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Regulation of mTOR and ERK Signaling in the Amygdala Through Proteolytic Modulation of PP2A Activity Following Auditory Fear Learning

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REGULATION OF mTOR AND ERK SIGNALING IN THE AMYGDALA THROUGH PROTEOLYTIC MODULATION OF PP2A ACTIVITY FOLLOWING AUDITORY FEAR LEARNING

by

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ABSTRACT
REGULATION OF mTOR AND ERK SIGNALING IN THE AMYGDALA THROUGH PROTEOLYTIC MODULATION OF PP2A ACTIVITY FOLLOWING AUDITORY FEAR LEARNING

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Under the Supervision of Professor Dr. Fred J. Helmstetter

The consolidation of fear memories is known to depend on a number of critical cellular processes including *de novo* protein synthesis and 26S proteasome-dependent protein degradation following auditory fear conditioning (Jarome *et al*., 2011; Kwapis *et al.*, 2011). Early work has suggested that protein degradation, mediated by the ubiquitin proteasome system (UPS), may regulate the requirement for *de novo* protein synthesis during memory consolidation (Jarome & Helmstetter, 2014). However, the precise way in which the UPS is able to regulate mechanisms of protein synthesis remain unclear. In the present set of experiments, we investigated the role of the protein phosphatase 2A (PP2A) in mediating the interaction between the UPS and learning-induced mechanisms of protein synthesis during fear memory consolidation. Here we show that post-training administration of the PP2A inhibitor, okadaic acid (OA), has no effect on auditory fear memory consolidation in the amygdala. Interestingly, we also found that simultaneous, intra-amygdala infusions of the proteasome inhibitor, clasto-lactacystin β-lactone (BLAC), and OA can prevent the memory impairment that results from proteasome inhibition alone. However, in a final experiment we found that inhibition of PP2A and proteasome activity is not sufficient to rescue the BLAC-induced reduction of

phosphorylated ERK seen 60 min after auditory fear conditioning. Together, these data suggest that PP2A may mediate the interaction between the UPS and mechanisms of learning-induced protein synthesis, outside of the ERK signaling pathway, during the consolidation of auditory fear memories in the amygdala.

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LIST OF ABBREVIATIONS

BLAC = clasto-lactacystin β-lactone

4EBP1 = 4E-binding protein 1

aCSF = artificial cerebral spinal fluid

ANI = anisomycin

AP5 = (2R)-amino-5-phosphonovaleric acid

BDNF = brain-derived neurotrophic factor

BLA = basolateral nucleus of the amygdala

CAMKII = Ca^{2+}/calmodulin-dependent protein kinase

CREB = cAMP response element binding protein

CS = conditioned stimulus

DFC^{W} = weak delay fear conditioning

DMSO = dimethyl sulfoxide

eIF4E = eukaryotic initiation factor 4E

ERK 1/2 = extracellular signal-regulated kinase 1 and 2

FKBP12 = FK506 binding protein 12

FRB = FKBP12-rapamycin binding domain

GABA = γ-Aminobutyric acid

HC VEH = homecage

K48 = lysine 48

L-LTP = long-lasting long-term potentiation

LTM = long term memory

MAPK = mitogen-activated protein kindase

MEK = mitogen/extracellular signal-regulated kinase

mTOR = mammalian target of rapamycin

mTORC1 = mammalian target of rapamycin complex 1

mTORC2 = mammalian target of rapamycin complex 2

NMDA = N-methyl-D-aspartate

OA = okadaic acid

p70S6K = p70 ribosomal S6 kinase

PKA = protein kinase A

PP2A = protein phosphatase 2A

PP2A-C = catalytic subunit of protein phosphatase 2A

raf = rapidly accelerated fibrosarcoma

ras = rat sarcoma

RISC = RNA-induced silencing complex

STM = short term memory

Thr-389 = threonine 389

TOR = target of rapamycin

UCS = unconditioned stimulus

UPS = ubiquitin proteasome system

VEH = vehicle

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Pavlovian fear conditioning has been an invaluable tool for investigating the neural mechanisms that support learning and memory. In this paradigm, an initially innocuous conditioned stimulus (CS) is paired with an aversive unconditioned stimulus (UCS). Over several pairings, the animal develops a fear memory of the CS so that presentations of the CS alone are enough to cause the subject to emit a fear response, termed the conditioned response (CR). In many cases, the magnitude or frequency of the fear response in the presence of the CS is used as an indicator of learning (Fanselow, 1980). While there are many variations of Pavlovian fear conditioning, standard auditory delay fear conditioning is one of the most well understood. This particular variation of fear learning is rapidly acquired, easy to measure and importantly, is known to rely on a well characterized neural circuit (Phillips & LeDoux, 1992; Sacchetti *et al.*, 1999).

It is generally accepted that auditory fear conditioning is critically dependent upon activity and synaptic plasticity in the amygdala (Fanselow and LeDoux, 1999; Wilensky *et al.*, 2006; Helmstetter *et al.*, 2008; Pape and Pare, 2010). Several early studies showed that neurotoxic lesions made to the amygdala severely impair the formation of an auditory fear memory (Helmstetter, 1992; Maren, 1999). Moreover, transient inactivation of the amygdala with the γ-Aminobutyric acid (GABA) receptor agonist, muscimol, before auditory or contextual fear conditioning significantly impairs auditory fear memory formation (Helmstetter & Bellgowan, 1994; Wilensky *et al.*, 1999). Together, these data demonstrate that selective activation of the amygdala is critical for fear learning.

Protein synthesis and long-term memory formation

Indeed, several cellular mechanisms have been identified to be necessary for fear memory acquisition and consolidation. For example, intra amygdala infusions of (2*R*)-amino-5-phosphonovaleric acid (AP5), a selective inhibitor of the N-methyl D-aspartate (NMDA) receptor, prior to auditory fear conditioning significantly impairs fear memory formation (Goosens & Maren, 2003). Furthermore, other studies have shown the importance of additional cellular mechanisms within the amygdala to the formation of auditory fear memories. For example, *de novo* protein synthesis has been repeatedly demonstrated to be critical to fear memory formation. The blockade of protein synthesis in the amygdala, dose-dependently impairs the formation and consolidation of both contextual and auditory fear memories (Schafe *et al.* 1999). In fact previous work has shown that both pre and post training infusions of the protein synthesis inhibitor, anisomycin, severely impair the acquisition and consolidation of fear memory, respectively (Schafe *et al.* 1999; Schafe & LeDoux, 2000; Kwapis *et al.*, 2011).

While anisomycin is an effective inhibitor of protein synthesis, blocking up to 50% of total mRNA translation *in vivo* (Parsons *et al.*, 2006), its use has been frequently criticized. In a 2008 paper, Rudy and colleagues disparage the use of anisomycin to investigate memory formation, citing a variety of non-specific, negative effects on cellular functioning as a result of anisomycin administration. Furthermore, anisomycin's method of action is through inhibition of peptidyl transferase activity of the 80S ribosome system, which subsequently prevents the elongation of nascent peptides. Since this method of action occurs at the level of the ribosome, anisomycin is able to inhibit protein synthesis initiated through several different signaling pathways. This creates difficulty in

identifying specific pathways involved in the initiation of activity-dependent mRNA translation. To address this, some studies have abandoned anisomycin in favor of translation inhibitors that act upon specific translation initiation signaling pathways such rapamycin, which is a selective inhibitor of the mammalian target of rapamycin complex 1 (mTORC1) signaling cascade.

The mammalian target of rapamycin (mTOR) is the mammalian orthologue of a highly conserved serine/threonine kinase, TOR (target of rapamycin), found in yeast. In mammals, the mTOR kinase can be associated with various scaffold and regulatory proteins to form two functionally distinct complexes (Costa-Mattioli & Monteggia, 2013). Association of mTOR with with the protein called raptor, forms the mTOR complex 1 (mTORC1), while association with the rictor protein forms the mTOR complex 2 (mTORC2). Studies have shown that mTORC1 signaling is critical for cell growth and proliferation through the initiation of cap-dependent translation, whereas mTORC2 function is involved in actin organization and polymerization (Hoeffer & Klann, 2010; Huang *et al.* 2013). In neurons, activation of mTORC1 occurs through its phosphorylation in response to a variety of synaptic plasticity associated events including stimulation of up-stream activators by brain-derived neurotrophic factor (BDNF) or NMDA receptor activation (Gong *et al.*, 2006; Slipczuk *et al.* 2009).

Activation of mTORC1 leads to subsequent phosphorylation of its downstream effectors, p70 ribosomal S6 kinase (p70S6K) and the 4E-binding protein (4EBP1). The mTOR-dependent phosphorylation of threonine-389 (Thr-389) on p70S6K results in its activation leading to subsequent ribosomal activation and mRNA translation. Furthermore, hyperphosphorylation of 4EBP1 by mTORC1 releases 4EBP1 from

eukaryotic translation initiation factor 4E (eIF4E), leading to cap-dependent mRNA translation (Raught *et al.,* 2001; Huang, Bjornsti, & Houghton, 2003). Importantly, mTORC1 activity is sensitive to inhibition by its name-sake inhibitor, rapamycin. When rapamycin is present, it complexes with the immunophilin, FKBP12. This complex is able to tightly bind to the FKBP12-rapamycin binding (FRB) domain on mTOR and subsequently inhibit the formation of mTORC1 thus impairing downstream signaling through this pathway (Kim *et al.* 2002; Huang, Bjornsti, and Houghton, 2003; Hoeffer & Klann, 2010). Importantly, the functionally distinct mTOR complex 2 (mTORC2) is rapamycin insensitive. This characteristic difference between mTORC1 and mTORC2 allows for the specific manipulation of signaling through mTORC1.

Several studies have demonstrated that activity-dependent mTORC1 signaling is critical for memory formation. In the dorsal hippocampus, post-training rapamycin infusions significantly impaired long term (LTM) but not short term memory (STM) formation for a one-trial inhibitory avoidance task and contextual fear conditioning (Bekinschtein *et al.* 2007; Gafford *et al.* 2011). Additionally, inhibition of mTORC1 in the dorsal hippocampus or amygdala dramatically impairs LTM but not STM consolidation following a novel-object recognition task (Jobim *et al.,* 2012). In the same vein, previous work from our lab has shown that intra-amygdala infusions of rapamycin selectively impair the formation of an auditory fear memory. Interestingly, the same manipulation results in significantly reduced p70S6k activation and subsequent mRNA translation (Parsons *et al.,* 2006). Although the rapamycin-dependent reduction in mRNA translation was found to be significantly smaller than the reduction induced by anisomycin, since rapamycin infusions were found to impair fear memory formation, it

can be concluded that mTORC1-dependent mRNA translation is critically involved in memory consolidation and synaptic plasticity.

In addition to mTOR signaling, the role of the mitogen-activated protein family of kinases (MAPKs) in memory formation and synaptic plasticity has grown considerably in the last decade. Members of this kinase family include but are not limited to the p38 MAP kinase, which is involved in stress-related signaling as a response to injury, and the extracellular signal-regulated kinases 1 and 2 (ERK 1/2; Roux & Blenis, 2004). Several studies have implicated ERK 1/2 signaling as a critical regulator of cell proliferation and survival (Roskoski, 2012). As such, it has garnered much attention as another potential initiator of mRNA translation involved in memory consolidation. In neurons, ERK 1/2 is activated the Ras-Raf-MEK-ERK pathway in response to various plasticity associated growth factors, neurotrophins and calcium influx (Kelleher *et al*., 2004; Roskoski, 2012). The involvement of ERK1/2 signaling in transcription has been well studied. Activation of this pathway can lead to the translocation of ERK 1/2 to the nucleus and subsequent activation of a variety of transcription factors including cAMP response element binding protein (CREB) and the ternary complex factor Elk-1 (Davis *et al.*, 2000).

However, ERK1/2 also maintains a critical role in initiating mRNA translation. This is characterized by ERK1/2-dependent phosphorylation of several downstream targets including 4EBP1, eukaryotic translation initiation factor 4E (eIF4E), and several ribosomal S6 kinases including p70 and p90 (p70S6K; p90RSK1; Roskoski, 2012). Interestingly, in the learning and memory literature manipulations that selectively knock-out or impair activation of any of the aforementioned ERK targets severely impairs memory formation and consolidation (Antion *et al.*, 2008; Hoeffer *et al.*, 2011). Together

this information identifies the ERK1/2 signaling cascade as another cellular pathway critical for memory formation and consolidation.

Early work by Schafe *et al.* (2000) showed that ERK phosphorylation was increased roughly 60 min after auditory fear conditioning. This time-course is similar to other pathways critical to memory formation such as mTORC1 (Parsons *et al.*, 2006; Reis & Helmstetter, preliminary data, see figure 2). Importantly, Schafe and colleagues found that preventing the activation of ERK in the amygdala with the MEK inhibitor U0126 significantly impaired the consolidation of auditory fear memory. In addition, the prevention of ERK activation with U0126 significantly impaired long-lasting long-term potentiation (L-LTP), an *in vitro* model of memory consolidation (Schafe *et al.*, 2000). Other studies have demonstrated similar memory impairments as a result of MEK inhibition with U0126 following a variety of behavioral paradigms such as auditory fear conditioning or inhibitory avoidance (Schafe *et al.*, 2000; Igaz *et al.*, 2006). Further, Kelleher *et al*. (2004) generated mutant mice that expressed an inactive but structurally viable form of MEK1, the ERK 1/2 kinase. Following contextual fear conditioning, the mutant mice demonstrated selective impairment in long-term contextual fear memory. In addition, these mutant mice had noticeable reductions in phospho-eIF4E, and the mTOR targets phospho-S6K and phospho-4EBP1 compared to the control mice. In support of this, others have shown that under certain conditions activation of ERK signaling is a prerequisite for the activation of mTOR (Fortress *et al.*, 2013). This further supports the role of ERK signaling in memory and suggests that there may be some level of cross-over between mTORC1 and ERK 1/2 signaling.

While there is clear evidence that signaling through mTOR and ERK is critical for mRNA translation-dependent memory consolidation, much less is known about how these mechanisms are regulated following learning. Many of the up-stream activators of mTOR and ERK have been identified. Indeed, selective manipulation of many of these activators before or soon after behavioral training significantly alters the activation of mTORC1 or ERK1/2 and produces measurable changes in long-term memory (Banko *et al.*, 2004; Merino & Maren, 2006; Chen *et al.*, 2011). Over-activation of translation regulating pathways has been strongly implicated in a variety of neuropathologies and disorders (Chong *et al.*, 2010). It is important to note that one way this over activation can be prevented is through the actions of negative regulators of these specific pathways.

One of the most common mechanisms of kinase inactivation is through desphosphorylation by various phosphatases such as calcineurin or protein phosphatase 2A (PP2A). When active, these enzymes persistently work to dephosphorylate their target substrates. However, constitutively active phosphatases would be detrimental to general cellular function in addition to preventing most activity-induced signaling like that required for memory formation. Therefore, phosphatase activity must be selectively regulated to allow for dynamic control of critical signaling pathways. One possibility is that the mTORC1 and ERK 1/2 signaling cascades are regulated by the ubiquitin-proteasome system (UPS) which modulates the activity of phosphatases that directly affect mTORC1 and ERK 1/2 signaling.

The ubiquitin-proteasome and memory consolidation

The UPS is a known modulator of cell homeostasis during a wide range of cellular conditions such as cell differentiation, oxidative stress, and changes in nutrient

levels (Schmidt & Finley, 2014). In this system, a small protein called ubiquitin is covalently attached to various proteins destined for degradation. This attachment of the ubiquitin molecule to specific substrates occurs through the action of an enzymatic pathway that consists of 3 families of enzymes, termed E1, E2, and E3 (Hershko and Ciechanover, 1998). Once a single ubiquitin is covalently attached, the pathway is then able to add additional ubiquitin molecules to the already substrate-bound ubiquitin. These successive ubiquitin molecules can be linked together at various lysine (K) residues of ubiquitin's C-terminus, thus creating a polyubiquitin chain. The specific lysine residue at which the ubiquitin molecules are linked can confer different functions of the polyubiquitin chain by acting as molecular signal for a variety of cellular processes (Deng *et al.,* 2000; Ye and Rape, 2009). Specifically, polyubiquitin chains of at least 4 ubiquitin moieties linked together at their lysine-48 (K48) residues is considered to be the typical recognition signal for proteolysis via the proteasome (Thrower *et al.*, 2000; Hegde, 2010). Proteins that are tagged with K48 polyubiquitin chains are targeted by the 26S proteasome and subsequently degraded.

Some early work has demonstrated a significant contribution of the UPS to synaptic plasticity and long-term potentiation through its regulation of transcription and translation repressors (Uphadya *et al.*, 2004; Dong *et al.*, 2008). More recently, work in our lab and others has shown that protein-degradation through the UPS is critical to memory formation and consolidation in several brain structures (Artinian, *et al.* 2008, Jarome *et al.* 2011, Reis *et al.* 2013), further supporting its role as a general regulator of memory consolidation. Jarome and colleagues (2011) found that degradation specific polyubiquitination was increased in the amygdala was significantly increased 60 min

after auditory fear conditioning and was maintained at this high level for at least 2 hrs after training. Further, this increase was found to be learning-induced and NMDA receptor dependent as pre-training amygdala infusion of the NMDA receptor antagonist Ifenprodil severely reduced the amount of degradation specific polyubiquitination. Moreover, previous work has revealed that proteasome activity, in addition to degradation specific polyubiquitination, is increased 60 minutes after training. Similarly the phosphorylation of target proteins in the mTOR and ERK1/2 signaling pathways is also increased 60 minutes post-training lending further support to the idea that these pathways are regulated by the UPS (Schafe *et al.* 2000; Parsons *et al.* 2006; Jarome *et al.* 2011).

Protein phosphatase 2A as a target of the ubiquitin-proteasome system

Interestingly, several targets of the 26S proteasome have been identified following learning such as the post-synaptic density scaffolding protein, Shank and the RNA-induced silencing complex (RISC), MOV10 (Jarome *et al.*, 2011). In cell culture, the catalytic subunit of the protein phosphatase 2A (PP2A-C) associates with the E3-ubiquitin ligase, MID1 (Du *et al.*, 2013). PP2A-C is then polyubiquitinated and subsequently degraded by the proteasome. Although this was identified in cell culture there are great potential implications to the learning and memory literature. Liu *et al.* (2011) showed that PP2A negatively affects the activity of the mTORC1 holoenzyme by disrupting the association of mTOR with raptor. Administration of a PP2A inhibitor okadaic acid (OA) prevents this disruption and promotes mTORC1 signaling. Inhibition of proteasome activity by MG132 significantly increased the level of PP2A-C and decreased the phosphorylation of down-stream targets of mTOR like S6 and 4EBP1

(Ghosh *et al.*, 2008; Liu *et al.*, 2011). Importantly, co-administration of MG132 and OA rescued the MG132 induced impairment in mTORC1 function (Liu *et al.*, 2011).

It is clear that both activity-dependent proteolysis through the UPS and *de novo* protein synthesis are critical mechanisms for the consolidation of auditory fear memories in the amygdala. However, it remains unclear whether these two primary mechanisms of memory formation are independent of each other or whether they functionally complement each other.

Much of the work investigating pathway interactions between the UPS and mTORC1 or ERK 1/2 signaling have used LTP or cell cultures as the primary model for investigation. In a preliminary study from our lab we investigated whether similar interactions occurred *in vivo* in an activity-dependent manner following auditory delay fear conditioning.

Preliminary Study 1 and 2

In this study, twenty-six male Long Evans rats were bilaterally implanted with stainless steel cannula aimed at the basolateral nucleus of the amygdala. Following a 7-day recovery period, the rats were trained in auditory delay fear conditioning in which there were four pairings of a 10-s white noise CS and a 1-s footshock UCS. Immediately after training, rats received bilateral infusions of the proteasome inhibitor, βlac (n=9), the protein synthesis inhibitor, anisomycin (ANI; n=8) or 2% DMSO in aCSF (n=9). Figure 1C shows the mean percent time freezing during the long-term memory test for the CS 24 hr after training. Post-training infusion of ANI or βlac into the amygdala significantly impairs freezing to the CS when presented 24 hrs after training. Consistent with previous studies, this data supports the idea that ubiquitin-proteasome mediated proteolysis is a critical component in the initial formation and consolidation of long-term fear memories.

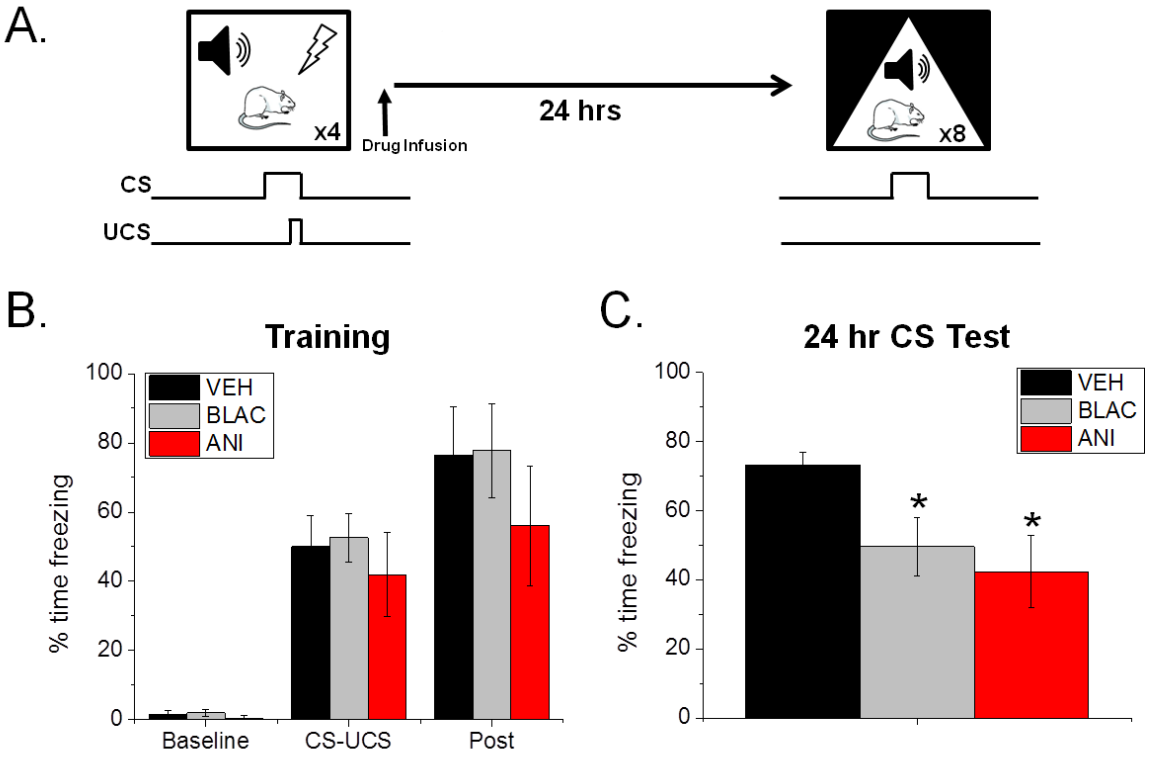

Figure 1: UPS-mediated proteolysis and *de novo* protein synthesis in the amygdala are critical for auditory fear memory formation. A) The behavioral training paradigm. B) The mean percent of time spent freezing during each period of the training session. C) Infusions of anisomycin or BLAC significantly impaired memory for the CS.

To investigate the effect of proteasome inhibition on signaling through mTOR and ERK 1/2, an additional twenty-five male Long Evans rats were bilaterally implanted with stainless steel cannula aimed at the BLA. Following a recovery period, rats were trained in 4-trial auditory delay fear conditioning. Immediately after training, rats received bilateral infusions of BLAC (n=9; 32 ng/µl; from Sigma Chemical, St. Louis, MO, USA) or VEH (n=8; 2% DMSO in aCSF). The remaining animals were not trained but did receive bilateral infusions of VEH and served as the home cage control group (HC VEH; n= 8). Approximately 60 min after training, animals were sacrificed and the

brains were removed. The amygdala from each animal was dissected, homogenized in lysis buffer and subsequently analyzed with western blots

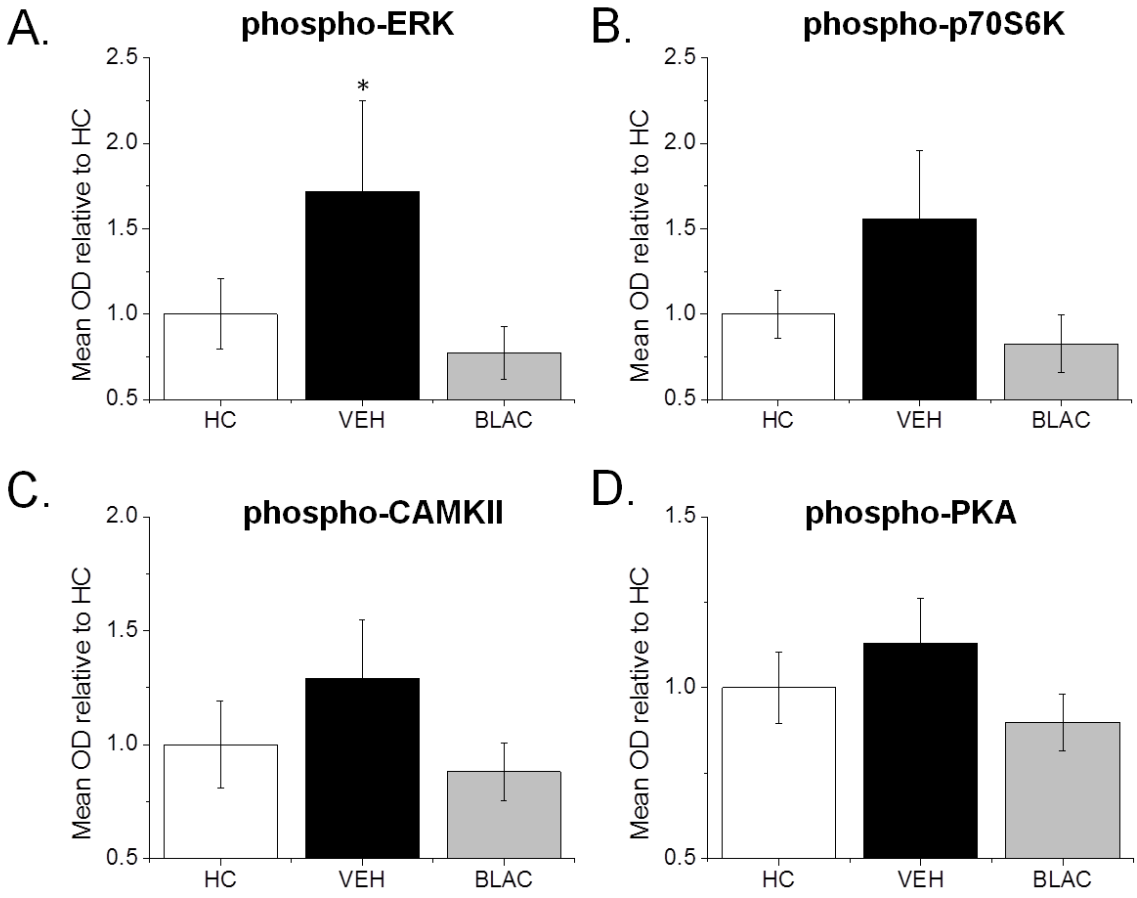

Figure 2: Post-training proteasome inhibition in the amygdala dramatically reduces the level of phospho-ERK 1/2 (A), phospho-p70S6K (B), phospho-CAMKII (C), and phospho-PKA (D) in the amygdala 60 minutes after training.

Interestingly, western blot analysis revealed significant alterations in the phosphorylation status of several signaling molecules critical to activity-dependent *de novo* protein synthesis, following proteasome inhibition. Figure 2 (A-D) shows a dramatic reduction in the level of phosphorylated ERK 1/2, phospho-p70S6K, phospho-CAMKII, and phospho-PKA approximately 60 min after training as a result of proteasome inhibition. This is the first evidence *in vivo* showing an interaction between

the ubiquitin-proteasome system and signaling mechanisms involved in translation initiation critical to synaptic plasticity. While this study is ongoing, the preliminary data strongly support previous literature and suggests that the UPS is regulating activity-dependent mRNA translation, necessary for memory consolidation, through the mTORC1 or ERK 1/2 signaling pathways.

If proteasome inhibition is positively regulating *de novo* protein synthesis through mTORC1 or ERK 1/2 activation as evidenced by proteasome inhibition-dependent reduction in mTORC1 or ERK1/2 signaling, then identifying a target protein common to these pathways as well as the UPS is critical. Data from our preliminary study and others suggests that UPS activity may be interacting with signaling cascades critical to translation initiation. Moreover, there is significant evidence supporting UPS regulation of PP2A. Given the interaction between PP2A and the mTORC1 and ERK 1/2 signaling pathways, PP2A might serve as an important link between the UPS and translation.

Therefore, we hypothesized that post-training inhibition of PP2A would 1) enhance memory formation and consolidation, 2) increase the activation/phosphorylation of down-stream effectors in translation signaling cascades, specifically in the mTORC1 and MAPK/ERK 1/2 pathways. Additionally, we predicted that simultaneous inhibition of PP2A and the 26S proteasome after training would rescue the memory deficits that are observed when proteasome activity is inhibited alone (Jarome *et al.*, 2011). Data supporting our hypotheses would identify a critical link between activity-dependent proteolysis and mRNA translation during memory formation and consolidation. These data will ultimately provide further evidence supporting the UPS as a critical regulatory

mechanism of *de novo* protein synthesis during memory consolidation and further extend our understanding of how memories are initially formed and consolidated.

Materials and Methods

Subjects

Subjects were 102 male, Long Evans rats weighing ~300-350 grams and were obtained from Harlan (Madison, WI). All animals were individually housed and given *ad libitum* access to food and water. The colony room was maintained on a 14:10 hr light/dark cycle with all experiments occurring during the light period. All procedures were approved by the University of Wisconsin-Milwaukee Institutional Animal Care and Use Committee and complied with the ethical guidelines of the National Institutes of Health (NIH).

Surgery

All animals that underwent surgery were implanted with bilateral, stainless steel guide cannulae (26 ga; Plastics One Inc) aimed at the basolateral nucleus of the amygdala (A.P. -2.9; M.L. $\pm$5.0; D.V. -7.0 from bregma). Coordinates were based on a rat brain atlas (Paxinos & Watson, 2007). Prior to surgery, each rat was anesthetized with isoflurane in 100% O_2 (4% induction, 2% maintenance). Cannulae were secured to the skull with a stainless steel screw, ethyl cyanoacrylate, and acrylic cement. Following surgery animals were returned to their homecage and given a 7 day recovery period before any subsequent behavioral test.

Conditioning apparatus

All conditioning sessions occurred in a set of four identical Plexiglas and stainless-steel chambers each housed inside a separate sound-attenuating box. Each outer

box was illuminated with a 7.5 watt house light and was ventilated with a small fan. The background noise level in each of these outer boxes ranged from 62-64 dB. The floors of the Plexiglas chambers in context A were made of evenly spaced stainless steel rods through which the footshock (UCS) was delivered. Between each set of rats, each chamber was cleaned and the inside wiped down with 5% ammonium hydroxide.

All behavioral tests were conducted in a shifted context (context B). The chamber floors in context B were composed of an opaque, white piece of plastic. The chambers of context B were wiped with 5% acetic acid before each test session.

Drug preparation and infusions

In experiments 2 and 3 rats were give intra-amygdala infusions of vehicle (2% dimethylsulfoxide (DMSO), in artificial cerebral spinal fluid (aCSF)), clasto-Lactacystin β-lactone (BLAC; 32 ng/μl), okadaic acid (OA; 100 nM), or BLAC + OA cocktail. Each infusion was given at a rate of 0.5 μl/min with a total volume of 0.5 ul/side. The injectors remained in the guide cannulae for 90 s to ensure sufficient diffusion of the drug. Following drug infusion, the obdurators were re-inserted into the cannulae and the animal was returned to its home cage.

Behavioral procedures

Experiment 1

Previous work in our lab has demonstrated that typical 4 trial delay fear conditioning (4 pairings of a 73dB white noise with a 1s, 1.0 mA footshock with a 90 s inter-trial interval) generally elicits high levels of freezing to the auditory cue when tested 24 hrs after training. We hypothesized that post-training inhibition of PP2A in the amygdala will enhance auditory fear memory consolidation. In order to detect a

behavioral enhancement, we needed to use a training paradigm that would elicit low levels of freezing in control animals when tested for fear to the CS 24 hr later. Therefore, this experiment sought to determine if reducing the amplitude of the footshock during fear conditioning was sufficient to produce moderate (~50% of the total CS presentation time) levels of freezing behavior at the 24 hr test. Moderate freezing behavior should allow for the detection of any potential behavioral enhancement or deficit in subsequent experiments. Animals in this experiment were trained with 4-trial delay fear conditioning with a footshock intensity of .2 mA (n=5), .5 mA (n=5), or 1.0 mA (n=5). Approximately 24 hr after training, animals were tested for fear to the auditory cue and training context.

Experiment 2

This experiment assessed whether 1) inhibition of proteasome activity in the amygdala impairs the formation and consolidation of an auditory fear memory in a weak-training (DFC^{W}) protocol 2) inhibition of PP2A enhances fear memory formation, and 3) simultaneous inhibition of PP2A and the 26S proteasome rescues the behavior deficit seen from proteasome inhibition alone. In this experiment 32 rats with chronic cannulae, aimed at the BLA, were trained in auditory fear conditioning as described above. Immediately after training, rats received bilateral infusions of the PP2A antagonist okadaic acid (n=8), the proteasome inhibitor BLAC (n=8), a cocktail of OA + BLAC (n=8), or vehicle (n=8). Twenty-four hours after training, all animals were tested for memory to the CS.

Experiment 3

This experiment was designed to address the possibility that 100 nM OA is insufficient to induce memory enhancement. Here 20 rats with chronic cannulae aimed at

the BLA underwent DFC^{w}. Immediately after training all animals received bilateral infusions of vehicle (n=5), 100 nM OA (n=5), 200 nM OA (n=5), or 400 nM OA (n=5). Twenty-four hours after training, all animals were tested for fear to the CS.

Experiment 4

This experiment examined the effects of post-training inhibition of PP2A, proteasome, or both on mTOR and ERK 1/2 activation in the amygdala 60 minutes after training. Thirty-five rats with chronic cannulae aimed at the BLA were used in this experiment. Twenty-eight rats were trained in auditory fear conditioning as described above. Immediately after training, rats received bilateral infusions of the either the PP2A antagonist okadaic acid (n=7), the proteasome inhibitor BLAC (n=7), a cocktail of OA + BLAC (n=7), or vehicle (n=7). Approximately 60 min after training, animals were euthanized and amygdala tissue was dissected, homogenized and subject to analysis via western blots (*see above for detailed method)*. A separate group of naive rats were removed from their home cages, given bilateral infusions of vehicle (n=7) and euthanized 60 min later. These animals served as a home cage training control. The degree of mTOR and ERK activation will be inferred by the relative level of phosphorylation of several down-stream effectors in the mTORC1 and ERK (1/2) pathways as determine through western blot probing. These targets include phosphorylated p70S6K, phospho-4EBP1, phospho-mTOR, phospho-ERK (1/2), phospho-CREB, and phospho-PKA.

Results:

Experiment 1

The first experiment was designed to determine fear conditioning parameters that would result in moderate levels of freezing to the auditory cue when tested 24 hr after

training. Here animals were trained with 4 trial DFC using either a .2 mA, .5 mA, or 1.0 mA footshock. Figure 1B shows the mean percentage of time spent freezing for each group during the three periods of training. An analysis of variance (ANOVA) revealed a main effect of group in the CS-UCS period ($F(2,12)=11.372$, $p=.002$) and in the post period ($F(2,9)=19.071$, $p=.001$). *Post hoc* analysis using Fisher's Least Significant Difference (LSD) further revealed that animals given 1.0 mA footshocks during training froze significantly more during the CS-UCS period than animals given .2 mA($p=.001$) or .5 mA ($p=.003$) footshocks. Furthermore, *post hoc* analyses revealed that animals given 1.0 mA shocks during training froze significantly more during the post period than did animals given .2 mA ($p<.001$) or .5 mA ($p=.002$) footshocks.

Figure 3C shows the mean percentage of time spent freezing for each group during presentations of the CS in the absence of a footshock. A one-way ANOVA revealed a main effect of group during the CS presentations ($F(2,21)=9.494$, $p=.001$). *Post-hoc* analysis using Fisher's LSD revealed that animals given .5 mA footshocks during training showed greater freezing to the CS 24 hrs after training than animals given .2 mA ($p=.052$) footshocks but froze less than animals given 1.0 mA ($p=.032$) footshocks.

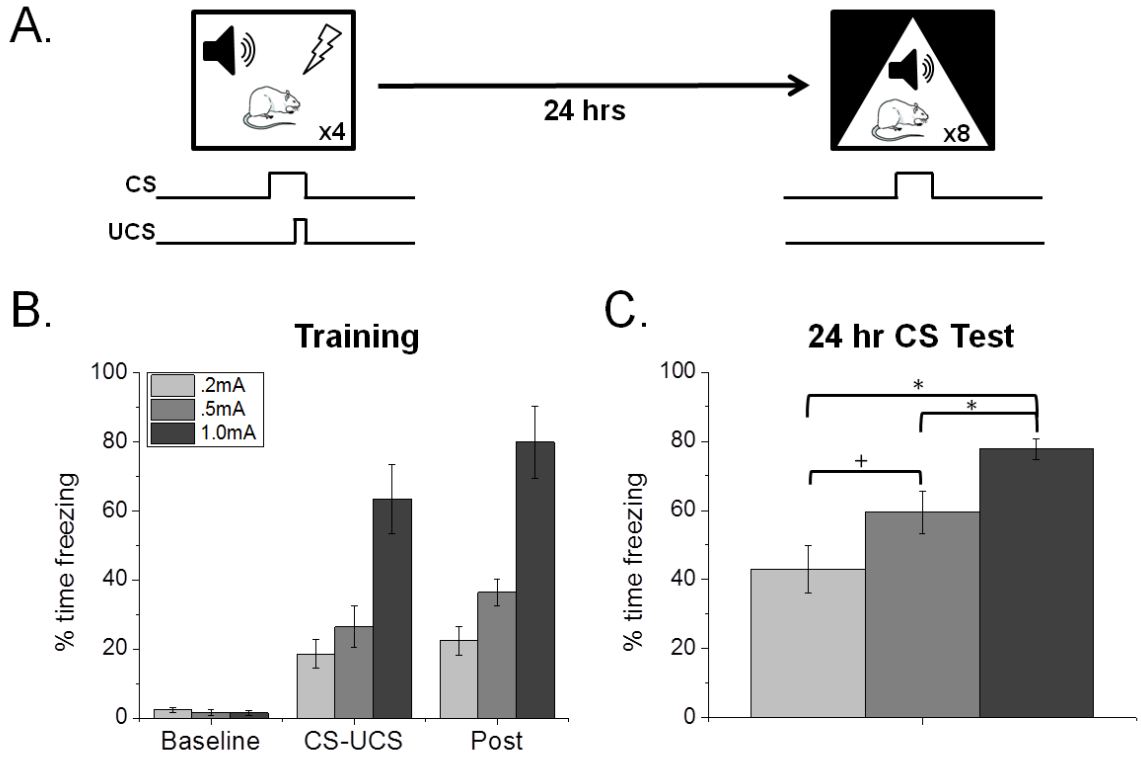

Figure 3: Shock intensity predicts CR magnitude. The percent time spent freezing during each period of training (A) and during the 24 hr CS test for each group. * p=.05; + p=.052.

Importantly, these results indicate that auditory delay fear conditioning using a 73 dB auditory CS and a .2 mA or .5 mA footshock is sufficient to produce a moderate level of freezing of approximately 50% when tested for fear to the CS 24 hrs after training. For the remaining experiments we chose to use a .3 mA footshock since our target amount of time spent freezing (50%) falls between the amount seen in the .2 mA (M=43) and .5 mA (M=59) groups.

Experiment 2

Next we wanted to investigate whether inhibition of PP2A in the amygdala enhances memory consolidation following DFC^{W} and if co-inhibition of PP2A and the proteasome is sufficient to abrogate the previously documented BLAC-induced behavior deficit (Jarome *et al.*, 2011). Here animals were trained with 4 trial DFC^{w} and were given

immediate post-training bilateral infusions of VEH, BLAC, OA, or OA + BLAC into the BLA. Figure 4B shows the mean percent of time spent freezing during each period of the training session. A repeated measures ANOVA revealed a main effect of training period ($F(1,3)=26.659$, $p<.001$) indicating that all of the rats learned the CS-UCS association during the training session. Importantly, no main effect of group was found in the baseline period ($F(3,28)=2.174$, $p=.113$), the CS-UCS period ($F(3,28)=.694$, $p=.564$), or in the post period ($F(3,28)=.390$, $p=.761$).

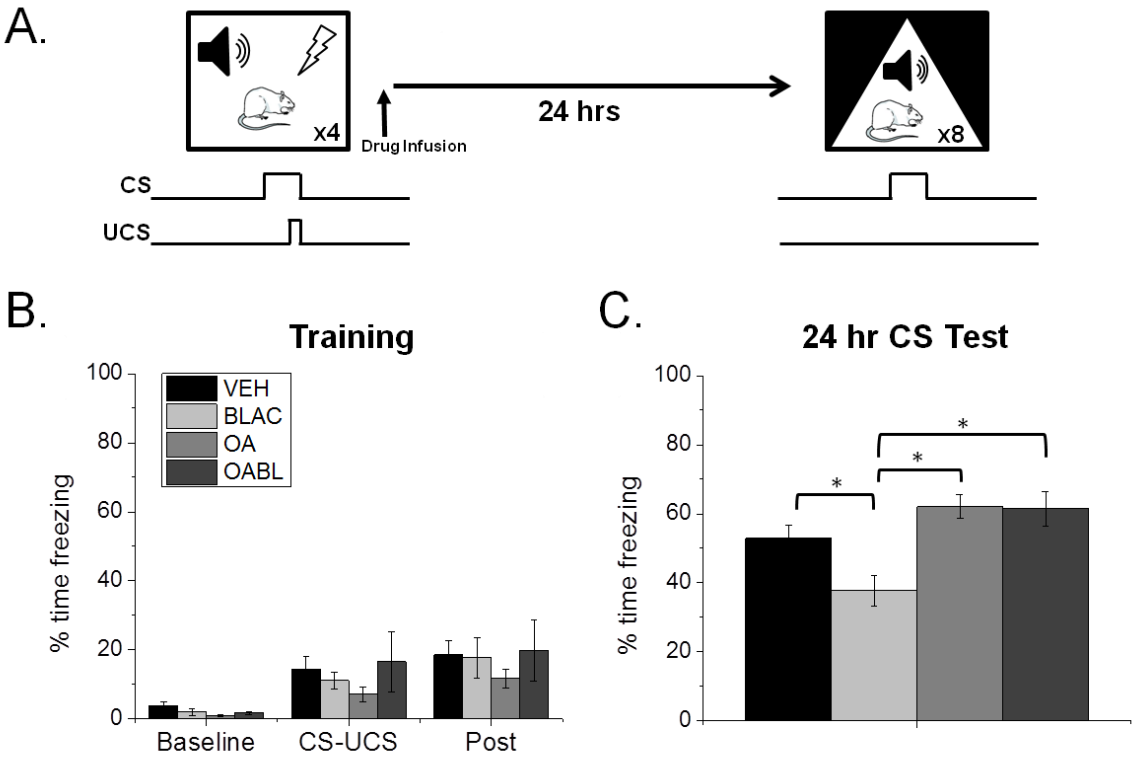

Figure 4: Co-infusion of BLAC and OA prevents memory deficits 24 hr after training. A) Schematic of the training and testing paradigm. B) Mean percent of time spent freezing for each group during each period of the training session. C) Mean percent of time spent freezing to 8 presentations of the CS.

Figure 4C shows the mean percentage of time spent freezing to the auditory cue during the CS test 24 hrs after training. A one-way ANOVA revealed a significant main

effect of group on freezing to the CS ($F(3,24)=7.294$, $p=.001$). In line with our preliminary data, Fisher's LSD further revealed that animals given post training infusions of Blac ($M=37.7$) froze significantly less during the CS presentations than animals given VEH ($M=52.9$; $p=.018$), OA ($M=62.1$; $p<.001$) or the cocktail of OA + BLAC ($M=61.5$; $p=.001$). However, in contrast with our hypothesis, post-training infusions of OA into the amygdala do not have an enhancing effect on fear memory consolidation since VEH freezing and OA freezing are not significantly different from each other. Nevertheless, these results do suggest that simultaneous inhibition of PP2A and the 26S proteasome in the amygdala, is sufficient to rescue the behavioral deficit observed in the Blac group as OA + BLAC animals froze significantly more than animals given infusions of BLAC alone.

Experiment 3

To address the possibility that the concentration of OA used in this experiment was not sufficient to induce memory enhancement, we trained rats with DFC^{w} and administered post-training amygdala infusions of OA at concentrations of 100 nM (n=5), 200 nM (n=5), 400 nM (n=5) or vehicle (n=5).

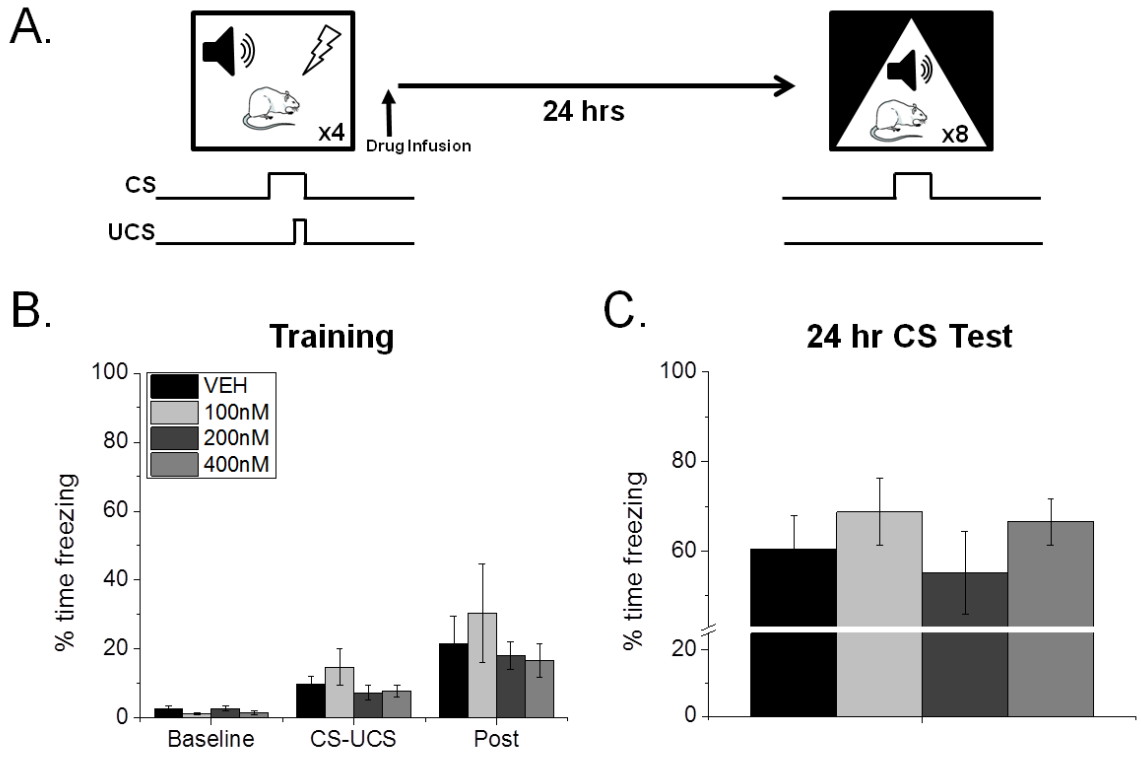

Figure 5: Increasing concentrations of OA do not effect memory consolidation. A) Schematic of the training and testing paradigm. B) Mean percent of time spent freezing during each period of the training session. C) Mean percent of time spent freezing to 8 presentations of the CS.

Figure 5B-C shows the mean percent of time spent freezing during training and the CS test 24 hrs after training. ANOVA revealed no significant main effect of dose during the baseline ($F(3,16)=1.483$, $p=.257$), CS-UCS ($F(3,16)=1.130$, $p=.367$) or the post ($F(3,16)=.479$, $p=.701$) period of the training session. Furthermore, there were no group differences in freezing to the auditory cue during the CS test ($F(3,16)=.676$, $p=.579$). This finding rules out the possibility that the lack of an OA-induced behavioral enhancement was due to an insufficient dosage of OA.

Experiment 4

Next we sought to investigate the effect of OA, BLAC, or OABL infusions into the amygdala on several cellular signaling pathways known to be involved in fear memory consolidation. Similar to experiment 2, rats were trained with 4 trial DFC^{W} and received immediate post-training infusions of either VEH, BLAC, OA, or OABL before being sacrificed 60 min after training for western blot analysis (Figure 6A).

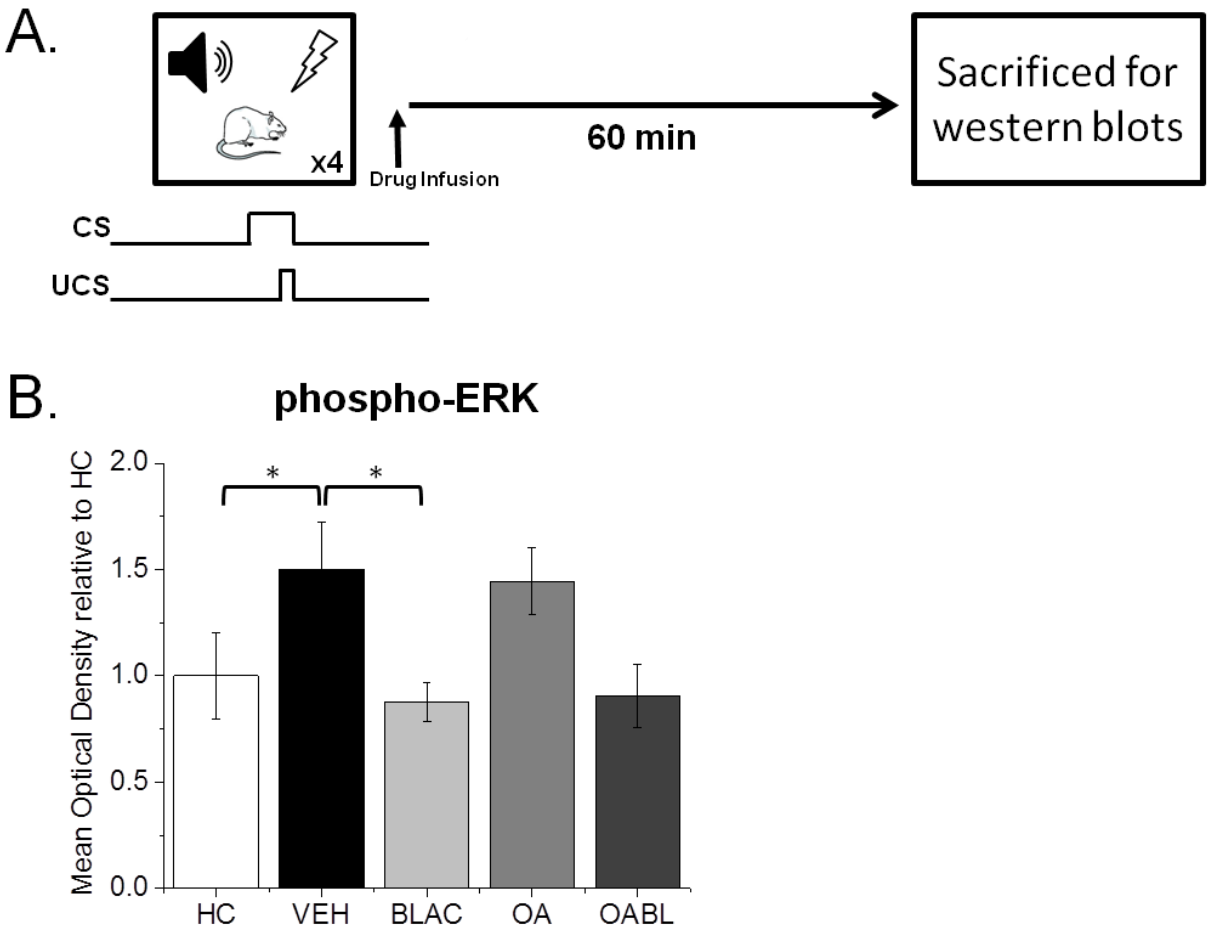

Figure 6: Post-training okadaic acid infusion has no effect on phosphorylated ERK A) Animals were trained in 4 trial DFC^{W} and sacrificed 60 min later for tissue analysis with western blots. B) Mean optical density of phosphorylated ERK. * indicated p=.05

While data analysis for this experiment is ongoing, ANOVA revealed a main effect of group ($F(4,27)=3.019$, $p=.035$) on the phosphorylation of ERK (Figure 6B). Fisher LSD *post hoc* analysis revealed an increase in phospho-ERK, nearing statistical significance, 60 min after training in VEH ($p=.081$) infused animals relative to HC control animals. Consistent with our preliminary data, BLAC infusion blocks the training-induced increase in phospho-ERK ($p=.036$). While the level of phospho-ERK is significantly attenuated following proteasome inhibition, simultaneous inhibition of PP2A and the 26S proteasome fails to prevent this BLAC-induced reduction, seen in the comparison of OABL ($p=.924$) to the BLAC. This is counter to our hypothesis and may suggest that the UPS modulates plasticity-related signaling pathways through multiple, pathway-specific regulatory mechanisms.

Discussion:

Here we present data suggesting that the regulation of PP2A by the UPS is critical for fear memory consolidation in the amygdala following auditory delay fear conditioning. Similar to earlier work, we found that inhibiting proteasome activity in the amygdala immediately after training with a weak DFC protocol impaired fear memory consolidation for the auditory cue. Furthermore we found that inhibition of PP2A activity was not sufficient to enhance memory consolidation but that simultaneous inhibition of PP2A and proteasome activity in the amygdala is able to block the behavioral deficit caused by proteasome inhibition. Together these data suggest that PP2A may be involved in mediating the interaction between the UPS and protein synthesis signaling.

Given the established role of PP2A as a negative regulator of the mTOR and ERK signaling pathways, we hypothesized that inhibition of PP2A would enhance mTOR and

ERK signaling and consequently enhance fear memory consolidation. Our results indicate that post-training infusion of OA into the amygdala does not enhance memory consolidation following DFC^{w}. Previous work using a conditioned taste aversion paradigm found that intra-amygdala infusions of OA enhanced memory only when administered 5 min prior to training but not when administered 5 min after training (Oberbeck *et al.*, 2010). This finding is consistent with other reports of temporally sensitive phosphatase activity for a variety of different learning models (Zhao *et al.*, 1995; Bennett *et al.*, 1996; Genoux *et al.*, 2002). Given that the present study did not investigate the effects of pre-training OA-infusions on fear memory, it is possible that post-training inhibition of PP2A falls outside of a window of phosphatase activity critical for the consolidation of fear memories. Future work is needed to evaluate the effects of pre-training intra-amygdala infusions of OA on fear memory formation and consolidation.

Earlier work has identified a critical role for protein synthesis and, more recently, UPS-mediated protein degradation in the consolidation of fear memories (Schafe *et al.*, 1999; Kwapis *et al.*, 2011; Jarome *et al.*, 2011). While the exact relationship between these two mechanisms remains unclear, recent evidence has suggested that UPS-mediated protein degradation may play a role in regulating activity-dependent protein synthesis during memory consolidation (Ehlers, 2003; Jarome *et al.*, 2011). Here we show that simultaneous blockade of UPS-mediated protein degradation and PP2A activity, in the amygdala, effectively prevents the BLAC-induced memory deficit. This finding is of particular interest given that OA was found to have no effect on the consolidation of an auditory fear memory, neither enhancing nor impairing the memory. Our results support

the idea that PP2A may be at least one substrate through which the UPS is able to modulate mechanisms of protein synthesis during memory consolidation.

In the same vein, we examined the effects of OA and OABL on various cellular signaling pathways known to be critically involved in memory consolidation. While this experiment is ongoing, early data suggests the UPS may differentially regulate mTOR and ERK signaling during memory consolidation. Here we demonstrate that inhibition of PP2A has no effect on ERK phosphorylation. This finding is in disagreement with our hypothesis and incongruent with early reports of PP2A-regulated ERK (Meng *et al.*, 2015). Since our preliminary data indicate reductions in phosphorylated ERK and p70S6K as a result of proteasome inhibition but also since PP2A inhibition has no effect on ERK, it is possible that the UPS differentially regulates mTOR and ERK signaling during memory consolidation.

While further work is needed to extend the findings presented here and to better delineate the differential regulation that the UPS may have on mTOR and/or ERK signaling, the data presented here strongly support the involvement of PP2A as an important regulatory element during the consolidation of fear memories.

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