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The Role of a CaMKII/PKA-Protein Degradation-GluR2 Pathway in the Control of Memory Updating Following Retrieval

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THE ROLE OF A CAMKII/PKA-PROTEIN DEGRADATION-GLUR2 PATHWAY IN
THE CONTROL OF MEMORY UPDATING FOLLOWING RETRIEVAL

by

Timothy J. Jarome

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ABSTRACT

THE ROLE OF A CAMKII/PKA-PROTEIN DEGRADATION-GLUR2 PATHWAY IN THE CONTROL OF MEMORY UPDATING FOLLOWING RETRIEVAL

by

Timothy J. Jarome

The University of Wisconsin-Milwaukee, 2013

Under the Supervision of Professor Fred J. Helmstetter

Reconsolidation is thought to be a process whereby consolidated memories can be modified following retrieval. However, very little is known about the molecular mechanisms that regulate this reconsolidation process. In the present series of experiments we tested if memories “destabilize” or become labile following retrieval through a specific signaling pathway. We found that retrieval of a contextual fear memory differentially increased proteasome activity in the amygdala and hippocampus and resulted in unique changes in AMPA receptor subunit expression in these brain regions. These changes were dependent on CaMKII activity, which was required for increases in Rpt6-S120 phosphorylation, proteasome activity and GluR2/3 in the amygdala and hippocampus. Interestingly, CaMKII-mediated protein degradation in the amygdala was critical for changes in proteasome activity and AMPA receptor subunit expression in the hippocampus, suggesting that protein degradation in the amygdala is critical for the reconsolidation of a contextual fear memory in the hippocampus.

Additionally, we found that auditory fear memories destabilize in the amygdala but reconsolidate in both the amygdala and hippocampus following retrieval, suggesting that the amygdala and hippocampus may interact to reconsolidate memories that are normally hippocampus-independent. Finally, we found that contextual information, but not prediction error, was the new information present at retrieval that controlled the destabilization and reconsolidation of a retrieved auditory fear memory. Collectively, these results suggest that fear memories undergo systems reconsolidation following retrieval where they destabilize through a specific cellular pathway mediated by CaMKII in the amygdala, and that contextual information may be the new information present at retrieval that controls the reconsolidation-dependent updating of fear memories in the amygdala.

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Pavlovian fear conditioning is widely used to study the neurobiology of learning and memory (Johansen, Cain, Ostroff & LeDoux, 2011). In this form of Pavlovian fear conditioning, a neutral conditioned stimulus, or CS, is paired with a noxious unconditioned stimulus, or UCS. After only a few pairings, the CS becomes associated with the UCS and acquires the ability to elicit the fear response associated with the UCS. In a typical auditory fear conditioning paradigm, an animal will learn to fear both the auditory cue which preceded an aversive footshock and the context in which the shock occurred. Memory for these two associations can then be assessed by placing the animal back into the training environment or exposing it to the auditory cue in a new environment, both in the absence of the shock, and assessing freezing behavior as an indication of fear (Fanselow, 1980). Memory for the CS-UCS relationships is robust and long lasting, making this an ideal paradigm for studying the molecular neurobiology of learning and memory.

The network of brain structures which support Pavlovian fear conditioning is relatively well understood. Information about the auditory CS is processed in cortical regions and the auditory thalamus while information about the contextual CS is processed in dorsal hippocampus (LeDoux, 2000). Lesioning the hippocampus will impair the acquisition of context fear conditioning, while leaving the auditory fear memory intact (Kim & Fanselow, 1992). Information about the auditory CS or context are processed in these sensory regions and convergence with UCS information in the lateral amygdala (LA) and lesioning the amygdala will completely abolish acquisition of both context and auditory fear conditioning (Phillips & LeDoux, 1992). The LA connects to the central

nucleus of the amygdala (CeA), which projects to hypothalamus and brainstem areas that control the conditioned responses such as freezing behavior (Johansen et al., 2011).

The role of protein synthesis in memory consolidation

Once acquired, fear memories go through a time-dependent process at the molecular level which is necessary to transfer the labile short-term memory (STM) into a stable long-term memory (LTM); a process known as memory consolidation (McGaugh, 2000). This consolidation process relies on a number of intracellular pathways and has been investigated using a combination of molecular, pharmacological and genetic approaches, most of which suggest that gene transcription and *de novo* protein translation are critical steps in the transfer of STM to LTM (for review see Jarome & Helmstetter, 2013; Johansen et al., 2011). The consolidation process starts with increases in intracellular levels of calcium, which is mediated by activation of NMDA-type glutamate receptors (Rodrigues, Schafe & LeDoux, 2001) and leads to activation of a number of intracellular signaling pathways. For example, autophosphorylation of Calcium-calmodulin dependent protein kinase II (CaMKII) is critical for memory stabilization following acquisition (Rodrigues, Farb, Bauer, LeDoux & Schafe, 2004). Additionally, inhibiting the Protein Kinase A (PKA), mitogen-activated protein kinase (MAPK) and PKC signaling pathways impair LTM formation (Abel, Nguyen, Barad, Deuel, Kandel & Bourchouladze 1997; Adams & Sweatt, 2002; Schafe & LeDoux, 2000). These proteins all phosphorylate the transcription factor CREB and genetic manipulation of CREB has shown that it is critical for memory consolidation (Kida, Josselyn, Pena de Ortiz, Kogan,

Chevere, Masushige & Silva, 2002). Supporting a role for gene transcription, broad spectrum inhibitors of RNA synthesis significantly impair LTM formation (Bailey, Kim, Sun, Thompson & Helmstetter, 1999; Parsons, Gafford, Baruch, Riedner & Helmstetter, 2006a). Additionally, broad spectrum inhibitors of protein synthesis, as well as specific inhibitors of the mTOR translational control pathway, impair LTM for a fear conditioning task (Parsons, Gafford & Helmstetter, 2006b; Schafe & LeDoux, 2000), and increases in protein synthesis have been reported in the amygdala following fear conditioning (Hoeffler et al., 2011), supporting that there is a need for *de novo* protein synthesis following the acquisition of Pavlovian fear memories.

The role of protein degradation in synaptic plasticity and memory consolidation

While it has been widely accepted that protein synthesis is a necessary step in the transfer of STM to LTM, recent evidence suggests that protein degradation is likely also important in LTM formation (Jarome & Helmstetter, 2013). However, very little is known about how protein degradation contributes to this same consolidation process. In mammals, the system that controls the majority of protein turnover is the ubiquitin-proteasome system (Hershko & Ciechanover, 1998). In this system, proteins get targeted for degradation by the covalent attachment of a small protein modifier called ubiquitin. This occurs in a three step process in which the target protein acquires 1-7 ubiquitin modifiers, which will link together at specific lysine residues, forming polyubiquitin chains (Bingol & Sheng, 2011; Fioravante & Byrne, 2011; Hegde, 2010). Target proteins that contain a lysine-48 polyubiquitin tag can then be captured and degraded by a large

multisubunit protein structure known as the 26S proteasome. The 26S proteasome consists of a catalytic 20S core and two 19S regulatory particles. The 20S core consists of two outer rings of alpha subunits surrounding to inner rings of beta subunits. The β 1, β 2, and β 5 subunits of the 20S regulate the chymotrypsin-like, trypsin-like and peptidylglutamyl-like activities of the proteasome complex. There are six ATPase subunits on the 19S caps, known as the Rpt subunits. The Rpt6 subunit has been shown to regulate increases in 20S catalytic activity following phosphorylation, suggesting that it is the main regulatory subunit for increased proteasome activity (Bedford, Paine, Sheppard, Mayer & Roelofs, 2010).

While the ubiquitin-proteasome system (UPS) is known to be involved in a variety of cellular processes, only recently has evidence begun emerging implicating the degradation function of this system in synaptic plasticity. For example, increased synaptic activity levels result in dynamic reorganization of the postsynaptic density (PSD) and this is dependent on increased ubiquitin-proteasome activity (Ehlers, 2003). Inhibiting proteasome activity not only prevented reorganization of the PSD in response to increased cellular stimulation, but it also dramatically altered phosphorylation states of downstream effectors such as CREB, indicating that the UPS may also have control over transcriptional and possibly translational processes. Consistent with this, some evidence also suggests that some forms of protein synthesis may be regulated by increased protein degradation (Banerjee, Neveu & Kosik, 2009). Importantly, inhibiting NMDA receptor activity can prevent activity-dependent increases in proteasome number and activity at synapses (Bingol & Schuman, 2006), suggesting that increases in proteasome activity occur specifically in response to activation of the postsynaptic neuron. Downstream of

NMDA receptors, proteasome activity has been shown to be regulated by both PKA and CaMKII (Bingol, Wang, Arnott, Cheng, Peng & Sheng, 2010; Djakovic, Schwarz, Barylko, DeMartino & Patrick, 2009; Djakovic, Marquez-Lona, Jakawich, Wright, Chu, Sutton & Patrick, 2012; Zhang, Hu, Huang, Toleman, Paterson & Kudlow, 2007).

Interestingly, both PKA and CaMKII positively regulate proteasome activity by phosphorylating the Rpt6 subunit at serine-120, suggesting that these two protein kinases may collaborate to regulate increases in proteasome activity.

Since PKA and CaMKII are both known to contribute significantly to various forms of synaptic plasticity and regulate proteasome activity *in vitro*, it seems likely that protein degradation may also be critically involved in learning-dependent synaptic plasticity. Consistent with this, inhibiting protein degradation can impair hippocampal long-term potentiation (LTP) and LTM for a spatial navigation task (Artinian, McGauran, De Jaeger, Mouldous, Frances & Roulet, 2008; Fonseca, Vabulas, Hartl, Bonhoeffer & Nagerl, 2006). To date, only a few studies have examined the role of protein degradation in fear memory formation, and the results have been conflicting. For example, some evidence suggests that protein degradation is critical for hippocampal-dependent fear memory formation (Lopez-Salon, Alonso, Vianna, Viola, Mello e Souza, Izquierdo, Pasquini & Medina, 2001), while others have not found such an effect with proteasome inhibitors (Lee et al., 2008). However, recent evidence from our lab suggests that protein degradation in the amygdala is critical for fear memory formation (Jarome, Werner, Kwapis & Helmstetter, 2011). We found NMDA-dependent increases in protein degradation, which were learning-dependent, and targeted synaptic scaffolding proteins and negative regulators of protein synthesis. Inhibiting the degradation of these proteins

with a proteasome inhibitor significantly impaired LTM formation to a similar degree as a broad spectrum protein synthesis inhibitor. This suggests that the amygdala relies on protein degradation for fear memory formation, likely to a similar degree as protein synthesis, indicating that increased UPS activity is critical for normal memory consolidation.

Memory reconsolidation

If the consolidation process is uninterrupted, then a memory is believed to become stable and no longer susceptible to disruption. At this point a memory is in the “maintenance” phase, which may rely on the atypical PKC isoform PKM ζ (Kwapis, Jarome, Lonergan & Helmstetter, 2009; Kwapis, Jarome, Gilmartin & Helmstetter, 2012; Parsons & Davis, 2011; Serrano, Friedman, Kenney, Taubenfield, Zimmerman, Hanna, Alberini, Kelley, Maren, Rudy, Yin, Sacktor & Fenton, 2009). Interestingly, evidence suggests that upon retrieval a once consolidated memory destabilizes and requires new protein synthesis in order to restabilize, a process known as reconsolidation (Nader, Schafe & LeDoux, 2000; Parsons et al., 2006a). Thus memory reconsolidation is believed to be a dynamic process in which an existing memory is again made labile so that new information can be incorporated into the original memory trace (Lee, 2008).

Memory reconsolidation can essentially be divided into two successive stages, destabilization and restabilization (Nader & Hardt, 2009). Restabilization is analogous to the consolidation process (for review see Tronson & Taylor, 2007). It requires a number of intracellular signaling pathways including ERK-MAPK, PKA, NF-kB, and mTOR-

mediated protein translation (Duvarci, Nader & LeDoux, 2005; Lubin & Sweatt, 2007; Parsons et al., 2006b; Tronson, Wiseman, Olausson & Taylor, 2006), though the role of new gene transcription remains controversial (Duvarci, Nader & LeDoux, 2008; Lee, Everitt & Thomas, 2004; Parsons et al., 2006a). These molecular mechanisms are believed to be necessary for the synaptic alterations that occur during the reconsolidation process which serve to restabilize or transfer the memory back to long-term storage within 6-hrs of retrieval (Nader et al., 2000) and inhibiting any of these mechanisms result in a permanent impairment in LTM for the original memory trace.

While protein synthesis is considered a critical step in the restabilization of a retrieved fear memory, very little is known about the mechanisms upstream of translation which regulate the initial destabilization of the memory trace. Some evidence suggests that NMDA receptor activity is necessary for memory destabilization, as inhibiting NMDA activity prior to retrieval will not impair reconsolidation but will prevent the effects of a protein synthesis inhibitor when both processes are simultaneously inhibited (Ben Mamou, Gamache & Nader, 2006). This suggests that the updating process starts with increases in NMDA receptor mediated synaptic transmission. Additionally, recent evidence suggests that UPS-mediated protein degradation also underlies memory destabilization and updating in the hippocampus and amygdala (Jarome et al., 2011; Lee, 2008; Lee et al., 2008). Furthermore, the increases in protein degradation were dependent on NMDA receptor activity at the time of retrieval, suggesting that protein degradation is downstream of NMDA receptor activity during the destabilization process (Jarome et al., 2011). This implies a pathway where memories are updated by activation of NMDA receptors, which signal increases in protein degradation that control the need

for new protein synthesis. Inhibiting either mechanism upstream of protein synthesis leaves the initial memory trace intact, but prevents the incorporation of new information (Lee, 2008).

A memory destabilization pathway

While it is possible then that memories are updated through a NMDA – UPS – translation pathway, it is unclear as to what specifically induces the need for new protein synthesis. Some recent evidence suggests that changes in AMPA receptor composition at the time of retrieval controls the ability of a retrieved fear memory to be updated (Clem & Huganir, 2010; Monfils, Cowansage, Klann & LeDoux, 2009; Rao-Ruiz, Rotaru, van der Loo, Mansvelder, Stiedl, Smit & Spijker, 2011). For example, retrieval of a contextual fear memory results in a time-dependent endocytosis of AMPAR subunits GluR1/2/3, which is observed from 1-4hrs after retrieval. This is the time at which a retrieved memory is labile, suggesting that the synaptic depotentiation which occurs following retrieval is due to loss of AMPAR subunits. Consistent with this, blocking endocytosis of GluR2 following retrieval prevented a temporary reduction in CA1 miniature excitatory postsynaptic currents (mEPSCs) and the ability of the retrieved fear memory to update. Interestingly, blocking GluR2 endocytosis also prevented an increase in GluR2 levels observed in the hippocampus 7-hrs after retrieval (Rao-Ruiz et al., 2011). Collectively, these results suggest that retrieval induces a biphasic change in AMPAR subunit composition, with both short-term and long-term changes in GluR2 levels which

reflect the time course of the reconsolidation process, and this is critical for memory updating.

The loss and subsequent increase in GluR1/2 suggests then that AMPAR trafficking and insertion is likely an important component of the late phase of the reconsolidation process. Consistent with this, a number of recent studies have shown that phosphorylation of GluR1-s845, a trafficking site, is important for memory reconsolidation (Jarome, Kwapis, Werner, Parsons, Gafford & Helmstetter, 2012; Monfils et al., 2009). Blocking pGluR-s845 in the amygdala prevents memory updating following retrieval (Clem & Huganir, 2010). Serine-845 is a PKA target site and is primarily involved in the insertion of AMPARs at the extrasynaptic site (Oh, Derkach, Guire & Soderling, 2006), suggesting that the trafficking of AMPAR subunits during the reconsolidation process could be mediated by PKA. Recent evidence suggests that PKA can bidirectionally regulate memory following retrieval (Tronson et al., 2006). This study showed that inhibiting PKA in the amygdala following fear memory retrieval resulted in persistent impairments in LTM, however, activating PKA after retrieval enhanced memory performance on later tests. This suggests then that PKA-dependent increases in AMPAR trafficking and insertion might be critical for memory restabilization.

Though it has been well established that GluR1-s845 is a PKA target site and an important regulator of AMPAR trafficking, some evidence suggests that PKA can also regulate proteasome activity *in vitro* (Zhang et al., 2007; Upadhyaya, Ding, Smith & Hegde, 2006). In mammals, it has been consistently reported that protein degradation is a critical regulator of memory destabilization following retrieval (Jarome et al., 2011; Lee et al.,

2008; Lee 2008; 2010). If PKA does regulate proteasome activity *in vivo*, then that would suggest that PKA should be a destabilization mechanism, however, evidence suggests that it is important in memory restabilization but not destabilization. These conflicting results make it unclear how then PKA is actually involved in the reconsolidation process and whether it's main contribution is to memory destabilization through ubiquitin-proteasome activity or memory restabilization through AMPAR insertion and regulation of translation.

Evidence suggests then that memory destabilization requires NMDA-mediated reduction in AMPAR subunits, followed by a PKA-dependent increase in AMPAR trafficking and insertion, though where protein degradation fits in this model is unclear. Interestingly, the proteasome has been shown to target synaptic scaffolding proteins GKAP and Shank following memory retrieval (Jarome et al., 2011; Lee et al., 2008). GKAP and Shank form receptor complexes in the PSD, which hold receptors at synapses (Mabb & Ehlers, 2010) suggesting that increased trafficking of AMPAR subunits might require the disassembly of the PSD. Consistent with this, recent evidence has shown that a deletion of Shank3 isoforms results in a reduction of GluR1 in the PSD and attenuates activity-dependent redistribution of GluR1 containing AMPARs (Wang et al., 2011). This indicates that protein degradation may contribute to memory destabilization through its actions on the PSD structure (Jarome et al., 2011; Lee et al., 2008), though currently no study to date has examined how blocking protein degradation following retrieval alters changes in PSD composition. Additionally, how protein degradation is actually regulated downstream of NMDARs following retrieval is currently unknown, and evidence suggests that PKA likely cannot be the primary regulator of proteasome activity during

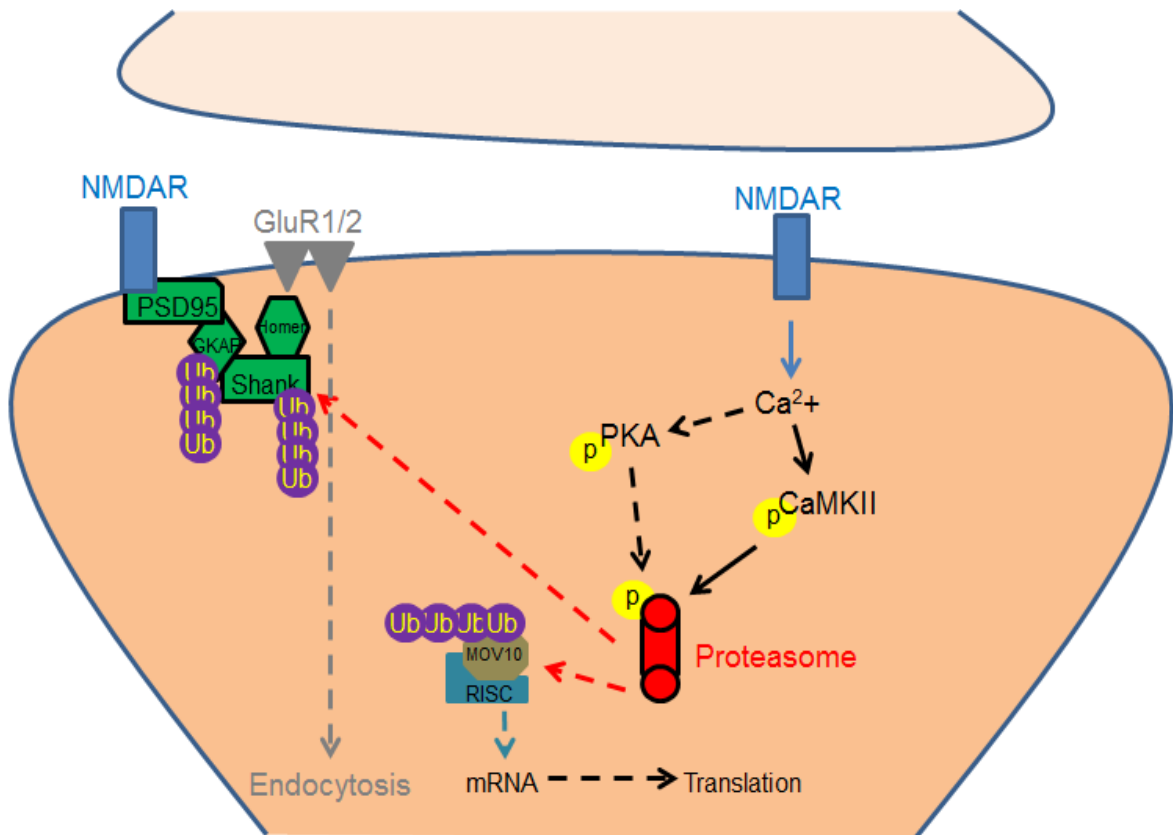


Figure 1. Destabilization model. Activation of NMDA receptors following retrieval leads to activation of Protein Kinase A (PKA) and Calcium/Calmodulin dependent protein kinase II (CaMKII), which both phosphorylate the proteasome on Rpt6 at Serine-120. The proteasome will then regulate PSD disassembly by degrading master scaffolds GKAP and Shank, which allows receptor changes at the synapse and leads to the temporary loss of GluR1/2 through endocytosis. The proteasome will also initiate the restabilization process by degrading translational repressors such as MOV10 and the production of new AMPAR subunits and synaptic scaffolds.

memory destabilization. One possibility is that proteasome activity is regulated by CaMKII, which has been shown to regulate proteasome activity by phosphorylation of the same Rpt6 site as PKA *in vitro* (Bingol et al., 2010; Djakovic et al., 2012;), suggesting that it could be involved in the destabilization process upstream of protein degradation. Interestingly, no study to date has examined the role of CaMKII in memory reconsolidation, though a role for it has been proposed (Tronson & Taylor, 2007). This suggests that memory destabilization may require a NMDA – PKA/CaMKII – UPS –

GluR2 reduction pathway (Figure 1). While several studies have implicated various components of this pathway in the reconsolidation process, no study to date has examined whether these mechanisms directly interact with each other following memory retrieval. In order to better understand what the functional role of reconsolidation is, we need a better understanding of what pathway induces a stored memory to transition from the maintenance to the labile phase.

While we know very little about what molecular mechanisms control memory destabilization following retrieval, we know even less about how a memory is simultaneously updated in multiple brain regions. For example, context fear memories undergo a protein synthesis dependent reconsolidation process in both the amygdala and hippocampus (Debiec, LeDoux & Nader, 2002; Mamiya, Fukushima, Suzuki, Matsuyama, Homma, Frankland & Kida, 2009). In both regions, protein degradation has been shown to underlie memory destabilization and the requirement for protein synthesis following retrieval (Jarome et al., 2011; Lee et al., 2008). Interestingly, the temporal profile of protein ubiquitination in the amygdala mirrors that of the hippocampus and the proteasome seems to target similar proteins following context fear memory retrieval, however, auditory fear memories, which require protein degradation for memory destabilization, do not reconsolidate in the hippocampus and have a different temporal profile for protein ubiquitination (Jarome et al., 2011). This suggests that the simultaneous destabilization of a context fear memory in the amygdala and hippocampus may rely on interplay between these two regions. To date, no study has examined if the simultaneous destabilization, and subsequent restabilization, of a contextual fear memory requires a direct interaction of the amygdala and hippocampus. Additionally, since

context fear memories do not seem to require the same maintenance molecule in the amygdala and hippocampus, it is possible that the mechanisms mediating memory destabilization in these two brain regions may be fundamentally different (Kwapis et al., 2009; Serrano et al., 2009).

Proteasome activity is increased in the amygdala following fear conditioning

In order to test this destabilization model, we first need a way to accurately and reliably measure proteasome activity in the amygdala. Currently, we know that protein polyubiquitination is increased in the amygdala following memory acquisition and retrieval (Jarome et al., 2011), however, we do not know how proteasome activity changes as a function of conditioning/retrieval. Since PKA and CaMKII act through the proteasome itself without direct actions on protein ubiquitination, it is critical to have a measure of actual proteasome activity in brain tissue homogenates. This can be achieved using an *in vitro* proteasome activity assay (Ehlers, 2003). In this assay, we quantified the rate at which functional proteasomes in our samples degraded a fluorogenic substrate of the UPS. Using this assay, we first assessed if fear conditioning resulted in reliable changes in proteasome activity in the amygdala. We found that proteasome activity gradually increased following fear conditioning, peaking at 4-hrs (Figure 2A). Increases in proteasome activity could occur due to 1) phosphorylation of proteasome subunits or 2) production of new proteasomes. To rule out the latter, we immunoblotted samples with an antibody against Rpt6, a major regulatory unit of the 19S proteasome. While we did observe increases in rpt6, they were transient and returned to near baseline levels by

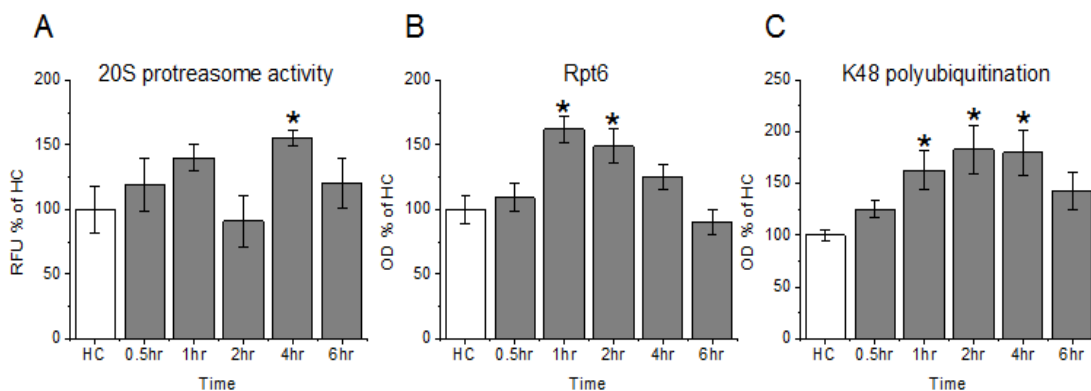


Figure 2. Proteasome activity is increased in the amygdala after fear conditioning. Animals were trained on auditory fear conditioning and amygdala lysates collected various times later for *in vitro* proteasome activity assays and protein analysis. (A) Proteasome activity was increased 4-hrs after conditioning. (B) Proteasome subunit Rpt6 transiently increased after fear conditioning, peaking at 1-hr and subsiding by 4-hrs. (C) Degradation-specific polyubiquitination was increased from 1-4hrs after fear conditioning. All analyses were planned comparisons against homecage (HC) control. * indicates that $P < .05$.

the time proteasome activity reached its peak (Figure 2B). This suggests that the peak increase in proteasome activity is likely due to post-translational modification of existing proteasome subunits. Furthermore, the increases in proteasome activity correlated with increases in degradation-specific polyubiquitination, which was detected using an antibody that recognizes Lys48-linked polyubiquitinated proteins (Figure 2C). This suggests that fear conditioning may dynamically regulate changes in UPS activity. To be sure that the observed increases in proteasome activity were CS-UCS specific, we collected amygdala homogenates 4-hrs after fear conditioning, CS or UCS exposure. Only the group that received the auditory cue paired with the footshock showed increases in proteasome activity (Figure 3A). Consistent with our previous study, this group did not show increased levels of Rpt6 but did have elevated levels of Lys48-linked polyubiquitinated proteins (Figures 3B and 3C). This suggests that the observed

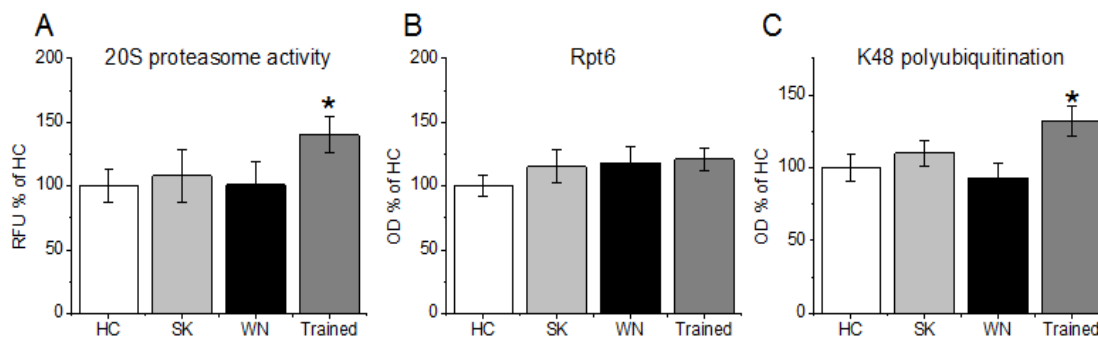


Figure 3. Increases in proteasome activity are specific to association of the auditory cue with shock.

Animals were trained on auditory fear conditioning (Trained) and amygdala lysates collected 4-hr later for *in vitro* proteasome activity assays and protein analysis. Separate control controls were either placed into the training context and immediately shocked and removed (SK) or exposed to the WN presentations without shock (WN). (A) Proteasome activity was increased only when the auditory cue was paired with shock. (B) There were no increases in proteasome subunit Rpt6. (C) Degradation-specific polyubiquitination was increased specifically in response to CS-UCS learning. All analyses were planned comparisons against homecage (HC)/SK/WN controls.

* indicates that $P < .05$.

increases in amygdala proteasome activity following conditioning were specific to the CS-UCS association and support previous work showing that functional proteasome activity was necessary for LTM formation in the amygdala (Jarome et al., 2011).

Both CaMKII and PKA regulate increases in proteasome activity following fear memory acquisition

Now that we can reliably quantify increases in proteasome activity in amygdala homogenates following fear conditioning, we need to demonstrate that an *in vivo* manipulation of intracellular signaling can affect *in vitro* proteasome activity assessed using our assay. So we tested whether CaMKII and PKA can regulate increases in proteasome activity in the amygdala following fear conditioning. Animals were trained

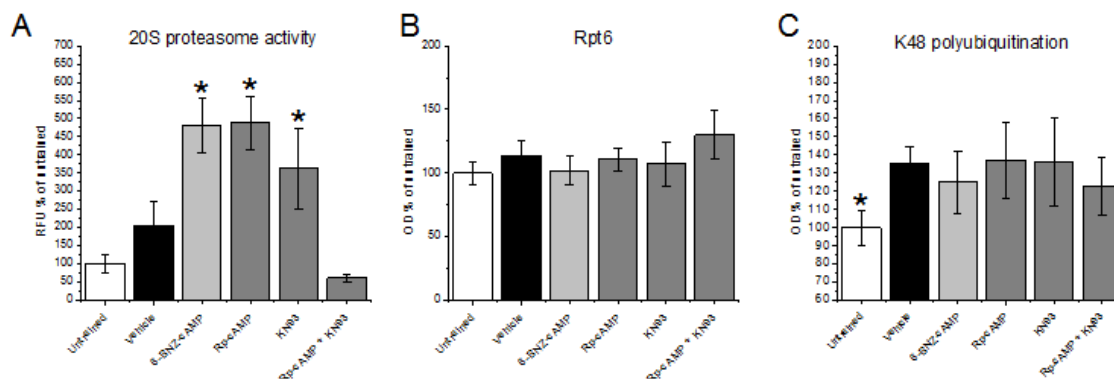


Figure 4. Both CaMKII and PKA are required for increases in proteasome activity following fear

conditioning. Animals were trained on auditory fear conditioning, infused, and amygdala lysates collected 4-hr later for *in vitro* proteasome activity assays and protein analysis. Separate controls were injected with vehicle without being trained, and sacrificed 4-hr later (Untrained). (A) Both activating (6-BNZ-cAMP) and inhibiting (Rp-cAMP) PKA drove proteasome activity relative to vehicle infused trained and untrained animals and inhibiting CaMKII (KN93) did not reduce the increase in proteasome activity, however, simultaneously blocking CaMKII and PKA abolished increases in proteasome activity. (B) There were no increases in proteasome subunit Rpt6. (C) Degradation-specific polyubiquitination was increased in all trained groups relative to untrained controls. All analyses were planned comparisons. * indicates that $P < .05$ from Untrained.

to auditory fear conditioning and received microinfusions of the PKA agonist 6-BNZ-cAMP, PKA antagonist Rp-cAMP, CaMKII inhibitor KN93, a cocktail of Rp-cAMP and KN93 or vehicle immediately after conditioning and euthanized 4-hrs later. A separate group of animals received vehicle infusions without training, and were euthanized 4-hrs later. Consistent with our previous studies, we found a general trend for fear conditioning to increase proteasome activity in the amygdala 4-hrs after conditioning (untrained vs trained). Enhancing PKA activity further drove proteasome activity, consistent with the interpretation that PKA targets the proteasome. Interestingly, inhibiting PKA did not block increases in proteasome activity as expected, but rather further drove proteasome activity relative to vehicle infused trained controls (Figure 4A). Additionally, blocking CaMKII did not reduce the increases in proteasome activity but

did result in a marginal increase in proteolytic activity. Interestingly, simultaneously inhibiting CaMKII and PKA abolished increases in proteasome activity. Furthermore, none of the manipulations altered total proteasome levels or protein ubiquitination (Figure 4B and 4C). So while enhancements in PKA are capable of driving proteasome activity, inhibiting PKA does not reduce proteasome activity indicating that PKA does not bidirectionally regulate proteasome activity. This suggests that while PKA may exert some influence over proteasome activity, it is not the only mechanism by which increases in proteasome activity are regulated. Consistent with this, inhibiting CaMKII by itself could not prevent increases in proteasome activity, but did abolish training-induced increases when inhibited in combination with a PKA inhibitor. Collectively, these results support previous research indicating that CaMKII and PKA likely target the proteasome at the same site (Bingol et al., 2010; Zhang et al., 2007) and suggest that both CaMKII and PKA regulate increases in proteasome activity and that in the absence of one the other becomes a more potent activator of proteasome activity. Considering that proteasome activity is higher at synapses than in the cytoplasm or nucleus (Upadhyaya et al., 2006) and CaMKII is the most abundant postsynaptic protein (Bingol et al., 2010), it is likely that CaMKII is the primary regulator of proteasome-dependent synaptic plasticity but proteasome activity can be driven by PKA in the absence of CaMKII. Consistent with this, we found that inhibiting CaMKII, but not PKA was sufficient to reduce the other two types of proteasome activity (Figure 5). These results indicate that CaMKII is the primary regulator of proteasome activity while PKA does modulate some types of proteasome activity.

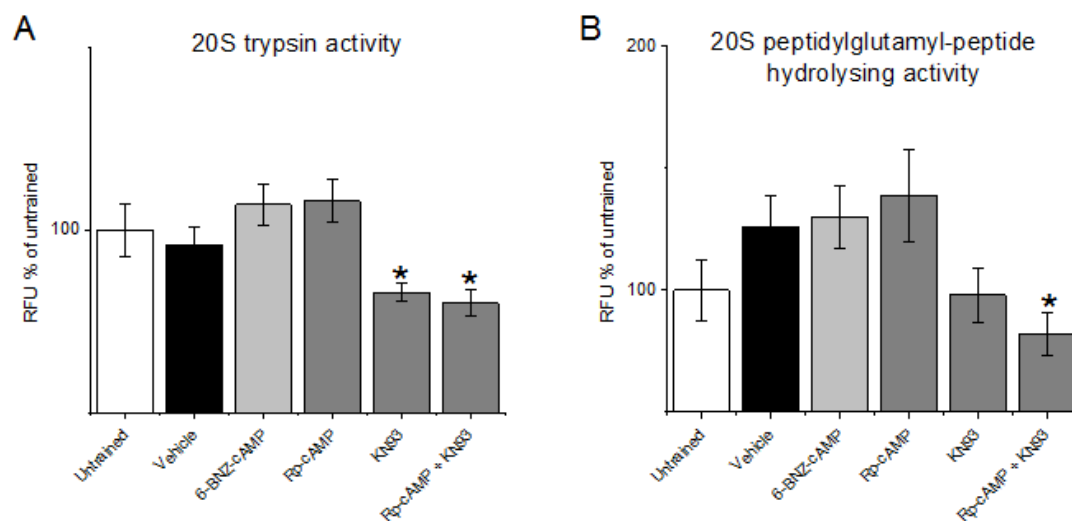


Figure 5. CaMKII, but not PKA, regulates proteasome trypsin-like and peptidylglutamyl-peptide hydrolysing-like activity. Animals were trained on auditory fear conditioning, infused, and amygdala lysates collected 4-hr later for *in vitro* proteasome activity assays and protein analysis. Separate controls were injected with vehicle without being trained, and sacrificed 4-hr later (Untrained). (A) Inhibiting CaMKII, but not PKA reduced basal levels of proteasome trypsin-like activity. (B) Inhibiting CaMKII, but not PKA, reduced increases in proteasome caspase-like activity. All analyses were planned comparisons. * indicates that $P < .05$ from Vehicle.

We have recently demonstrated that fear conditioning increases proteasome activity in the amygdala in a CaMKII- and PKA-dependent manner, suggesting that CaMKII and PKA may play a critical role in the regulation of protein degradation during long-term memory formation and storage. The purpose of the present series of experiments was to further evaluate the role of CaMKII and PKA in the regulation of proteasome activity following fear memory formation and retrieval. Additionally, the present series of experiments was designed to test if a retrieved fear memory undergoes identical reconsolidation processes in both the amygdala and hippocampus, and if reconsolidation itself occurred specifically in response to new information incorporated during retrieval. These ideas were tested in a series of experiments using a variety of

behavioral, pharmacological and molecular approaches, and the results indicate that 1) CaMKII is the primary regulator of proteasome activity and phosphorylation during fear memory formation and following retrieval, 2) the amygdala and hippocampus undergo distinct destabilization and reconsolidation processes for the same retrieved fear memory, 3) protein degradation in the amygdala regulates this systems reconsolidation process between the amygdala and hippocampus, 4) the retrieval of a hippocampus-independent auditory fear memory engages the amygdala for its destabilization but both the amygdala and hippocampus for its reconsolidation and 5) the new information present during the reconsolidation of an auditory fear memory is contextual novelty, but not prediction error, suggesting that the involvement of the hippocampus in this reconsolidation process may be to mediate memory “updating”.

Methods

Subjects

283 male Long Evans rats were obtained from Harlan (Madison, WI) weighing approximately 325-350 grams at time of arrival. All animals were housed individually in shoebox cages with free access to rat chow and water. The colony was maintained under a 14:10-hr light/dark cycle. All experiments took place during the lighted portion of the cycle. All procedures were approved by the University of Wisconsin-Milwaukee Institutional Animal Care and Use Committee and conducted within the ethical guidelines of the National Institutes of Health.

Cannula implantation surgery

For experiments in which animals received microinjections, animals were handled for several days prior to surgery. On the day of surgery, all animals were anesthetized with 2%–4% isoflurane in 100% O₂ and implanted with bilateral stainless steel 26-gauge cannulae aimed at the basolateral nucleus region of the amygdala (BLA; AP -2.8 mm, ML \pm -5.0 mm, DV -7.2 mm) or the dorsal hippocampus (DH; AP -3.5mm, ML \pm -2.6mm, DV -3.0mm) using stereotaxic coordinates relative to bregma. Cannulae were secured to the skull with stainless steel screws, superglue, and dental acrylic. Rats were given a recovery period of at least 7 d before behavioral testing.

Drug preparation and infusion procedure

Rats received bilateral infusions into the amygdala or dorsal hippocampus. The total volume of infusion (0.5 μ l/side BLA; 1.0 μ l/side DH) were given over 60-s, and the injection cannula remained in place an additional 90-s to ensure diffusion away from the injector tip. The injection cannula were cut to extend approximately 0.5mm beyond the guide cannula. Rats were returned to their homecages after infusions. The specific PKA inhibitor myristoylated Protein Kinase Inhibitor 14-22 amide (myr-PKI, 4 μ g/ μ l; EMD Biosciences) and the specific CaMKII inhibitor myristoylated autocalmitide-2 related inhibitory peptide (myr-AIP, 6ng/ μ l; Enzo Life Sciences) were dissolved in distilled H₂O. The proteasome inhibitor β lac (32ng/ μ l; Sigma) was dissolved in 2% DMSO in distilled H₂O. These dosages were determined based on prior studies (Jarome et al.,

2011; Ma, Abel & Hernandez., 2009; Ota, Monsey, Wu & Schafe, 2010; Ouyang et al., 2008; Tinsley et al., 2009).

Apparatus

Fear conditioning was conducted in a set of four Plexiglas and stainless steel observation chambers housed in sound-attenuating chambers (Context A). The floor consisted of 18 stainless steel bars 18mm in diameter spaced 12mm apart and connected to a shock generator. Ventilation fans produced 62-64dB of background noise. Each chamber was equipped with a speaker centered in the middle of one end of the chamber. Before the testing of the animals, each chamber was wiped with 5% ammonium hydroxide solution. Context B was used for context-shift experiments and had a variety of differences from Context A, including textured floors, infrared lighting and 5% acetic acid smell.

General behavioral procedures

All animals were allowed 1-week to recover following surgery. Animals were then acclimated to the transport and restraint and injection procedures for 3-days. On each day, each rat was gently restrained in a towel for several minutes. During this time, the infusion pump to be used during the experiment was turned on to habituate the animals to the sound it produces. For experiments using rats without cannula, the animals received 3 days of acclimation to the transport procedure only. For context fear

conditioning, animals were placed into context A and after a 2-min baseline period, received 5 unsignaled shocks (1.0mA/1-s) followed by a 60-s intertrial interval. After a 2-min post-shock period, the animals were removed from the chambers. The next day, animals were placed back into the training chamber for 90-s in the absence of shock to reactivate the memory or in novel context B for 90-s as a control.

For auditory fear conditioning, animals were placed into context A and after a 6-min baseline period, received 4 white noise (72dB, 10-s)- shock (1.0mA/1-s) pairings with a 90-s intertrial interval. After a 4-min post-shock period, the animals were removed from the chambers. The next day, animals were placed into novel context B and after a 90-s baseline, presented with a 30-s white noise presentation in the absence of shock to reactivate the memory. Animals were then removed from the chamber and returned to their homecages. For 50% reinforcement auditory fear conditioning, animals were placed into context A and after a 6-min baseline, received 4 white noise (72dB, 10-s)- shock (1.0mA/1-s) pairings (WN-SK) and 4 white noise (72dB, 10-s) only presentations (WN) with a 90-s intertrial interval. The WN-SK and WN presentations were given in a pseudorandom order (WN-SK, WN, WN-SK, WN-SK, WN, WN, WN-SK, WN). After a 4-min post-shock period, the animals were removed from the chambers. In cases where animals received “retrieval pre-exposure” the day prior to training, they were placed in novel context B and after a 90-s baseline presented with a 30-s white noise presentation in the absence of shock.

Procedure Experiment 1

The first experiment examined if CaMKII or PKA regulated proteasome activity in the amygdala during memory consolidation. Experiment 1 used 30 animals, consisting of 3 groups with 10 animals per group. Animals were implanted with bilateral cannula aimed at the amygdala 1-week prior to behavioral testing. All animals were trained to auditory fear conditioning as described above and received immediate post-training infusions of vehicle (n = 10), myr-PKI (n = 10), or myr-AIP (n = 10) into the amygdala and euthanized 4-hrs later. Amygdala whole cell lysates were then collected and analyzed using proteasome activity assay and western blotting.

Procedure Experiment 2

The second experiment examined if the amygdala and hippocampus underwent similar reconsolidation processes for a contextual fear memory. Experiment 2 used 62 animals, consisting of 5 groups with 12-13 animals per group, all of which underwent context fear conditioning as described in the general behavioral procedures. One of these 5 groups was euthanized on day 2 without retrieval and served as the No Retrieval (NR) control group (n = 12). The animals in the other 4 groups received a brief retrieval on day 2 and then were euthanized 1- (n = 12), 1.5- (n = 12), 2- (n = 13), or 7-hrs (n = 13) later. In all cases, the amygdala and dorsal hippocampus were dissected, crude synaptosomal membrane fractions obtained, and analyzed using proteasome activity assays and western blotting.

Procedure Experiment 3

The third experiment tested if the amygdala and hippocampus both required CaMKII for increases in proteasome activity following memory retrieval within the same animal. Experiment 3 used 29 animals, consisting of 3 groups with 9-10 animals per group, all of which underwent context fear conditioning. One of these 3 groups was euthanized on day 2 without retrieval and served as the No Retrieval (NR) control group (n = 9). The animals in the other 2 groups were given a retrieval to the training context (n = 10) or a novel context (n = 10) on day 2 and euthanized 1.5-hrs later. In all cases, the amygdala and dorsal hippocampus were dissected, crude synaptosomal membrane fractions obtained, and analyzed using proteasome activity assays and western blotting.

Procedure Experiment 4

The fourth experiment tested if CaMKII regulates proteasome activity in the amygdala following memory retrieval and if protein degradation in the amygdala regulates reconsolidation in the hippocampus. Experiment 4 used 33 animals, consisting of 4 groups with 8-9 animals per group. Animals were implanted with bilateral cannula aimed at the amygdala 1-week prior to behavioral testing. All 4 groups will undergo context fear conditioning as described in the general behavioral procedures. One group received an injection of vehicle on day 2 and euthanized without retrieval (~1.5-hrs later), which served as the No Retrieval (NR) control group (n = 8). The animals in the other 3 groups received a brief context retrieval on day 2 followed by injections of vehicle (n = 8), myr-AIP (n = 9), or β lac (n = 8) and then euthanized 1.5-hr later. In all cases, the

amygdala and dorsal hippocampus were dissected, crude synaptosomal membrane fractions obtained, and analyzed using proteasome activity assays and western blotting.

Procedure Experiment 5

The fifth experiment tested if CaMKII regulates proteasome activity in the hippocampus following memory retrieval and if protein degradation in the hippocampus regulates reconsolidation in the amygdala. Experiment 5 used 31 animals, consisting of 4 groups with 7-8 animals per group. Animals were implanted with bilateral cannula aimed at the dorsal hippocampus 1-week prior to behavioral testing. All 4 groups underwent context fear conditioning as described in the general behavioral procedures. One group received an injection of vehicle on day 2 and euthanized without retrieval (~1.5-hrs later), which served as the No Retrieval (NR) control group (n = 8). The animals in the other 3 groups received a brief context retrieval on day 2 followed by injections of vehicle (n = 8), myr-AIP (n = 8), or β lac (n = 7) and then euthanized 1.5-hr later. In all cases, the amygdala and dorsal hippocampus were dissected, crude synaptosomal membrane fractions obtained, and analyzed using proteasome activity assays and western blotting.

Procedure Experiment 6

The sixth experiment examined whether auditory fear memories destabilize and reconsolidate in both the amygdala and hippocampus following retrieval. Experiment 6 used 32 animals, consisting of 3 groups with 10-11 animals per group, all of which

underwent auditory fear conditioning as described in the general behavioral procedures. One of these 3 groups was euthanized on day 2 without retrieval and served as the No Retrieval (NR) control group (n = 10). The animals in the other 2 groups received a brief auditory CS retrieval on day 2 and then were euthanized 1.5- (n = 11) or 2-hrs (n = 11) later. In all cases, the amygdala and dorsal hippocampus was dissected, crude synaptosomal membrane fractions obtained, and analyzed using proteasome activity assays and western blotting.

Procedure Experiment 7

The seventh experiment tested what the new information was present at retrieval that led to the reconsolidation-dependent “updating” of an auditory fear memory and if both the amygdala and hippocampus showed changes in protein expression that selectively occurred following the presentation of this new information. Experiment 7 used 66 animals, consisting of 5 groups with 13-14 animals per group. One group was trained to auditory fear conditioning and then euthanized on day 2 without retrieval. This group served as the no retrieval (NR) control group (n = 13). Another group of animals was trained to auditory fear conditioning and the following day received a brief auditory CS retrieval event and euthanized 2-hrs later (n = 13); this was the 100% reinforcement group (100%). Group 3 was given a “retrieval pre-exposure” session, trained to auditory fear conditioning the next day, and given a brief auditory CS retrieval and euthanized 2-hrs later on day 3 (n = 14); this was the 100% reinforcement plus retrieval pre-exposure group (100% + pre). Group 4 was trained to 50% reinforcement auditory fear

conditioning and the following day received a brief auditory CS retrieval and euthanized 2-hrs later (n = 13); this was the 50% reinforcement group (50%). The final group was given a “retrieval pre-exposure” session, trained to 50% reinforcement auditory fear conditioning the next day, and given a brief auditory CS retrieval and euthanized 2-hrs later on day 3 (n = 13); this was the 50% reinforcement plus retrieval pre-exposure group (50% + pre). In all cases, the amygdala and dorsal hippocampus was dissected, crude synaptosomal membrane fractions obtained, and analyzed using proteasome activity assays and western blotting.

Crude synaptosomal membrane and whole cell lysate preparation

Animals were overdosed on isoflurane and the brain rapidly removed and immediately frozen on dry ice. Amygdala and dorsal hippocampus tissue were then dissected out by blocking the brain in a rat brain matrix (Harvard Apparatus, Holliston, MA). For whole cell lysates, tissue was homogenized in lysis buffer (50mM Tris-HCl, 150mM NaCl, 1mM EDTA, 1mM NaF, 10 ml 10% SDS, 1 mM sodium orthovanadate, 1 $\mu\text{g}/\mu\text{l}$ PMSF, 1 $\mu\text{g}/\mu\text{l}$ leupeptin, and 1 $\mu\text{g}/\mu\text{l}$ aprotinin) and centrifuged at 4,000rpm for 20-min. The supernatant was collected and measured using a Bradford protein assay kit (BioRad, Hercules, CA). For crude synaptosomal membrane preparation, tissue samples were homogenized in TEVP with 320mM sucrose plus Roche protease inhibitor complete tablet and centrifuged at 1000 x g for 10-min at 4°C. The supernatant was collected and centrifuged at 10,000 x g for 10-min at 4°C. The resulting pellet was denatured in lysis buffer (50mM Tris-HCl, 150mM NaCl, 1mM EDTA, 1mM NaF, 10 ml 10% SDS, 1 mM

sodium orthovanadate and Roche protease inhibitor complete tablet) and centrifuged at 15,000 x g for 5-min at 4°C. The supernatant was collected and measured using a Bradford protein assay kit.

20S proteasome activity assay

Samples (10µg) were diluted in DDH₂O and mixed with reaction buffer (250mM HEPES, pH 7.5, 5mM EDTA, 0.5% NP-40, 0.01% SDS, 5mM ATP). Fluorogenic peptide Suc-LLVY-AMC (chymotrypsin-like activity; Millipore), Bz-VGR-AMC (trypsin-like activity; Enzo Life Sciences), and z-LLE-AMC (peptidylglutamyl-like activity; Enzo Life Sciences) were then added to the samples according to the manufactures instructions. The reaction was incubated at 37°C for 2-hrs and fluorescence monitored every 30-min at 360 (excitation)/ 460 (emission) on a monochromatic plate reader (Synergy H1; Biotek). The peak fluorescence was taken for the subsequent analysis, which was 30-min (Bz-VGR-AMC and z-LLE-AMC) or 2-hrs (Suc-LLVY-AMC). For the *in vitro* manipulation of CaMKII, samples were pre-incubated with the CaMKII inhibitor AIP (10µM) for 30-min at 37°C prior to the addition of the fluorescent substrate. Protein free blanks were used and an AMC standard curve was produced.

Antibodies

Primary antibodies included K48 polyubiquitin (1:1000; Millipore), Rpt6 (1:500; Enzo Life Sciences), Actin (1:1000; Cell Signaling), CaMKII phospho-T286 (1:1000;

Abcam), CaMKII (1:1000; Abcam), GluR1 phospho-S845 (1:1000; Millipore), GluR1 (1:1000; Millipore), GluR2 (1:1000; NeuroMab) and GluR3 (1:1000; Millipore). The phosphorylated Rpt6-Serine120 rabbit polyclonal antibody was generated commercially (ProSci) against a synthetic peptide [NH₂-CALRND(pS)YTLHK-OH] as described previously (Djakovic et al., 2012).

Western blotting

Samples (10µg) were loaded on 7.5% TGX gels, ran through SDS-PAGE and transferred using a Turbo Transfer System (Biorad). Membranes were incubated in 3% milk in TBS + 0.1% Tween-20 (blocking buffer) for 1-hr at room temperature, followed by overnight incubation in antibody in 3% BSA in TBS + 0.1% Tween-20. Membranes were then washed and incubated in secondary antibody (1:20,000; Millipore for goat anti-rabbit, Santa Cruz for goat anti-mouse) in blocking buffer for 60-min. Following a final wash, membranes were incubated in enhanced chemiluminescence substrate (SuperSignal West Dura, Thermo) for 5-min and images developed using a CCD-camera based system (GBOX Chemi XT-4; Syngene) and analyzed using GeneTools software.

Conditioned fear responses

The activity of each rat was recorded on digital video and the amount of movement was determined by frame-by-frame changes in pixels using FreezeScan 1.0 software (CleverSys, Reston, VA). The automatic scoring parameters are chosen such

that the scored activity matches hand-scoring methods previously used in our lab to measure freezing (Parsons, Gafford & Helmstetter, 2010), which is defined as a lack of all movement other than that necessary for respiration.

Statistical analyses

For quantitative protein assays, mean pixel density was calculated for each sample and taken as a percentage of the vehicle (Experiment 1) or no retrieval (Experiments 2-7) control group. For proteasome activity assays, each raw fluorescence reading was standardized to the generated AMC standard curve for that plate and taken as a percentage of the vehicle (Experiment 1) or no retrieval (Experiments 2-7) control group. Data was analyzed using Analysis of Variance (ANOVA), Fisher Least Significant Difference (LSD) post hoc tests and pairwise comparisons where appropriate. Outliers were determined if a sample was two or more standard deviations from the group mean.

Hypotheses

Hypothesis 1. CaMKII, but not PKA, activity is critical for Rpt6-S120 phosphorylation and proteasome activity during long-term memory formation. This hypothesis is tested in Experiment 1.

Hypothesis 2. The amygdala and hippocampus will show similar changes in proteasome activity and AMPAR subunit expression following the retrieval of a contextual fear memory. These changes should be observed as increases in proteasome activity at 1.5-

hrs and decreases in AMPAR subunit expression from 1 to 2-hrs after retrieval, which are followed by increases in AMPAR subunit expression at 7-hrs. This hypothesis is tested in Experiment 2.

Hypothesis 3. CaMKII activity is critical for retrieval-dependent increases in proteasome activity in the amygdala and hippocampus. This hypothesis is tested in Experiments 3, 4, and 5.

Hypothesis 4. Protein degradation is critical for retrieval-dependent changes in AMPAR subunit expression in the amygdala and hippocampus. This hypothesis is tested in Experiments 4 and 5.

Hypothesis 5. The amygdala and hippocampus interact during the reconsolidation of a contextual fear memory, and this is a bidirectional relationship. This hypothesis is tested in Experiments 4 and 5.

Hypothesis 6. The amygdala, but not the hippocampus, will show changes in proteasome activity and AMPAR subunit expression following the retrieval of a hippocampus-independent auditory fear memory. This hypothesis is tested in Experiment 6.

Hypothesis 7. Both new contextual information and prediction error trigger the reconsolidation-dependent updating of a retrieved fear memory. This hypothesis is tested in Experiment 7.

Results

CaMKII, but not PKA, regulates proteasome phosphorylation and activity during fear memory formation in the amygdala

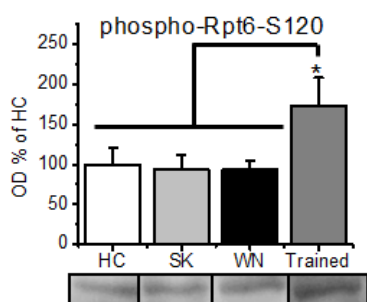


Figure 6. Fear conditioning increases

the phosphorylation of Rpt6-S120 in

the amygdala. Animals were trained on

auditory fear conditioning and amygdala

lysates collected 4-hr later. Samples

were probed with an antibody against

phosphorylated 19S proteasome subunit

Rpt6-S120. Fear conditioning resulted in

learning-specific increases in Rpt6-S120

phosphorylation. Analysis was a planned

comparison. * indicates that $P < .05$.

CaMKII and PKA are known to regulate proteasome activity through the phosphorylation of the 19S regulatory subunit Rpt6 at Serine-120 (S120) *in vitro* (Djakovic et al., 2009; Zhang et al., 2007).

Additionally, phosphorylation of S120 is sufficient to

drive proteasome-dependent changes in synaptic

strength and new dendritic spine growth in cultured

hippocampal neurons. In our preliminary

experiments, we found that fear conditioning led to

learning-specific increases in proteasome activity in

the amygdala (Figure 3), but it is unknown if

behavioral training also increases the phosphorylation

of S120. To test this, we commercially generated an

antibody which could recognize Rpt6 only when phosphorylated at S120 and probed our

samples with this antibody. We found that fear conditioning resulted in learning-specific

increases in the phosphorylation of S120 ($t_{(35)} = 2.847, p < .01$; Figure 6). This suggested

that both proteasome phosphorylation and activity are increased in the amygdala

following fear conditioning.

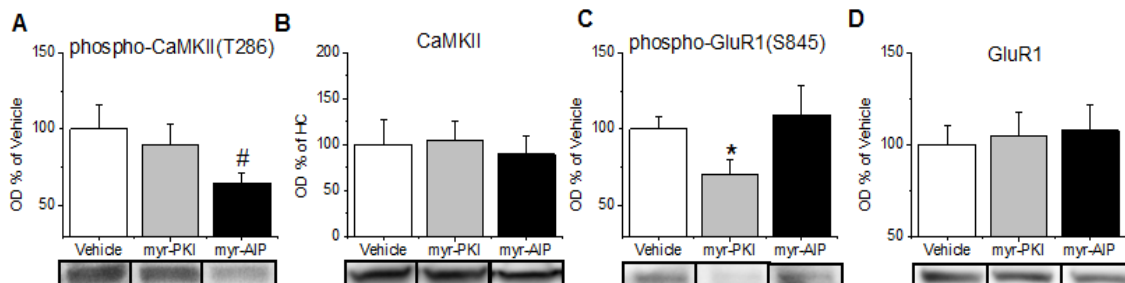


Figure 7. Specific blockade of CaMKII and PKA signaling in the amygdala following fear conditioning. Animals were trained on auditory fear conditioning, infused, and amygdala lysates collected 4-hr later for amygdala proteasome assays and protein analysis. (A-B) Inhibiting CaMKII (myr-AIP), but not PKA (myr-PKI), reduced phosho-CaMKII (A) but not total CaMKII (B) protein expression. (C-D) Inhibiting PKA, but not CaMKII, reduced phosho-GluR1(S845) (C) but not total GluR1 (D). All analyses were planned comparisons. * indicates that $P < .05$ from Vehicle. # indicates that $P = .06$ from Vehicle.

In our preliminary experiments we found that both CaMKII and PKA were involved in the regulation of proteasome activity in the amygdala following fear conditioning (Figures 4 and 5), however, the effectiveness of the pharmacological inhibitors were not consistent across the different types of proteasome activity. This could have been due to the non-selective nature of the inhibitors used. To more directly test the role of CaMKII and PKA in the regulation of proteasome activity during fear memory formation, in Experiment 1 we specifically blocked CaMKII or PKA signaling in the amygdala following fear conditioning using myristolyated peptides. First, we confirmed the effectiveness of these peptides at specifically blocking CaMKII or PKA activity (Figure 7). We found that the CaMKII inhibitor myr-AIP reduced the phosphorylation of CaMKII-T286 ($t_{(27)} = 1.964$, $p = .06$; Figure 7A), the autophosphorylation site known to regulate proteasome activity *in vitro* (Djakovic et al., 2012), but not total CaMKII ($t_{(26)} = 0.325$, $p = .748$; Figure 7B) relative to vehicle and PKA inhibitor groups. Conversely, the PKA inhibitor myr-PKI reduced the

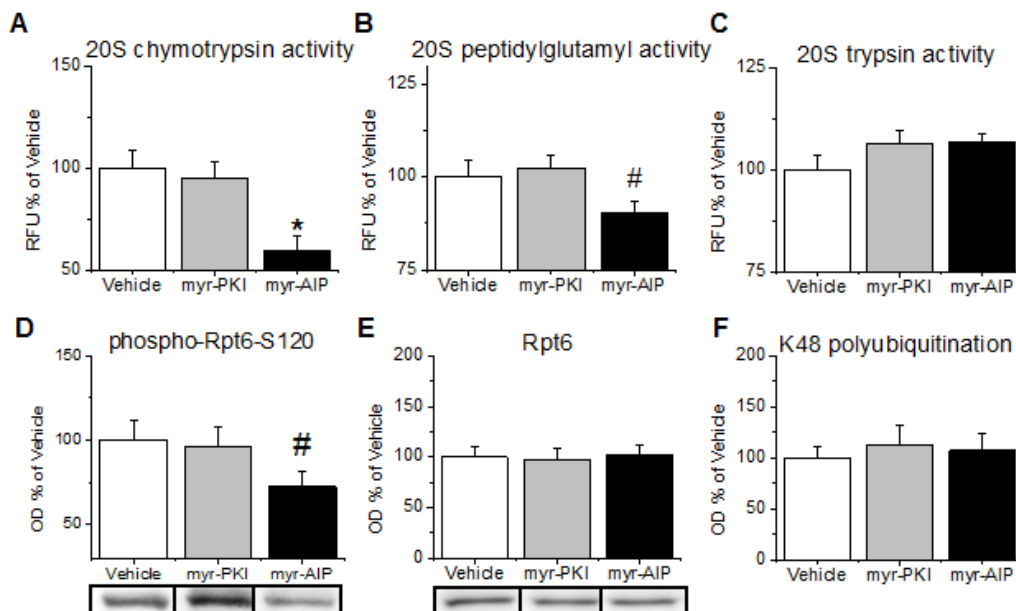


Figure 8. CaMKII, but not PKA, regulates proteasome activity and Rpt6-S120 phosphorylation

following fear conditioning. Animals were trained on auditory fear conditioning, infused, and amygdala lysates collected 4-hr later for amygdala proteasome assays and protein analysis. (A-C) Inhibiting CaMKII (myr-AIP), but not PKA (myr-PKI), reduced proteasome chymotrypsin (A) and peptidylglutamyl (B), but not trypsin (C), activity following fear conditioning. (D-E) Inhibiting CaMKII, but not PKA, reduced Rpt6-S120 phosphorylation (D) but not total Rpt6 (E). (F) Neither inhibitor altered K48 polyubiquitination levels. * indicates that $P < .05$ from Vehicle. # indicates that $P = .07$ from Vehicle.

phosphorylation of GluR1-S845 ($t_{(27)} = 2.066$, $p < .05$; Figure 7C), a PKA target site, but not total GluR1 expression ($t_{(27)} = 0.20$, $p = .740$; Figure 7D) relative to vehicle and CaMKII inhibitor groups. These results indicate that our inhibitors were effective at specifically inhibiting CaMKII and PKA activity.

Next, we tested if CaMKII or PKA were involved in the regulation of proteasome activity in the amygdala following fear conditioning (Figure 8). We found a main effect for drug on proteasome chymotrypsin activity ($F_{(2, 24)} = 3.330$, $p = .053$; Figure 8A).

Fisher *post hoc* tests revealed that the CaMKII inhibitor, but not the PKA inhibitor, reduced proteasome activity relative to vehicle infused trained controls. Additionally, similar results were found for proteasome peptidylglutamyl activity ($F_{(2, 27)} = 2.881, p = .073$; Figure 8B), though neither inhibitor altered proteasome trypsin activity ($F_{(2, 27)} = 1.879, p = .172$; Figure 8C). These results suggest that CaMKII, but not PKA, regulates proteasome activity during fear memory consolidation in the amygdala. Since increases in proteasome activity are regulated by phosphorylation of S120, we tested if the CaMKII inhibitor, but not the PKA inhibitor, regulated S120 phosphorylation following fear conditioning. We found that the CaMKII inhibitor reduced phosphorylated S120 levels ($t_{(26)} = 1.890, p = .07$; Figure 8D) relative to the vehicle and PKA inhibitor groups, but did not alter total Rpt6 ($t_{(27)} = 0.238, p = .841$; Figure 8E) or K48 polyubiquitination ($t_{(25)} = 0.024, p = .981$; Figure 8F) levels. Collectively, these results suggest that CaMKII, but not PKA, regulates increases in Rpt6-S120 phosphorylation and proteasome activity during the formation of long-term fear memories in the amygdala.

Distinct changes in proteasome activity in the amygdala and hippocampus following the retrieval of a contextual fear memory.

If CaMKII regulates proteasome activity during memory consolidation in the amygdala, we next wanted to know if CaMKII also regulates protein degradation during memory reconsolidation. Additionally, we also wanted to test whether a specific fear memory simultaneously reconsolidates in multiple interacting brain regions. To examine both of these processes, we first examined changes in proteasome activity at amygdala

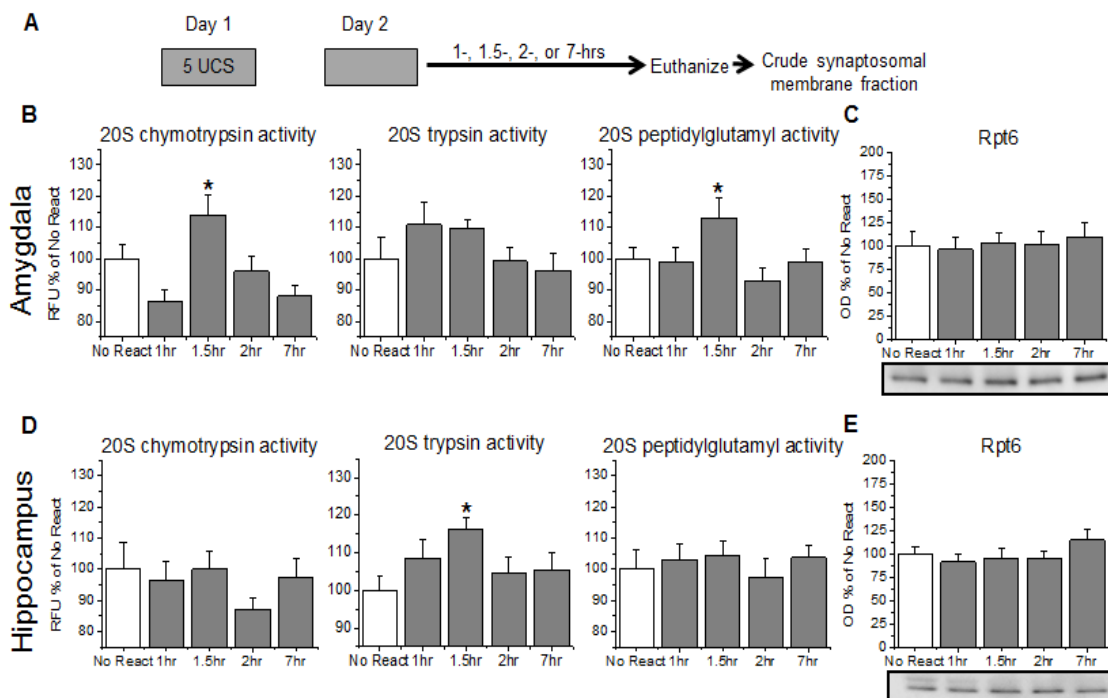


Figure 9. Distinct, temporally linked increases in proteasome activity at amygdala and dorsal hippocampus

synapses following the retrieval of a contextual fear memory. (A) Animals were trained to contextual fear conditioning and given a brief retrieval event the following day. Amygdala and dorsal hippocampus crude synaptosomal membrane fractions were then collected 1-, 1.5-, 2-, or 7-hrs after retrieval ($n = 12-13$ per group). (B) Proteasome chymotrypsin and peptidylglutamyl but not trypsin activities were increased in the amygdala 1.5-hr after retrieval. (C) There were no changes in total Rpt6 levels. (D) Only proteasome trypsin, but not chymotrypsin or peptidylglutamyl, activity was increased in the hippocampus 1.5-hrs after retrieval. (E) There were no changes in total Rpt6 levels. * $P < .05$.

and dorsal hippocampus synapses following the retrieval of a contextual fear memory, since this type of memory has been shown to undergo a protein degradation and protein synthesis dependent reconsolidation process in both the amygdala and hippocampus (Jarome et al., 2011; Lee et al., 2008). We trained animals to context fear conditioning, gave them a brief retrieval the following day and collected amygdala and dorsal hippocampus crude synaptosomal membrane fractions at time points both within (1-, 1.5-, and 2-hrs) and outside (7-hrs) the reconsolidation window (Figure 9A). We then measured proteasome activity using our *in vitro* proteasome activity assay. In the

amygdala, we main effects for time after retrieval for proteasome chymotrypsin ($F_{(4,56)} = 5.631, p = .001$) and peptidylglutamyl activities ($F_{(4,53)} = 2.312, p = .07$), but not proteasome trypsin activity ($F_{(4,52)} = 1.440, p = .234$). Fisher LSD post hoc tests revealed that memory retrieval increased both proteasome chymotrypsin ($p < .05$) and peptidylglutamyl ($p = .07$), but not trypsin, activities 1.5-hrs after retrieval relative to the no retrieval controls (Figure 9B), though there were no changes in total proteasome number between any of the groups ($F_{(4,55)} = 0.136, p = .968$; Figure 9C). Interestingly, in the hippocampus we found a much different pattern of proteasome activity (Figure 9D). We did not find main effects for time following retrieval for proteasome chymotrypsin ($F_{(4,57)} = 0.741, p = .568$), trypsin ($F_{(4,53)} = 1.745, p = .154$), or peptidylglutamyl ($F_{(4,56)} = 0.359, p = .837$) activities. Despite this, Fisher LSD *post hoc* tests did reveal an increase in proteasome trypsin-like activity ($p = .015$) 90-min after contextual fear memory retrieval relative to no retrieval controls without any change in total proteasome number between groups ($F_{(4,50)} = 1.004, p = .414$; Figure 9E). These results support previous studies suggesting that protein degradation is increased in the amygdala and hippocampus following the retrieval of a contextual fear memory (Jarome et al., 2011; Lee et al., 2008), and demonstrate that these changes in protein degradation coincide. Importantly, despite this simultaneous change in proteasome activity in both regions, the overall characteristics of the increased proteasome activity differed in both regions suggesting that the amygdala and hippocampus undergo simultaneous, biochemically distinct destabilization processes following the retrieval of a contextual fear memory.

Different changes in AMPA receptor subunit expression in the amygdala and hippocampus following the retrieval of a contextual fear memory.

We found that contextual fear memories simultaneously destabilize in both the amygdala and hippocampus as indicated by retrieval-dependent increases in proteasome activity. Next, we examined if amygdala and hippocampus synapses underwent similar reconsolidation processes by measuring changes in the expression of AMPA receptor subunits following the retrieval of a contextual fear memory (Figure 10A). In the amygdala, we did not find main effects for time following retrieval for the expression of the GluR1 ($F_{(4,54)} = 1.698, p = .164$), GluR2 ($F_{(4,51)} = 1.181, p = .330$), or GluR3 ($F_{(4,55)} = 1.533, p = .206$) subunits. To determine if there were transient changes in expression of these AMPA receptor subunits (Rao-Ruiz et al., 2011), we did pairwise comparisons for each time point relative to no retrieval controls. We found that GluR1 levels decreased 2-hrs ($t_{(1,54)} = -2.237, p = .03$), but not 1- ($t_{(1,54)} = -0.34, p = .735$), 1.5- ($t_{(1,54)} = -0.871, p = .387$), or 7-hrs ($t_{(1,54)} = -1.659, p = .103$) after retrieval, suggesting a transient loss of this subunit. Interestingly, we found that GluR2 levels increased 1.5-hrs ($t_{(1,51)} = 2.121, p = .034$), but not 1- ($t_{(1,51)} = 0.781, p = .438$), 2- ($t_{(1,51)} = 1.137, p = .261$), or 7-hrs ($t_{(1,51)} = 1.121, p = .268$) after retrieval, while there were no changes in the expression in GluR3 at any of the time points [1-hr ($t_{(1,55)} = 0.794, p = .431$), 1.5-hr ($t_{(1,55)} = -0.065, p = .948$), 2-hrs ($t_{(1,55)} = -1.562, p = .124$) and 7-hrs ($t_{(1,55)} = 0.244, p = .808$)]. Additionally, there were no changes in Actin expression at any of the time points [1-hr ($t_{(1,57)} = -0.354, p = .725$), 1.5-hr ($t_{(1,57)} = -0.655, p = .515$), 2-hrs ($t_{(1,57)} = -0.428, p = .670$) and 7-hrs ($t_{(1,57)} = -0.893, p = .376$)]. These results suggest that the retrieval of a contextual fear memory results in transient bidirectional changes in the expression of

these AMPA receptor

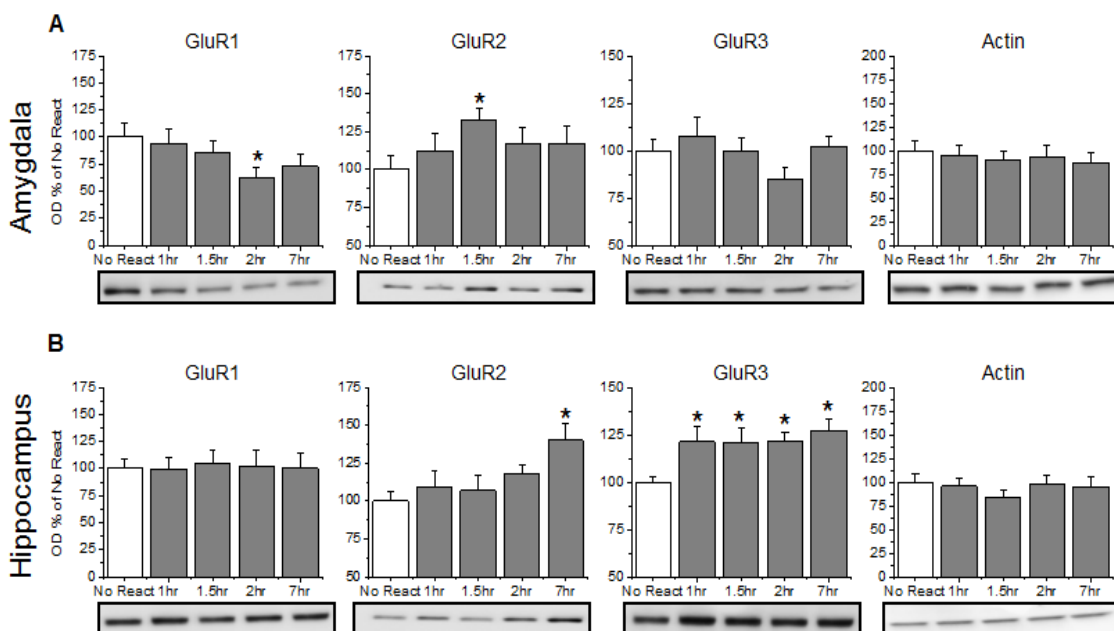


Figure 10. Distinct, temporally disparate changes in AMPA receptor subunit expression at amygdala and dorsal hippocampus synapses following the retrieval of a contextual fear memory. Animals were trained to contextual fear conditioning and given a brief retrieval event the following day. Amygdala and dorsal hippocampus crude synaptosomal membrane fractions were then collected 1-, 1.5-, 2-, or 7-hrs after retrieval ($n = 12-13$ per group). (A) There was a reduction in GluR1 at 2-hrs and an increase in GluR2 at 1.5-hrs after retrieval in the amygdala. (B) There was an increase in GluR2 at 7-hrs and a rapid and persistent increase in GluR3 at 1-, 1.5-, 2-, and 7-hrs after retrieval in the hippocampus. * $P < .05$.

subunits in the amygdala, which return to basal levels by the completion of the reconsolidation process.

In the hippocampus, the retrieval-induced changes in the expression of the AMPA receptor subunits were significantly different than that of the amygdala (Figure 10B). We found main effects for time following retrieval for GluR2 ($F_{(4,55)} = 3.158, p = .021$) and GluR3 ($F_{(4,52)} = 2.619, p = .045$), but not the GluR1 ($F_{(4,57)} = 0.030, p = .998$) subunit. To determine if there were transient changes in expression of these AMPA receptor subunits, we did pairwise comparisons for each time point relative to no retrieval

controls. We found that GluR2 levels selectively increased 7-hrs ($t_{(1,55)} = 3.225, p = .002$), but not 1- ($t_{(1,55)} = 0.713, p = .479$), 1.5- ($t_{(1,55)} = 0.526, p = .601$), or 2-hrs ($t_{(1,55)} = 1.460, p = .150$) after retrieval, suggesting a delayed increase of this subunit.

Interestingly, we found that GluR3 levels increased 1- ($t_{(1,52)} = 2.364, p = .022$), 1.5- ($t_{(1,52)} = 2.254, p = .028$), 2- ($t_{(1,52)} = 2.325, p = .024$), and 7-hrs ($t_{(1,52)} = 3.072, p = .003$) after retrieval, suggesting a rapid and persistent increase of this subunit, while there were no changes in the expression in GluR1 at any of the time points [1-hr ($t_{(1,57)} = -0.041, p = .968$), 1.5-hr ($t_{(1,57)} = 0.255, p = .800$), 2-hrs ($t_{(1,57)} = 0.153, p = .879$) and 7-hrs ($t_{(1,57)} = 0.027, p = .978$)]. Additionally, there were no changes in Actin expression at any of the time points [1-hr ($t_{(1,53)} = -0.334, p = .740$), 1.5-hr ($t_{(1,53)} = -1.264, p = .212$), 2-hrs ($t_{(1,53)} = -0.153, p = .879$) and 7-hrs ($t_{(1,53)} = -0.387, p = .701$)]. The changes in the GluR2 and GluR3 at 7-hrs are consistent with previous studies (Rao-Ruiz et al., 2011) and may reflect a long-term change in synaptic strength as a result of the reconsolidation process. These results suggest that the retrieval-induced changes in AMPA receptor subunit expression in the amygdala and hippocampus are distinct, with the amygdala showing transient changes that occur within the reconsolidation window (1-2hrs) and the hippocampus showing more persistent changes that remain elevated after the reconsolidation process has completed (7hrs). Collectively, these results indicate that while memory destabilization processes in the amygdala and hippocampus coincides, the reconsolidation processes in these regions are both temporally and structurally different and suggests that the amygdala and hippocampus may interact to destabilize and reconsolidate a contextual fear memory.

***In vitro* manipulation of CaMKII can reverse retrieval-induced changes in proteasome activity in both the amygdala and hippocampus**

In our previous experiment we found that retrieval of a context fear memory resulted in destabilization processes in the amygdala and hippocampus that coincided, but destabilization and reconsolidation processes that had distinct biochemical signatures. We next wanted to further characterize the simultaneous destabilization processes in the amygdala and hippocampus by testing if they were regulated by a similar mechanism. To test this, we trained animals to context fear conditioning and then briefly exposed them to the training context or a novel context the following day. To confirm that this paradigm resulted in a context-specific reconsolidation process in the amygdala and hippocampus, we examined the expression of GluR2 in the amygdala and GluR3 in the hippocampus 1.5-hrs after memory retrieval. In the amygdala, we found that the animals exposed to the training context during retrieval showed a significant increase in GluR2 expression ($t_{(1,25)} = 2.257, p = .033$; Figure 11A), but not Rpt6 ($t_{(1,26)} = 0.141, p = .889$; Figure 11B) or Actin ($t_{(1,26)} = -0.492, p = .627$; Figure 11C), relative to no retrieval and novel context (B) exposure groups, suggesting that the retrieval-induced increases in AMPA receptor subunit expression in the amygdala is specific to exposure to the training context. Similar results were observed in the hippocampus where we found that the animals exposed to the training context during retrieval showed an increase in GluR3 expression ($t_{(1,24)} = 1.832, p = .079$; Figure 11A), but not Rpt6 ($t_{(1,23)} = -0.037, p = .971$; Figure 11B) or Actin ($t_{(1,26)} = 0.699, p = .491$; Figure 11C), relative to no retrieval and novel context exposure groups, suggesting that the retrieval-induced increases in AMPA receptor subunit expression in the hippocampus is specific to exposure to the training context.

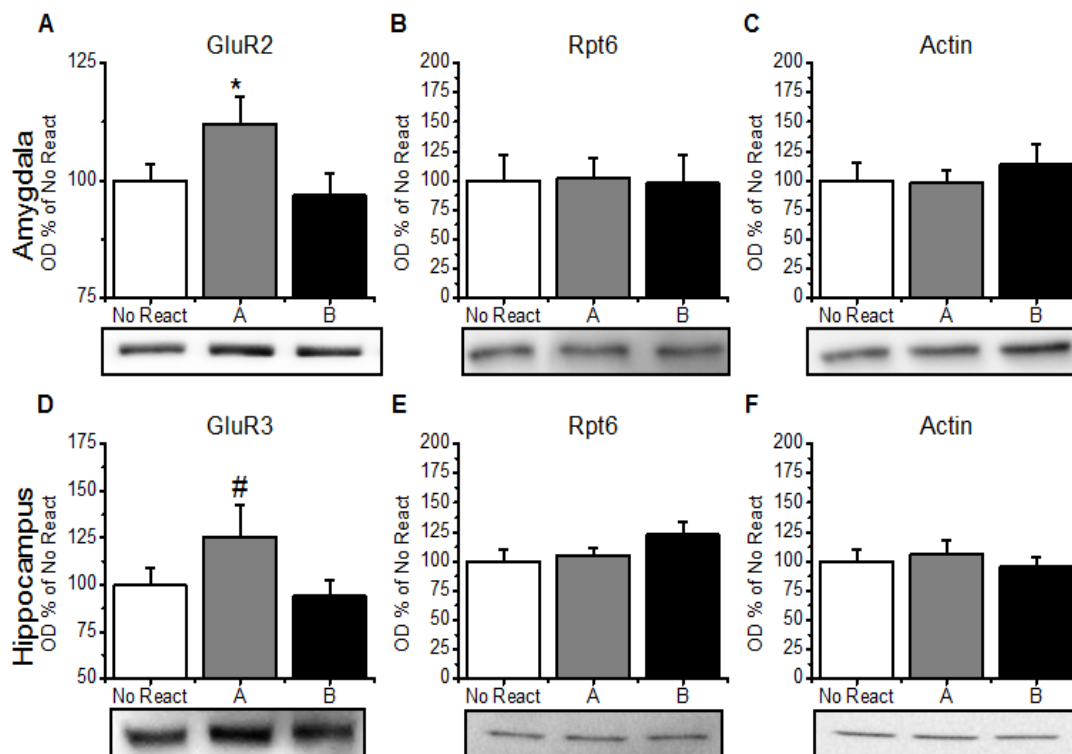


Figure 11. Context-specific changes in AMPA receptor subunit expression following memory retrieval. Animals were trained to contextual fear conditioning and were exposed to the training context or a novel context the following day. Amygdala and dorsal hippocampus crude synaptosomal membrane fractions were then collected 1.5-hrs after retrieval ($n = 9-10$ per group). (A-C) Only the animals exposed to the training context during retrieval had an increase in GluR2 (A), but not Rpt6 (B) or Actin (C), in the amygdala. (D-F) Only the animals exposed to the training context during retrieval had an increase in GluR3 (D), but not Rpt6 (E) or Actin (F), in the hippocampus. * $P < .05$. # $P = .079$.

These results suggest that the reconsolidation processes in the amygdala and hippocampus are specific to retrieval of the appropriate contextual fear memory.

We next tested if the amygdala and hippocampus destabilization processes are regulated by a similar mechanism. To test this, we measured *in vitro* proteasome activity at amygdala and hippocampus synapses in the absence or the presence of a CaMKII inhibitor (AIP) 1.5-hrs after the retrieval, since CaMKII has been shown to regulate proteasome activity (Bingol et al., 2010; Djakovic et al., 2009; 2012; Hamilton et al.,

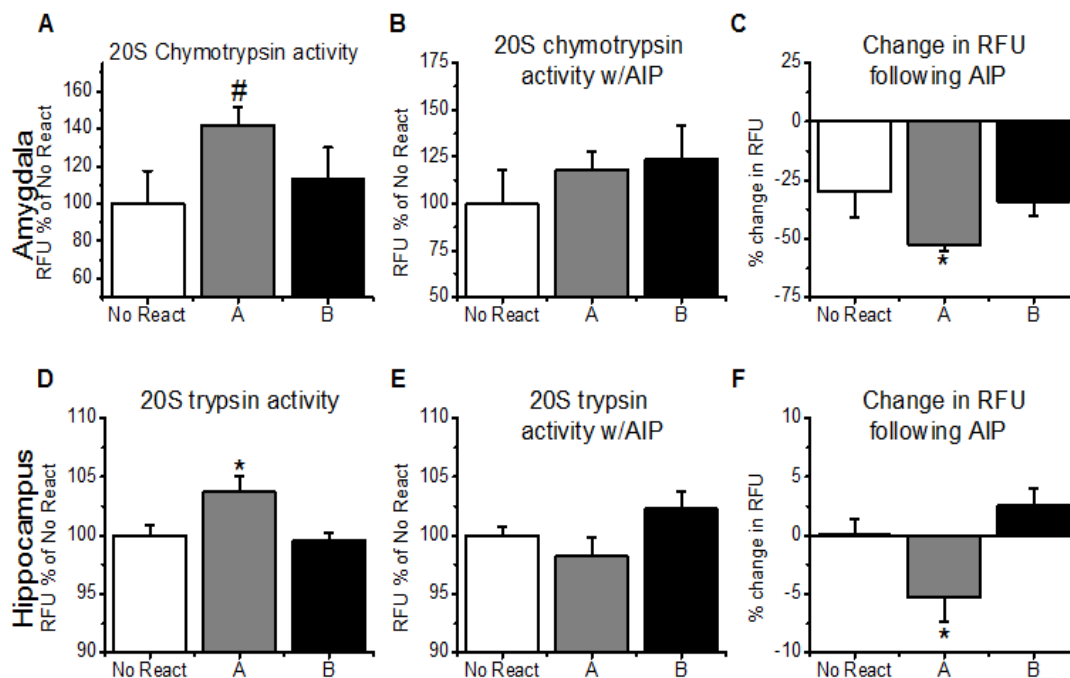


Figure 12. Inhibiting CaMKII *in vitro* can reverse retrieval-induced increases in proteasome activity in the amygdala and hippocampus. Animals were trained to contextual fear conditioning and were exposed to the training context or a novel context the following day. Amygdala and dorsal hippocampus crude synaptosomal membrane fractions were then collected 1.5-hrs after retrieval ($n = 9-10$ per group). (A-C) Only the animals exposed to the training context during retrieval had an increase in proteasome activity in the amygdala (A), but this was reversed by incubating samples with the CaMKII inhibitor AIP (B-C). (D-F) Only the animals exposed to the training context during retrieval had an increase in proteasome activity in the hippocampus (D), but this was reversed by incubating samples with the CaMKII inhibitor AIP (E-F). * $P < .05$. # $P = .085$.

2012). We found that in the absence of AIP, proteasome activity was increased in both the amygdala ($t_{(1,23)} = 1.802, p = .085$) and hippocampus 90-min ($t_{(1,24)} = 2.953, p = .007$) after memory retrieval relative to no retrieval and novel context exposure controls (Figure 12A and 12D). Interestingly, this effect was completely reversed in both the amygdala ($t_{(1,23)} = 0.343, p = .735$) and hippocampus ($t_{(1,24)} = -1.669, p = .108$) when the samples were pre-incubated with AIP for 30-min (Figure 12B and 12E). Consistent with this, the group that received exposure to the training context during retrieval showed a

significantly greater reduction in proteasome activity in the amygdala ($t_{(1,23)} = -2.127, p = .044$) and hippocampus ($t_{(1,24)} = -3.266, p = .003$) in the presence versus the absence of AIP (Figure 12C and 12F). These results demonstrate that *in vitro* manipulation of CaMKII signaling can reverse the retrieval-induced changes in proteasome activity in both the amygdala and hippocampus, suggesting that CaMKII likely mediates the simultaneous memory destabilization processes in these regions.

Amygdala protein degradation is critical for reconsolidation in the hippocampus

Our previous two experiments demonstrated that while the amygdala and hippocampus undergo similar destabilization processes for the same memory, the biochemical signatures of their reconsolidation processes are distinct. We next wanted to test if the amygdala and hippocampus directly interacted to destabilize and reconsolidate a retrieved fear memory. Animals were implanted with chronic cannula aimed at the basolateral amygdala and trained to our contextual fear conditioning and retrieval paradigm. Following retrieval, they received intra-amygdala infusions of the proteasome catalytic inhibitor β lac, the CaMKII inhibitor myr-AIP or vehicle and amygdala and dorsal hippocampus crude synaptosomal membrane fractions were collected 1.5-hrs later (Figure 13A). In the amygdala, we found that vehicle-infused animals showed a significant increase in proteasome chymotrypsin-like activity following retrieval relative to the β lac, myr-AIP and vehicle-infused no retrieval control groups ($t_{(1,26)} = 2.019, p = .054$; Figure 13B). Additionally, we found similar results for proteasome peptidylglutamyl-like ($t_{(1,28)} = 2.074, p = .047$) and trypsin-like ($t_{(1,28)} = 1.879, p = .071$)

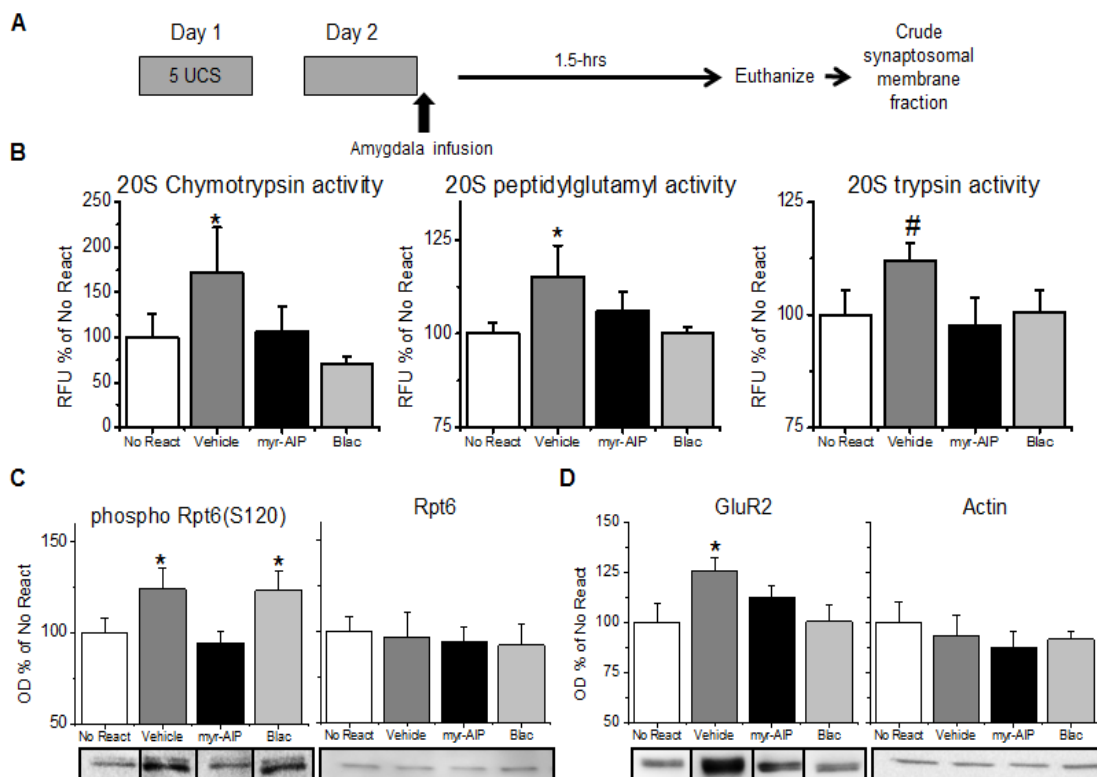


Figure 13. Inhibiting CaMKII in the amygdala prevents retrieval-induced increases in proteasome activity, Rpt6-S120 phosphorylation and GluR2 in the amygdala. (A) Animals were trained to contextual fear conditioning, given a brief retrieval the following day and microinfused with vehicle, the CaMKII inhibitor myr-AIP or proteasome inhibitor β lac. Amygdala and dorsal hippocampus crude synaptosomal membrane fractions were then collected 1.5-hrs after retrieval ($n = 8-9$ per group). (B) Both the CaMKII inhibitor and the proteasome inhibitor prevented increases in proteasome chymotrypsin, peptidylglutamyl and trypsin activity in the amygdala. (C) The CaMKII inhibitor, but not the proteasome inhibitor, prevented increases in Rpt6-S120 phosphorylation without altering total Rpt6 levels (D) Both the CaMKII inhibitor and the proteasome inhibitor prevented increases in GluR2 in the amygdala. * $P < .05$. # $P = .071$.

activities. This result suggests that CaMKII mediates increases in proteasome activity in the amygdala following memory retrieval. Consistent with this, we found an increase in Rpt6-S120 phosphorylation in the vehicle-infused and β lac groups relative to the no retrieval and myr-AIP groups ($t_{(1,27)} = 2.681, p = .012$; Figure 13C) without any change in total Rpt6 levels ($t_{(1,28)} = -0.258, p = .798$), supporting the theory that Rpt6-S120 phosphorylation is upstream of increases in proteasome catalytic activity in the amygdala

following memory retrieval. These results suggest that CaMKII regulates Rpt6-S120 phosphorylation and proteasome activity in the amygdala following memory retrieval. Interestingly, we found that the vehicle-infused animals showed a significant increase in GluR2 expression relative to the β lac, myr-AIP and no retrieval groups ($t_{(1,28)} = 2.397, p = .023$; Figure 13D), without any change in Actin expression ($t_{(1,27)} = -0.016, p = .987$). This result suggests that changes in the expression of the AMPA receptor subunits is dependent on CaMKII-mediated protein degradation in the amygdala following memory retrieval, and that inhibiting the memory destabilization process prevents reconsolidation from occurring.

We next tested if the amygdala regulates synaptic plasticity in the hippocampus following memory retrieval. Similar to the amygdala, the vehicle-infused animals showed a significant increase in proteasome trypsin-like activity in the hippocampus relative to the β lac, myr-AIP and no retrieval groups ($t_{(1,26)} = 2.076, p = .048$; Figure 14A). This suggests that protein degradation in the amygdala is necessary for retrieval-induced memory destabilization in the hippocampus. Consistent with this, we found a significant increase in Rpt6-S120 phosphorylation ($t_{(1,25)} = 3.164, p = .004$; Figure 14B) without any change in total Rpt6 levels ($t_{(1,29)} = 1.115, p = .274$). Additionally, we found a trend for an increase in GluR3 ($t_{(1,29)} = 1.798, p = .083$; Figure 14C) in the hippocampi of vehicle-infused animals relative to the β lac, myr-AIP and no retrieval groups, without any change in Actin ($t_{(1,27)} = -0.045, p = .964$). These results demonstrate that CaMKII-mediated protein degradation in the amygdala regulates memory destabilization and reconsolidation in both the amygdala and hippocampus following retrieval.

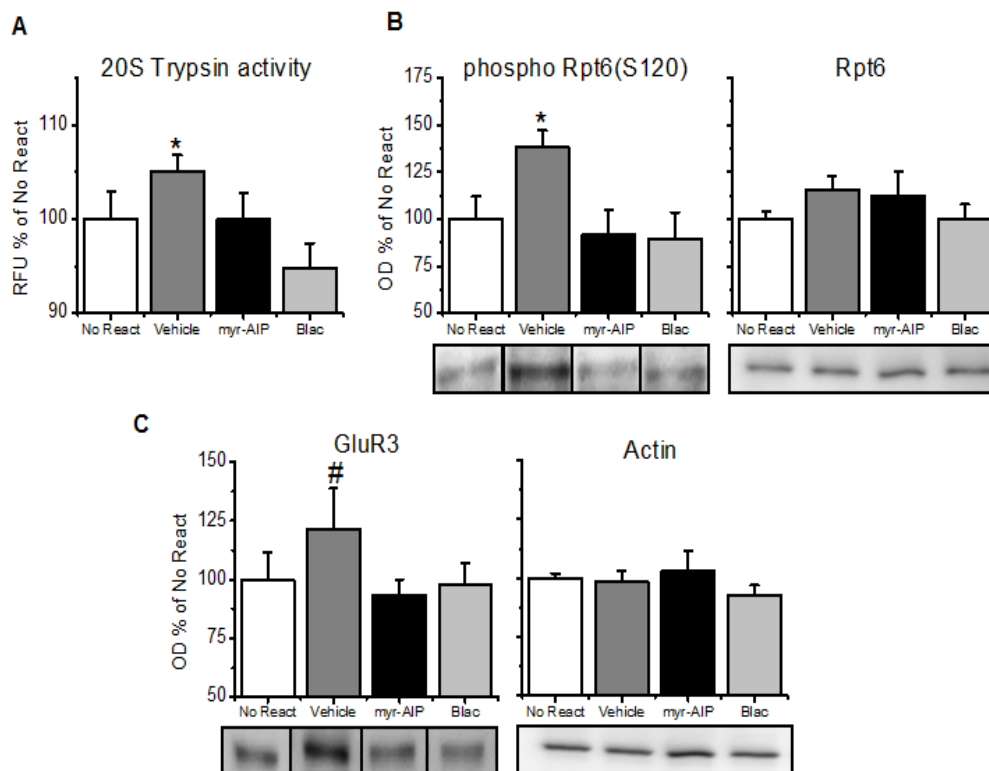


Figure 14. Inhibiting CaMKII in the amygdala prevents retrieval-induced increases in proteasome activity, Rpt6-S120 phosphorylation and GluR3 in the hippocampus. Animals were trained to contextual fear conditioning, given a brief retrieval the following day and microinfused with vehicle, the CaMKII inhibitor myr-AIP or proteasome inhibitor blac into the amygdala. Amygdala and dorsal hippocampus crude synaptosomal membrane fractions were then collected 1.5-hrs after retrieval ($n = 8-9$ per group). (A) Both the CaMKII inhibitor and the proteasome inhibitor prevented increases in proteasome trypsin activity in the hippocampus. (B) Both the CaMKII inhibitor and the proteasome inhibitor prevented increases in Rpt6-S120 phosphorylation in the hippocampus. (C) Both the CaMKII inhibitor and the proteasome inhibitor prevented increases in GluR3 in the hippocampus. * $P < .05$. # $P = .083$.

Hippocampal protein degradation does not regulate reconsolidation in the amygdala

In the previous experiment we found that retrieval-and-CaMKII dependent increases in proteasome activity in the amygdala were critical for memory destabilization and reconsolidation in the hippocampus, suggesting that amygdala protein degradation regulates a systems level reconsolidation process between the amygdala and

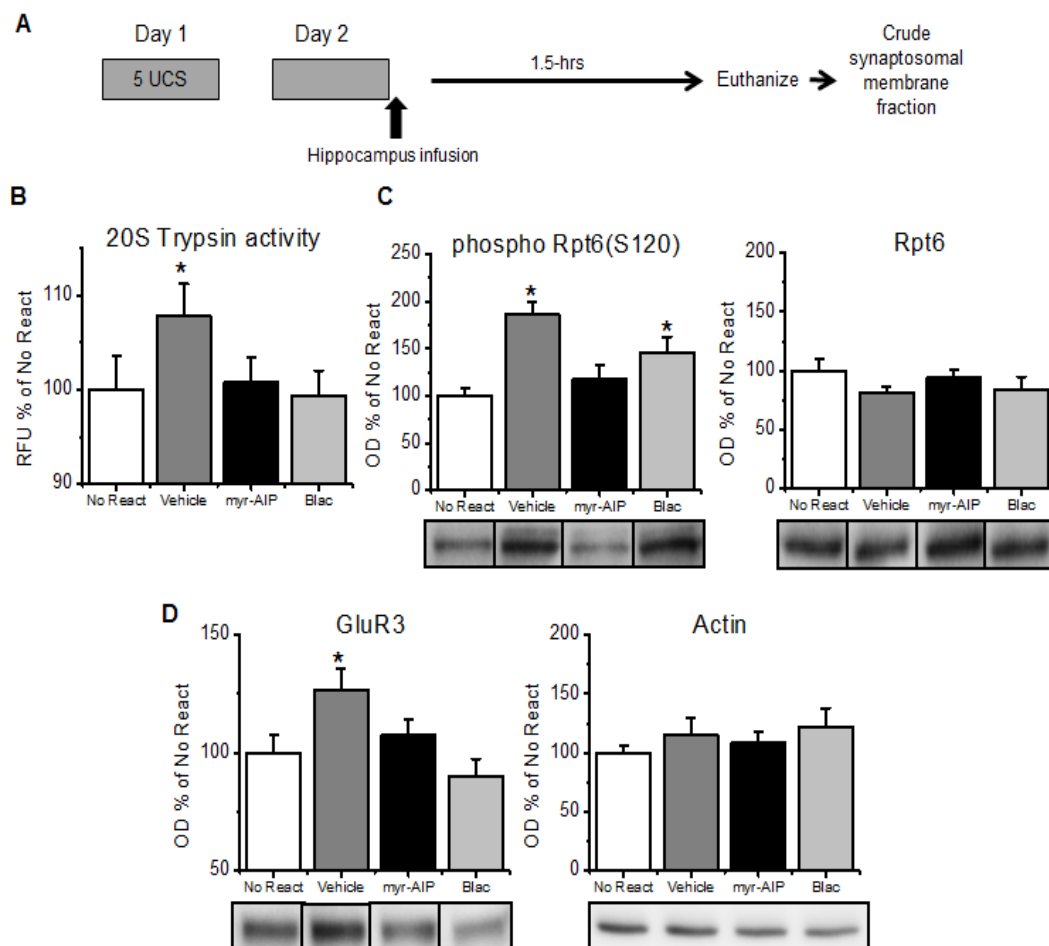


Figure 15. Inhibiting CaMKII in the hippocampus prevents retrieval-induced increases in proteasome activity, Rpt6-S120 phosphorylation and GluR3 in the hippocampus. (A) Animals were trained to contextual fear conditioning, given a brief retrieval the following day and microinfused with vehicle, the CaMKII inhibitor myr-AIP or proteasome inhibitor blac into the hippocampus. Amygdala and dorsal hippocampus crude synaptosomal membrane fractions were then collected 1.5-hrs after retrieval ($n = 7-8$ per group). (B) Both the CaMKII inhibitor and the proteasome inhibitor prevented increases in proteasome trypsin activity in the hippocampus. (C) The CaMKII inhibitor, but not the proteasome inhibitor, prevented increases in Rpt6-S120 phosphorylation without altering total Rpt6 levels (D) Both the CaMKII inhibitor and the proteasome inhibitor prevented increases in GluR3 in the hippocampus. * $P < .05$.

hippocampus. We next wanted to test if the relationship was bidirectional. To test this, we implanted animals with chronic cannula aimed at the dorsal hippocampus and trained to our contextual fear conditioning and retrieval paradigm. Following retrieval, they

received intra-hippocampus infusions of the proteasome catalytic inhibitor β lac, the CaMKII inhibitor myr-AIP or vehicle and amygdala and dorsal hippocampus crude synaptosomal membrane fractions were collected 1.5-hrs later (Figure 15A). In the hippocampus, we found that vehicle-infused animals showed a significant increase in proteasome trypsin-like activity following retrieval relative to the β lac, myr-AIP and vehicle-infused no retrieval control groups ($t_{(1,24)} = 2.188, p = .039$; Figure 15B). This result suggests that similar to the amygdala, CaMKII mediates increases in proteasome activity in the hippocampus following memory retrieval. Consistent with this, we found an increase in Rpt6-S120 phosphorylation in the vehicle-infused and β lac groups relative to the no retrieval and myr-AIP groups ($t_{(1,24)} = 2.172, p = .04$; Figure 15C) without any change in total Rpt6 levels ($t_{(1,24)} = -0.45, p = .656$), supporting the theory that Rpt6-S120 phosphorylation is upstream of increases in proteasome catalytic activity in the hippocampus following memory retrieval. These results suggest that CaMKII regulates Rpt6-S120 phosphorylation and proteasome activity in the hippocampus following memory retrieval. Interestingly, we found that the vehicle-infused animals showed a significant increase in GluR3 expression relative to the β lac, myr-AIP and no retrieval groups ($t_{(1,25)} = 3.139, p = .004$; Figure 15D) without any change in Actin expression ($t_{(1,26)} = 0.357, p = .724$). This result suggests that changes in the expression of the AMPA receptor subunits is dependent on CaMKII-mediated protein degradation in the hippocampus following memory retrieval, and that inhibiting the memory destabilization process prevents reconsolidation from occurring.

We next tested if the hippocampus regulates synaptic plasticity in the amygdala following memory retrieval. Interestingly, we found that manipulation of the

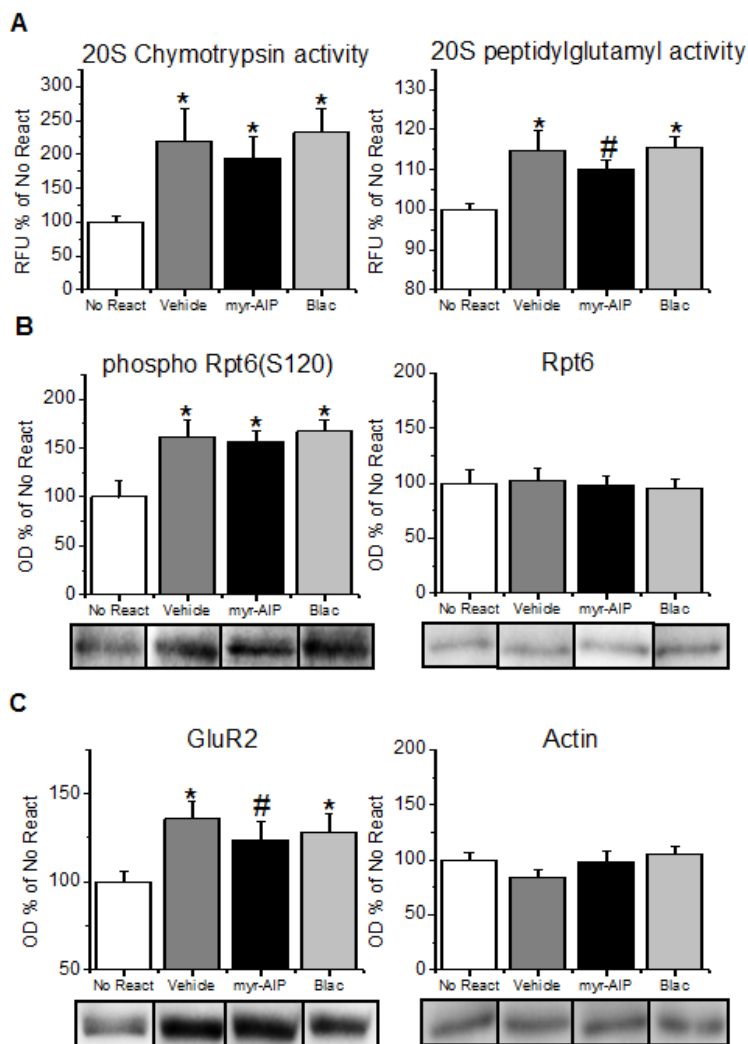


Figure 16. Inhibiting CaMKII in the hippocampus does not alter reconsolidation in the amygdala. Animals were trained to contextual fear conditioning, given a brief retrieval the following day and microinfused with vehicle, the CaMKII inhibitor myr-AIP or proteasome inhibitor β lac into the hippocampus. Amygdala and dorsal hippocampus crude synaptosomal membrane fractions were then collected 1.5-hrs after retrieval ($n = 7-8$ per group). (A-C) Neither the CaMKII inhibitor nor the proteasome inhibitor prevented increases in proteasome activity (A), Rpt6-S120 phosphorylation (B) or GluR2 (C) in the amygdala. * $P < .05$. # $P < .07$.

reconsolidation process in the hippocampus had no effect on synaptic plasticity in the amygdala. We found a main effect for proteasome chymotrypsin-like activity in the amygdala ($F_{(3,25)} = 3.223, p = .040$; Figure 16A). Fisher LSD *post hoc* tests revealed a significant retrieval-induced increase in vehicle-, β lac- and myr-AIP-infused groups relative to controls. Additionally, similar results were obtained for proteasome

peptidylglutamyl-like activity ($F_{(3,24)} = 3.846, p = .022$; Figure 16A). Consistent with this, we found main effects for Rpt6-S120 phosphorylation ($F_{(3,24)} = 4.224, p = .016$; Figure 16B) and GluR2 expression ($F_{(3,26)} = 2.835, p = .058$; Figure 16C) in the amygdala, but not for total Rpt6 ($F_{(3,26)} = 0.081, p = .970$; Figure 16B) or Actin ($F_{(3,26)} = 1.265, p = .307$; Figure 16C). Fisher LSD *post hoc* tests revealed a significant retrieval-induced increase in vehicle-, β lac- and myr-AIP-infused groups relative to controls. This suggests that protein degradation in the hippocampus is necessary for retrieval-induced memory destabilization in the hippocampus; however, retrieval-induced synaptic plasticity in the hippocampus does not regulate the systems reconsolidation process between the amygdala and hippocampus. Since the amygdala had transient changes in AMPA receptor subunit expression that occurred during the reconsolidation window and the hippocampus had delayed, long-term changes in AMPA receptor subunit expression that peaked after the completion of the reconsolidation process and the amygdala controlled reconsolidation in the hippocampus, this suggests that the amygdala may be the primary regulator of the destabilization process following retrieval while the hippocampus may be primarily involved in the retrieval-dependent “updating” of the memory trace.

Auditory fear memories destabilize in the amygdala but not the hippocampus after retrieval

In the previous experiments we found that the amygdala and hippocampus directly interacted to reconsolidate a retrieved contextual fear memory, suggesting that

contextual fear memories undergo a systems reconsolidation process. Contextual fear memories require both the amygdala and hippocampus for their formation and long-term storage. Consistent with this, we found that contextual fear memories destabilize in both the amygdala and hippocampus following retrieval. However, this suggests that systems reconsolidation between the amygdala and hippocampus likely only occurs if the memory required both regions for its initial consolidation. To test this idea, we examined if the amygdala and hippocampus also show complimentary changes in proteasome activity and AMPA receptor subunit expression following the retrieval of an auditory fear memory, which is a hippocampus-independent memory. We trained animals to auditory fear conditioning, gave them a brief retrieval the following day and collected amygdala and dorsal hippocampus crude synaptosomal membrane fractions 1.5- and 2-hrs later (Figure 17A). We first tested if the memory destabilized in the amygdala and hippocampus using our *in vitro* proteasome activity assay. In the amygdala, we found main effects for proteasome chymotrypsin ($F_{(2,26)} = 4.463, p = .022$) and peptidylglutamyl activity ($F_{(2,27)} = 4.133, p = .027$). Fisher LSD post hoc testes revealed that memory retrieval increased proteasome chymotrypsin and peptidylglutamyl activity 2-hrs, but not 1.5-hrs, after retrieval (Figure 17B). This increase is slightly delayed from what we saw in the amygdala following the retrieval of a contextual fear memory suggesting that the temporal dynamics of the reconsolidation process in the amygdala can vary depending on the type of fear memory retrieved, which is consistent with previous findings from our lab (Jarome et al., 2011). Additionally, we found a main effect for both phosphorylated

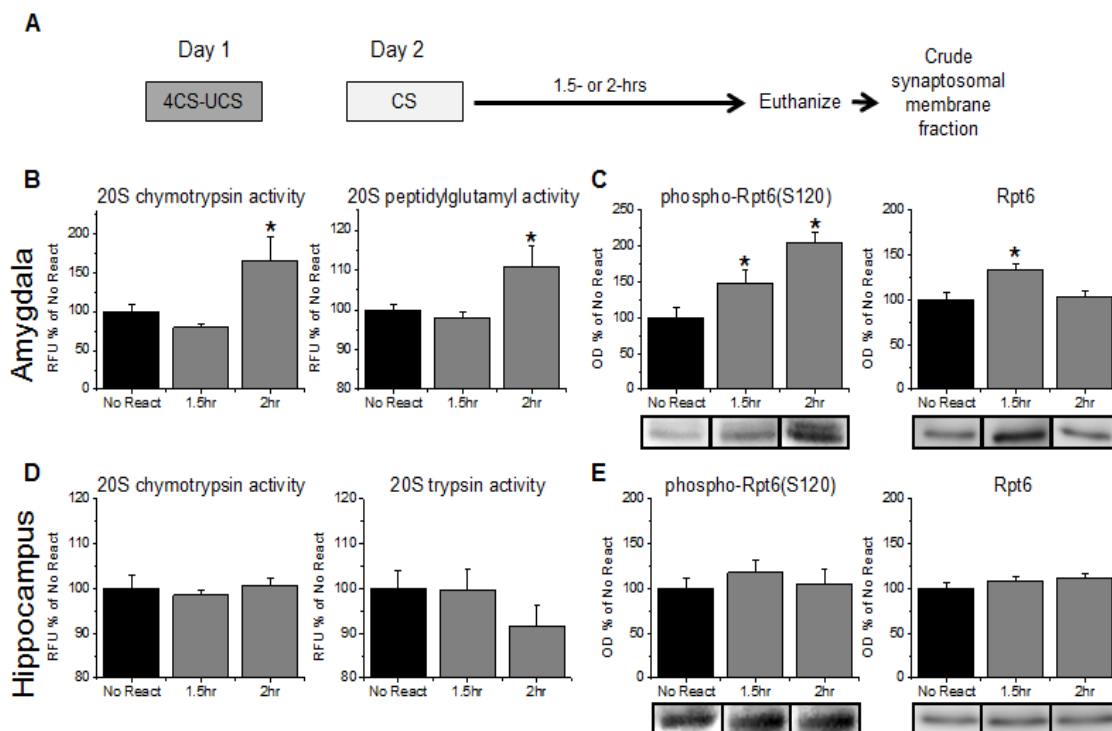


Figure 17. Auditory fear memories destabilize in the amygdala but not the hippocampus following retrieval. (A)

Animals were trained to auditory fear conditioning, given a brief retrieval the following day and amygdala and dorsal hippocampus crude synaptosomal membrane fractions were then collected 1.5- or 2-hrs after retrieval ($n = 10-11$ per group). (B) Proteasome chymotrypsin and peptidylglutamyl activity were increased in the amygdala 2-hrs after retrieval. (C) Phosphorylated Rpt6-S120 was increased at 1.5- and 2-hrs after retrieval in the amygdala, with transient increases in total Rpt6 at 1.5-hrs. (D) There were no changes in proteasome activity in the hippocampus after retrieval. (E) There were no changes in phosphorylated Rpt6-S120 or total Rpt6 in the hippocampus after retrieval. * $P < .05$.

Rpt6-S120 ($F_{(2,23)} = 11.645$, $p < .001$) and total Rpt6 ($F_{(2,25)} = 6.458$, $p = .005$) in the amygdala (Figure 17C). Fisher LSD post hoc tests revealed increases in Rpt6-S120 phosphorylation at 1.5-hrs after retrieval, with a significantly larger increase at 2-hrs. Interestingly, we found a significant increase in total Rpt6 levels at 1.5-hrs, but not 2-hrs, after retrieval. This suggests that the increase in Rpt6-S120 phosphorylation at 1.5-hrs, a time when proteasome activity was not increased, was likely due to elevated total Rpt6 levels and may reflect a non-proteolytic function of the 19S proteasome. These results

suggest that a retrieved auditory fear memory destabilizes in the amygdala. In the hippocampus, we did not find main effects for proteasome chymotrypsin ($F_{(2,25)} = 0.294$, $p = .748$) or trypsin activity ($F_{(2,29)} = 1.193$, $p = .318$; Figure 17D). Additionally, we did not observe changes in phosphorylated Rpt6-S120 ($F_{(2,29)} = 0.400$, $p = .674$) or total Rpt6 levels ($F_{(2,29)} = 0.836$, $p = .444$; Figure 17E). Collectively, these results suggest that an auditory fear memory destabilizes in the amygdala but not the hippocampus following retrieval, supporting the theory that the consolidation of auditory fear memories is independent of the hippocampus.

Distinct changes in AMPA receptor subunit expression in the amygdala and hippocampus following the retrieval of an auditory fear memory.

We found that an auditory fear memory destabilized in the amygdala but not the hippocampus following retrieval. Next, we wanted to confirm that the hippocampus was not involved in the reconsolidation of the auditory fear memory by examining AMPA receptor subunit expression in both regions. In the amygdala, we found main effects for GluR1 ($F_{(2,25)} = 3.287$, $p = .054$) and GluR2 ($F_{(2,26)} = 3.459$, $p = .047$), but not GluR3 ($F_{(2,28)} = 2.096$, $p = .142$) expression (Figure 18A). Fisher LSD post hoc tests revealed decreases in GluR1 and GluR2 expression at 1.5- and 2-hrs after retrieval, with trends for reductions in GluR3 at the same times but we did not find any changes in Actin expression ($F_{(2,29)} = 0.267$, $p = .768$; Figure 18B). These results suggest that there is a transient loss of AMPA receptor at amygdala synapses following the retrieval of an

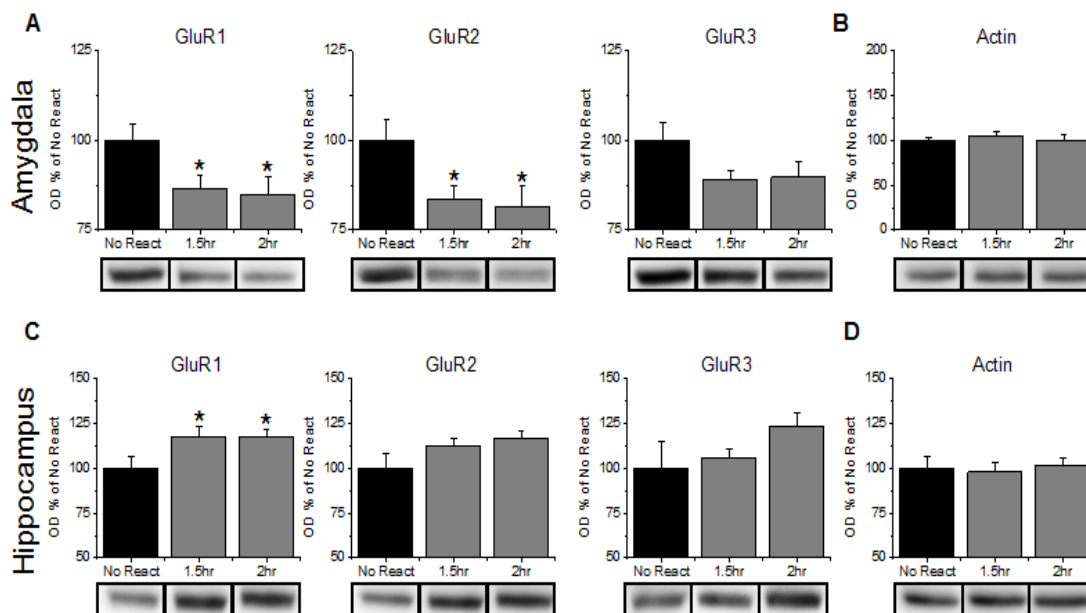


Figure 18. Distinct. Temporally linked changes in AMPA receptor subunit expression in the amygdala and hippocampus following the retrieval of an auditory fear memory. Animals were trained to auditory fear conditioning, given a brief retrieval the following day and amygdala and dorsal hippocampus crude synaptosomal membrane fractions were then collected 1.5- or 2-hrs after retrieval ($n = 10-11$ per group). (A) GluR1, GluR2, and GluR3 levels decreased in the amygdala 1.5- and 2-hrs after retrieval. (B) There was no change in Actin expression. (C) GluR1 levels were increased at 1.5- and 2-hrs in the hippocampus, and there were trends for increases in GluR2 and GluR3 at these time points. (D) There were no changes in Actin expression. * $P < .05$.

auditory fear memory, a result drastically different than what we saw in the amygdala following the retrieval of a contextual fear memory. This suggests that the amygdala can undergo characteristically distinct reconsolidation processes for different types of fear memories. Interestingly, in the hippocampus we found a main effect for GluR1 ($F_{(2,26)} = 3.194, p = .058$), but not GluR2 ($F_{(2,26)} = 2.007, p = .155$), GluR3 ($F_{(2,28)} = 1.499, p = .241$) and Actin ($F_{(2,27)} = 0.098, p = .907$) expression following retrieval of the auditory fear memory (Figure 18C and 18D). Fisher LSD post hoc tests revealed a significant increase in GluR1 expression 1.5- and 2-hrs after retrieval of the auditory fear memory. Collectively, these results suggest that while auditory fear memories destabilize in the

amygdala but not the hippocampus, both regions are involved in the reconsolidation of the memory. These results would lend to the existence of a systems reconsolidation between amygdala and hippocampus for the retrieved auditory fear memory.

Additionally, considering that memory destabilization and reconsolidation are thought to be mutually exclusive processes, this suggests that the hippocampus may be involved specifically in the retrieval-dependent updating of memory content for the auditory fear memory (see discussion).

Contextual novelty, but not prediction error, regulates the reconsolidation-dependent updating of retrieved fear memories

In the previous experiments we provided evidence that outline cellular and systems mechanisms by which consolidated memories destabilize and reconsolidate following retrieval. Specifically, our results suggest a novel pathway by which memories destabilize following retrieval, through CaMKII-mediated phosphorylation of Rpt6-S120 and increased proteasome catalytic activity. Importantly, we show for the first time that retrieved memories undergo a systems reconsolidation process where retrieval-dependent plasticity in the hippocampus is dependent on CaMKII-mediated protein degradation in the amygdala. This suggests that retrieved fear memories reconsolidate and “update” in a distributed network of brain regions that are regulated by protein degradation in the amygdala. However, while the prevalent theory is that reconsolidation mediates memory updating, it is unknown what that new information is under normal retrieval conditions.

Some recent evidence has shown that retrieval can strengthen contextual-based fear memories, suggesting that reconsolidation may incorporate new contextual information into the memory trace (de Oliveira Alvares, Crestani, Cassini, Haubrich, Santana & Quillfeldt, 2013; Inda, Muravieva & Alberini, 2011). More recent evidence indicates that reconsolidation occurs in response to an error in the CS-UCS contingency, suggesting that prediction error may be the major factor underlying the reconsolidation-dependent updating of fear memories (Diaz-Mataix, Ruiz Martinez, Schafe, LeDoux & Doyere, 2013; Sevenster, Beckers & Kindt, 2013). However, the studies examining prediction error have always presented the UCS during retrieval, so it is unknown if prediction error controls memory reconsolidation under normal retrieval conditions in which the UCS is not presented. Additionally, all of the studies that have suggested that contextual information may be the new information incorporated into the memory trace during reconsolidation have used context-based fear conditioning tasks, making it unclear if other non-contextual based memories reconsolidate due to new contextual information. Additionally, the latter studies did not manipulate the contextual information present during retrieval, suggesting that the retrieval-dependent strengthening of the memories could have been due to another variable.

In our final experiment, we tested what new information is present at the time of memory retrieval that controls the destabilization and reconsolidation of an auditory fear memory, contextual novelty or prediction error. We did this by manipulating what the animals learned immediately prior to and/or during training. We used auditory fear conditioning since 1) the discrete cue allows precise control over the CS-UCS contingency during training and 2) we found that these memories destabilize in the

amygdala, but reconsolidate both in the amygdala and hippocampus which allows independent measures of memory reconsolidation and updating. We manipulated two different parts of the animals training experience, prediction error and retrieval novelty/contextual information (Figure 19A). To manipulate prediction error, two groups of animals were trained to 50% reinforcement during conditioning and compared to animals that received 100% reinforcement. This type of partial reinforcement manipulates the CS-UCS contingency so that the CS has an equal chance of being followed by the UCS as it does not being followed by the UCS, which allows us to prevent prediction error during retrieval without presenting the UCS (Haselgrove, Aydin & Pearce, 2004; Jenkins & Stanley, 1950). To control for memory strength, all groups received an equal number of shock presentations but the 50% reinforcement groups received twice the number of CS presentations. To manipulate retrieval novelty, we exposed two groups of animals to the retrieval parameters the day prior to fear conditioning. On the day after training, four groups received a retrieval event using the same parameters and amygdala and dorsal hippocampus crude synaptosomal membrane fractions were collected 2-hrs later. This resulted in five groups, 100% with no retrieval (No React), 100% reinforcement without pre-exposure (100%), 100% reinforcement with pre-exposure (100% + Pre), 50% reinforcement without pre-exposure (50%) and 50% reinforcement with pre-exposure (50% + Pre). During fear conditioning, we found main effects for time ($F_{(1,61)} = 2988.493, p < .001$) and condition ($F_{(4,61)} = 7.430, p < .001$), and we found a time by conditioning interaction ($F_{(4,61)} = 3.663, p = .010$). Fisher LSD post hoc tests revealed that the 50% reinforcement groups, regardless of pre-exposure, froze significantly more than the no retrieval and 100% reinforcement groups (Figure

19B).

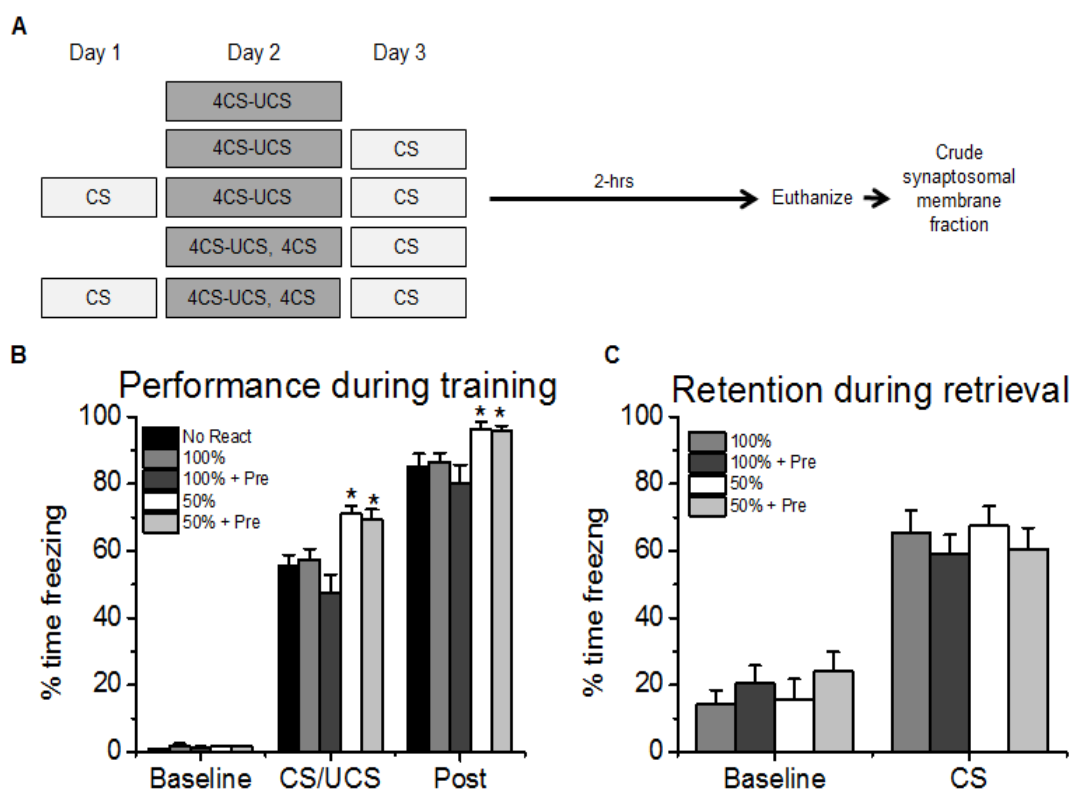


Figure 19. Retrieval pre-exposure and partial reinforcement do not result in altered memory retention during

retrieval. (A) Animals were trained to 100% or 50% reinforcement auditory fear conditioning, given a brief retrieval the following day and amygdala and dorsal hippocampus crude synaptosomal membrane fractions were then collected 2-hrs after retrieval ($n = 13-14$ per group). Two groups of animals were given pre-exposure to the retrieval conditions the day prior to training. (B) The 50% reinforcement groups froze significantly more than the 100% groups during training. (C) There were no differences in memory retention between groups during the retrieval session. $* P < .05$.

However, during the retrieval session we found a main effect for time ($F_{(1,49)} = 195.351$, $p < .001$) but not condition ($F_{(3,49)} = 0.066$, $p = .978$) and there was not a time by condition interaction ($F_{(3,49)} = 1.559$, $p = .211$; Figure 19C). This suggests that while the unique training conditions resulted in differential performance during the training session, the animals' retention of the task was equivalent. This indicates that any

differences seen in our molecular measures are likely not due to differences in performance between the different groups.

We next examined changes in memory destabilization in the amygdala and hippocampus by measuring *in vitro* proteasome activity. In the amygdala, we found main effects for proteasome chymotrypsin ($F_{(4,57)} = 2.622, p = .044$) and peptidylglutamyl ($F_{(4,58)} = 2.890, p = .030$) activities (Figure 20A). Fisher LSD post hoc tests revealed that proteasome chymotrypsin activity was increased after retrieval relative to no retrieval controls and both pre-exposure groups but not the 50% (no pre-exposure) group. This suggests that the novelty of the CS in the new context during retrieval, but not prediction error, governs the destabilization of the retrieved fear memory. Consistent with this, Fisher LSD post hoc tests revealed that proteasome peptidylglutamyl activity was significantly higher in the 100% reinforcement group relative to the 100% + Pre group, but not the 50% reinforcement group. Additionally, we did not have any changes in proteasome number in the amygdala ($F_{(4,58)} = 0.819, p = .519$; Figure 20B) or in proteasome trypsin activity in the hippocampus ($F_{(4,57)} = 0.386, p = .818$; Figure 20C). These results suggest that the novelty of the CS occurring in the new context during retrieval is the new information present during retrieval that controls the reconsolidation-dependent “updating” of an auditory fear memory, and indicates that memories are likely undergoing modification during memory reconsolidation.

Next, we examined changes in the expression of AMPA receptor subunits following retrieval of the auditory fear memory. In the amygdala, we found a main effect for GluR2 ($F_{(4,52)} = 3.903, p = .008$), but not GluR1 expression ($F_{(4,54)} = 1.219, p = .314$), and a trend for a main effect on GluR3 expression ($F_{(4,53)} = 2.233, p = .078$). Fisher LSD

post hoc tests revealed a significant reduction in GluR2 and GluR3 expression and a trend for a reduction in GluR1 expression in animals receiving 100% reinforcement without

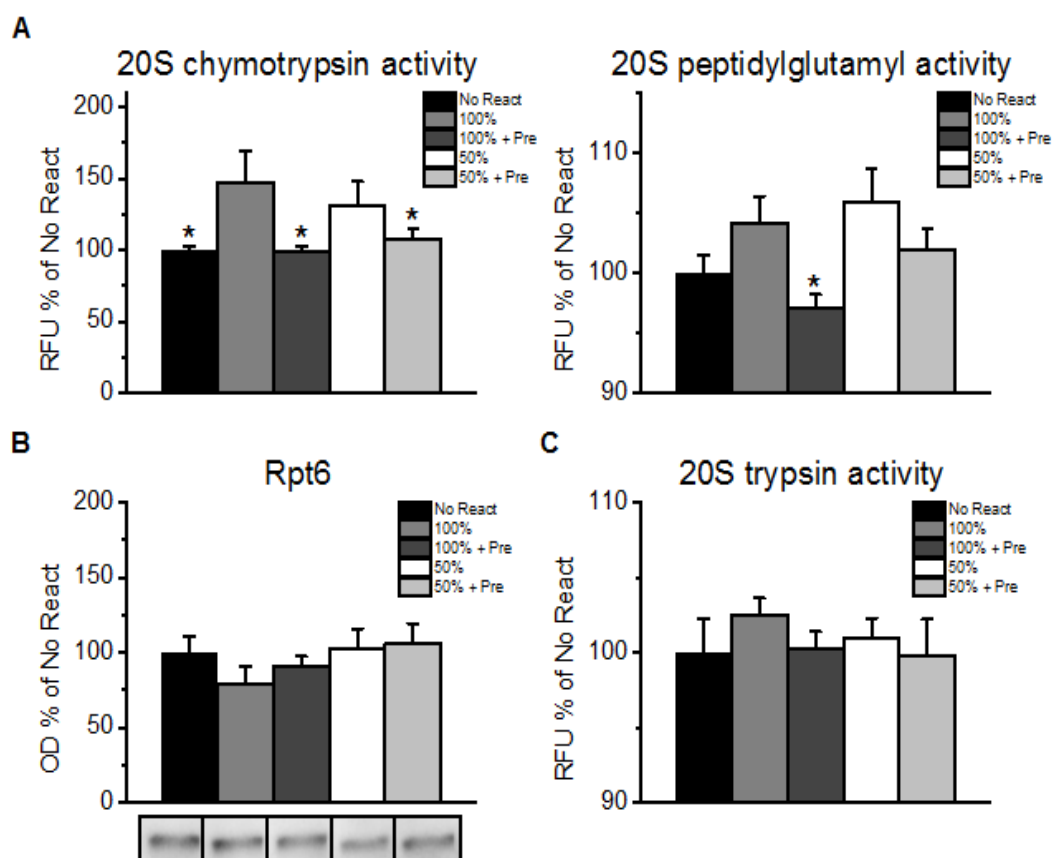


Figure 20. Contextual novelty, but not prediction error, regulates the destabilization of a

retrieved fear memory in the amygdala. Animals were trained to 100% or 50% reinforcement

auditory fear conditioning, given a brief retrieval the following day and amygdala and dorsal

hippocampus crude synaptosomal membrane fractions were then collected 2-hrs after retrieval (n =

13-14 per group). Two groups of animals were given pre-exposure to the retrieval conditions the day

prior to training. (A-C) Eliminating contextual novelty, but not prediction, prevented increases in

proteasome activity in the amygdala (A, B) without altering total proteasome number (C). (D) None of

the manipulations increased proteasome activity in the hippocampus. * $P < .05$.

pre-exposure relative to no retrieval controls (Figure 21A). Interestingly, pre-exposing

animals to the retrieval conditions completely alleviated the reduction in GluR2 and

GluR3 in the 100% reinforcement group, without altering the reductions in GluR1. Additionally, animals trained to 50% reinforcement without pre-exposure showed a significant reduction in GluR2 relative to no retrieval controls that was completely rescued by the retrieval pre-exposure. There were no difference in Actin expression ($F_{(4,58)} = 0.239, p = .915$; Figure 21B). These results indicate that changes in GluR2 expression following memory retrieval strongly correlate with the presence of new information at the time of retrieval, suggesting that changes in GluR2 expression may be critical for the proper reconsolidation-dependent updating of a retrieved fear memory.

In the hippocampus we did not find main effects for GluR1 ($F_{(4,58)} = 1.405, p = .244$; Figure 21C), GluR3 ($F_{(4,60)} = 0.553, p = .698$) or Actin expression ($F_{(4,57)} = 0.223, p = .925$; Figure 21D). Since contextual novelty during retrieval but not prediction error controlled changes in proteasome activity and AMPA receptor subunit expression in the amygdala, we tested whether it also controlled changes in GluR1 expression in the hippocampus following retrieval. Consistent with the amygdala, a planned comparison revealed a significant increase in GluR1 expression in 100% and 50% reinforcement groups without pre-exposure relative to no retrieval controls and pre-exposure groups ($t_{(1,58)} = 2.291, p = .026$). This suggests that eliminating the new information present at retrieval that controlled the destabilization and reconsolidation of the fear memory in the amygdala prevented changes in AMPA receptor subunit expression in the hippocampus, supporting the idea that the hippocampus may be selectively involved in the updating of the auditory fear memory. Collectively, these results suggest that the novelty of the CS occurring in a new context during retrieval (i.e., contextual novelty), but not prediction error, is the new information present at the time of retrieval that controls the

destabilization and reconsolidation of an auditory fear memory, and provides the first evidence that fear memories do undergo “updating” under normal retrieval conditions.

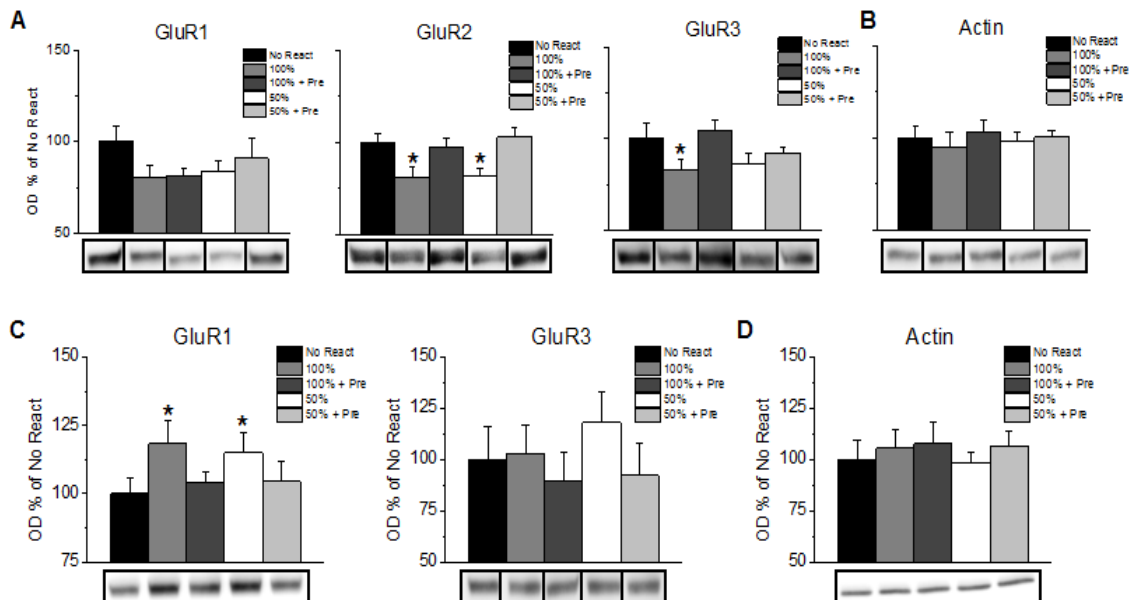


Figure 21. Contextual novelty, but not prediction error, regulates the reconsolidation of a retrieved fear memory in the amygdala. Animals were trained to 100% or 50% reinforcement auditory fear conditioning, given a brief retrieval the following day and amygdala and dorsal hippocampus crude synaptosomal membrane fractions were then collected 2-hrs after retrieval ($n = 13-14$ per group). Two groups of animals were given pre-exposure to the retrieval conditions the day prior to training. (A-B) Eliminating contextual novelty, but not prediction, prevented decreases in GluR2 and GluR3, but not GluR1, in the amygdala. (B) There were no changes in Actin expression in the amygdala. (C) Eliminating contextual novelty, but not prediction, prevented increases in GluR1, but not GluR3, in the hippocampus. (D) There were no changes in Actin expression in the hippocampus. * $P < .05$.

Discussion

Collectively, the present series of experiments revealed that 1) fear conditioning increases both Rpt6-S120 phosphorylation and proteasome activity in a CaMKII, but not PKA, dependent manner in the amygdala, 2) retrieval of a contextual fear memory resulted in temporally linked but characteristically distinct destabilization processes in the amygdala and hippocampus, 3) the retrieval of a contextual fear memory resulted in

temporally separate and unique changes in AMPA receptor subunit expression in the amygdala and hippocampus, 4) CaMKII regulates increases in Rpt6-S120 phosphorylation, proteasome activity and GluR2/3 in the amygdala and hippocampus following the retrieval of a contextual fear memory, 5) protein degradation in the amygdala regulates the “systems reconsolidation” of a contextual fear memory between the amygdala and hippocampus, 6) the retrieval of an auditory fear memory engages both the amygdala and hippocampus and 7) contextual novelty, but not prediction error, is the new information present during retrieval that controls the reconsolidation-dependent updating of an auditory fear memory. These results provide evidence to support updated cellular models of memory consolidation and reconsolidation that include protein degradation, the existence of a systems reconsolidation process, and that reconsolidation does mediate memory updating under normal retrieval conditions.

The regulation of protein degradation during memory consolidation

Numerous studies have supported the theory that the formation of long-term fear memories requires *de novo* gene transcription and protein synthesis (Bailey et al., 1999; Parsons et al., 2006b; Schafe & Ledoux, 2000) and increases in protein synthesis have been reported following fear conditioning (Hoeffler et al., 2011). Importantly, this requirement for new gene transcription and translation has been reported for a variety of different fear-based memory tasks across several different brain regions (for review, see Jarome & Helmstetter, 2013), supporting the theory that at the cellular level memory consolidation is dependent on protein synthesis and the upstream signaling pathways

which regulate it in multiple brain regions. As a result, the prevalent hypothetical cellular model of memory consolidation suggests that activation of NMDA receptors at the time of behavioral training triggers increased activity of intracellular signaling pathways which regulate the transcriptional and translational processes necessary for long-term memory formation (Johansen et al., 2011). However, this model does not account for the potential role of protein degradation.

In recent years, numerous studies have begun to support a role for protein degradation in the initial consolidation of fear memories. For example, protein degradation has been shown to be involved in the formation of conditioned taste aversion, auditory, contextual and inhibitory avoidance fear memories (Felsenberg, Dombrowski & Eisenhardt, 2012; Jarome et al., 2011; Lopez-Salon et al., 2001; Pick, Malumbres & Klann, 2012; Pick, Wang, Mayfield & Klann, 2013; Rodriguez-Ortiz, Balderas, Saucedo-Alquicira, Cruz-Castaneda & Bermudez-Rattoni, 2011). Despite these emerging findings, the current cellular model of memory consolidation does not account for protein degradation being involved in the consolidation process. One potential reason for this is that it is currently unknown if protein degradation is directly interacting with the transcriptional and translation control pathways that we know to be important in memory consolidation or if it is occurring as a parallel, independent process. One way to remedy this is by determining how protein degradation is regulated during the consolidation process and if the same intracellular signaling pathways that are thought to regulate gene transcription and protein translation are involved in the regulation of protein degradation during long-term memory formation. In cell cultures, both PKA and CaMKII have been shown to regulate protein degradation through their actions on the proteasome (Bingol et

al., 2010; Djakovic et al., 2009; 2012; Hamilton et al., 2012), though this link has never been established *in vivo*.

In the present series of experiments, we found that fear conditioning lead to increases in proteasome activity in the amygdala. Interestingly, this increase in proteasome activity correlated with increased phosphorylation of the proteasome regulatory subunit Rpt6-S120, a CaMKII and PKA target site *in vitro*. Importantly, we found that specifically manipulating CaMKII, but not PKA, signaling in the amygdala following fear conditioning prevented this increase in proteasome activity and reduced the phosphorylation of Rpt6-S120 without altering protein polyubiquitination levels. This result indicates that CaMKII, in addition to its potential regulation of gene transcription, is a critical regulator of protein degradation during long-term memory formation in the amygdala, suggesting a novel role for CaMKII during the consolidation process. However, PKA, which can regulate proteasome activity *in vitro*, is not involved in the regulation of protein degradation during the memory consolidation process. This result lends to an updated hypothetical model of cellular consolidation in which protein degradation may be a central component of the consolidation process that links upstream signaling to the downstream transcriptional and translational processes (Jarome & Helmstetter, 2013). In this hypothetical model (Figure 22), fear conditioning leads to the activation of NMDA receptors which increase protein polyubiquitination levels (Jarome et al., 2011), likely through increased activity of the ubiquitin ligases. Proteasome activity is then increased through NMDA-CaMKII mediated phosphorylation of Rpt6-S120. The proteasome then can target and degrade a variety of proteins

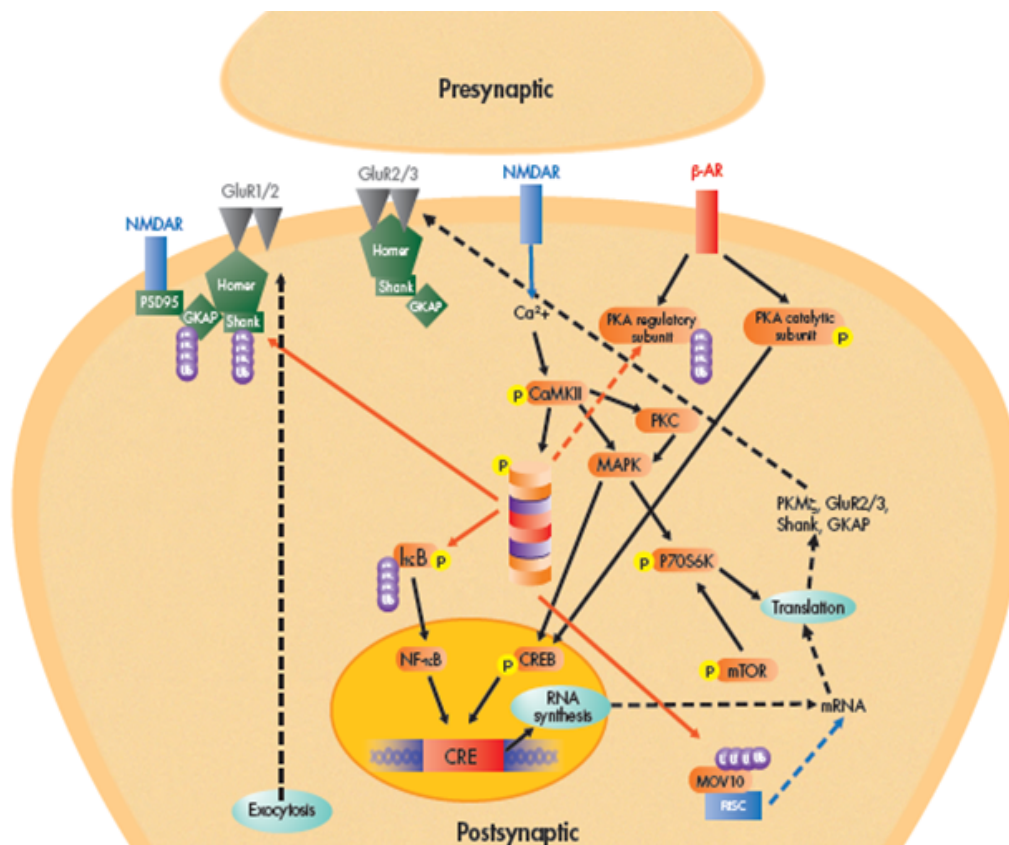


Figure 22. Cellular model of memory consolidation. New memories are formed by the coordinated activation of the UPS, which regulates transcriptional and translational control proteins to promote increases in gene transcription and protein synthesis necessary for normal memory formation. In this hypothetical model, protein polyubiquitination is increased through a NMDA-dependent mechanism and proteasome activity is increased by NMDA-CaMKII mediated phosphorylation of the 19S subunit Rpt6 at Serine-120. The proteasome then can regulate *de novo* gene transcription and protein translation through the removal of transcriptional and translational repressors and proteasome-dependent degradation of “master” scaffolds such as Shank could control alterations to the postsynaptic structure, allowing long-term changes in synaptic strength. This model suggests that protein degradation could be a major regulator of memory consolidation by linking upstream signaling mechanisms to the downstream transcriptional and translational processes thought to be important in long-term memory formation.

involved in transcriptional (Lopez-Salon et al., 2001; Upadhy, Smith & Hegde, 2004) or translational control (Banerjee et al., 2009; Jarome et al., 2011), and the regulation of the

synaptic structure (Jarome et al., 2011). This model suggests that protein degradation may not only link upstream signaling to the downstream transcriptional and translational processes, but that it may actually be a primary regulator of gene transcription and protein synthesis during long-term memory formation. Future research will have to examine the downstream predictions of this model in more detail.

The regulation of protein degradation during memory reconsolidation

Numerous studies have shown that upon retrieval once consolidated memories destabilize and require *de novo* protein synthesis in order to restabilize, a process referred to as memory reconsolidation (Jarome et al., 2011; 2012; Nader et al., 2000; Parsons et al., 2006a; 2006b). At the cellular level, reconsolidation does require some of the same mechanisms as the initial consolidation process does, however, reconsolidation is not simply a recapitulation of consolidation (Alberini, 2005). Despite this, recent evidence suggests that protein degradation is involved in the both consolidation and reconsolidation of auditory and contextual fear memories in the amygdala (Jarome et al., 2011). Interestingly, while protein degradation is critical for the long-term storage of the memory during consolidation, it actually regulates the lability or destabilization of the memory trace following retrieval (Jarome et al., 2011; Lee et al., 2008). This suggests that while protein degradation is involved in both the consolidation and reconsolidation processes, the functional significance of this protein degradation process may differ between these two different stages of memory storage. In the present series of experiments, we found that protein degradation was regulated by a similar mechanism

following both memory acquisition and retrieval. Specifically, we found that CaMKII regulated increases in both Rpt6-S120 phosphorylation and proteasome activity during memory consolidation and reconsolidation. In combination with our previous studies examining NMDA-receptor mediated regulation of protein degradation following memory acquisition and retrieval (Jarome et al., 2011), these results suggest that protein degradation is regulated by a NMDA-CaMKII-dependent process during both memory consolidation and reconsolidation in the amygdala. This would suggest that protein degradation is initiated by similar mechanisms following behavioral training and retrieval, and indicates a general pathway by which memory storage is regulated at the cellular level. These results provide support for a newer hypothetical model of cellular reconsolidation (Figure 23), in which NMDA-CAMKII-dependent increases in protein degradation following retrieval regulate the “destabilization” of a consolidated memory through the disassembly of the postsynaptic structure (Jarome & Helmstetter, 2013; Kaang & Choi, 2012).

One interesting finding from our study was that changes in AMPA receptor expression following retrieval were dependent on proteasome activity. These results are in agreement with recent evidence demonstrating that protein degradation can regulate reductions in the expression of GluR2 following the retrieval of a cocaine reward memory (Ren, Liu, Xue, Ding, Xue, Zhai & Lu, 2013) and suggests that one potential function of protein degradation during the reconsolidation process is to regulate changes to the postsynaptic density (Jarome & Helmstetter, 2013). Consistent with this, the proteasome is known to target the receptor scaffold Shank following memory retrieval (Jarome et al., 2011; Lee et al., 2008). Whether the retrieval-and-proteasome dependent

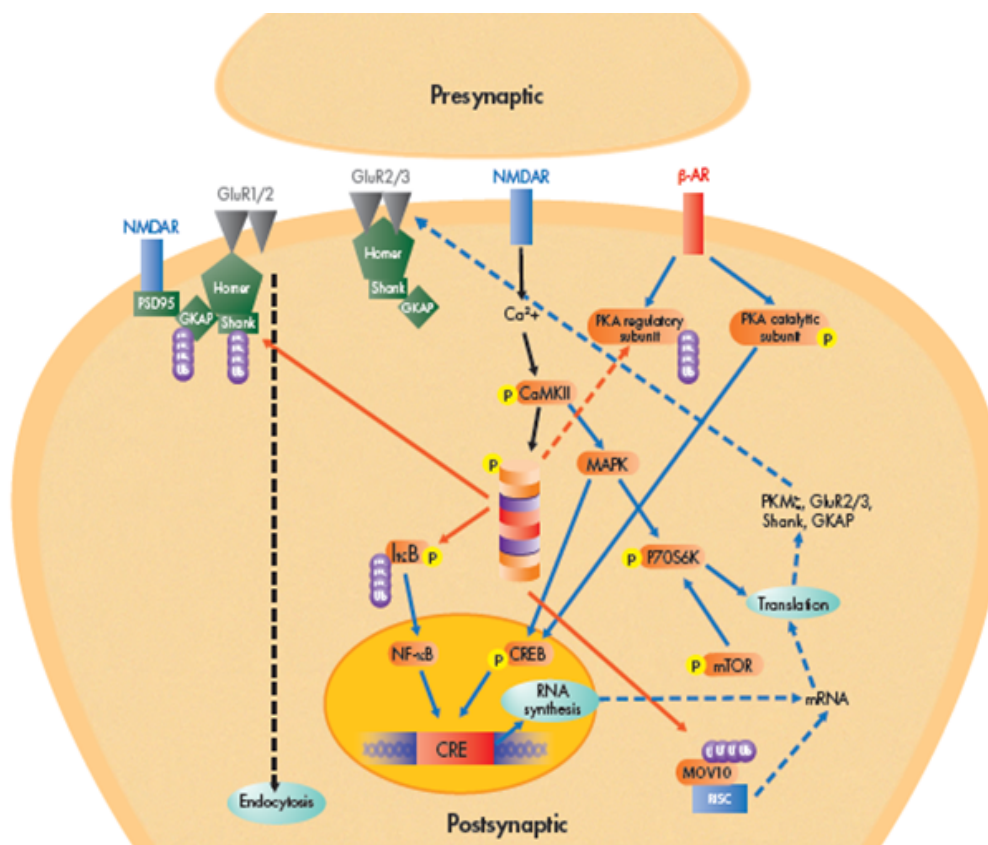


Figure 23. Cellular model of memory reconsolidation. Retrieved consolidated memories are modified by the coordinated activation of the UPS, which controls the destabilization of the memory. In this hypothetical model, protein polyubiquitination is increased through a NMDA-dependent mechanism and proteasome activity is increased by NMDA-CaMKII mediated phosphorylation of the 19S subunit Rpt6 at Serine-120. The proteasome then can regulate the need for *de novo* protein translation by disassembling the synaptic structure through the degradation of “master” scaffolds such as Shank, allowing new long-term changes in synaptic strength. Additionally, the UPS could directly regulate increases in gene transcription and protein translation, but these would occur as a consequence of memory destabilization initiated by disassembly of the synaptic structure. This model suggests that protein degradation could be a major regulator of memory reconsolidation by controlling the ability of synapses to be plastic following retrieval, allowing modification of previously consolidated memories.

changes in AMPA receptor subunit expression is dependent on the degradation of Shank following retrieval will be of interest in future studies.

Systems reconsolidation

Both the amygdala and hippocampus have been shown to be important in the reconsolidation of various different fear memories (Finnie & Nader, 2012). For example, contextual fear memories are known to reconsolidate in both the amygdala and hippocampus (Debiec et al., 2002; Jarome et al., 2011; Lee et al., 2008; Mamiya et al., 2009), while inhibitory avoidance and auditory fear memories reconsolidation only in the amygdala (Jarome et al., 2012; Milekic, Pollonini & Alberini, 2007; Nader et al., 2000; Taubenfeld, Milekic, Monti & Alberini, 2001). Currently, it is unknown if the amygdala and hippocampus interact to destabilize and reconsolidate a retrieved fear memory through a “systems reconsolidation” process. Contextual fear memories are known to undergo a protein degradation and protein synthesis dependent reconsolidation process in both the amygdala and hippocampus following retrieval (Jarome et al., 2011; Lee et al., 2008), and these two brain regions interact for the proper consolidation of contextual fear memories following their acquisition (Calandreau, Trifilieff, Mons, Costes, Marien, Marighetto, Micheau, Jaffard & Desmedt, 2006; Coelho, Ferreria, Soares & Oliveira, 2013; Huff, Frank, Wright-Hardesty, Sprunger, Matus-Amat, Higgins & Rudy, 2006), suggesting that the amygdala and hippocampus may interact to reconsolidate retrieved contextual fear memories.

In the present study, we found that the amygdala and hippocampus show unique changes in proteasome activity and AMPA receptor subunit expression following the retrieval of a contextual fear memory. We found that the amygdala and hippocampus

have temporally linked increases in proteasome activity, which peak 1.5-hrs after retrieval. This overlapping increase in proteasome activity across the two brain regions is consistent with previous studies showing that changes in degradation-specific polyubiquitination peak at similar times in the amygdala and hippocampus (Jarome et al., 2011; Lee et al., 2008). Additionally, the increase in proteasome activity in both regions returned to baseline by 2-hrs, supporting that the destabilization process is complete within 2-hrs of memory retrieval (Jarome et al., 2011). Interestingly, though these increases in proteasome activity occurred simultaneously, we found unexpected differences in the type of proteolytic activity increased across the two regions. In the amygdala we found increases in proteasome chymotrypsin and peptidylglutamyl activity, while in the hippocampus we found an increase in proteasome trypsin activity. This suggests that while the retrieval-dependent protein degradation processes in the amygdala and hippocampus are similar, they are not identical.

Consistent with this similar but unique destabilization process in the amygdala and hippocampus, we unexpectedly found very specific changes in AMPA receptor subunit expression that differed in both timing and appearance in both regions. In the amygdala, we found transient, bidirectional changes in AMPA receptor subunit expression, characterized by decreases in GluR1 and increases in GluR2. Importantly, both of these changes were reversed by the completion of the reconsolidation process (6-hrs+). In the hippocampus we found more delayed and persistent changes in AMPA receptor subunit expression, characterized by delayed increased in GluR2 and rapid and persistent increases in GluR3. Interestingly, the increases in subunit expression were specific to (GluR2) or still present at (GluR3) 7-hrs after retrieval, a time point outside

the completion of the reconsolidation process. This suggests that the changes in AMPA receptor subunit expression selectively occurred during reconsolidation in the amygdala and were largely confined to the post-reconsolidation window in the hippocampus.

These complimentary changes in AMPA receptor subunit expression between the amygdala and hippocampus may reflect a systems reconsolidation process, where the amygdala and hippocampus directly interact to correctly destabilize, reconsolidate and update the retrieved fear memory.

Since the amygdala had earlier changes in AMPA receptor subunit expression than the hippocampus, this may suggest that the amygdala regulates the hippocampus during the reconsolidation of a retrieved contextual fear memory. Consistent with this, we found that inhibiting CaMKII in the amygdala not only prevented increases in proteasome activity and AMPA receptor subunit expression in the amygdala, but also in the hippocampus. This suggests that the amygdala regulates the reconsolidation process in the hippocampus following the retrieval of a contextual fear memory. Interestingly, unlike memory consolidation, this regulatory relationship between the amygdala and hippocampus was not bidirectional as we found that manipulation of CaMKII in the hippocampus selectively altered proteasome activity and AMPA receptor subunit expression in the hippocampus without altering the retrieval-dependent increases in these molecules in the amygdala. Collectively, these results suggest that in addition to cellular reconsolidation, retrieved fear memories can also undergo systems reconsolidation that is regulated by protein degradation in the amygdala.

One question that remains is if this systems reconsolidation process is selective to contextual fear memories, the only fear memory known to reconsolidate in multiple brain

regions. However, some evidence suggests that this systems reconsolidation process may exist for other types of fear memories. For example, we found that even though auditory fear memories, a hippocampus-independent memory, destabilize in the amygdala but not the hippocampus following retrieval, both the amygdala and hippocampus show changes in AMPA receptor subunit expression. This would suggest that while the hippocampus is not involved in the consolidation or destabilization of auditory fear memories, it may be involved in its reconsolidation. Importantly, it is likely that the amygdala regulates the hippocampus in this case as well since destabilization and reconsolidation are mutually exclusive, where destabilization must occur for a memory to reconsolidate. Consistent with this, inhibitory avoidance memories, which reconsolidate in the amygdala but not the hippocampus, can undergo retrieval-dependent memory strengthening that is dependent on the hippocampus (Chen, Stern, Garcia-Osta, Saunier-Rebori, Pollonini, Bambah-Mukku, Blitzer & Alberini, 2011). These results suggest that the amygdala and hippocampus may interact to regulate the systems reconsolidation of fear memories in general, and that the amygdala may be the primary site regulating this systems reconsolidation process.

Retrieval-dependent changes in AMPA receptor subunit expression

While the initial reports of mechanisms of memory reconsolidation focused primarily on potential regulators of transcription and translation (e.g., Debiec et al., 2002; Duvarci et al., 2005; Nader et al., 2000; Tronson et al., 2006), more recently there has been a rise in interest in retrieval-dependent changes in AMPA receptor subunit

phosphorylation and expression as a marker of reconsolidation-mediated long-term synaptic modification. For example, retrieval of an auditory fear memory increases the phosphorylation of the AMPA receptor subunit GluR1-S845, which correlates with memory destabilization (Jarome et al., 2012). Phosphorylation of GluR1 at Serine845 is associated with AMPA receptor trafficking, suggesting that memory retrieval results in changes in AMPA receptor subunit expression. Consistent with this, several recent studies have shown both transient and persistent changes in AMPA receptor subunit expression following retrieval (Clem & Hugarir, 2010; Hong, Kim, Kim, Lee, Ko, Nader, Kaang, Tsien & Choi, 2013; Rao-Ruiz et al., 2011), suggesting that changes in subunit expression may be a marker of the reconsolidation process.

The primary focus of studies examining retrieval-dependent changes in AMPA receptor subunit expression has been changes in GluR2 levels as GluR2-containing AMPA receptors are calcium-impermeable and GluR2-lacking AMPA receptors are calcium-permeable (Derkach, Oh, Guire & Soderling, 2007). For example, one study found that the presence of GluR2-lacking AMPA receptors in the amygdala at the time of retrieval regulated the reconsolidation-dependent updating of an auditory fear memory (Clem & Hugarir, 2010), suggesting that calcium-permeable AMPA receptors regulate the reconsolidation process. However, another recent study found that there was a greater presence of GluR2-containing AMPA receptors following memory consolidation while memory retrieval resulted in a transient increase in GluR2-lacking AMPA receptors in the amygdala and inhibiting this exchange of calcium-impermeable to calcium-permeable receptors prevented memory destabilization (Hong et al., 2013). Interestingly, the retrieval-dependent increase in GluR2-lacking AMPA receptors

reversed with time and inhibiting the exchange of receptors back to GluR2-containing prevented the reconsolidation of the memory. This suggests that an exchange between GluR2-containing and GluR2-lacking receptors in the amygdala regulates the destabilization and reconsolidation of a retrieved fear memory. Consistent with this, in the hippocampus retrieval of a contextual fear memory results in a transient loss of GluR2-containing AMPA receptors which is necessary for persistent increases in GluR2-containing AMPA receptors after the reconsolidation process has completed (Rao-Ruiz et al., 2011), supporting that the reconsolidation process requires changes in the presence of GluR2-containing AMPA receptors.

The present series of experiments contribute to this growing literature examining changes in AMPA receptor subunit expression following retrieval. Following the retrieval of a context fear memory, we found increases in the expression of the AMPA receptor subunits GluR2 and GluR3 in the hippocampus at 7-hrs. These results partially replicate those of Rao-Ruiz and colleagues (2011). In their study, they found a transient loss of GluR1, GluR2 and GluR3 receptors at 1-2-hrs after retrieval in the hippocampus that was followed by increases in GluR2 and GluR3 receptors at 7-hrs. The lack of reductions in GluR1, GluR2 and GluR3 expression in our study was surprising, but may be attributed to procedural differences. For example, Rao-Ruiz used a relatively weak training protocol while ours is a strong protocol that results in substantially higher freezing and strength of conditioning has been shown to be a boundary condition on the reconsolidation process (Wang, de Oliveira Alvares & Nader, 2009). Additionally, Rao-Ruiz and colleagues used a 3-min retrieval session, while ours was only 90-sec. While our 90-sec retrieval does result in a protein synthesis dependent reconsolidation process

(Gafford, Parsons & Helmstetter, 2011; Jarome et al., 2011), several studies have shown that retrieval length can result in different cellular mechanisms for memory reconsolidation (Lee et al., 2008; Suzuki, Josselyn, Frankland, Masushige, Silva & Kida, 2004). Nonetheless, both our study and that of Rao-Ruiz and colleagues found retrieval-dependent increases in GluR2 and GluR3 subunits at 7-hrs, a time when the reconsolidation process is thought to be complete, suggesting that there are long-term changes in AMPA receptor subunit expression in the hippocampus following the retrieval of a contextual fear memory. Importantly, we extend the results of Rao-Ruiz and colleagues by demonstrating that the amygdala also shows retrieval-dependent changes in AMPA receptor subunit expression. Interestingly, these changes are temporally unique and there is a transient increase in GluR2 expression, though at a much earlier time point. In combination with our pharmacological data demonstrating that the amygdala regulates the hippocampus following the retrieval of a contextual fear memory, these results indicate that the amygdala and hippocampus interact to properly reconsolidate a contextual fear memory and suggest that changes in AMPA receptor subunit expression, particularly GluR2, may be a critical regulator of this reconsolidation process.

For auditory fear memories, we provide the first study directly examining changes in AMPA receptor subunit expression in the amygdala following the retrieval of an auditory fear memory and found that memory retrieval resulted in a reduction in GluR1, GluR2 and GluR3 expression in the amygdala. These results extend previous electrophysiology experiments (Hong et al., 2013) by showing that the retrieval of an auditory fear memory results in a transient loss of GluR2 receptors in the amygdala. Interestingly, we found reductions in GluR1 and GluR3 as well, suggesting a

depotentiation of synapses in the amygdala (Rao-Ruiz et al., 2011). This result contradicts that of Hong and colleagues and Clem and Huganir (2010) as both did not report changes in synaptic strength and ours suggests a decrease in synaptic strength following retrieval. While the reason for these discrepant findings among the three studies examining retrieval-dependent changes in AMPA receptor subunit expression in the amygdala is unclear, they do add to the growing literature that suggests that retrieval-dependent changes in AMPA receptor subunit expression in the amygdala regulates the reconsolidation of auditory fear memories.

Unique reconsolidation processes in the amygdala

One interesting result from our experiments is that the amygdala shows unique changes in proteasome activity and AMPA receptor subunit expression following the retrieval of an auditory or a contextual fear memory. While it is known that the amygdala is a critical site of plasticity for the reconsolidation of both auditory and contextual fear memories (e.g., Gafford et al., 2011; Jarome et al., 2011; Nader et al., 2000), few studies have examined if these reconsolidation processes are identical. Here, we found that the biochemical signatures for both the destabilization and restabilization processes of auditory and contextual fear memories differed in the amygdala. These results support previous findings that the reconsolidation processes for these different memories are not identical in the amygdala (Jarome et al., 2011), however, it is unknown why these processes differ. One possibility is that these changes result from the differential presynaptic input from sensory areas between these two conditioning

paradigms during the consolidation process. For example, contextual information is processed in the hippocampus and projects to the basal nucleus of the amygdala while auditory CS information is processed in the auditory thalamus and projects to the lateral nucleus (Kim & Fanselow, 1992; LeDoux, 2000; Phillips & LeDoux, 1992; Romanski & LeDoux, 1992). While not examined in the present study, this suggests that the different biochemical signatures of the reconsolidation process may be due to the contribution of different amygdala nuclei. Future studies should examine the specific contribution of the different amygdala nuclei to the destabilization and reconsolidation of retrieved auditory and contextual fear memories.

Reconsolidation-dependent memory updating

Reconsolidation is thought to be a dynamic process whereby consolidated memories can be modified following retrieval (Alberini, 2011). Consistent with this, several studies have shown that reconsolidation can mediate memory strengthening or updating following retrieval. For example, additional learning in a contextual fear memory paradigm requires reconsolidation mechanisms in the hippocampus (Lee, 2008) and IGF-II mediated enhancement of an inhibitory avoidance memory is dependent on memory retrieval (Chen et al., 2011). Additionally, the “reconsolidation-update” effect where memories become weakened or erased following extinction training is dependent on reconsolidation (Monfils et al., 2009; Schiller, Monfils, Raio, Johnson, LeDoux & Phelps, 2010). Furthermore, recent studies have found that memory retrieval promotes the precision and strengthening of a contextual fear memory and an inhibitory avoidance

memory (de Oliveira Alvares et al., 2013; Inda et al., 2011). However, to date, only a few studies have shown that reconsolidation occurs specifically in response to the presence of new information during retrieval. One study found that prediction error controls the destabilization of a retrieved fear memory, and if no error is predicted in the CS-UCS contingency then the memory does not undergo reconsolidation (Sevenster et al., 2013). Consistent with this, one recent study found that reconsolidation in rats only occurred if the timing of the CS-UCS relationship changed during retrieval (Diaz-Mataix et al., 2013). As a result, it is widely believed that reconsolidation mediates memory updating following retrieval. However, it is currently unknown what new information is present at the time of retrieval that actually drives this updating of fear memories, prediction error or contextual information. All the prior studies that manipulated prediction error presented the UCS during retrieval (Diaz-Mataix et al., 2013; Sevenster et al., 2013), making it difficult to know what the new information was that is present during the retrieval session under normal retrieval conditions. Additionally, while contextual information can drive memory updating on contextual-based hippocampus-dependent memory tasks (de Oliveira Alvares et al., 2013; Inda et al., 2011), it is unknown if this interpretation can account for other non-contextual based hippocampus-independent memory tasks. In the present experiment, we tested whether prediction error or contextual information was the new information present during retrieval that controlled the destabilization and reconsolidation of fear memories by manipulating specific parts of the animals training experience, allowing us to use normal retrieval session parameters. Interestingly, we found that contextual information but not prediction error controlled the destabilization and reconsolidation of a retrieved auditory

fear memory, a non-context based hippocampus-independent memory task. In combination with previous results (de Oliveira Alvares et al., 2013; Inda et al., 2011), this suggests that new contextual information present during retrieval is the primary regulator of memory reconsolidation in the amygdala.

This result calls into question why prediction error can regulate memory reconsolidation under certain circumstances (Diaz-Mataix et al., 2013; Sevenster et al., 2013). One possibility is that prediction error provides more novel information about the CS when the UCS occurs during the retrieval session. For example, the absence of the UCS may not be enough information on its own to suggest that the CS-UCS contingency has changed unless the CS is continually presented in the absence of the UCS. This interpretation is consistent with data showing that reconsolidation mediates memory strengthening under single retrieval conditions (Chen et al., 2011; de Oliveira Alvarez et al., 2013; Inda et al., 2011) but memory erasure under multiple retrieval conditions (Clem & Huganir, 2010; Monfils et al., 2009; Rao-Ruiz et al., 2011; Schiller et al., 2010). Conversely, new contextual information may provide a better predictor of potential CS occurrence. Additionally, continued exposure to the training context or the new context that the CS occurred in could promote the precision and thus persistence of memory content. Consistent with this, exposing animals to a second auditory CS retrieval in the same context twice in a short period of time prevents memory destabilization while presenting the second retrieval 24-hrs later still results in normal memory destabilization (Jarome et al., 2012) and re-exposure to the training context can prevent the time-dependent loss of discrimination for a contextual fear memory (de Oliveira Alvares et al., 2013). Collectively, evidence such as this suggests that contextual information may be a

better predictor of the CS-UCS relationship than the absence of the UCS during the retrieval session, indicating that contextual novelty/information at the time of retrieval may be the primary regulator of memory destabilization and reconsolidation.

Conclusion

For years numerous studies have tried to elucidate the molecular mechanisms that regulate the reconsolidation of fear memories and how reconsolidation modifies existing memories. Here, we have added to this growing literature by demonstrating that the same cellular pathway which regulates the initial consolidation of fear memories also regulates their reconsolidation as we found that fear memories destabilize following retrieval through a CaMKII-Rpt6(S120)-protein degradation-GluR2 pathway. Importantly, this pathway regulated memory destabilization in multiple brain regions and changes in GluR2 expression correlated with both the destabilization (amygdala) and restabilization (hippocampus) phases of the reconsolidation process for a contextual fear memory. Additionally, we demonstrate for the first time that fear memories undergo a systems reconsolidation process which is regulated by a protein degradation-dependent cellular reconsolidation process in the amygdala, suggesting that memories undergo retrieval-dependent modification in multiple brain regions simultaneously. Finally, we found evidence to suggest that under normal retrieval conditions contextual information, but not prediction error, triggers the destabilization and reconsolidation of a retrieved fear memory. Collectively, these results suggest that not only is memory reconsolidation a dynamic process that regulates memory updating following retrieval, but that this

reconsolidation process occurs throughout a distributed network of interconnected brain regions that rely on CaMKII-mediate protein degradation in the amygdala.

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Wang, X., McCoy, P.A., Rodriguez, R.M., Pan, Y., Je, H.S., Roberts, A.C., Kim, C.J., Berrios, J., Colvin, J.S., Bousquet-Moore, D., Lorenzo, I., Wu, G., Weinberg, R.J., Ehlers, M.D., Philpot, B.D., Beaudet, A.L., Wetsel, W.C. & Jiang, Y.H. (2011). Synaptic dysfunction and abnormal behaviors in mice lacking major isoforms of Shank3. *Human Molecular Genetics*, 20, 3093-3108.

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CURRICULUM VITAE

Timothy J. Jarome

Place of birth: Niles, OH

Education

Ph.D., University of Wisconsin-Milwaukee, August 2013
Major: Psychology (Neuroscience)

M.S., University of Wisconsin-Milwaukee, December 2010
Major: Psychology (Neuroscience)

B.A., Kent State University, May 2006
Major: Psychology

Dissertation Title: The Role of a CaMKII/PKA-Protein Degradation-GluR2 Pathway in the Control of Memory Updating Following Retrieval

Awards and Honors

UWM Psychology Graduate Research Award (for 2012 paper)	2013
American Psychological Foundation – Ruth G. and Joseph D. Matarazzo Research Award (for 2013)	2012
National Institute of Health – Ruth L. Kirschstein Individual Predoctoral National Research Service Award (3-year fellowship)	2010 - 2013
1 st Place Research Presentation, AGSIP (UWM) symposium	2010
UWM Student Travel Award	2008, 2009, 2010
The Pavlovian Society Student Poster Award	2008
Chancellor's Graduate Student Award	2006, 2007
Kent State University – Graduated <i>Summa Cum Laude</i>	2006
Kent State University Academic Scholarship	2002 - 2006

Publications

1. **Jarome, T.J.**, Kwapis, J.L. & Helmstetter, F.J. (in preparation). Contextual information drives the reconsolidation-dependent updating of retrieved fear memories.
2. **Jarome, T.J.**, Kwapis, J.L. & Helmstetter, F.J. (in preparation). Amygdala protein degradation is a critical regulator of systems reconsolidation.
3. **Jarome, T.J.**, Kwapis, J.L., Hallengren, J.J., Wilson, S.M. & Helmstetter, F.J. (submitted). The ubiquitin-specific protease 14 (USP14) is a critical regulator of long-term memory formation. *Neurobiology of Learning and Memory*
4. Kwapis, J.L., **Jarome, T.J.**, Lee, J.L., Gilmartin, M.R. & Helmstetter, F.J. (submitted). Extinguishing complex fear engages the retrosplenial cortex rather than the amygdala. *Neurobiology of Learning and Memory*
5. **Jarome, T.J.**, Kwapis, J.L., Ruenzel, W.L. & Helmstetter, F.J. (submitted). CaMKII, but not Protein Kinase A, regulates Rpt6 phosphorylation and proteasome activity during long-term memory formation. *Frontiers in Behavioral Neuroscience*
6. **Jarome, T.J.** & Lubin, F.D. (2013). Histone lysine methylation: Critical regulator of memory and behavior. *Reviews in the Neurosciences*, epub ahead of print.
7. **Jarome, T.J.** & Helmstetter, F.J. (2013). The ubiquitin-proteasome system as a critical regulator of synaptic plasticity and long-term memory formation. *Neurobiology of Learning and Memory*, epub ahead of print.

8. Kwapis, J.L., **Jarome, T.J.**, Gilmartin, M.R. & Helmstetter, F.J. (2012). Intra-amygdala infusion of the protein kinase Mzeta inhibitor ZIP disrupts context fear memory. *Neurobiology of Learning and Memory*, 98(2), 148-153.
9. **Jarome, T.J.**, Kwapis, J.L., Werner, C.T., Parsons, R.G., Gafford, G.M. & Helmstetter, F.J. (2012). The timing of multiple retrieval events can alter GluR1 phosphorylation and the requirement for protein synthesis in fear memory reconsolidation. *Learning and Memory*, 19(7), 300-306.
10. Kwapis, J.L., **Jarome, T.J.**, Schiff, J. & Helmstetter, F.J. (2011). Memory consolidation in both trace and delay fear conditioning is disrupted by intra-amygdala infusion of the protein synthesis inhibitor anisomycin. *Learning and Memory*, 18, 728-732.
11. **Jarome, T.J.**, Werner, C.T., Kwapis, J.L. & Helmstetter, F.J. (2011). Activity dependent protein degradation is critical for the formation and stability of fear memory in the amygdala. *PLoS One*, 6(9), e24349. DOI: 10.1371/journal.pone.0024349.
12. Lonergan, M.E., Gafford, G.M., **Jarome, T.J.** & Helmstetter, F.J. (2010). Time-dependent expression of Arc and zif268 after acquisition of fear conditioning. *Neural Plasticity*, e139891. DOI: 10.1155/2010/139891
13. **Jarome, T.J.**, Kwapis, J.L., Nye, S.N. & Helmstetter, F.J. (2010). Introgression of Brown Norway chromosome 1 onto Fawn Hooded Hypertensive background rescues long-term fear memory deficits. *Behavior Genetics*, 40(1), 85-92.

14. Lee, T., **Jarome, T.**, Li, S-J., Kim, J.J. & Helmstetter, F.J. (2009). Chronic stress selectively reduces hippocampal volume in rats: a longitudinal MRI study. *Neuroreport*, 20, 1554-1558.
15. Kwapis, J.L., **Jarome, T.J.**, Lonergan, M.E. & Helmstetter, F.J. (2009). Inhibition of protein kinase M ζ erases an established fear memory in the amygdala but not the hippocampus. *Behavioral Neuroscience*, 123(4), 844-850.

Poster Presentations/Abstracts

1. **Jarome, T.J.**, Kwapis, J.L. & Helmstetter, F.J. (2013). Contextual novelty, but not prediction error, is the new information present at retrieval that controls the reconsolidation-dependent updating of an auditory fear memory. Poster to be presented at the annual meeting of the Society for Neuroscience in November, 2013.
2. **Jarome, T.J.**, Kwapis, J.L., Ruenzel, W.L. & Helmstetter, F.J. (2012). Distinct changes in synaptic protein degradation and AMPA receptor composition in the amygdala and dorsal hippocampus following the retrieval of a context fear memory. Poster presented at the annual meeting of the Society for Neuroscience in October, 2012.
3. Reis, D.S., **Jarome, T.J.** & Helmstetter, F.J. (2012). Degradation specific polyubiquitination is increased in the amygdala and prefrontal cortex following the acquisition of auditory delay or trace fear conditioning. Poster presented at the annual meeting of the Society for Neuroscience in October, 2012.

4. Kwapis, J.L., **Jarome, T.J.**, Gilmartin, M.R., Lee, J.L. & Helmstetter, F.J. (2012). Dissociable roles of the amygdala and retrosplenial cortex in delay and trace fear extinction. Poster presented at the annual meeting of the Society for Neuroscience in October, 2012.
5. **Jarome, T.J.**, Kwapis, J.L., Ruenzel, W.L. & Helmstetter, F.J. (2012). CaMKII regulates proteasome-dependent increases in GluR2 in the amygdala during fear memory reconsolidation. Poster presented at the annual meeting of the Pavlovian Society in September, 2012.
6. Kwapis, J.L., **Jarome, T.J.**, Lee, J.L., Gilmartin, M.R. & Helmstetter, F.J. (2012). Extinction of trace fear conditioning requires the retrosplenial cortex but not the amygdala. Poster presented at the annual meeting of the Pavlovian Society in September, 2012.
7. **Jarome, T.J.**, Ruenzel, W.L. & Helmstetter, F.J. (2011). Fear conditioning increases the amount of proteasome subunit Rpt6 and lysine-48 linked polyubiquitinated proteins in the amygdala. Poster presented at the annual meeting of the Society for Neuroscience in November, 2011.
8. Kwapis, J.L., **Jarome, T.J.**, Gilmartin, M.R. & Helmstetter, F.J. (2011). The basolateral amygdala may be required for delay but not trace fear extinction. Poster presented at the annual meeting of the Society for Neuroscience in November, 2011.
9. **Jarome, T.J.**, Ruenzel, W.L., Kwapis, J.L. & Helmstetter, F.J. (2011). Temporally graded increases in proteasome number and activity in the amygdala

following fear conditioning. Poster presented at the annual meeting of the Pavlovian Society in September, 2011.

10. Kwapis, J.L., **Jarome, T.J.**, Gilmartin, M.R. & Helmstetter, F.J. (2011). Trace fear consolidation, but not extinction, may require the basolateral amygdala. Poster presented at the annual meeting of the Pavlovian Society in September, 2011.
11. **Jarome, T.J.**, Werner, C.T., Kwapis, J.L., Lonergan, M.E. & Helmstetter, F.J. (2010). Protein degradation controls protein synthesis and synaptic remodeling in the amygdala during the formation and stability of long-term fear memories. Poster presented at the annual meeting of the Society for Neuroscience in November, 2010.
12. Werner, C.T., **Jarome, T.J.**, Kwapis, J.L., Parsons, R.G., Gafford, G.M. & Helmstetter, F.J. (2010). The timing of multiple retrieval events can reverse synaptic GluR1 phosphorylation and alter the requirement for protein synthesis in fear memory reconsolidation. Poster presented at the annual meeting of the Society for Neuroscience in November, 2010.
13. Kwapis, J.L., Schiff, J.C., **Jarome, T.J.**, Lonergan, M.E., Gilmartin, M.R. & Helmstetter, F.J. (2010). The basolateral amygdala may use different neural mechanisms for delay and trace fear conditioning. Poster presented at the annual meeting of the Society for Neuroscience in November, 2010.
14. **Jarome, T.J.**, Werner, C.T., Kwapis, J.L., Lonergan, M.E. & Helmstetter, F.J. (2009). Proteasome-dependent protein degradation is critical for long-term

memory formation in the amygdala. Poster presented at the annual meeting of the Pavlovian Society in October, 2009.

15. **Jarome, T.J.**, Werner, C.T. & Helmstetter, F.J. (2009). Enhancements in protein degradation following the acquisition of auditory and context fear memories. Poster presented at the annual meeting of the Society for Neuroscience in October, 2009.
16. Kwapis, J.L., Gilmartin, M.R., **Jarome, T.J.**, Lonergan, M.E. & Helmstetter, F.J. (2009). The maintenance of hippocampal-dependent fear conditioning does not depend on protein kinase ζ (PKM ζ) in the hippocampus. Poster presented at the annual meeting of the Society for Neuroscience in October, 2009.
17. **Jarome, T.J.**, Kwapis, J.L., Parsons, R.G., Gafford, G.M. & Helmstetter, F.J. (2008). Altered gene expression and a change in the requirements for protein synthesis following multiple retrieval events in fear memory reconsolidation. Poster presented at the annual meeting of the Society for Neuroscience in November, 2008.
18. Kwapis, J.L., **Jarome, T.J.**, Parsons, R.G. & Helmstetter, F.J. (2008). Inhibition of protein kinase ζ erases an established fear memory in the amygdala but not the hippocampus. Poster presented at the annual meeting of the Society for Neuroscience in November, 2008.
19. Helmstetter, F.J., Lee, T., **Jarome, T.**, Li, S.-J. & Kim, J.J. (2008). Chronic stress selectively reduces hippocampal volume in rats. Poster presented at the annual meeting of the Society for Neuroscience in November, 2008.

20. **Jarome, T.J.**, Kwapis, J.L., Parsons, R.G., Gafford, G.M. & Helmstetter, F.J. (2008). The timing of multiple retrieval events can alter gene expression and change the requirements for protein synthesis in fear memory reconsolidation. Poster presented at the annual meeting of the Pavlovian Society, September, 2008.
- **Pavlovian Poster Award Recipient****
21. Kwapis, J.L., **Jarome, T.J.**, Parsons, R.G. & Helmstetter, F.J. (2008). Inhibition of protein kinase M ζ erases an established fear memory in the amygdala but not the hippocampus. Poster presented at the annual meeting of the Pavlovian Society, September, 2008.
22. **Jarome, T.J.**, Karbowski, J.L. & Helmstetter, F.J. (2007). Multiple retrieval events can change the requirements for protein synthesis in fear memory reconsolidation. Poster presented at the annual meeting of the Society for Neuroscience in November, 2007.
23. Kerr, A., **Jarome, T.**, Berggren K., Wolter, M., Koenigs, K., Nye, S.H., Helmstetter, F.J. & Swain, R.A. (2007). Enhanced learning and behavioral adaptation in the FHH.BN1 consomic rat. Poster presented at the annual meeting of the Society for Neuroscience in November, 2007.
24. Berggren, K., Kerr, A., Wolter, M., Koenigs, K., **Jarome, T.**, Nye, S.H., Helmstetter, F.J. & Swain, R.A. (2007). Impaired learning and behavioral adaptation in the SSBN.13 consomic rat. Poster presented at the annual meeting of the Society for Neuroscience in November, 2007.
25. Nye, S.H., Wolter, M., Koenigs, K., Dahley-Vernon, A., Kerr, A.L., Berggren, K.L., **Jarome, T.**, Helmstetter, F.J. & Swain, R.A. (2007). Chromosomal

substitution strains of rat for studying human psychological traits. Slide presentation presented at the annual meeting of the Society for Neuroscience, November, 2007.

26. **Jarome, T.J.**, Karbowski, J.L. & Helmstetter, F.J. (2007). The timing of multiple retrieval events can change the requirements for protein synthesis in fear memory reconsolidation. Poster presented at the annual meeting of the Pavlovian Society, October, 2007.
27. Kwapis, J.L., **Jarome, T.J.**, Parsons, R.G. & Helmstetter, F.J. (2007). Inhibition of protein kinase Mzeta in the amygdala erases an established fear memory. Poster presented at the annual meeting of the Pavlovian Society, October, 2007.
28. Barnes, G.W., Bogart, A.R., **Jarome, T.** & Riccio, D.C. (2006). "Changes in Velocity of Movement as a Measure of Memory Loss Following Passive-Avoidance Training. Part I: Behavioral Aspects." Presented at the 19th Annual Tri State Psychology Conference at IUPUI on April 7, 2006.
29. Bogart, A. R., Barnes, G.W., **Jarome, T.** & Riccio, D.C. (2006). "Changes in Velocity of Movement as a Measure of Memory Loss Following Passive-Avoidance Training. Part II: Computer Analysis in Image Processing." Presented at the 19th Annual Tri State Psychology Conference at IUPUI on April 7, 2006.

Invited Talks

1. University of Alabama at Birmingham (2013): Molecular mechanisms of memory consolidation and reconsolidation: The role of protein degradation.
(Invited by Dr. Farah Lubin)

2. New York University (2013): Molecular mechanisms of memory reconsolidation: From protein degradation and synthesis to systems reconsolidation. (Invited by Dr. Cristina Alberini)
3. Pavlovian Society (2011): Temporally graded increases in proteasome number and activity in the amygdala following fear conditioning (Invited by Dr. Fred Helmstetter).

Colloquia and Symposia

1. Amygdala protein degradation controls systems reconsolidation of contextual fear memories. Presented in part of a Data Blitz for Dr. Jarrod Lewis-Peacock (Princeton University). January 2013.
2. Protein degradation as a critical regulator of long-term memory formation and stability. Neuroscience and Physiology Brown Bag, University of Wisconsin-Milwaukee. November 2012.
3. Protein kinase A and CaMKII regulate increases in proteasome activity during fear memory formation. Presented at the 14th annual Graduate Research Symposium. April 2012.
4. Dynamic regulation of proteasome activity in the amygdala during fear memory formation. Presented in part of a Data Blitz for Dr. Brad Postle (UW-Madison). March 2012.
5. Dynamic changes in ubiquitin-proteasome activity in the amygdala following fear memory acquisition and retrieval. Presented in part of a Data Blitz for Dr. Neil Cohen (University of Illinois). October 2011.

6. Protein degradation is critical for the formation of long-term fear memories in the amygdala. Presented in part of a Data Blitz for Dr. Bruce McEwen (Rockefeller University). May 2011.
7. Protein degradation is critical for the consolidation of fear memory in the amygdala. Presented in part of a Data Blitz for Dr. Gregory Quirk (Univ. of Puerto Rico). March 2011.
8. The role of protein degradation in the consolidation, reconsolidation and extinction of fear memories. Neuroscience and Physiology Brown Bag, University of Wisconsin-Milwaukee. February 2011.
9. Protein degradation is critical for the formation and stability of long-term fear memories in the amygdala. Presented at the 12th annual Graduate Research Symposium. April 2010. ***First place AGSIP research presentation***
10. Protein degradation is critical for the formation of long-term fear memory in the amygdala. Presented in part of a Data Blitz for Dr. Tom Carew (UC-Irvine). March 2010.
11. A role for proteasome-dependent protein degradation in the formation and stability of long-term fear memories. Neuroscience and Physiology Brown Bag, University of Wisconsin-Milwaukee. October 2009.
12. The timing of multiple retrieval events can alter gene expression and change the requirement for protein synthesis in fear memory reconsolidation. Presented in part of a Data Blitz for Dr. Howard Eichenbaum (Boston University), October 2008.

13. Multiple Retrieval Events, Altered Gene Expression, and a Change in the Requirement for Protein Synthesis in Fear Memory Reconsolidation. Presented at the 10th Annual Graduate Research Symposium. April 2008.
14. Multiple Retrieval Events can Change the Requirement for Protein Synthesis in Fear Memory Reconsolidation. Neuroscience and Physiology Brown Bag. University of Wisconsin-Milwaukee. December 2007.
15. The Effects of Translation-Inhibition on Multiple Fear Memory Reconsolidation Trials. Presented at the 9th Annual Graduate Research Symposium. April 2007.
16. A Review of Molecular Mechanisms for Fear-Memory Reconsolidation. Neuroscience and Physiology Brown Bag. University of Wisconsin-Milwaukee. February 2007.

Membership in Professional Associations

- Pavlovian Society Student Member 2007 - Present
- Society for Neuroscience Student Member 2006 - Present
- Midwestern Psychological Association Student Member 2006 - Present
- Sigma XI Member 2006 - Present
- Psi Chi Member 2004 - Present
- Golden Key Honor Society Member 2004 - Present

Professional Positions

1. Postdoctoral Fellow, University of Alabama-Birmingham Fall 2013 – Present
Mentor: Dr. Farah D. Lubin, Ph.D.
Fall 2013 - Present
2. National Institute of Health
Predoctoral Fellow (Mentor: Dr. Fred J. Helmstetter, Ph.D.)
Summer 2010 - 2013

3. Guest Lecturer – University of Wisconsin Milwaukee

Neurobiology of Learning and Memory, *Protein degradation and synaptic plasticity (Spring 2013)*
 Introductory Psychology, 1 class (Fall 2006)

4. Teaching Assistant – University of Wisconsin Milwaukee

Research Methods (Dr. Marcellus Merritt)	Spring 2010
Research Methods (Dr. Susan Lima)	Fall 2009
Research Methods (Dr. Marcellus Merritt)	Spring 2009
Research Methods (Dr. Susan Lima)	Fall 2008
Physiological Psychology (Dr. James Moyer, Jr.)	Spring 2008
Physiological Psychology (Dr. James Moyer, Jr.)	Fall 2007
Introductory Psychology (Chris Flessner)	Spring 2007
Introductory Psychology (Chris Flessner)	Fall 2006

5. Research Assistant Positions – University of Wisconsin Milwaukee

Graduate Research Assistant (Dr. Fred Helmstetter)	August 2013
Graduate Research Assistant (Dr. Fred Helmstetter)	Summer 2010
Graduate Research Assistant (Dr. Fred Helmstetter)	Summer 2009
Graduate Research Assistant (Dr. Fred Helmstetter)	Summer 2008
Graduate Research Assistant (Dr. Fred Helmstetter)	Summer 2007
Part-time Research Assistant (PhysioGenix Inc.)	Summer 2007

Ad Hoc Reviewer

Neurobiology of Learning and Memory