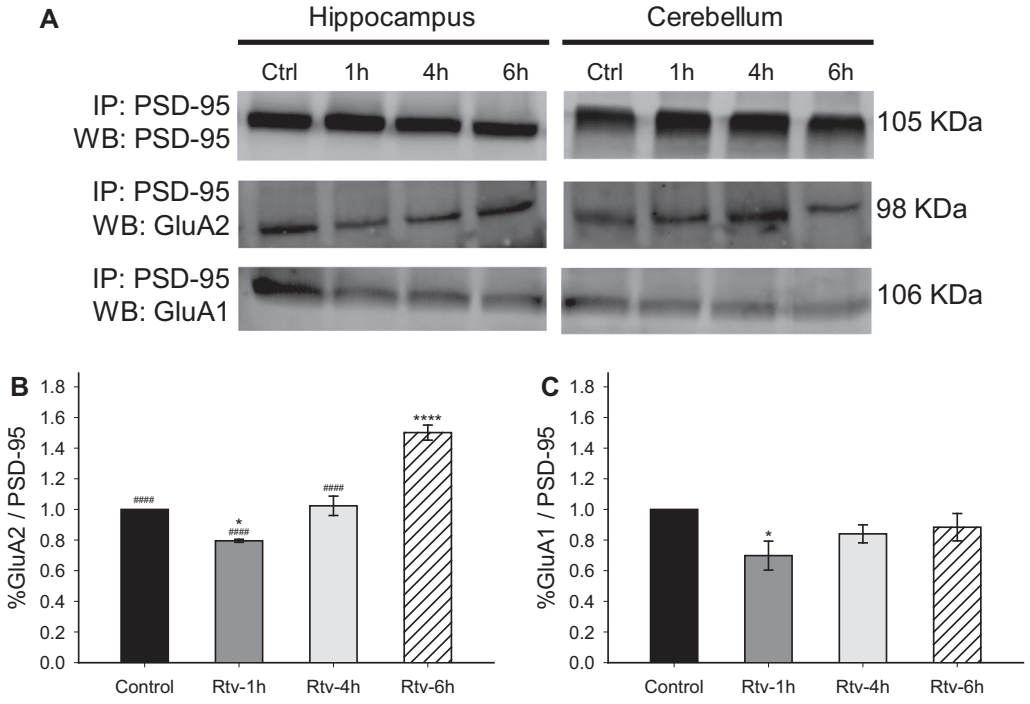

Fig. 2. Immunoblotting of precipitated PSD-95 fraction shows the biphasic pattern of interaction of the target receptors at the postsynaptic density. A. Immunoblots indicate altered synaptic receptor interaction with PSD-95 at different time points in Hippocampus (area of interest) and Cerebellum (control area to show that the changes are not global) compared to control groups. Numbers on left side of each panel indicate the molecular weights of each probed protein. Each group had an n > 3. B and C. Percent ratio of expressed protein (GluA2 and GluA1, respectively) vs. PSD-95. All data are presented as mean ± SEM. Asterisks (*) represent differences from Control, hashtags (#) represent differences from Rtv-6h. * = p < 0.05, ** = p < 0.01, *** = p < 0.005, **** = p < 0.001 (equivalent representation of statistical significance for comparisons against Rtv-6h).

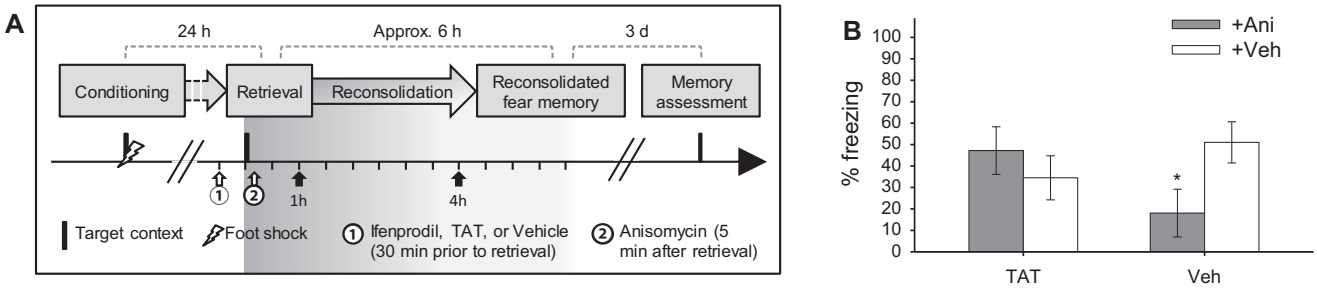

Fig. 3. A presents the antagonist manipulations. During conditioning, a 3-min context exposure (represented by small rectangles above the timeline) was paired with delivery of electric footshock (lightning symbol). Memory of the conditioning trial was assessed as percent time freezing in the conditioning context 24 h later in a second 3-min session. Animals received vehicle (Veh) or TAT-GluA2-3Y (TAT) 1 h prior to retrieval, and vehicle or Anisomycin (Ani) 5 min after retrieval (*ns* = 6–8). Animals were euthanized for biochemical and electrophysiological experiments 1 or 6 h after retrieval (upward arrows), or reassessed for memory of the conditioning episode 3 d later. The shaded area represents the progression of reconsolidation, from highest destabilization of memory (darker portion) to memory restabilization (lighter portion). B. Presents freezing 3d after retrieval and drug manipulations. Anisomycin resulted in decreased retrieval of the conditioning memory (Veh + Ani *vs.* Veh + Veh, $p < 0.05$), showing anisomycin's amnestic effect. TAT-GluA2-3Y ameliorated the amnestic effect of Anisomycin, making freezing in the TAT + Ani group equivalent to the Veh + Veh group, $p > 0.25$. Data are presented as mean ± SEM. * = $p < 0.05$ (against Veh + Ani group).

Animals in the Rtv condition, which experienced shock in the test context, exhibited more freezing than animals in the Control condition, which had an equivalent experience but received no shocks, $F_{1,74} = 41.08$, $p < 0.001$. Freezing levels in the Control group were equivalent to those in the NoRtv group, which received shocks in a second context, $F_{1,31} < 1$, confirming the validity of the Control treatment (Fig. 1A and B). After retrieval, animals were randomly assigned to the 1 h, 4 h, or 6 h condition (*ns* = 14–18) and euthanized at the predetermined time following retrieval. Freezing was equivalent in these groups, $F_{2,70} = 1.16$, and continued to be significantly higher for animals in the Rtv than the Control conditions, $F_{1,70} = 40.74$, $p < 0.001$. There was no interaction between these two factors, $F_{2,70} < 1$.

3.2. Temporal changes in synaptic GluA1 and GluA2 receptor expression during reconsolidation

Rao-Ruiz et al. observed altered AMPA receptor surface expression in the hippocampus in a mouse model of reconsolidation (Rao-Ruiz et al., 2011). To confirm the role of synaptic AMPA receptors in recall of memory at 1, 4, and 6 h post retrieval in our rat model, hippocampal synaptic GluA1 and GluA2 receptor expression was assessed by co–immunoprecipitation with PSD-95 fraction 1, 4, and 6 h after retrieval (Conditions Rtv-1h, Rtv-4h, and Rtv-6h). Although synaptic receptor expression can be assessed through multiple procedures, including surface receptor quantification (Rao-Ruiz et al., 2011) and synaptosomal studies, we assessed AMPA receptors via PSD-95 pull down since synaptic

receptors are activated more significantly when they interact with the scaffolding-PSD complex, and PSD-associated receptor expression levels may vary during the reconsolidation process. With this strategy, surface expression levels exclude receptors from the extrasynaptic zones (Feng, Raghavachari, & Lisman, 2011; Gomperts, 1996; Hrabetova et al., 2000; Kochlamazashvili et al., 2010; Petralia et al., 2010; Simon, Hepburn, Chen, & De Schutter, 2014). With this strategy, we can better assess the role of the active pool of AMPA receptors interacting with the PSD complex during reconsolidation of fear memory.

Memory retrieval resulted in altered interactions of AMPA receptor subunits with PSD-95 in the hippocampus, as compared to a control area (i.e., the cerebellum; Fig. 2), highlighting the fact that this biphasic wave of receptor expression is unique to the hippocampus. These altered patterns were specific to retrieval of an associative memory, and were distinct from those observed at equivalent time points in animals that had previously experienced the context without shock (the Control condition). Immunoprecipitation of PSD-95 and associated GluA1 and GluA2 subunits showed marked alterations throughout the reconsolidation period in the hippocampus. In comparison to the Controls, GluA1 receptor interaction with PSD-95 changed as reconsolidation progressed, $F_{3,9} = 3.90$, $p < 0.05$, with GluA1 expression decreasing 1 h after reconsolidation (Rtv-1h, $p < 0.05$), and recovering to become equivalent to Controls in the Rtv-4h and Rtv-6h groups, $ps > 0.33$ (Fig. 2C, $n = 3$ from 5 to 6 rats). GluA2 receptor expression was similarly impaired through reconsolidation, $F_{3,8} = 54.83$, $p < 0.001$. In the Rtv-1h group, GluA2 receptor expression levels were lower than in the Control, Rtv-4h, and Rtv-6h groups, $ps < 0.05$, 0.05, and 0.001, respectively. GluA2 expression was equivalent to Controls in Rtv-4h, $p = 0.98$, and above Control levels in Rtv-6h, $p < 0.001$ (Fig. 2B, $n = 3$ from 5 to 6 rats). Importantly, none of these effects were observed in the immunoblotting experiments performed with homogenates from the cerebellum (Fig. 2A). Thus, our data suggest that retrieval of a fear memory is associated with distinct patterns of GluA1 and GluA2 receptor interaction with PSD-95 in the hippocampus.

3.3. Blocking GluA2 endocytosis attenuates retrieval-induced memory destabilization

Since GluA2 receptors seemed to have a unique biphasic expression pattern in the hippocampus (PSD-95 interaction, as described above for active synaptic receptors and elsewhere for all surface receptors (Rao-Ruiz et al., 2011), manipulating GluA2 receptor expression after memory retrieval should alter the functional consequences of retrieval on memory. One of the classical consequences of memory retrieval is increased susceptibility of the retrieved memory to amnestic manipulations (*cf.* Misanin et al., 1968). For example, administration of the protein synthesis inhibitor Anisomycin shortly after memory retrieval decreases subsequent recall of the retrieved memory (Rodriguez-Ortiz et al., 2008). However, if destabilization of memory is prevented, Anisomycin should have little impact on subsequent memory recall. Hence we decided to investigate the effect of controlled inhibition of GluA2 receptor endocytosis (as receptors were less available or endocytosed in our PSD pull down studies) on amnesic effects of Anisomycin in spontaneous recovery of fear.

One day after contextual fear conditioning, animals received either Anisomycin (Ani) or vehicle (Veh) 5 min after the memory retrieval session. For all animals, the retrieval session was preceded by administration of the GluA2 endocytosis blocker TAT-GluA2-3Y (TAT) or vehicle 30 min prior to exposure to the context (Fig. 3A, $ns = 6–8$). This resulted in four groups: Veh+Veh, Veh+Ani, Veh+TAT, and TAT+Ani (group names represent treatment prior + following memory retrieval, respectively). Memory of the conditioning episode was assessed after a 3 d washout period. At this 3 d test, memory of the conditioning episode was not independently determined by whether animals received anisomycin or TAT-GluA2-3Y, $Fs_{1,23} < 1.0$, but whether these two factors interacted, $F_{1,23} = 4.69$, $p = 0.05$ (Fig. 3B). Planned pairwise comparisons revealed that Anisomycin effectively disrupted memory of the conditioning event, with the Veh+Ani group exhibiting less conditioned freezing than the Veh+Veh group, $F_{1,23} = 5.04$, $p < 0.05$. Administration of TAT-GluA2-3Y ameliorated the amnestic effect of Anisomycin, making this group equivalent to the Veh+Veh and TAT+Veh groups, $ps > 0.25$ (Fig. 3B, $ns = 6–8$). Hence our results suggest that blocking the initial GluA2 endocytosis at the early hours of reconsolidation has the ability to interfere with retrieval-induced memory destabilization.

3.4. Basal synaptic transmission is altered during the reconsolidation period

Maintenance of LTP, a cellular substrate of learning and memory, is associated to short-lived reduced expression of GluA1 AMPA receptors at the synapse, followed by GluA2 receptor insertion (Gomperts, 1996; Niethammer, Kim, & Sheng, 1996; Plant et al., 2006). Furthermore, removal of GluA2-containing AMPA receptors appears to be one of the molecular basis of LTD, another form of synaptic plasticity associated with low frequency stimulation at the synapses (Ashby et al., 2004). Mounting evidence suggests a biphasic wave of GluA2 receptors during the reconsolidation window; this observation was confirmed by our PSD-95 pull down studies. We also demonstrated that controlled interference in such receptor expression pattern leads to disruption of retrieval-induced memory destabilization. However, it is still not clear how such receptor expression trends impact the synaptic plasticity changes that may provide a physiological basis of such outcomes. Normal expression and activity of GluA1 and GluA2 receptors are essential for proper synaptic transmission; thus, it is likely that altered synaptic interaction of these receptor subunits with PSD-95 leads to altered synaptic transmission during reconsolidation which may lead to distinct plasticity traits. Stimulus-response experiments were conducted in slices from rodents sacrificed 1, 4, and 6 h after retrieval to assess how basal synaptic transmission is affected by the observed alterations in glutamate receptor expression. All groups were sensitive to changes in stimulus intensity, $F_{6,48} = 66.59$, $p < 0.001$, and intensity interacted with time since retrieval, $F_{18,48} = 2.24$, $p < 0.05$ (Fig. 4C, n = 3). This interaction reflects the observation that basal synaptic transmission was impaired in Rtv-1h compared to the remaining groups, which did not differ from each other, suggesting that synaptic communication might be compromised in the early stages of reconsolidation.

3.5. Synaptic plasticity is consistent with the biphasic wave of hippocampal AMPA receptor expression initiated by memory retrieval

The interaction of post-synaptic GluA1 and GluA2 receptors with the PSD-95 scaffolding complex is important for the receptors' activity and stabilization at the surface of the neurons. Inhibition of this interaction can lead to altered synaptic physiology and plasticity in the hippocampus and other areas associated with the development and maintenance of fear memories (e.g. the amygdala). Such impaired interaction can also lead to changes in other types of glutamate receptor activity and downstream signaling (Gomperts, 1996; Niethammer et al., 1996). In our model (focused on the hippocampus), the interaction between glutamate receptors and the PSD complex changed as a function of time since retrieval and impairing receptor endocytosis had distinct effects on retrieval-induced memory destabilization. To determine if such changes play a role in altered synaptic plasticity, we measured

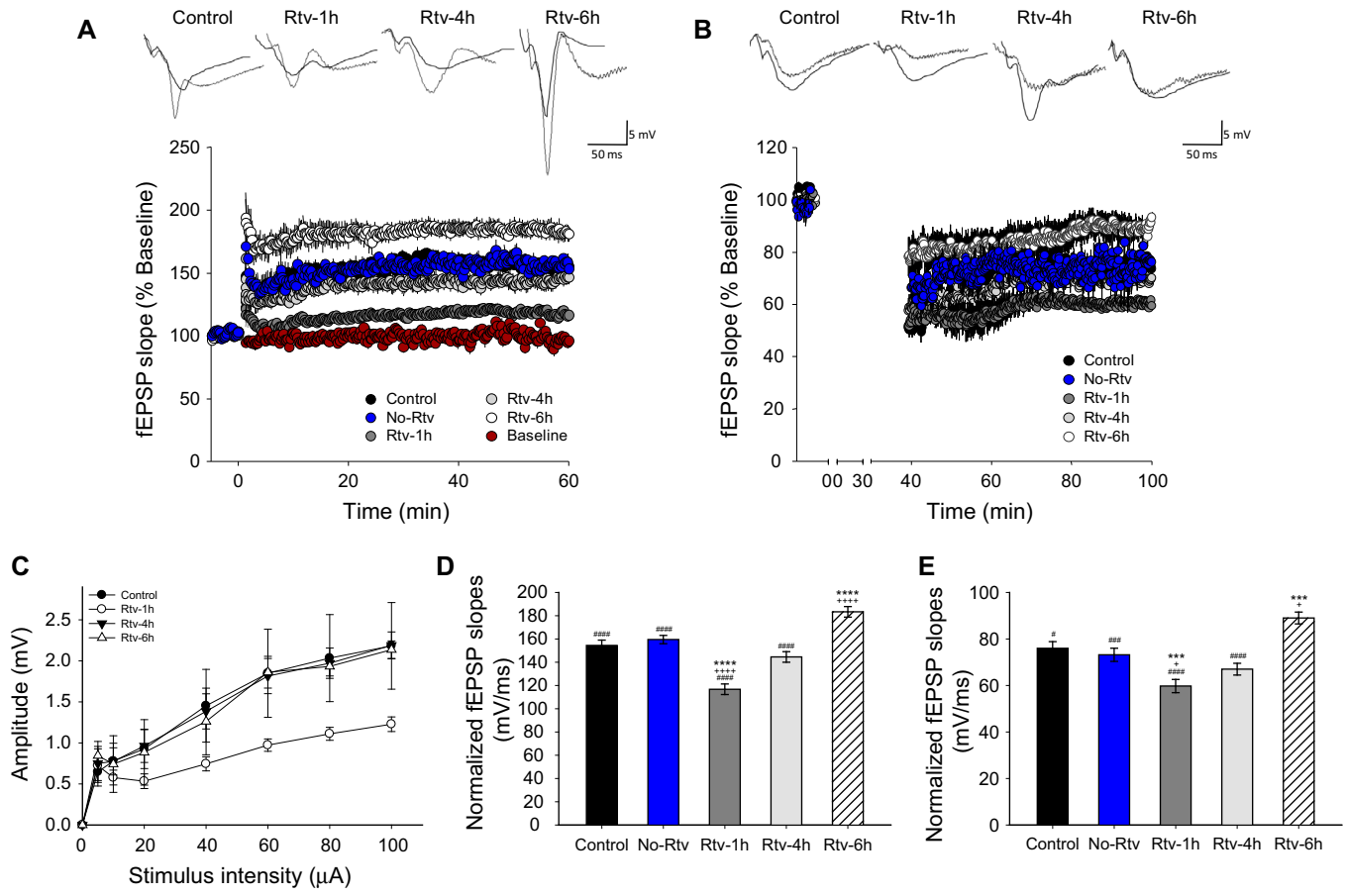

Fig. 4. LTP and LTD from CA3-CA1 pathway of the hippocampus during reconsolidation. A. Compared to Controls receiving no shock during conditioning (which did not differ from subjects receiving shock in a context different from the context of retrieval, No-Rtv), TBS-induced LTP was impaired 1 h after retrieval (Rtv-1h, n = 5), returned to control levels 4 h after retrieval (Rtv-4h, n = 5), and was expressed significantly above controls 6 h after retrieval (Rtv-6h, n = 5). Representative traces were collected before and after LTP induction (within first 5 min). Baseline was recorded for 55–60 min without induction to verify stability of fEPSP slope recording (n = 8). B. Dual-LFS induced LTD was increased 1 h after retrieval (Rtv-1h, n = 5), returned to control levels 4 h after retrieval (Ret-4h, n = 5), and decreased to near baseline levels 6 h after retrieval (Rtv-6h, n = 5). Representative traces were obtained as described for LTP. Recordings were conducted 55–60 min post induction. C. Basal synaptic transmission recorded from CA3-CA1 pathway of the hippocampus was impaired shortly after retrieval (Rtv-1h), and was normalized to Control levels at 4 and 6 h after retrieval (Rtv-4h and Rtv-6h). Slopes were calculated from fEPSPs generated from n = 3 animals for each group. Data are presented as mean ± SEM (brackets). A Group X Stimulus Intensity ANOVA revealed a main effect of stimulus intensity, $F_{6,48} = 66.59$, $p < 0.001$, and an interaction, $F_{18,48} = 2.24$, $p < 0.05$. D and E. Normalized fEPSP slopes obtained from the last 5 min of recording for LTP and LTD, respectively. All data are presented as mean ± SEM. Asterisks (*) represent differences from control, hashtags (#) differences from Rtv-6h, and crosses (+) differences from No-Rtv. * = p < 0.05, ** = p < 0.01, *** = p < 0.005, **** = p < 0.001 (equivalent representation of statistical significance for comparisons against Rtv-6h and No-Rtv). For all panels, traces shown are calibrated at a 5 mV/50 ms scale.

LTP, in the Shaffer Collateral CA3-CA1 pathway of the hippocampus 1–2, 4–5, and 6–7 h after retrieval of the conditioned fear memory (slices were obtained from animals sacrificed 1, 4 and 6 h after reconsolidation, respectively). Measures were obtained 55–60 min post induction using TBS, and were compared against Control and No-Rtv (animals were sacrificed at equivalent time points *ns* = 8). The average fEPSP slope, computed as a percentage of baseline, differed with time since memory retrieval, $F_{4,23} = 28.78$, $p < 0.001$. Post-hoc analyses confirmed that animals in the Rtv-1h group exhibited impaired LTP (<20%) as compared to all other groups, *ps* < 0.005 (Fig. 4A and D, *n* = 5). LTP recovered over time; the Rtv-4h group did not differ from Control and No-Rtv groups (>50%), *ps* > 0.10; however, Rtv-4h still differed from the Rtv-6h group, $p < 0.001$. Indeed, the Rtv-6h group exhibited higher maintenance of LTP (>80%; Fig. 4A and D) than all other groups, *ps* < 0.005. This pattern of time-dependent recovery of LTP might be a result of the biphasic wave of GluA2 receptor expression observed, in which GluA2 receptor interaction with PSD-95 increased to a level above control 6 h after retrieval.

Although LTP was significantly altered during the reconsolidation period, the observed LTP patterns do not provide information on whether such plasticity changes are unidirectional or bidirectional. To determine whether altered LTP was associated with inverse changes in LTD, low frequency-mediated LTD was induced in hippocampal slices to test the nature of plasticity during reconsolidation. This protocol revealed changes in LTD as a function of time since retrieval, $F_{4,17} = 17.05$, $p < 0.001$ (Fig. 4B and E, *n* = 5). Tukey post-hoc comparisons revealed a long-term reduction of the fEPSP slope in the Rtv-1h group (>30%), as compared to the Control (<30%), No-Rtv (<30%) and Rtv-6h (approx. 10%) groups, *ps* < 0.05. fEPSP slopes gradually approached control levels 4 h after retrieval (Rtv-4h did not differ from Rtv-1h, Control, or No-Rtv, *ps* > 0.17) and were well below control levels 6 h after retrieval (Rtv-6h differed from all groups, *ps* < 0.05).

Our data suggest memory retrieval triggers a biphasic wave of GluA2 receptor expression with associated changes in synaptic plasticity (LTP/LTD) that last for the duration of the reconsolidation period. The immediate consequences of memory retrieval are a decrease in expression of GluA1 and GluA2, with an associated impairment in LTP and enhancement in LTD (1 h condition) that normalizes to baseline levels midway through the reconsolidation period (4 h condition). However, this normalized activity is

followed by a rebound increase in GluA2 activity, which is accompanied by increased LTP and decreased LTD expression (reversal in plasticity traits). Thus, it seems that both GluA1 and GluA2 subunits of AMPA receptor play important roles in determining the pattern of synaptic plasticity observed during memory reconsolidation.

3.6. Controlled inhibition of GluA2 endocytosis leads to increased LTP and decreased LTD during reconsolidation

Our data points that preventing GluA2 endocytosis during the early stages of reconsolidation prevents, at least in part, the memory from becoming unstable and protects it from modification, such as disruption by amnestic agents (Fig. 3A and B). It seems reasonable to assume that disruptions of retrieval-induced changes in AMPA receptor expression should have important effects on synaptic plasticity in conjunction with other signaling mechanisms (Fig. 4A–E). To confirm this hypothesis, GluA2 endocytosis was manipulated following the procedure detailed above (administration of TAT-GluA2-3Y or TAT-GluA2-3A 30 min prior to memory retrieval). Animals were euthanized and LTP assessed 1 and 4 h after retrieval (Rtv-1h and Rtv-4h). LTP levels were dependent on time since retrieval, $F_{1,23} = 22.45$, $p < 0.001$, and were also dependent on the compound administered (TAT-3Y, >40%; TAT-3A < 20%; or no drug Rtv-1h, <20%), $F_{2,23} = 16.15$, $p < 0.001$. More importantly, these two factors interacted to determine LTP levels, $F_{2,23} = 8.52$, $p < 0.005$ (Fig. 5A, B, D and E). Post-hoc Tukey tests revealed that LTP levels were significantly increased by TAT-GluA2-3Y 1 h after retrieval, as compared to both Rtv-1h and TAT-GluA2-3A groups, $ps < 0.001$; there was, however, no change in LTP 4 h after retrieval (>30% for TAT-3A and TAT-3Y), $ps > 0.86$ (Fig. 5A, B, D and E, $n > 3$). Other laboratories (Rao-Ruiz et al., 2011) have reported that disruption of GluA2 endocytosis after retrieval failed to alter miniature AMPAR currents after approximately 4 h, suggesting that an initial decrease in GluA2 receptors is critical for the occurrence of reconsolidation. To assess this assumption, we assessed LTP 6h after retrieval in an additional group of animals that received TAT-GluA2-3Y. Preventing GluA2 endocytosis also prevented overexpression of GluA2 during the final stages of reconsolidation, $F_{2,22} = 23.45$, $p < 0.001$. LTP levels were stable in the TAT-GluA2-3Y groups, in sharp contrast with the dramatic impairment in LTP observed in the Rtv-1h group, and the enhancement observed in the Rtv-6h group (Fig. 5A, B, D and E).

Altered LTD during reconsolidation also appears to be dependent on initial GluA2 endocytosis. TAT-GluA2-3Y administration attenuated LTD 1 h after memory retrieval (<5%), $F_{2,9} = 91.07$, $p < 0.001$, as compared to both TAT-GluA2-3A (<30%) and no drug Rtv-1h (<30%), $ps < 0.001$ (Fig. 5C and F, $n > 3$). Thus, interruption of the retrieval-induced wave of GluA2 receptors interferes with the otherwise occurring patterns of LTP and LTD, and could play a major role in memory destabilization effects after retrieval (Figs. 3 and 4A and B). Taken together, our results suggest that retrieval-induced GluA2 endocytosis plays a critical role on the cascading chain of events that occur through the reconsolidation period (Figs. 4 and 5). However, the role of other glutamate receptor subtypes and interaction between different pathways might also have significant contributions in the synaptic phenomenon observed. Those mechanisms should be a matter of future investigation.

4. Discussion

The present studies used a contextual fear conditioning preparation in which rats were exposed to a novel environment paired with a fear-inducing stimulus. During a retrieval trial, animals were placed back in the context and memory of the conditioning experience was assessed via conditioned freezing. Contextual fear conditioning is dependent on hippocampal function (Phillips & LeDoux, 1992) and provides an ideal model to investigate events underlying memory reconsolidation in this area. Rao-Ruiz et al. (2011) reported that a biphasic wave of AMPA receptor activity in the hippocampus is important for memory reconsolidation in a mice model of fear, and that initial impaired expression of AMPA receptor is critical for reconsolidation to occur. However, the physiological and plastic events that are responsible behind such effects of the receptor expression pattern have yet not been investigated. Following retrieval of conditioned fear memory, we analyzed the role of GluA1 and GluA2 receptor-mediated hippocampal plasticity during reconsolidation, a period of approximately 6 h following memory retrieval. This study was used as the basis for a novel model of time-based synaptic plasticity changes during the reconsolidation period, in which retrieval-induced alterations of AMPA receptor expression were accompanied by altered synaptic plasticity (Fig. 4). This model was inspired by the observation that modulation of GluA2 activity (using the endocytosis blocker TAT-GluA2-3Y) could prevent the altered synaptic plasticity otherwise triggered by memory retrieval, and preventing the memory destabilization that characterizes the onset of memory reconsolidation.

4.1. Role of AMPA receptors in reconsolidation

Assessment of receptor expression in the present studies (also see Rao-Ruiz et al., 2011) suggested that there are distinct stages during the reconsolidation process, each characterized by a specific pattern of receptor interaction with PSD-95. During the initial stage (in our study, 1 h after retrieval), GluA1/2 expression was impaired in the hippocampus, compared to controls that were exposed to a familiar place but with no associative learning memories to retrieve, and compared to a control site that is not part of the primary fear learning circuit (i.e., cerebellum). This suggests that decreased synaptic expression of GluA1/2 in the hippocampus plays an important role in altered balance of plasticity observed at the onset of reconsolidation (Figs. 3–5). GluA1 receptor interaction with PSD-95 normalized to control levels later in the reconsolidation process (4–6 h after retrieval), but GluA2 receptors followed a different pattern of recovery. GluA2 receptors gradually normalized (4 h after retrieval) and then overexpressed as the memory became restabilized (6 h after retrieval). Thus, although GluA1 alterations appear critical for the onset of reconsolidation, a biphasic wave of GluA2 activity appears critical for the progression of reconsolidation.

4.2. Balance of LTP and LTD during reconsolidation

Synaptic plasticity is expressed in two main forms, LTP and LTD. A controlled balance between these two different forms of plasticity is considered crucial for short- and long-term memory storage. The present study provides a model of how the interaction of LTP and LTD, as well as the balance between these two processes is crucial during the process of memory reconsolidation. In our studies, LTP and LTD manifested opposing patterns (increased LTP at a time point had correspondingly decreased LTD at a particular time point) through the memory destabilization and restabilization periods, with these patterns being closely related to AMPA receptor activity (Fig. 4). Our underlying assumption is that retrieval is associated with a sensitive period of synaptic plasticity. This assumption is partially based on reports that LTP after retrieval is not sensitive to protein synthesis inhibitors unless re-stimulation occurs during the maintenance phase (Bear & Abraham, 1996). This hypothesis is further guided by our own behavioral experiments

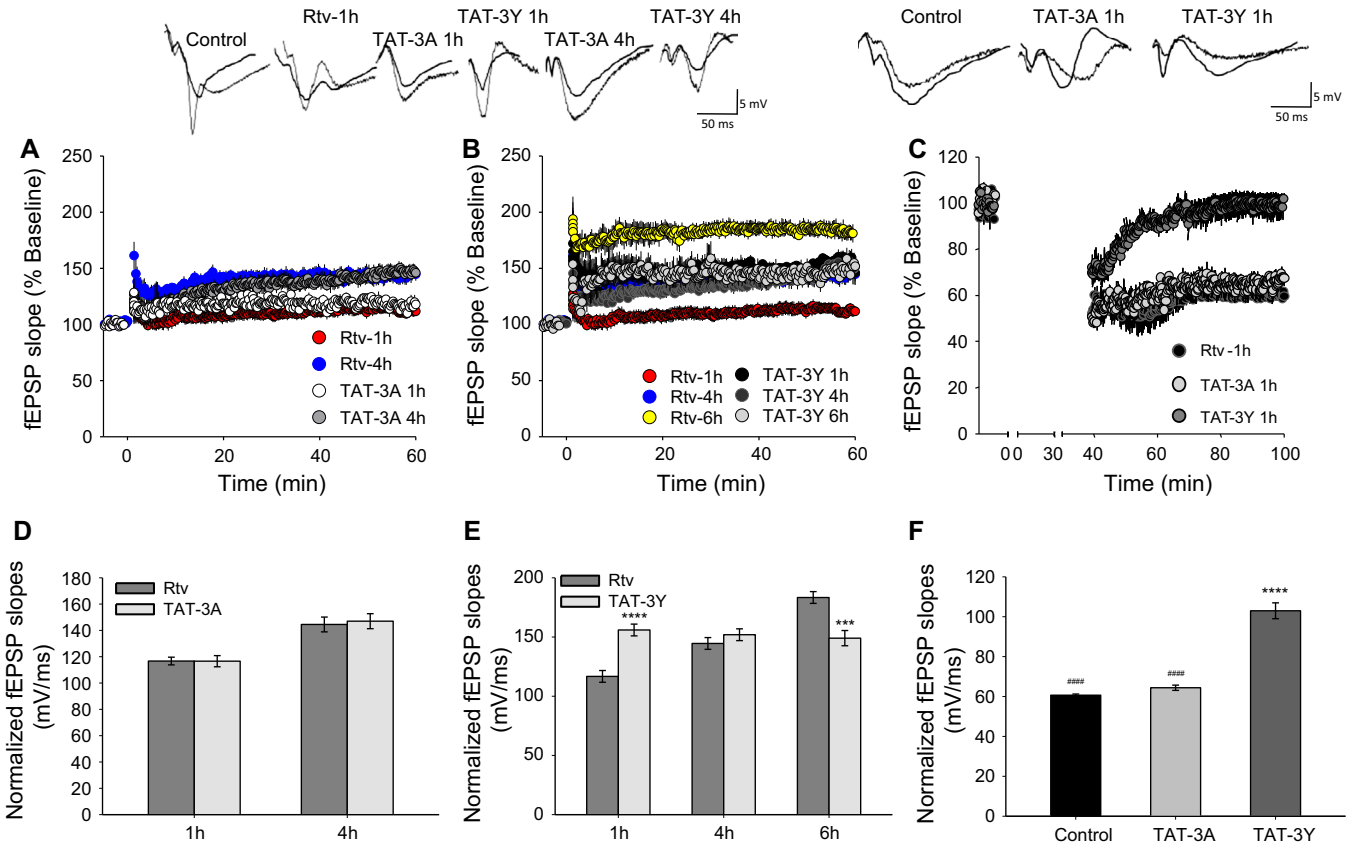

Fig. 5. LTP and LTD recorded from CA3-CA1 pathway of the hippocampus 1 h after memory retrieval after inhibition of GluA2 endocytosis with TAT-3Y, control treatment with a scrambled peptide (TAT-3A), or no treatment (Rtv). A and B. TAT-3A did not produce any changes relative to no treatment (Rtv) 1 or 4 h after retrieval (Panel A), whereas TAT-3Y administration prevented retrieval-induced alterations in LTP otherwise observed following memory retrieval (Rtv condition; Panel B). Representative traces were collected before and after LTP induction (within first 5 min), smooth traces represent the period before induction, while rough traces represent the period after induction. C. Dual-LFS induced LTD was at Rtv-1h levels 1 h after administration of TAT-3A and decreased to baseline levels after administration of TAT-3Y. Representative traces were obtained as described for LTP. Recordings were conducted 55–60 min post induction. D–F. Normalized fEPSP slopes obtained from the last 5 min of recording for LTP (Panels D and E) and LTD (Panel F). All data are presented as mean ± SEM. For all measures, n > 3. (*) asterisks represent differences from the Rtv condition at each time point, (#) hashtags represent differences from TAT-3Y. *** = $p < 0.005$; **** = $p < 0.001$ (equivalent representation of statistical significance for comparisons against Rtv-1h and TAT-3Y).

showing that blockade of retrieval-induced endocytosis of GluA2 receptors plays a role in decreasing the amnesic effects of Anisomycin, resulting in a strong memory. The present study suggests that impaired LTP is a physiological marker of the initiation of memory reconsolidation (Rtv-1h), and the reconsolidation process is accompanied by a gradual normalization (Rtv-4h) and overexpression (Rtv-6h) of LTP. These changes in LTP expression are accompanied by an initial increase in LTD expression (Rtv-1h), followed by a gradual normalization (Rtv-4h) and reduction (Rtv-6h) of LTD. All observed plasticity changes are consistent with changes in AMPA receptor expression. GluA1/2 receptor expression, which decreased shortly after retrieval, is involved in induction and maintenance of LTP/LTD. Hence GluA2 receptor biphasic wave must be crucial for LTP/LTD waves post retrieval, as can be inferred from our blockade of GluA2 receptor blockade experiments. However, the role of NMDA receptors and other downstream signaling pathways, either dependent or independent of AMPA receptor functionality, might also play an important role during memory reconsolidation in the hippocampus (Fig. 6). Recent studies (Holehonnur et al., 2016) have observed that increases in the GluN2A/2B (NMDA receptor subunits) ratio in the amygdala are important for retrieval-mediated destabilization of memory and, hence, retrieval-mediated memory modification. This increased GluN2A/2B ratio also inhibits retrieval-induced phosphorylation of GluA1 in the amygdala and affects plasticity, suggesting that an interaction of AMPA and NMDA receptors may be relevant for memory reconsolidation (Holehonnur et al., 2016).

4.3. Plastic effects of interfering with receptor interaction during reconsolidation

Functionally, impaired LTP and increased LTD correlates with the period of memory destabilization at the basis of retrieval-induced memory distortions (Auber, Tedesco, Jones, Monfils, & Chiamulera, 2013; Kwapis et al., 2011; Misanin et al., 1968; Rodriguez-Ortiz et al., 2008; Tronson & Taylor, 2007), and preventing this initial alteration of synaptic plasticity should make the retrieved memory impervious to these manipulations. Induction of LTP is characterized by an initial period of expression of GluA2-lacking AMPA receptors (Romberg et al., 2009); thus, it seemed likely that preventing GluA2 endocytosis would have profound effects on memory reconsolidation. Thus, retrieval-induced endocytosis of GluA2 receptors was blocked with TAT-GluA2-3Y and an amnestic agent (Anisomycin, a protein synthesis inhibitor known to disrupt reconsolidation of recently retrieved memories; Rodriguez-Ortiz et al., 2008) was administered during the critical memory destabilization period. Controlled blockade of GluA2 endocytosis reduced the amnesic effects of Anisomycin, resulting in a robust memory that was evident even 3d after the initial retrieval episode (this was not the case for control subjects). These

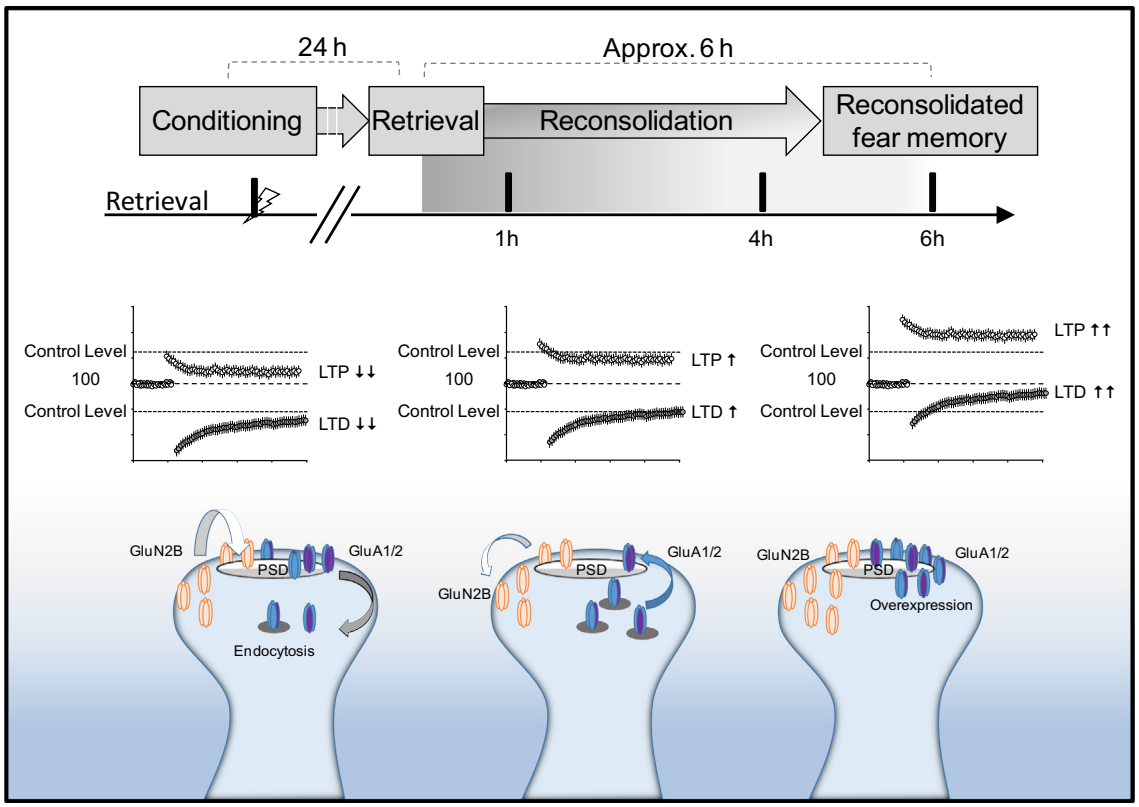

Fig. 6. Model for bidirectional synaptic plasticity (LTP and LTD) during memory reconsolidation of a conditioned fear memory, and its relationship with receptor expression and trafficking. Shortly after retrieval (1 h assessment), there is a downregulation of GluA1 and endocytosis of GluA2 AMPA receptors. This pattern of receptor activity leads to an increase in LTD and decrease in LTP. As reconsolidation progresses (4 h assessment), GluA1 and GluA2 receptor expression approach control levels, leading to a normalization of LTP and LTD. As reconsolidation comes to an end (6 h assessment), LTP is overexpressed while LTD decreases to baseline levels. GluA1 AMPA receptors remain at a control levels on the surface, while GluA2 AMPA receptors are overexpressed. This effect is possibly a delayed reaction to the initial downregulation of the particular receptor subtype during the destabilizing period of reconsolidation. Controlled inhibition of endocytosis of GluA2 AMPA receptors led to increased LTP and decreased LTD shortly (1 h) after retrieval, but had no effects later during the reconsolidation period (4 h after retrieval). These observations suggest that LTP and LTD mechanisms plays an important role in determining synaptic plasticity during memory reconsolidation, and these effects are mediated partially by GluA2 AMPA receptor activity. Further investigation is warranted to understand role of GluN2B receptors in memory reconsolidation.

behavioral observations gave a mechanistic clue for synaptic mechanisms during reconsolidation which was further validated in our field recording experiments (Fig. 4). These behavioral observations were also consistent with TAT-GluA2-3Y abolishing the retrieval-induced deficits in LTP otherwise observed 1 h after memory retrieval, and reducing LTD to control levels. The short half-life of the compound (90 min; Rao-Ruiz et al., 2011) further suggests that its effects on LTP/LTD is due to their ability to interfere with initial receptor expression patterns rather than a lingering effect of the drug. Consistent with this assumption, preventing alterations in plasticity during the initial portion of the reconsolidation period also prevented enhancements in LTP otherwise observed at the culmination of the reconsolidation period (Rtv-6h), suggesting that initial sensitive period of synaptic plasticity may have long-term consequences for memory reconsolidation. These experiments bring a significant advancement of understanding the physiological traits of reconsolidation that has been so far not studied. The effect of interfering with receptor expression on amnesic effects of anisomycin further helps us understand the importance of both phenomena in reconsolidation.

5. Conclusion

The occurrence of memory destabilization as a consequence of retrieval has been known for almost 50 years (*cf.* Misanin et al., 1968). More recently, there has been a renewed interest in reconsolidation processes because of the possibility that the content of a retrieved memory can be altered by new information acquired while the memory is unstable. For example, Monfils et al. (2009), Rao-Ruiz et al. (2011), Schiller et al. (2010), Sorg, Todd, Slaker, and Churchill (2015) reported that treatments attenuating fear conditioning (i.e., extinction) administered shortly after memory retrieval can dramatically decrease the likelihood of fear relapsing at a later time (fear relapse is a ubiquitous behavioral observation after extinction due to the passage of time or *spontaneous recovery*; Rescorla, 2001, 2004; Sorg et al., 2015). The applications of such an approach are clear, since reconsolidation of fear-producing memories (e.g., of an event that signals a threatening event) with a new memory could provide a powerful tool to address anxiety (e.g., phobias) and trauma-related disorders (e.g., post-traumatic stress disorder [PTSD]) by changing the content of the fear memory. However, the utility of this approach is limited. Although the process of reconsolidation has received great attention (Besnard, Caboche, & Laroche, 2012; Einarsson, Pors, & Nader, 2014; Tronson & Taylor, 2007), it is still unclear whether memory alterations during the reconsolidation period changes the to-be reconsolidated memory, or results in the formation of new memories with common retrieval links (Tronson & Taylor, 2007). Furthermore, manipulations intended to alter memories during the reconsolidation period are not effective in all cases (Auber et al., 2013). A full characterization of the physiological processes that underlie memory reconsolidation can provide a framework upon which such manipulations can be more successful. The present study provides a thorough description of the synaptic plasticity changes that

occur during the reconsolidation period, along with a conceptualization of the mechanisms that lead to such changes, providing the first unified model of glutamate receptor expression and synaptic plasticity across the different stages of the reconsolidation period.

Our findings suggest that the process of reconsolidation is characterized by bidirectional synaptic plasticity changes predominantly determined by postsynaptic mechanisms. They also show how depolarization of synapses change over time during memory reconsolidation, in processes where altered waves of AMPA receptor expression in hippocampal synapses play a significant role. These changes in the balance of LTP and LTD may reflect a natural way for hippocampal synapses to control various components of the to-be-reconsolidated memory, such as content or strength. However, given the importance of amygdala in fear memory reconsolidation, investigating system level interactions between the amygdala and hippocampus and the role of GluN2B receptors in our current model is a necessary future step in this research. Furthermore, the present observations need to be complemented with a full characterization of the role of NMDA receptors on memory destabilization, restabilization, and potential alterations of memory during the reconsolidation period (as also reported in Holehonnur et al., 2016). A detailed understanding of the processes triggered by memory retrieval is an important first step to fully characterize the process of memory reconsolidation and potentially lead to development of Fig. 6. Model for bidirectional synaptic plasticity (LTP and LTD) during memory reconsolidation of a conditioned fear memory, and its relationship with receptor expression and trafficking. Shortly after retrieval (1 h assessment), there is a downregulation of GluA1 and endocytosis of GluA2 AMPA receptors. This pattern of receptor activity leads to an increase in LTD and decrease in LTP. As reconsolidation progresses (4 h assessment), GluA1 and GluA2 receptor expression approach control levels, leading to a normalization of LTP and LTD. As reconsolidation comes to an end (6 h assessment), LTP is overexpressed while LTD decreases to baseline levels. GluA1 AMPA receptors remain at a control levels on the surface, while GluA2 AMPA receptors are overexpressed. This effect is possibly a delayed reaction to the initial downregulation of the particular receptor subtype during the destabilizing period of reconsolidation. Controlled inhibition of endocytosis of GluA2 AMPA receptors led to increased LTP and decreased LTD shortly (1 h) after retrieval, but had no effects later during the reconsolidation period (4 h after retrieval). These observations suggest that LTP and LTD mechanisms plays an important role in determining synaptic plasticity during memory reconsolidation, and these effects are mediated partially by GluA2 AMPA receptor activity. Further investigation is warranted to understand role of GluN2B receptors in memory reconsolidation therapeutics that increase or prevent destabilization, modification, or restabilization of memory afterretrieval (reconsolidation model, Fig. 6).

Financial disclosure

Portions of this work was supported by NSF-EPSCOR summer fellowship granted to S.B.

Significance statement

Memory retrieval results in memory destabilization, rendering the memory suitable for modification as the memory is restabilized (reconsolidated). The studies described here investigated the time-dependent electrophysiological and biochemical processes that take place during the reconsolidation period, and how interfering with these processes alters memory reconsolidation. We also show the dependence of reconsolidation on new protein synthesis, especially during the initial hours of the reconsolidation window. Although the biochemical signature of AMPA receptor expression during reconsolidation has been previously demonstrated, this work has developed a synaptic plasticity model that can be used to guide behavioral and pharmacological interventions where long-term memory modification is desirable, such as in several neurodegenerative disorders.

Author contribution

Subhrajit Bhattacharya: Performed experiments, analyzed data, wrote manuscript.
Whitney Kimble: Performed experiments.
Manal Buabeid: Wrote manuscript.
Dwipayan Bhattacharya: Performed experiment.
Jenna Bloemer: Wrote manuscript.
Ahmad Alhowail: Analyzed data.
Miranda Reed: Edited manuscript.
Muralikrishnan Dhanasekaran: Wrote manuscript.
Martha Escobar: Designed and performed experiments, wrote manuscript.
Vishnu Suppiramaniam: Designed experiments and wrote manuscript.

Acknowledgement

The authors do not have any personnel other than the authors listed to acknowledge. The work was solely funded by departmental funding from Dr. Suppiramaniam's startup grant.

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