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Functionally Distinct Pools of Calcineurin Contribute to Depotentiation-like Synaptic Changes in the Lateral Amygdala During Auditory Fear Extinction

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by

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ABSTRACT

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Until recently, auditory fear extinction was not thought to modify substrates involved in the storage of the original auditory fear memory. Evidence now suggests that extinction results in the reversal of the fear conditioning-induced potentiation of thalamic inputs to the lateral amygdala. However, little is known about the molecular mechanisms that support this depotentiation of synaptic strength. Here we present behavioral and molecular evidence in support of the contribution of two distinct pools of the protein phosphatase calcineurin to depotentiation-like changes in lateral amygdala AMPA receptor trafficking during auditory fear extinction. Calcineurin protein that exists prior to the onset of extinction training is required for the reduction in conditional fear responses during the extinction session, whereas calcineurin protein that is synthesized during the extinction session is involved in the long-term retention of extinction learning. Furthermore, the pre-existing pool of calcineurin mediates endocytosis of GluR2-containing AMPARs, whereas the newly translated pool of calcineurin mediates reductions in AMPAR-stabilizing protein PSD-95. These results suggest that extinction involves the concerted actions of pre-existing and newly translated calcineurin to induce and stabilize AMPA receptor-mediated reductions in synaptic strength in the lateral amygdala.

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LIST OF ABBREVIATIONS

4t	4 trial (retrieval session)
40t	40 trial (extinction session)
AMPAR	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor
Ani	anisomycin
BA	basal nucleus of the amygdala
BL	refers to the stimulus free baseline period of auditory fear conditioning
CaN	calcineurin
CeL	central nucleus of the amygdala, lateral division
CeM	central nucleus of the amygdala, medial division
CR	conditional response
CS	conditional stimulus
IL	infralimbic cortex
ITC	intercalated cell masses
ITCd	dorsal cluster of intercalated cell masses
ITCv	ventral cluster of the intercalated cell masses
LA	lateral nucleus of the amygdala
LTP	long-term potentiation
mPFC	medial prefrontal cortex
OD	optical density
ODN	oligodeoxynucleotide
PL	prelimbic cortex
Post	refers to stimulus free period after CS-UCS pairings during auditory fear conditioning
Pre-Ret	pre-retrieval group

Ser	serine
Scr	scrambled oligodeoxynucleotide
T-LA	thalamic inputs to the lateral amygdala
UCS	unconditional stimulus
Veh	vehicle solution

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Introduction

Though associative auditory fear memories are robust and long-lasting, memory retrieval, via re-exposure to the feared stimulus, can modify both neural substrates and behavioral responses associated with the memory. For example, in the absence of an ensuing aversive event, prolonged or repeated exposure to the feared auditory stimulus can result in fear extinction, or the attenuation of the learned fear response (Myers & Davis, 2007). Though auditory fear extinction has long been conceptualized as the formation of a new inhibitory memory, recent work suggests that it may also act directly on the substrates of the original fear memory (Quirk & Mueller, 2008; Kim et al., 2007; Maren, 2014). Specifically, auditory fear extinction has been found to produce a reversal of a subset of fear conditioning-induced changes in the lateral nucleus of the amygdala (Kim et al., 2007). Relative to the well-characterized regulation of lateral amygdala plasticity during auditory fear conditioning, the nature and function of plasticity in this region during auditory fear extinction remain poorly understood. However, several lines of evidence implicate signaling mediated by the protein phosphatase calcineurin as a critical component of the reversal of fear conditioning-induced plasticity (Lin et al., 2003a,b; Merlo et al., 2014). Therefore, the present study used a combination of behavioral and molecular techniques to characterize the role of calcineurin in lateral amygdala plasticity during auditory fear extinction.

Auditory Fear Conditioning

The behavioral and neural changes that accompany the formation of an auditory fear memory provide the background on which auditory fear extinction occurs. To create an auditory fear memory, an initially neutral auditory stimulus (conditional stimulus; CS), is paired with an aversive stimulus (unconditional stimulus; UCS), like a foot shock. After several pairings, the animal learns that the CS predicts the UCS, and will exhibit a conditional fear response (CR), such as freezing, to subsequent presentations of the CS in absence of the UCS.

The initial encoding of the auditory fear memory occurs during conditioning as information about the auditory CS and the somatosensory UCS converge on neurons in the lateral nucleus of the amygdala (LA) (Schafé, Doyere, & LeDoux, 2005). While both the insular cortex and the intralaminar complex of the thalamus send somatosensory information to the LA, the critical UCS pathways remains undefined (Shi & Davis, 1999; Brunzell & Kim, 2001). In contrast, the auditory CS pathways are better characterized. Information about the auditory CS is transmitted to the LA through a fast, direct thalamic pathway and a slower, indirect pathway that travels from the thalamus to the auditory cortex prior to traveling to the LA (Quirk, Armony, & LeDoux, 1997). The coincident timing of weak CS input with the strong UCS input to the LA is proposed to strengthen auditory thalamus-LA (T-LA) and auditory cortex-LA (C-LA) synapses through the induction of associative long-term potentiation (LTP) (Tsvetkov et al., 2002; Maren, 2005). Within the 24 hour period following fear conditioning, the fear memory undergoes consolidation, in which protein synthesis-dependent cellular changes occur to reinforce the potentiation of these synapses to support the long-term storage of the new memory (Schafe et al., 2001).

While the LA is believed to be the primary site of encoding and storage of the CS-UCS association, the production of the CR requires information arriving at the LA to be transmitted to the central nucleus of the amygdala (CeM), which in turn sends projections to several structures that mediate defensive behavioral responses, including the hypothalamus and periaqueductal grey (LeDoux et al., 1988; Pare & Duvarci, 2012). There are multiple indirect pathways through which the LA can drive CeM activation. LA neurons send excitatory projections to the lateral division of the central nucleus of the amygdala (CeL), possibly to an identified population of PKCδ-negative cells known as “CeL-ON cells” (Haubensak et al., 2010). These PKCδ-negative cells form inhibitory connections with a population of PKCδ-positive cells known as “CeL-OFF cells” that tonically inhibit the CeM. Thus, the LA-driven activation of CeL-ON cells results in the disinhibition of the CeM and, consequently, robust expression of conditioned fear responses. Additionally, the LA activates the dorsal cluster of intercalated cells (ITCd), a

mass of GABAergic interneurons located between the basolateral amygdala complex and the central nucleus of the amygdala (Royer, Marina, & Pare, 1999; Ehrlich et al., 2009). Activation of ITCd cells may remove inhibition of the CeM mediated by the ventral cluster of ITC cells (ITCv). The LA also sends excitatory projections to the basal nucleus of the amygdala (BA), which in turn sends excitatory projections to the CeM, resulting in additional facilitation of CeM output (Pare & Duvarci, 2012). Thus, while expression of the CR is facilitated by LA potentiation, it also depends on the appropriate coordination of several intrinsic amygdala circuits. Importantly, distinction between the potentiation of thalamic and cortical input to the LA and the multi-synaptic pathways through which LA activity can drive output from the CeM have played a significant role in reconciling behavioral and neurobiological features of auditory fear extinction.

Auditory Fear Extinction is New Learning

To induce auditory fear extinction, the CS is presented repeatedly in absence of the UCS (collectively referred to as the *extinction training session*). When freezing is used an as index of conditioned fear, extinction learning is typically assessed at two different times. The first assessment, *within-session extinction*, measures the gradual reduction in CS-evoked freezing over the course of the extinction training session. The second assessment, the *long-term retention of extinction*, measures the continued suppression of freezing when the extinguished CS is presented 24 hours or more following extinction training.

Despite the observed attenuation of the CR, extinction does not induce forgetting or unlearning of the CS-UCS association. Rather, the CR can still be expressed following extinction in absence of explicit repairing of the CS and UCS. For example, *renewal* of the CR is observed when an extinguished CS is presented in a context other than the one in which it was extinguished (Bouton, 1993). Furthermore, exposure to stress, such as an unsignaled shock, can induce *reinstatement* of the CR

(Rescorla & Heth, 1975). Most strikingly, the simple passage of time, in the absence of additional manipulation, can induce the *spontaneous recovery* of the CR (Rescorla, 2004). Given this persistence of CS-UCS association, extinction is most commonly characterized as the formation of a new CS-No UCS memory that inhibits the expression of the original CS-UCS memory.

Neurobiological studies of auditory fear extinction have enriched this conceptualization of extinction as new learning. Central to these studies are the multiple pathways throughout the subnuclei of the amygdala that contribute to the regulation of CeM output. Specifically, amygdala neurons within these pathways receive input from a number of extrinsic structures that, following extinction, play key roles in determining whether the CR will be expressed or suppressed (Pare and Duvarci, 2012). Critically, this extrinsically-guided regulation of amygdala output provides a mechanisms that could account for extinction-induced suppression of the CR without degradation of the substrates of the original fear memory.

One key set of inputs to the amygdala that facilitate extinction learning come from the infralimbic (IL) division of the medial prefrontal cortex. During auditory fear extinction, input from the IL to the BA drives the potentiation of BA-ITCv synapses (Amano, Unal, & Pare, 2010; Senn et al., 2014). Excitation of ITCv cells increases inhibition of the CeM, favoring the suppression of the CR (Duvarci & Pare, 2014). In addition, the ventral hippocampus (VH) contributes indirectly to the modulation of amygdala activity. During extinction recall, the VH drives activity in the IL and local-circuit GABAergic inhibition in the PL, which favors the suppression of fear (Rosas-Vidal et al., 2014; Santini et al., 2008). Collectively, this extended network of structures is believed to support the long-term storage of extinction learning and suppress the CR during extinction recall by inhibiting the neural activity associated with the CS-UCS association.

However, renewal of the CR following extinction does not simply involve the disinhibition of LA-driven amygdala output. It also requires additional inputs that facilitate CeM output. For example, VH- and PL-driven activation of the BA is necessary for fear renewal (Herry et al., 2008; Orsini et al., 2011; Jin & Maren, 2015). Additionally, the dorsal hippocampus-dependent activation of the LA has been found to be critical for renewal (Hobin, Maren, & Goosens, 2003; Maren & Hobin, 2007). Therefore, while the CS-UCS association remains intact following extinction, there is data to suggest that the critical substrates supporting the recall of the auditory fear memory have been modified.

Auditory Fear Extinction Modifies Substrates Involved in the Storage of the Auditory Fear Memory

Evidence suggests that extinction can modify the substrates supporting the storage of the original fear memory. In contrast to the strong, CS-evoked responses of LA neurons following fear conditioning, extinction induces a significant reduction in CS-evoked firing in the LA (Quirk et al., 1995). While the active inhibition of LA neurons cannot be ruled out as a contributing factor, there is growing evidence to suggest this reduced responsivity to the CS is at least partially attributable to the reversal of conditioning-induced changes at a subset of LA neurons. Specifically, auditory fear extinction may induce *depotentiation* (an electrophysiological phenomenon referring to the depression of a previously potentiated synapse to basal levels of strength) at thalamic inputs to the lateral amygdala (Kim et al., 2007; Barrionuevo, Schottler, & Lynch, 1980). For example, one study found that simultaneous stimulation of efferents from the IL and auditory thalamus to the lateral amygdala resulted in depotentiation of T-LA inputs and an extinction-like suppression of fear response to the CS (Park & Choi, 2013). Another study demonstrated that low frequency paired-pulse stimulation in fear conditioned rats resulted in depotentiation of T-LA synapses, while depotentiation was occluded in rats that had also received extinction (Kim et al., 2007).

It is possible that T-LA depotentiation is critical in reducing rapid, CS-evoked excitation of the LA, in favor of slower, cortically-derived modulation of CS-evoked behavior. However, empirically-based knowledge regarding the form and function of depotentiation as it occurs in auditory fear extinction remains very limited. Therefore, a critical initial step in understanding this form of plasticity is to characterize the molecular mechanisms underlying the induction and expression of T-LA depotentiation.

Depotentiation-Like Synaptic Changes in Extinction

Because α-amino-3-hydroxy-5-methyl-4-isoxazoleproprionic acid receptors (AMPARs) mediate the majority of fast excitatory transmission in the central nervous system, the dynamic regulation of the number, subunit composition, and phosphorylation state of these receptors is a critical mechanism underlying bidirectional change in synaptic efficacy (McCormark, Stornetta, & Zhu, 2006; Rumpel et al., 2005).

Regulation of AMPARs in LTP and Auditory Fear Conditioning

In the lateral amygdala, both LTP and auditory fear conditioning result in the enhanced synaptic expression of AMPARs (Shukla et al., 2014; Takahasi et al., 2003). More specifically, homomeric AMPARs, composed of two GluR1 subunits, are rapidly trafficked into the synapse (Kauer & Malenka, 2006; Rumpel et al., 2005; Shi et al., 2001; Yeh, et al., 2006). These receptors are calcium permeable and are functionally regulated by phosphorylation of serine 831 and serine 845 to increase channel conductance and open probability, respectively (Oh & Derkach, 2005; Lee et al., 2013). Therefore, activity-dependent trafficking of these receptors to the synapse may confer additional potential for plasticity. In addition, phosphorylation of serine 845 is also known to provide a signal for the recruitment of additional AMPARs to the synapses, possibly acting as a tagging mechanism for synapses that will undergo learning-induced, long-term strengthening (Kauer & Malenka, 2006; Man et al., 2007). Gradually, these homomeric receptors are replaced by GluR2-containing AMPARs (McCormack et al,

2006; Takahashi, Svoboda, & Malinow, 2003). Consistent with the hypothesized role of GluR2-containing AMPARs in synaptic stability and memory maintenance, the presence of the GluR2 subunit confers several properties to the AMPAR, including calcium impermeability and an apparent insensitivity to modulation of channel conductance and open probability through phosphorylation of serine 831 and serine 845 on the GluR1 subunit, respectively (Oh & Derkach, 2005; Benke et al., 1998; Roche et al., 1996; Migues et al., 2010). Therefore, upregulation of Glur2-containing AMPARs increases postsynaptic responsivity to neurotransmission while reducing potential sources of destabilizing calcium influx.

An additional factor critical for the long-lasting enhancement in AMPAR expression is the upregulation of certain scaffolding proteins in the postsynaptic density (Opazo, Sainlos, & Choquet, 2012; Xu et al., 2008; Chung et al., 2000). Because AMPARs are highly mobile and prone to lateral diffusion away from the synapses, scaffolding proteins interact both directly and indirectly with AMPARs maintain their localized expression (Isaac et al., 2007). Among these proteins, PSD-95 expression has a particularly strong correlation with long-lasting changes in AMPAR expression (Colledge et al., 2003). The enhancement of scaffolding protein PSD-95 at active synapses in the lateral amygdala synapses following both LTP and fear conditioning is proposed to serve a dual role in activity-dependent synaptic strengthening (Ehrlich & Malinow, 2004; Mao et al., 2013). Initially, newly inserted PSD-95 may act as a tag that guides the targeted insertion of AMPARs (Opazo et al., 2012). Subsequently, PSD-95 anchors AMPARs stabilize the increase in receptor expression (Chen et al., 2011; Yudowski et al., 2013). Collectively, this coordinated upregulation of AMPAR and scaffolding protein expression is critical for long-lasting enhancements in synaptic strength.

Regulation of AMPARs in Depotentiation and Auditory Fear Extinction

Conversely, the coordinated downregulation of AMPAR and scaffolding protein expression is a major component of depotentiation (Carroll et al., 2001; Derkach et al., 2007; Corea, Doucet, & Fon,

2009). Critically, there are emerging similarities between the regulation of AMPARs in the LA during depotentiation and auditory fear extinction. For example, both depotentiation and extinction require regulated AMPAR endocytosis mediated by interactions with sequences on the C terminus of GluR2 (Kim et al., 2007; Dalton et al., 2008).

However, it must be noted that AMPAR endocytosis is not specific to extinction or depotentiation. Certain memory retrieval conditions, typically involving relatively brief re-exposure to the CS, can induce an active plastic process called *reconsolidation* at T-LA synapses (Nader, Schafe, & LeDoux, 2000; Kim et al., 2010). Reconsolidation begins with a destabilization phase, which is characterized, in part, by the endocytosis of GluR2-containing AMPARs (Jarome, 2013; Hong et al., 2013). During subsequent restabilization, however, AMPARs are reinserted into the membrane and ultimately, both T-LA potentiation and CR expression are maintained (Hong et al., 2013). In contrast, auditory fear extinction is associated with long-lasting reductions in GluR1 and GluR2 expression that remain observable at for at least 24 hours following extinction training (Kim et al., 2007). Moreover, reductions in PSD-95 expression, which are associated with long-term reductions in AMPAR expression, have only been reported following extinction training (Mao et al., 2013). Therefore, extinction may be more uniquely defined by both the occurrence and persistence of reduced AMPAR expression.

A Multifaceted Role for Calcineurin in the Mediation of Depotentiation-Like Plasticity

Calcineurin (CaN) is an abundantly expressed, serine-threonine phosphatase. It is the only neuronal phosphatase directly activated by calcium and is therefore a prime candidate for initiating activity-dependent changes in synaptic strength (Klee, Crouch, & Krinks, 1997; Baumgäretel & Mansuy, 2012). Multiple studies have established a critical role for CaN-mediated signaling in the amygdala during depotentiation and auditory fear extinction. Relatively rapid (within approximately 10 minutes) increases in CaN protein levels and CaN enzymatic activity in the amygdala are observed following both

depotentiation and auditory fear extinction (Lin et al., 2003a; b; Beattie et al., 2000). Electrophysiological studies have demonstrated that CaN is necessary for depotentiation in a number of brain structures, including the amygdala (Jouvenceau et al., 2003; Kang-Park et al., 2003; Lin et al., 2003a). Furthermore, inhibition of CaN prevents the induction of depotentiation in the amygdala using the same stimulation parameters that attenuate the expression of a previously consolidated fear memory (Lin et al., 2003a). Similarly, behavioral studies have shown that inhibition of CaN in the amygdala impairs extinction learning (Merlo et al., 2014; Lin et al., 2003b).

CaN-Mediated Regulation of AMPAR Expression

A number of studies outside the extinction literature have established roles for CaN in both stimulating AMPAR endocytosis as well as limiting the synaptic re-insertion of AMPARs. For example, CaN has been shown to activate protein tyrosine phosphatase STEP61, which is required for mGluR-stimulated endocytosis of GluR1/2 AMPARs (Goebel-Goody & Lombroso, 2012). In addition, CaN may directly or indirectly (via protein phosphatase 1) dephosphorylate serine 845 on GluR1 to limit activity-dependent recruitment of AMPARs to the surface (Lee et al., 2000; Sanderson et al., 2012). Finally, CaN has been shown to mediate the degradation of PSD-95, which may restrict the targeted reinsertion of AMPARs following endocytosis (Colledge et al., 2003; Xu et al., 2008). However, the specific substrates targeted by CaN during auditory fear extinction remain largely uncharacterized.

Functionally Distinct Pools of CaN?

Several recent observations have provided some basis to suggest that auditory fear extinction involves at least two distinct pools of CaN in the amygdala. As first observed by Merlo and colleagues (2014), these pools may be distinguishable by whether the protein existed prior to the onset of extinction training or whether the protein was synthesized following the onset of extinction training.

Newly translated CaN may be involved aspects of plasticity underlying the persistence of extinction learning. In support of this, inhibiting the translation of new CaN protein during extinction training has been shown to disrupt the long-term retention of extinction, without effecting within-session extinction (Merlo et al., 2014). Furthermore, the synthesis of new CaN protein has been observed to selectively occur following extinction-inducing, but not reconsolidation-inducing, retrieval sessions (Merlo et al., 2014). This is consistent with evidence of differential regulation of protein synthesis in reconsolidation and extinction, such that a certain set of proteins are translated to restabilize the memory in reconsolidation whereas a distinct set of proteins are translated, some of which may help maintain certain elements of the memory in a "deconsolidated" state in extinction (Tronson et al., 2012; de la Fuente et al., 2011; Pedreira & Maldonado, 2003). Together, these observations suggest that the translation of new calcineurin protein may underlie at least part of the differences between extinction and reconsolidation related to protein synthesis and that this pool of CaN may be preferentially involved in changes that stabilize the state of reduced synaptic strength, such as the dephosphorylation of serine 845 and degradation of PSD-95.

In contrast, pre-existing CaN may be involved in a more general destabilization process associated with memory retrieval. Blockade of CaN activity in the amygdala prevents retrieval-induced changes in learned behavior in both extinction and reconsolidation procedures. Inhibition of CaN activity has been found to prevent within-session auditory fear extinction, as well as the retrieval-induced strengthening of an inhibitory avoidance memory (Lin et al., 2003b; Fukushima et al., 2014). Furthermore, specifically implicating a role for pre-existing CaN, the inhibition of CaN activity prior to brief memory retrieval also protects against the amnestic effects of protein synthesis inhibitors (Fukushima et al., 2014). Therefore, the pre-existing pool of CaN may be rapidly induced following memory retrieval to induce a set of initial, destabilizing changes to the synaptic structure, such as the endocytosis if AMPARs.

Summary and Hypotheses

Extinction is a complex phenomenon involving both potentiation and depotentiation of synapses at different loci throughout a critical neural circuit**.** Several lines of evidence suggest a potential role for CaN activity in driving depotentiation of thalamic inputs to lateral amygdala, possibly through the regulation of AMPAR endocytosis. However, data concerning specific molecular mechanisms of extinction-induced depotentiation are limited. Furthermore, it is unclear whether the proposed distinction between pre-existing and newly translated CaN corresponds to dissociable elements of extinction-related plasticity. Therefore, the overarching aim of the present study was to address whether there are functionally distinct pools of CaN that contribute to depotentiation-like synaptic changes in the lateral amygdala during auditory fear extinction. Specifics aims included establishing the critical window of CaN synthesis and activity (Aim 1) and providing a behavioral (Aim 2) and molecular (Aim 3) characterization of the roles of pre-existing and newly translated CaN. It was predicted 1) that pre-existing CaN is critical for within-session extinction and mediates AMPAR endocytosis, whereas 2) newly translated CaN is critical for the long-term retention of extinction, and mediates changes that may limit the reinsertion of the AMPARs, such as the dephosphorylation of serine 845 or the loss of PSD-95.

Materials & Methods

Subjects and Surgery

Subjects were 214 adult male Long-Evans rats (300-400g; Harlan, Madison, WI). Animals were housed individually in shoe-box cages with food and water available *ad libitum*. The colony room was maintained under a 14:10 hour light/dark cycle. All experimental procedures were approved by the University of Wisconsin-Milwaukee Institutional Animal Care and Use Committee.

Following 3 days of handling, animals were anesthetized with 4% isoflurane and maintained at 2.5% isoflurane. Animals were implanted with bilateral 26-gauge stainless steel guide cannulae (Plastics One, Roanoke, VA) aimed at the basolateral amygdala using stereotaxic coordinates (-3.0 mm posterior; ±5.1 mm lateral; -7.3 mm ventral) relative to bregma (Paxinos & Watson, 2007). Animals were allowed to recover for seven days prior to behavioral training.

Conditioning Apparatus

Fear conditioning occurred in set of 4 Plexiglas chambers with stainless-steal floors connected to a shock generator (Context A) concealed in a sound-attenuating box. A speaker was centered at one end of each of the 4 chambers. Each chamber was illuminated with white light. A ventilation fan produced approximately 63 dB of background noise. Between animals, chambers were cleaned with a 5% ammonium hydroxide solution.

Extinction, retrieval, and fear retention testing occurred in a novel context (Context B) in a separate room. Context B had Plexiglas floors, infrared lighting, and a ventilation fans providing approximately 58 dB of background noise. Between animals, the chambers were cleaned with a 5% acetic acid solution.

Drug and Antisense Oligodeoxynucleotide Infusions

Rats received bilateral infusions into the amygdala. Injector tips extended 0.5 mm beyond the end of the guide cannulae, resulting in a final ventral coordinate of -7.8 mm relative to bregma. Anisomysin (125 µg/µl; Sigma Aldrich) dissolved in 20% DMSO diluted in ddH_20 was infused 15 minutes prior or immediately following extinction or retrieval. FK506 (10 µg/µl; Sigma Aldrich) dissolved in 100% DMSO was infused 15 minutes prior to or immediately following extinction or retrieval. A cocktail of two desalted, phosphoriothiate end-capped, antisense oligodeoxynucleotides (ODNs) targeting the CaN A subunit (CaN A1 antisense sequences: 5'-CTC GGA CAT CTC CAG TCA-3'; CaN A2 antisense sequence: 5'-

CTC CGG GGC GGC CAT GCT-3') or two scrambled ODNs (CaN A1 scrambled sequence: 5'-GTC GCA GAT CCT CCA ACT -3'; CaN A2 scrambled sequence: 5'-GCT CGT TAG CCG GCG CGC-3') suspended in sterile saline (10 nmol/µl) was infused 2.5 hours prior to extinction or retrieval (as described in Merlo et al., 2014). All drugs were infused at a total volume of 0.5 µl per side at a rate of 0.5 µl per minute. Injectors were left in place for an additional 90 seconds to ensure diffusion away from the tip.

Sequences for the ODNs were obtained from a previous report, in which sequences showed no off-target alignment in a BLAST database search (Merlo et al., 2014).

Behavioral Procedures

3 days prior to behavioral training (Day 1), animals were transported, gently restrained, and acclimated to the sound of the infusion pump to minimize stress and novelty of the injection procedure. On day 1, rats were fear conditioned in Context A. Conditioning involved a 6 minute baseline followed by four white noise (72 dB, 10 s)-shock (1mA, 1s) pairings separated by a 90 second intertrial interval. Animals were removed from the context following a 4 minute post-shock period and returned to their homecages. Following fear conditioning, *strength-matched* experimental groups were created by using the combined average of freezing during the CS-UCS presentations and the post-shock period, such that each group representing each level of each factor exhibited statistically similar freezing during conditioning. On day 2, rats received either an extinction or brief retrieval session in Context B. Rats were transported to the injection room and given an infusion of anisomycin or vehicle 15 minutes prior to extinction or retrieval, FK506 or vehicle 15 minutes prior to extinction or retrieval, or an infusion of CaN ODN 2.5 hours prior to extinction or retrieval. Extinction consisted of a 1 minute baseline followed by 40 white noise (72 dB, 30s) presentations separated by an intertrial interval of 60 seconds. Following a 1 minute post-CS period, animals were removed from the context and returned to their homecages. Retrieval consisted of a 1 minute baseline followed by 4 white noise (72dB, 30s) presentations separated

by an intertrial interval of 60 seconds. Following a 55 minute post-CS period, animals were removed from the context and returned to their homecages. On day 3, rats underwent auditory fear retention testing in Context B. The test involved 1 minute baseline followed by 8 white noise (72 dB, 30s) presentations separated by an intertrial interval of 60 seconds.

Percent time freezing during the white noise presentations was used to assess the conditional fear response. Freezing was defined as the absence of movement with the exception of those required for respiration. Throughout the behavioral sessions, a digital video observation system (FreezeScan, CleverSys Inc, Reston, VA, USA) continuously scored each rat as freezing or active. Percent time spent freezing, as measured by FreezeScan, was used to assess behavioral performance during fear conditioning and retention testing. Freezing during extinction and retrieval session was hand-scored because animals tend to lie down by the end of the extinction session, which would be incorrectly scored as freezing by FreezeScan. To hand-score behavior, each animal was scored as either freezing (=1) or not freezing (=0) once every 5 seconds through the extinction or retrieval session. Percent time spent freezing was derived from the sum of 5 second binds scored as freezing divided by the total number of 5 second bins scored.

Histological Verification

Rats were sacrificed after completion of the auditory fear retention test with an overdose of isoflurane and transcardially perfused with saline followed by 10% buffered formalin. Brains were be removed and stored in buffered formalin for at least 24 hours before soaking in 30% sucrose formalin for an additional 24 or more hours. 40 µm coronal tissue sections containing the amygdala were mounted on slides and stained with cresyl violet to aid in the visualization of the injection site. Animals with injection sites outside of the amygdala were excluded from the analyses.

Calcineurin Activity Assay

Rats were sacrificed with an overdose of isoflurane and decapitated. Brain were removed, frozen on dry ice, and stored at -80°C until use. Amygdala tissue was collected from each brain. Lateral and basal amygdala tissue were not separated here as they were for western blot experiments given the larger amount of protein required for this assay (as described by Lin et al., 2003b). The activity assay was performed with the calcineurin cellular activity assay kit (Enzo Life Sciences). Tissue samples were washed in TBS and homogenized in lysis buffer (BML-KI35). Desalting resin (BML-KI100) was rehydrated overnight and centrifuged at 800 *g* for 3 minutes at 4°C in the desalting column. After discarding the flow through, tissue sample were added to each column and centrifuged at 800 *g* for 3 minutes at 4°C to remove free phosphate from the tissue. Protein content of each sample was determined using the 660nm protein assay (ThermoFisher) and samples were diluted to a concentration of 0.96µg/µl to prevent precipitation during the assay.

Standard curve sample wells were prepared in duplicate with seven 1:1 serial dilutions of phosphate standard (BML-KI132-0500) with 1X assay buffer (500 µl of 2x assay buffer [BML-KI128] diluted with 500 µl dH_2O) and a 1X assay buffer blank. For each tissue sample, 3 wells were prepared to measure background phosphate release (20 µl of dH_2O and 25 µl of 2X assay buffer [BML-KI128] with calmodulin), total phosphatase activity (10 µl of dH_2O r and 25 µl of 2X assay buffer with calmodulin), and total phosphatase activity without CaN activity (10 µl of dH_2O and 25 µl of 2X EGTA buffer [BML-KI136]). 10 µl of phosphopeptide substrate (BML-P160) were added to each well, with the exception of the background control and standards wells. The samples were allowed to equilibrate to room temperature for 10 minutes. To initiate the assay, 5 µl of sample were added to their respective wells. After incubation for 30 minutes at 37°C, 100 µl of biomol green reagent (BML-AK111-9090) was added to all wells. Color was allowed to develop for 25 minutes at room temperature before reading OD_{620nm} on a microplate reader (BioTek).

A standard curve was created by plotting OD_{620nm} vs. nmol PO_4 for the phosphate standards. The slope and Y-intercept were used to calculate the amount of phosphate released (phosphatase activity) with the equation: Phosphate released = $(OD_{620nm} – Y_{int})$/slope. The background phosphate was subtracted from each sample well and specific CaN activity was determined using the equation: CaN activity = total phosphatase activity – total phosphatase activity less CaN activity.

Synaptosomal Fractions

Rats were sacrificed with an overdose of isoflurane and decapitated. Brains were removed, frozen on dry ice, and stored at -80°C until dissected by hand. Careful efforts, aided by anatomical templates (Paxinos & Watson, 2007), were made to isolate tissue from the lateral nucleus of the amygdala. However, given practical limitations of hand dissection, the final tissue sample likely included some portions of adjacent amygdala nuclei. Tissue was homogenized in TEVP buffer (.157 g tris, .0042 g sodium fluoride, 1 ml sodium orthovanadate, .038 g EDTA, .038 g EGTA, 2 tables complete protease inhibitor, 10.944 g sucrose in ddH_2O). For samples that would also be used for whole cell protein analysis, 18 µl of homogenate was transferred to a separate tube, lysed in homogenization buffer, and stored at -80°C until used. Remaining samples were centrifuged at 1,000 *g* for 10 minutes at 4°C to remove nuclei and large debris. The supernatant was removed and spun at 10,000 *g* for 10 minutes at 4°C to separate cytoplasmic and synaptosomal fractions. The resulting pellet was re-suspended and lysed homogenization buffer (.605 g tris, .876 g NaCl, 10 ml sodium fluoride, .0042 g sodium fluoride, 1 ml sodium orthovanadate, 2 tablets complete protease inhibitor in ddH_2O) and stored at -80°C until use.

Western Blotting

Following determination of protein content using the 660nm protein assay (Pierce), 7.5 µg of each whole-cell lysate or synaptosomal fraction was mixed with 2x Laemmli sample buffer (BioRad) and ran on a 7.5% TDX gel (BioRad). Proteins were transferred to PVDF membranes**.** Membranes were

incubated in blocking buffer (3.75 ml 4X TBS, .75 g dry milk, 15 µl Tween-20 in 12.5 ml ddH_2O) for 60 minutes at room temperature before being exposed to primary antibody diluted in primary buffer (5 ml 4X TBS, 1 g BSA, 20 µl Tween-20 in 15 ml ddH_2O) overnight at 4°C. Membranes were then incubated in 15 ml blocking buffer with anti-rabbit, anti-goat, or anti-mouse secondary antibody (1:20,000; Santa Cruz BioTech) for 60 minutes at room temperature before exposure to a SuperSignal West Dura chemiluminescence solution (Thermo Fisher) for 5 minutes. Protein bands were imaged with the Gbox Chemi XT4 imager (SynGene) and optical density of each band was quantified with Gene Tools software (SynGene). Optical densities for each protein band were normalized to the optical density of the β-Actin band on an animal-by-animal basis.

Primary antibodies included pan-CaN A (1:1000; Cell Signaling), phospho-GluR1 (ser 845) (1:1000; Cell Signaling), total GluR1 (1:1000; Cell-Signaling), total Glur2 (1:1000; Santa Cruz BioTech), PSD-95 (1:1000; Santa Cruz BioTech), and β-Actin (1:1000; Cell Signaling).

Statistical Analysis

Group differences were assessed using independent samples t test, two way ANOVA or mixed-model ANOVA, as appropriate. An exception to this was the western blot and CaN activity assay experiments in Aim 1. The unbalanced design of these experiments produced a non-sensical interaction term when analyzed by two-way ANOVA. Therefore, analysis was carried out using one-way ANOVAs. In all two-way ANOVAs, *drug* and *behavioral group* (referring to the retrieval condition) were used as between-subjects factors. In mixed model ANOVAs, *drug* condition was used as the between-subjects factor and *retrieval bin* (referring to discrete time points within the extinction training session) was used as the within-subject factor. Post hoc tests were conducted with Dunnett's method to compare each group to a control group. An exception to this was the CaN activity assay data, in which the Bonferroni correction was used to compare a pre-selected set of groups. Observations greater than 2 standard

deviations from the group mean were considered outliers. A p-value less than 0.05 was considered significant in all cases.

Procedure: Aim 1

Aim 1 established central parameters relevant to the subsequent experiments. To define a window of behavioral sensitivity to pharmacological manipulation, animals were trained with auditory fear conditioning in context A. Animals were split into strength-matched experimental groups. Twenty-four hours post-training, animals were given extinction training in context B. Animals received bilateral amygdala infusions of anisomycin or vehicle (immediately following extinction or 15 minutes prior to extinction) or FK506 or vehicle (immediately following extinction). One day following extinction training, animals were given an auditory fear retention test in context B to assess fear to the auditory CS.

To verify translation of new CaN protein in the amygdala during extinction training, cannulated animals were trained with auditory fear conditioning in context A and divided into strength-matched groups. Twenty-four hours following fear conditioning, animals received bilateral amygdala infusions of anisomycin or vehicle (15 minutes prior to extinction training) or CaN ODN or scrambled ODN (2.5 hours prior to extinction training). Animals were sacrificed immediately following the end of the extinction session. Expression of CaN protein in whole-cell amygdala homogenate from each animal was quantified using western blots.

To assess the time course of CaN activity during training, cannulated animals were trained with auditory fear conditioning in context A and divided into strength-matched groups. Twenty-four hours post-training, animals received bilateral amygdala infusions of vehicle 15 minutes prior to extinction training. Animals were sacrificed at one of five time-points throughout the extinction session: 0 CS's (0 min), 10 CS's (15 min), 20 CS's (30 min), 30 CS's (45 min), and 40 CS's (60 min). Amygdala tissue from each animal was used in the CaN activity assay to determine CaN activity at each time point. An

additional group of rats was infused with FK506 15 minutes prior to the extinction session and sacrificed following 0 CS's (0 minutes) or 20 CS's (30 minutes) to verify pharmacological inhibition of endogenous CaN activity.

Procedure: Aim 2

Aim 2 examined the behavioral impact of inhibiting CaN activity in the amygdala prior to extinction and aimed to delineate the effects of newly translated versus pre-existing CaN. Cannulated rats were trained with auditory fear conditioning in context A. Animals were split into strength-matched experimental conditions. Twenty-four hours post-training, animals received infusions of vehicle, FK506, scrambled ODN, or CaN ODN and were then given either an extinction or retrieval session in context B. Twenty-four hours later, animals were tested for fear to the CS in Context B.

Procedure: Aim 3

Aim 3 assessed CaN-dependent changes in expression and phosphorylation state of AMPARs and AMPAR-associated proteins in the lateral amygdala following extinction training. Cannulated rats were trained with auditory fear conditioning in context A. Animals were split into strength-matched experimental groups. The next day, animals received infusions of FK506 or vehicle (15 minutes prior to the extinction or retrieval session) or CaN ODN or scrambled ODN (2.5 hours prior to the extinction or retrieval session). Immediately after the conclusion of extinction or retrieval, animals were sacrificed. A separate group of rats were injected with vehicle or the scrambled ODN twenty-four hours after auditory fear conditioning for use as a pre-extinction baseline. These animals were sacrificed 76 minutes (vehicle) or 3 hours and 31 minutes (scrambled ODN) following injection without receiving an extinction or retrieval session. These time points were chosen to match the timing between injection and sacrifice to animals that underwent extinction and retrieval sessions. Lateral amygdala tissue was dissected and

synaptosomal fractions were obtained from each sample. Tissue fractions underwent Western blot analysis for changes in total GluR1, total GluR2, phosphorylated GluR1 (ser845), and PSD-95.

Results

A representative cannula placement is shown in Figure 1.

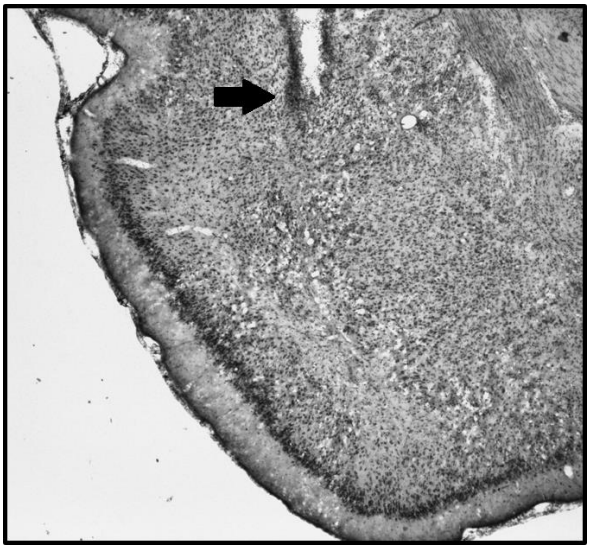


Figure 1. Representative amygdala cannula placement. Photomicrograph of cannula placement.

Aim 1 Extinction Induces Increases in CaN Protein and Activity Levels

We first conducted a series of tests to characterize critical periods of CaN translation and activity following the onset of extinction training.

1.1 The Critical Window of Amygdala Protein Synthesis Occurs Within the 40 CS (60 Minute) Extinction Session

Despite evidence that new CaN protein in the amygdala is translated during extinction training, post-extinction insensitivity to protein synthesis inhibitors has also been reported (Lin et al., 2003b,c; Duvarci, ben Mamou, & Nader, 2006). Therefore, we sought to determine a time window of sensitivity to protein synthesis inhibition.

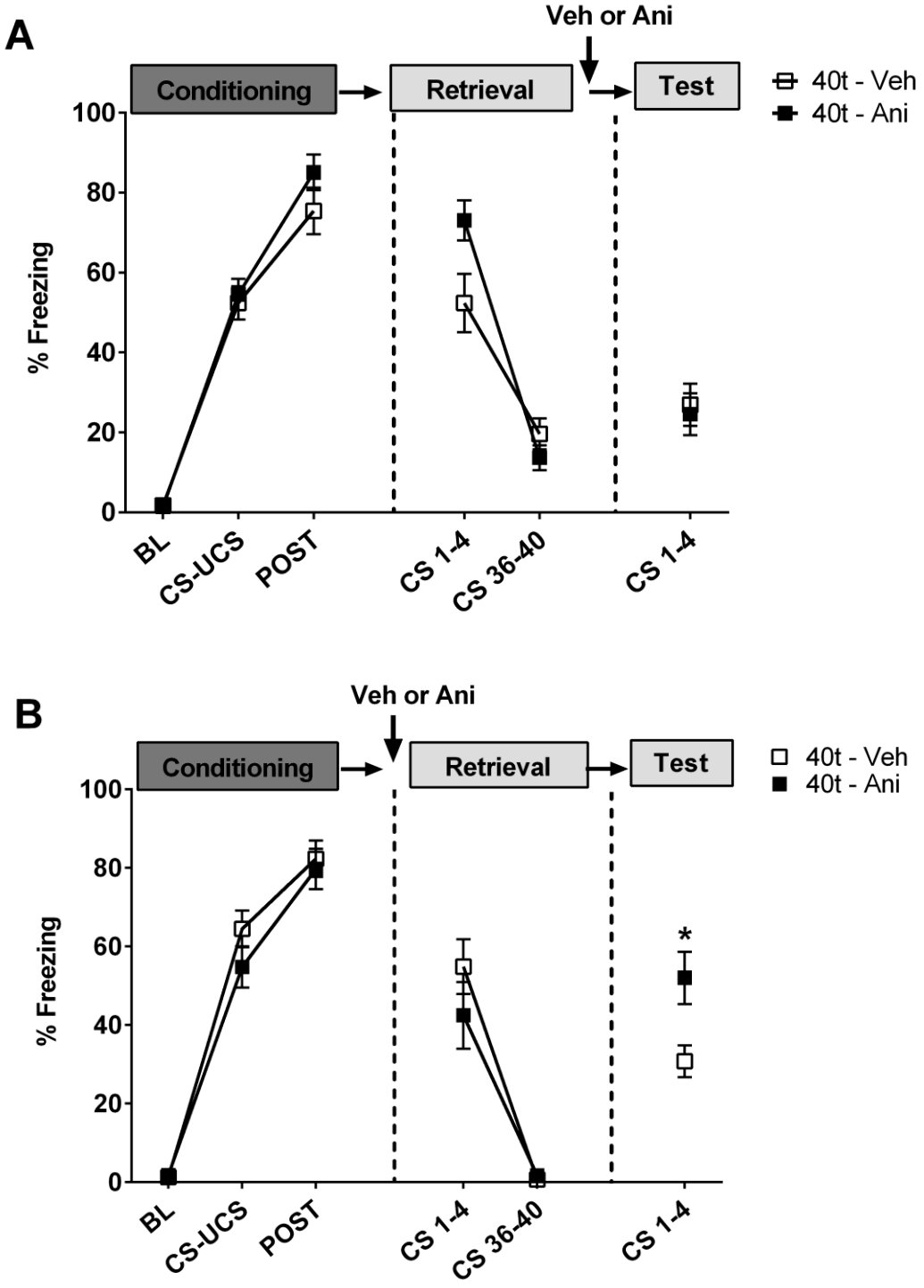
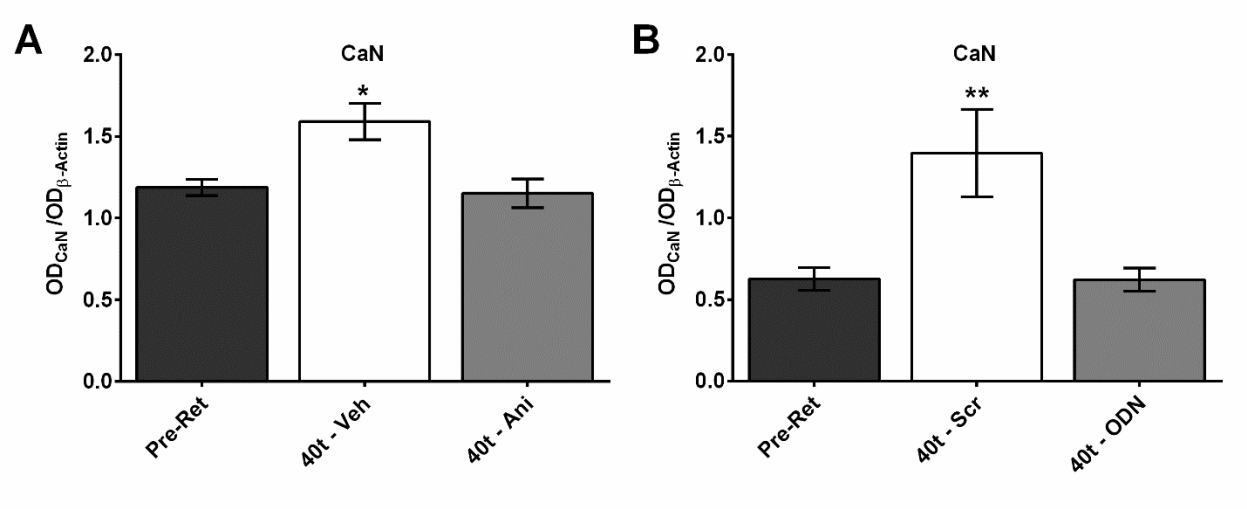


Figure 2. The critical window of amygdala protein synthesis occurs within the 40 CS (60 minute) extinction session. (A) Post-extinction infusion of protein synthesis inhibitor anisomycin in the amygdala did not effect performance at the extinction retention test. (B) Pre-extinction infusion of anisomycin in the amygdala disrupted the long-term retention of extinction but did not effect within-session extinction. All data represent mean (± SEM). *p<.05.

To test whether the long-term retention of extinction could be disrupted with post-extinction inhibition of protein synthesis, strength-matched *drug* conditions were created prior to the experimental manipulation based on average time spent freezing during the 4 CS-UCS pairings and the 4 minute post-CS-UCS period of fear conditioning (*independent samples t test:* t(10) = 0.97, p>.05) (Fig 2A). The following day, rats received bilateral amygdala infusions of either the general protein synthesis inhibitor anisomycin (n=6) or vehicle (n=6) immediately following a 40-trial (40t) extinction session in a novel context. During the drug-free extinction session, there was a significant decreases in freezing between the first 4 CS presentations and the last 4 CS presentations (*mixed-model ANOVA:* F(1, 10) = 206.71, p<.001), with no significant differences between the drug conditions*(*F (1, 10) = 0.66, p>.05). While there was a significant *CS Bin*Drug* interaction (F(1,10) = 20.59, p<.01), this effect was driven by the moderately higher level of freezing during the first 4 CS presentations in the anisomycin group relative to the vehicle group (p< .10). During the last 4 CS presentations of the extinction session, there were no differences in freezing between the drug conditions (p>.05). Twenty-four hours later, animals were returned to the extinction context for a CR retention test. There were no significant differences in freezing behavior between the anisomycin and vehicle groups (*independent samples t test:* t(10) = 0.53, p>.05).

Next, we tested whether pre-extinction infusions of anisomycin would disrupt the long-term retention of extinction. Strength-matched *drug* conditions were created prior to the experimental manipulation based on average time spent freezing during the 4 CS-UCS pairings and the 4 minute post-CS-UCS period of fear conditioning (*independent samples t test:* t(10) = 1.55; p>.05) (Fig 2B). The following day, rats received bilateral amygdala infusions of either anisomycin (n=6) or vehicle (n=6) 15 minutes prior to a 40t extinction session in a novel context. During the extinction session, there were significant decreases in freezing between the first 4 CS presentations and the last 4 CS presentations in both groups of animals (*mixed-model ANOVA:* F(1, 10) = 32.64, p<.001), with no significant differences

between drug conditions overall *(*F (1, 10) = .66**,** p>.05) or at either individual time point in the extinction session(F (1, 8) = 0.15, p>.05). Twenty-four hours later, animals were returned to the extinction context for a CR retention test. Anisomycin-injected animals froze significantly more to the CS than vehicle injected animals (*independent samples t test:* t(10) = 2.73; p<.05).



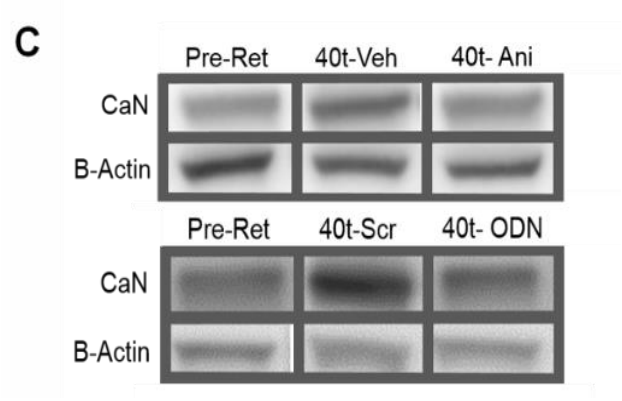


Figure 3. Extinction induces synthesis of new CaN protein in the amygdala. (A) Extinction-induced increases in CaN expression were blocked by pre-extinction infusions of the general protein synthesis inhibitor anisomycin and (B) by a CaN-targeted antisense ODN.
(C) Representative protein bands from Western blots. All data represent mean (± SEM). *p<.05. **p<.01 relative to the pre-retrieval group.

To confirm that anisomycin was preventing extinction-induced translation of CaN, animals that received pre-extinction infusions of anisomycin (n=5) or vehicle (n=6) were sacrificed immediately following 40t extinction to examine CaN expression in the amygdala (Fig 3A). An additional group of animals received vehicle injections (n=5) 24 hours following fear conditioning and were sacrificed 76

minutes later. There were significant differences in CaN expression in amygdala whole-cell lysates between the groups (*one-way ANOVA:* $F(2,13) = 7.45$; $p<.01$). Follow-up tests revealed that, compared to vehicle-infused animals that did not receive extinction training, animals who received vehicle infusions prior to extinction had significantly greater levels of amygdala CaN expression ($p<.05$) whereas this increase was not observed in animals infused with anisomycin prior to extinction ($p>.05$).

Finally, to assess whether a similar knockdown of CaN translation could be achieved using a CaN ODN, animals that received pre-extinction infusions of the CaN ODN (n=8) or scrambled ODN (n=8) were sacrificed immediately following 40t extinction to examine CaN expression in the amygdala (Fig 3B). An additional group of animals received scrambled ODN injections (n=8) 24 hours following fear conditioning and were sacrificed approximately 3.5 hours later. There were significant differences in CaN expression in amygdala whole-cell lysates between the groups (*one-way ANOVA:* $F(2,21) = .004$). Follow-up tests revealed that, compared to scrambled ODN-infused animals that did not receive extinction training, animals who received scrambled ODN infusions prior to extinction had significantly greater levels of amygdala CaN expression ($p<.01$) whereas this increase was not observed in animals infused with the CAN ODN prior to extinction ($p>.05$).

1.2 The Critical Window of Amygdala CaN Activity Occurs Within the 40 CS (60 Minute) Extinction Session

Although the results of the anisomycin experiments suggested that translation of CaN is not required in the amygdala following extinction training, this did not preclude the possibility that CaN activity is critical following extinction training. Therefore, we next tested whether post-extinction infusions of the CaN activity inhibitor FK506 would disrupt the long-term retention of extinction (Fig 4A).

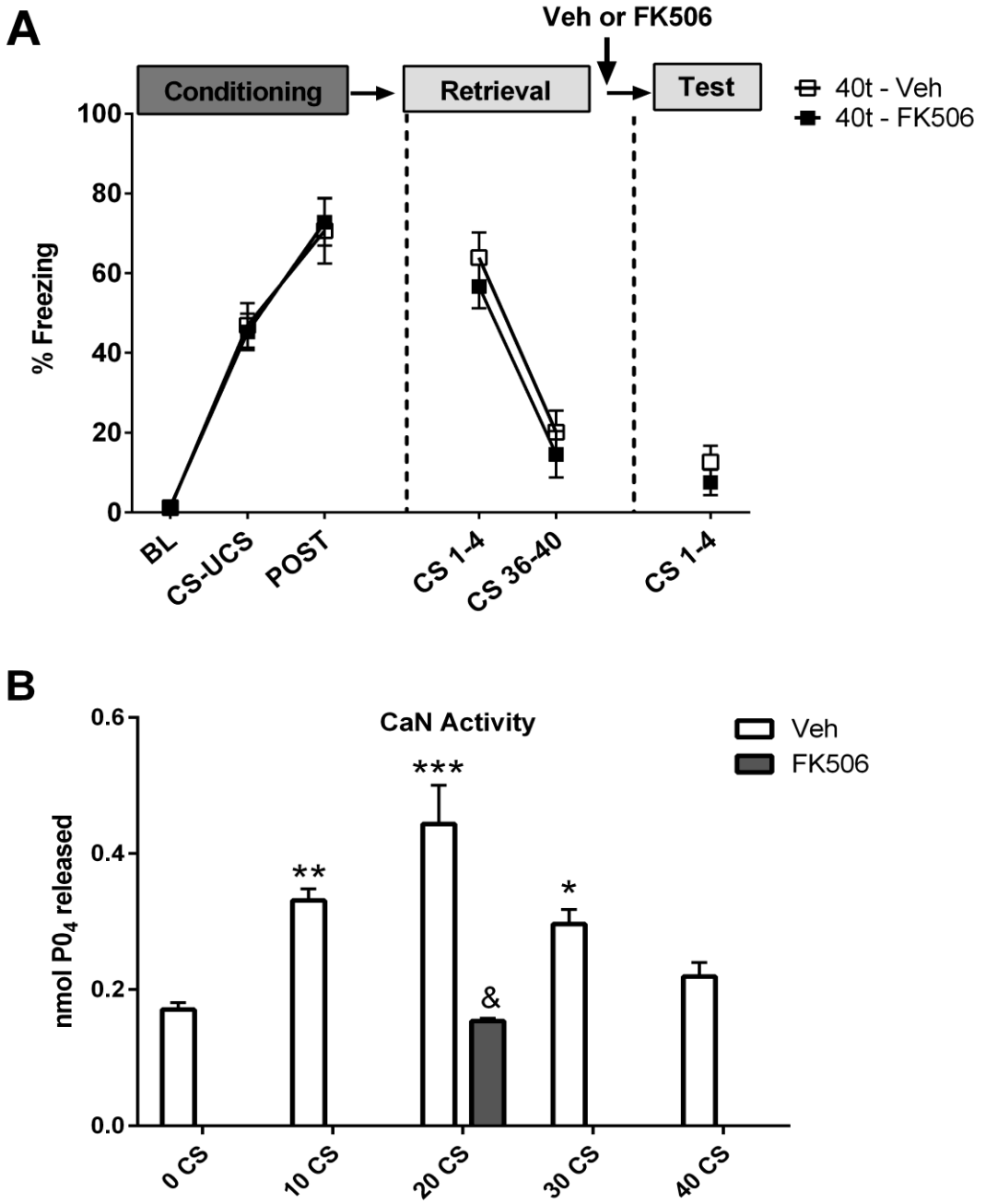


Figure 4. The critical window of amygdala CaN activity occurs within the 40 CS (60 minute) extinction session. (A) Post-extinction infusion of CaN inhibitor FK506 in the amygdala did not effect performance at test. (B) Amygdala CaN activity is significantly increased immediately following 10 and 20 CS presentations. However, CaN activity returns to approximately basal levels by the 40th CS presentation. Pre-extinction infusion of FK506 blocks the elevation in CaN activity observed following 20 CS presentations (± SEM). *p<.05,**p<.01 and ***p<.001 relative to the 0 CS group. $^{\&}$p<.001 relative to the 20 CS vehicle group.

Strength-matched *drug* conditions were created prior to the experimental manipulation based on average time spent freezing during the 4 CS-UCS pairings and the 4 minute post-CS-UCS period of fear conditioning (*independent samples t test:* t(8) = 0.01; p>.05).The following day, rats received bilateral amygdala infusions of either FK506 (n=5) or vehicle (n=5) 15 minutes immediately following a 40t extinction session in a novel context. During the drug-free extinction session, there were significant decreases in freezing between the first 4 CS presentations and the last 4 CS presentations in both groups of animals (*mixed-model ANOVA:* F (1, 8) = 37.77, p<.001), with no significant differences between drug conditions overall (F (1, 8) = 1.54, p>.5) or at either individual time point in the extinction session; *:*(F(1, 8) = 0.15, p>.05). Twenty-four hours later, animals were returned to the extinction context for a CR retention test. There were no significant differences in freezing behavior between the FK506 and vehicle groups (*independent samples t test:* t(8) = 0.99; p>.05).

Given the apparent general insensitivity to post-extinction manipulations, we next determined the time course of CaN activity through the extinction session (Fig 4B). 24 hours following fear conditioning, animals were injected with vehicle prior to a 0 (n=4), 10 (n=4), 20 (n=4), 30 (n=4) or 40 (n=4) CS extinction session. Additional animals received injections of FK506 (n=4) prior to a 20 CS extinction session to test the efficacy of the drug at inhibiting CaN activity. All groups were sacrificed immediately following the conclusion of their respective extinction sessions and amygdala tissue was used to determine CaN activity. There were significant differences in CaN activity between groups (*one-way ANOVA:* F(5,18) = 15.91, p<.001). Follow up tests revealed that, relative to the 0 CS group, there were significant increases in the 10 CS (p<.01), 20 CS (p<.001), and 30 CS (p<.05) vehicle groups. In contrast, CaN activity in the 20 CS FK506 group was not different than the 0 CS vehicle group (p>.05) and was significantly lower than the 20 CS vehicle group (p<.001).

Collectively, these findings indicate that the window of translation and elevated activity of CaN occurs within the boundaries of our 40 trial, 60 minute extinction session. With these experimental

parameters, post-extinction pharmacological manipulation of the amygdala did not produce detectable deficits in the long-term retention of fear extinction. Therefore, all subsequent experiments were conducted using pre-extinction drug infusions.

Aim 2 Pre-Existing and Newly Translated CaN Have Dissociable Behavioral Roles in Extinction

It has previously been suggested that distinct pools of CaN, defined by whether the protein existed prior to the onset of extinction training or whether the protein is translated following onset of extinction training, differentially mediate extinction-related processes (Merlo et al., 2014). To test this hypothesis, we compared the behavioral effects of pre-extinction infusion of pharmacological agents that inhibited either the activity of CaN (FK506) or the translation of CaN (CaN antisense ODN).

2.1 Inhibition of CaN Activity in the Amygdala Results in Deficits in Within-Session Fear Extinction and the Long-Term Retention of Extinction

To test this effects of inhibiting CaN activity in the amygdala during extinction, strength-matched *drug x behavioral* conditions were created prior to the experimental manipulation based on average time spent freezing during the 4 CS-UCS pairings and the 4 minute post-CS-UCS period of fear conditioning (*two-way ANOVA:* $F_{drug}(1,35) = .07$, $p>.05$; $F_{behavioral\ group}(1,35) = 1.52$, $p > .05$; $F_{drug\ x\ behavioral\ group}(1,35) = 0.03$, $p>.05$) (Fig 5A). The following day, rats received bilateral amygdala infusions of either FK506 or vehicle 15 minutes prior to a 40t extinction (FK506: n = 13; vehicle: n = 14) or 4t retrieval (FK506: n = 6; Vehicle: n= 6) session in a novel context. There were no group differences during the first 4 CS presentations due to *drug* condition (*two-way ANOVA:* $F(1,35) = 0.0004$, $p<.05$), *behavioral group* ($F(1,35) = 0.60$), or a *drug x behavioral group* interaction ($F(1,35) = 0.1481$, $p>.05$). Within the 40t groups, there were significant main effects for both drug (*mixed-model ANOVA:* $F(1,35) = 5.97$, $p<.05$) and retrieval CS bin ($F(1,35) = 13.49$, $p=.001$) and a significant *drug x CS bin* interaction;($F(1,25) = 9.24$, $p<.01$). Follow up tests revealed a significant decrease in freezing between the first and last four CS

presentations of retrieval in the 40t vehicle group ($p<.001$) but not the 40t FK506 group ($p>.05$). In addition, the 40t FK506 group froze significantly more than the 40t vehicle group during the last 4 CS presentations of retrieval ($p<.01$). Twenty-four hours later, animals were returned to the extinction context for a CR retention test. There was a significant main effect of drug (*two-way ANOVA:* $F(1,35) = 5.48$, $p<.05$) and behavioral group ($F(1,35) = 4.66$, $p<.05$), and a significant *drug x behavioral group* interaction;($F(1,35) = 4.59$, $p<.05$). Follow up tests revealed that groups of animals frozen significantly more than than the 40t vehicle group (40t veh vs. 40t FK506: $p<.001$; 40t veh vs 4t veh: $p<.05$; 40t veh vs 4t FK506: $p<.01$).

2.2 Inhibition of Calcineurin Translation in the Amygdala Selectively Impairs the Long-Term Retention of Extinction

To test the effects of inhibiting CaN translation in the amygdala during extinction, strength-matched *drug x behavioral* conditions were created prior to the experimental manipulation based on average time spent freezing during the 4 CS-UCS pairings and the 4 minute post-CS-UCS period of fear conditioning (Fig 5B). Though there was a significant main effect for behavioral group (*two-way ANOVA:* $F(1, 18) = 6.05$, $p<.05$), where rats in the 40t extinction condition showed significantly greater freezing during training than those in the 4t retrieval condition, there were not a significant main effect for *drug* or ($F(1,18) = 0.79$, $p > .05$ a significant *drug x behavioral group* interaction $F(1,18) = 0.001$, $p>.05$). Therefore, these groups were accepted as strength-matched.

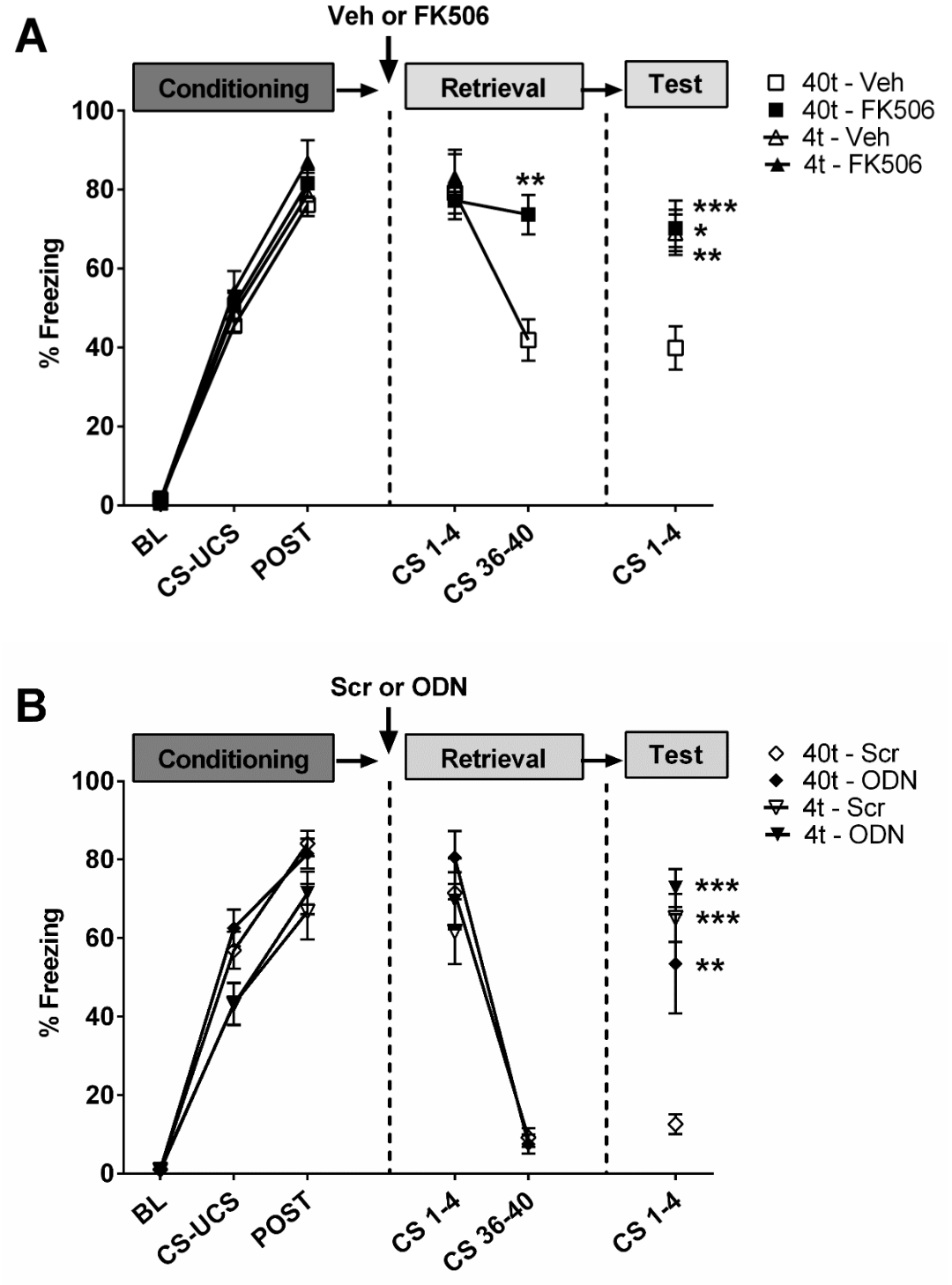


Figure 5. Inhibition of CaN activity or CaN translation in the amygdala produce distinct patterns of deficits in extinction. (A)Inhibition of CaN activity impairs within-session extinction and the long-term retention of extinction whereas (B) inhibition of CaN translation selectively impairs the long-term retention of extinction. All data represent mean (± SEM). *p<.05, **p<.01, ***p<.001 relative to the 40t vehicle group.

The following day, rats received bilateral amygdala infusions of either the CaN ODN or scrambled ODN 2.5 hours prior to a 40t extinction (CaN ODN: n=5; Scrambled ODN: n=5) or 4t retrieval (CaN ODN: n=6; Scrambled ODN: n=6) session in a novel context. There were no group differences during the first 4 CS presentations between any of the four groups due to *drug* condition (*two-way ANOVA:* $F(1,18) = 1.60$, $p>0.5$), *behavioral group* ($F(1,18) = 2.14$, $p>.05$), or a *drug x behavioral group interaction* ($F(1,18) = .21$, $p>.05$). Within the 40t groups, there was a significant effect of retrieval CS bin (*mixed-model ANOVA:* $F(1,8) = 314.07$, $p<.001$). Follow up tests revealed that 40t Scr ($p<.01$) and 40t ODN ($p<.01$) animals exhibited a significant decrease in freezing between the first and last four CS presentations of retrieval. Importantly, there was no significant main effect for *drug* (*mixed-model ANOVA:* $F(1,8) = 2.02$, $p>.05$). While there was some suggestions of a *drug x retrieval CS bin* interaction ($F(1,8) = 5.34$, $p=.05$), this is likely due to the somewhat elevated freezing of the 40t ODN group during the first four CS presentations of retrieval. However, there were no significant difference between drug conditions during the final 4 CS presentations of the 40t extinction session ($p>.05$). Twenty-four hours later, animals were returned to the extinction context for a CR retention test. There was a significant main effect of drug (*two-way ANOVA:* $F(1,18) = 11.33$, $p<.01$) and behavioral group ($F(1,18) = 24.77$, $p<.001$), and a significant *drug x behavioral group* interaction ($F(1,18) = 5.31$, $p<.05$). Follow up tests revealed all other groups of animals froze significantly more than animals in the 40t scrambled ODN group (40t Scr ODN vs. 40t CaN ODN: $p<.01$; 40t Scr ODN vs 4t Scr ODN: $p<.001$; 40t Scr ODN vs 4t CaN ODN: $p<.001$).

Aim 3 Pre-Existing and Newly Translated CaN Mediate Distinct Aspects of Depotentiation-Like Synaptic Changes in the Lateral Amygdala

Given the distinct impairments in extinction observed following amygdala infusions of FK506 and the CaN antisense ODN, we next probed for differences in depotentiation-like changes in synaptosomal proteins following the inhibition of CaN activity and translation during extinction. While acute changes in AMPARs surface expression are thought to be important for the induction of synaptic plasticity and are likely behaviorally relevant as well, the up- or down-regulation of scaffolding proteins like PSD-95 tend to be indicative of longer-term changes in AMPAR expression and synaptic strength (Ehlers, 2000; Colledge et al., 2003; Kim et al., 2007; Dalton et al., 2007). Therefore, we predicted that inhibition of CaN activity would prevent extinction-induced reductions in AMPAR and PSD-95 expression in synaptosomal preparations of amygdala tissue, whereas selective inhibition of CaN translation would preserve these initial AMPAR dynamics but prevent the loss of PSD-95 expression.

3.1 Inhibition of CaN Activity Prevents Extinction-Induced Reductions in Synaptosomal Expression of GluR2 and PSD-95

To test whether inhibition of CaN activity would prevent reduced expression of AMPARs and PSD-95, fear-conditioned animals were injected with either FK506 or vehicle 15 minutes prior to a 40t extinction (FK506: n=8; Vehicle: n=8) or 4t retrieval (FK506: n=7; Vehicle: n=7) session and were sacrificed 60 minutes after the onset of the first CS. An additional group of fear conditioned rats (n=8) was used to establish basal protein levels after conditioning but prior to any re-exposure to the CS. Twenty-four hours after conditioning, these animals received vehicle infusions and were returned to their homecage for 76 minutes before being sacrificed. LA synaptosomal fractions were used to characterize extinction-induced changes in AMPAR and PSD-95 expression.

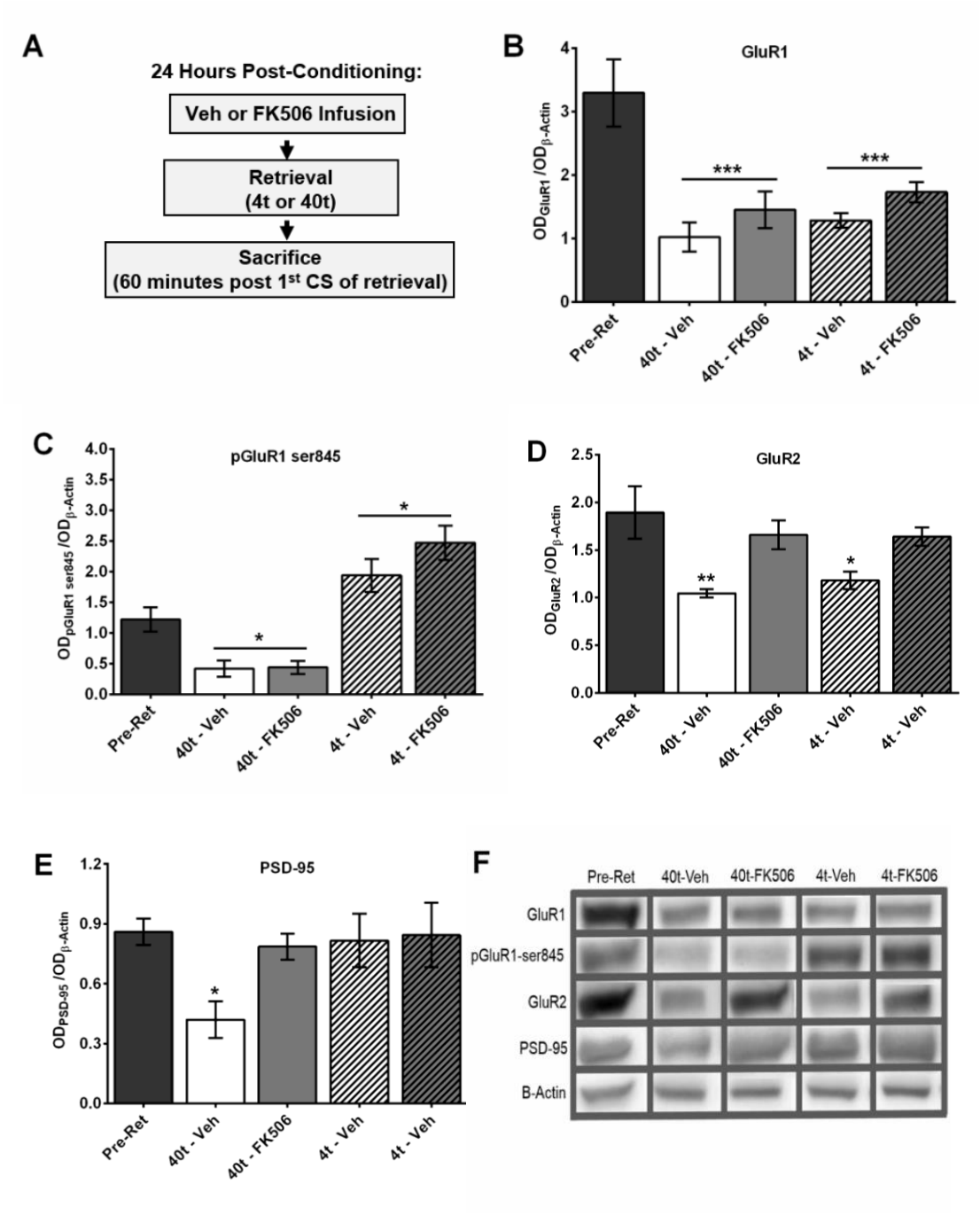


Figure 6. Inhibition of CaN activity prevents changes in synaptosomal expression of GluR2 and PSD-95 in the lateral amygdala. (A) Schematic of experimental procedure. Inhibition of CaN activity does not effect (B) reduction of GluR1 or (C) bidirectional changes in the phosphorylation of serine 845 on GluR1 following extinction or retrieval. Inhibition of CaN activity (D) prevents reductions in GluR2 following extinction or retrieval and E) prevents the extinction-specific loss of PSD-95. F) Representative protein bands from Western blots. All data represent mean (± SEM). *p<.05, **p<.01, ***p<.001 relative to the pre-retrieval group.

There was a significant effect of behavioral group on total GluR1 expression (*two-way ANOVA:* $F(2,32) = 15.86$, $p<.001$) (Fig 6B). However, there was no drug effect ($F(1, 32) = 1.85$, $p>.05$) and no significant *drug x behavioral group* interaction ($F(1, 32)= 0.001$, $p>.05$). Follow up tests revealed a significant reduction in GluR1 in the 40t and 4t groups relative to the pre-retrieval group (pre-ret vs. 40t: $p<.01$; pre-ret vs 4t: $p<.05$). Similar to the total synaptosomal expression of GluR1, there was a significant main effect of behavioral condition on the total amount of GluR1 subunits phosphorylated at serine 845 (*two-way ANOVA:* $F(2,30) = 23.45$, $p<.001$) without a significant main effect of drug condition ($F(1, 30) = 0.92$, $p>.05$ or a significant *drug x behavioral group* interaction ($F(1, 30)= 0.76$, $p>.05$) (Fig 6C). However, in contrast to the unidirectional change in total GluR1 following retrieval, post-hoc comparisons revealed that, regardless of drug infusion, animals in the 4t retrieval condition expressed significantly increased level of phosphorylated Glur1 relative to the pre-retrieval control group (Pre-Ret vs. 4t: $p<.05$), whereas animals in the 40t extinction condition exhibited significantly reduced level of phosphorylated GluR1 (Pre-Ret vs 40t: $p<.05$).

There was a significant effect of behavioral group (*two-way ANOVA:* $F(2, 31) = 8.22$, $p<.001$) and drug condition ($F(1,31) = 10.84$, $p<.01$) on GluR2 expression (Fig 6D). However, there was no significant *drug x behavioral group* interaction ($F(1, 31) = 0.46$, $p>.05$). Follow up tests revealed a significant reduction in GluR2 relative to baseline in the 40t and 4t vehicle groups (pre-ret vs 40t veh: $p<.001$; pre-ret vs 4t veh: $p<.01$) but no significant reduction in the 40t and 4t FK506 groups (pre-ret vs 40t FK506: $p>.05$; pre-ret vs 4t FK506: $p>.05$). There were also significant differences in levels of PSD-95 as a function of drug (*two-way ANOVA:* $F(2, 31) = 5.84$, $p<.01$) and behavioral group ($F(1,31) = 5.36$, $p<.05$), as well as a significant *drug x behavioral group* interaction($F(1, 31) = 4.18$, $p<.05$) (Fig 6E). Follow up tests revealed a significant reduction in PSD-95 relative to baseline in the 40t vehicle group only (pre-ret vs 40t veh: $p<.05$; pre-ret vs 40t FK506: $p>.05$; pre-ret vs 4t veh: $p>.05$; pre-ret vs 4t FK506: $p>.05$).

3.2 Inhibition of CaN Translation Preserves AMPAR Trafficking but Prevents Extinction-Induced Reductions in Synaptosomal Expression of PSD-95

To test whether inhibition of CaN translation would prevent reduced expression PSD-95, but preserve the observed CaN-dependent reduction in GluR2 in the amygdala following extinction, fear-conditioned animals were injected with either the CaN antisense ODN or the scrambled ODN 2.5 hours prior to a 40t extinction (CaN ODN: n=8; Scr ODN: n=8) or 4t retrieval (CaN ODN: n=8; Scr ODN: n=8) session and were sacrificed 60 minutes after the onset of the first CS. An additional group of fear conditioned rats (Pre Ret: n=8) was used to establish basal protein levels after conditioning but prior to any re-exposure to the CS. Twenty-four hours after conditioning, these animals received scrambled ODN infusions and were returned to their homecage for 3 hours and 31 minutes before being sacrificed. LA synaptosomal fractions were used to assess extinction-induced changes in AMPAR and PSD-95 expression.

Consistent with the results obtained with FK506, there was a significant effect of behavioral group on total GluR1 expression (*two-way ANOVA:* $F(2,34) = 6.26$, $p<.01$); but no significant effect of drug ($F(1, 34) = .13$, $p>.05$) and no significant *drug x behavioral group* interaction ($F(1, 34)= .67$, $p>.05$) (Fig 7B). Follow up tests revealed a significant reduction in GluR1 in all behavioral groups relative to the pre-retrieval group (pre-ret vs. 40t: $p<.01$; pre-ret vs 4t: $p<.01$). There was also a significant main effect of behavioral condition on the total amount of GluR1 subunits phosphorylated at serine 845 (*two-way ANOVA:* $F(2,30) = 18.40$, $p<.001$)but no significant main effect of drug condition ($F(1, 30) = 0.50$, $p>.05$) and no significant *drug x behavioral group* interaction ($F(1, 30)= 0.02$, $p>.05$) (Fig 7C). Post-hoc comparisons revealed that significantly increased levels of phosphorylated GluR1 in the retrieval condition relative to the pre-retrieval control group (Pre-Ret vs. 4t: $p<.05$), whereas there was a trend towards dephosphorylation of serine 845 in the extinction condition (Pre-Ret vs 40t: $p<.10$).

There was a significant effect of behavioral group (*two-way ANOVA:* F (1, 34) = 9.60, p<.001) on GluR2 expression. In contrast to findings with FK506, there was no significant effect of drug condition (F(1,34) = .88, p>.05) on GluR2 expression (Fig 7D). In addition, there was no significant *drug x behavioral group* interaction (F (1, 34) = 0.90, p>.05). Follow up tests revealed a significant reduction in GluR2 in all groups relative to the pre-retrieval group (pre-ret vs. 40t: p<.01; pre-ret vs 4t: p<.01). Despite the absence of drug effects on AMPAR expression, there were significant differences in levels of PSD-95 as a function of drug (*two-way ANOVA:* F(2, 33) = 5.13, p<.05) and behavioral group (F (1,33) = 4.92, p<.05), as well as a trend toward a significant *drug x behavioral group* interaction(F (1, 33) = 3.87, p<.10) (Fig 7D). Follow up tests revealed a significant reduction in PSD-95 relative to baseline in the 40t Scr group only (pre-ret vs 40t Scr: p<.01; pre-ret vs 40t ODN: p>.05; pre-ret vs 4t Scr: p>.05; pre-ret vs 4t ODN: p>.05).

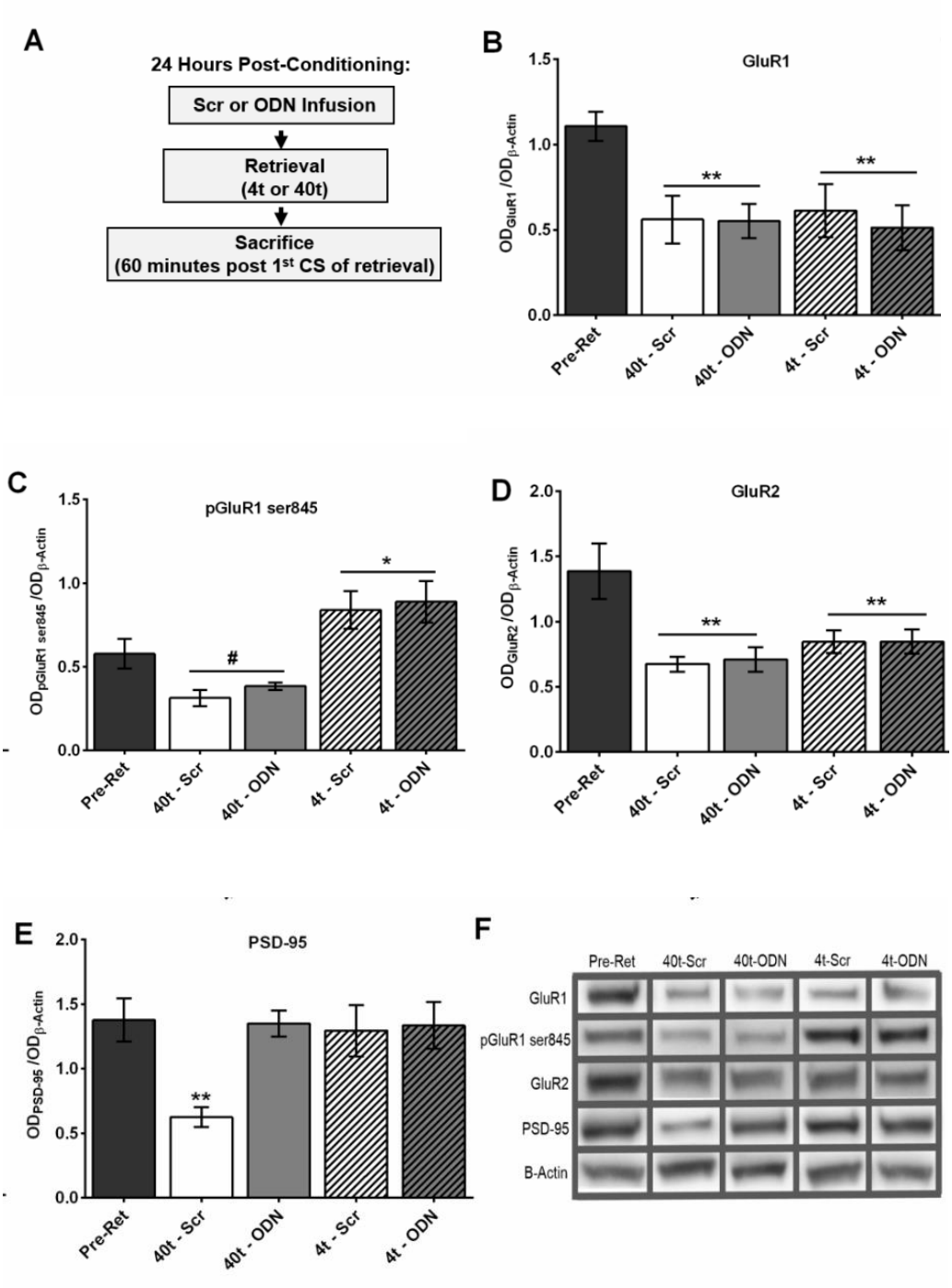


Figure 7. Inhibition of CaN translation selectively prevents the reduction in PSD-95 in the lateral amygdala. (A) Schematic of experimental procedure. Inhibition of CaN translation does not effect (B) reduction of GluR1 following extinction or retrieval, (C) the enhanced phosphorylation of serine 845 on GluR1 retrieval, or D) the reduction in GluR2 following extinction or retrieval. E) Inhibition of CaN translation prevents the extinction-specific loss of PSD-95. F) Representative protein bands from Western blots. All data represent mean (± SEM). #<.01 *p<.05, **p<.01 relative to the pre-retrieval group.

Discussion

The present series of experiments addressed whether functionally distinct pools of CaN contribute to depotentiation-like synaptic changes in the lateral amygdala during auditory fear extinction. The results support a critical and complex role for CaN in the amygdala during auditory fear extinction. Surprisingly, the critical window of CaN-mediated signaling in the amygdala was found to be relatively brief, where CaN protein levels increase and CaN activity levels rise, peak, and fall within the bounds of the 40 CS (60 minute) extinction session. Despite this brief time window, CaN is involved in both short- and long-term extinction-related changes in conditioned fear expression. Inhibition of CaN activity from all potential sources with FK506 prevented the typical reduction in the CR over the course of the extinction session and high-levels of CS-induced freezing continued to be observed at the long-term extinction retention test. However, selective interference with extinction-induced translation of CaN using a CaN-targeted ODN did not affect within-session extinction but impaired the long-term retention of fear extinction. In agreement with these distinct behavioral deficits, inhibition of all pools of CaN activity disrupted extinction-induced GluR2-containing AMPAR endocytosis and the reduction in PSD-95 expression, whereas inhibition of newly translated CaN prevented the reduction in PSD-95 while leaving AMPAR dynamics intact. Collectively, these results support a tightly regulated balance between pre-existing CaN, which may facilitate the induction of behavioral and molecular change, and newly translated CaN, which may contribute to the long-term stability of extinction.

The preliminary aim of this study was to characterize periods of extinction during which there would be sensitivity to disruption of CaN-mediated signaling in the amygdala. In line with previous findings, both CaN protein levels and activity increase during extinction training (Merlo et al., 2014; Lin et al 2003b,c). More unexpected was that CaN activity both peaked and subsequently declined within the extinction session. While a portion of pre-existing CaN protein is anchored in the PSD near calcium

channels, which allows rapid activation and inactivation in response to calcium influx, it is unclear how the synthesis, activation, and inactivation of new CaN protein are also accomplished within this same timeframe (Dodge & Scott, 2003). Though CaN activity was not measured at any time point beyond the conclusion of the 40 CS (60 minute) extinction session, it is unlikely that there is a subsequent wave of CaN activity, as post-extinction injection of FK506 failed to disrupt extinction. Alternatively, given evidence that CaN is locally translated in dendrites during fear extinction, it is possible that CaN is synthesized quickly and in close enough proximity to the cell membrane to respond to extinction-related signaling on the time scale observed here (Lin et al., 2003c).

The narrow time window of critical CaN activity precluded the possibility of varying drug injection time to dissociate the roles of pre-existing and newly translated CaN. Therefore, for the second aim of this study, two different pharmacological agents were employed to probe for unique contributions of these pools. Effects that were common to both pharmacological conditions were interpreted as dependent on newly translated CaN, where findings unique to the FK506 condition were considered to be dependent on pre-existing CaN. As predicted, newly translated CaN was found to be selectively involved in the long-term retention of extinction whereas pre-existing CaN was found to be critical for within-session changes in CR expression. Importantly, inhibition of either pool did not disrupt the expression or maintenance of the CR in the brief retrieval condition. Together, these observations support distinct roles of pre-existing and newly translated CaN in extinction-related behavioral changes.

In contrast to the present behavioral findings, it was recently reported that inhibition of CaN activity from all pools via systemic injections of FK506 produces deficits in within-session, but not the long-term retention of extinction (Almeida-Correa et al., 2015). Because the current study used amygdala-targeted micro-infusions of FK506, differences in the efficacy and spread of the CaN inhibitor due to the method of delivery may relate to the nature of deficits in extinction learning. Given that extinction requires distinct forms of plasticity in multiple brain structures, it is possible that systemic

delivery of FK506 alters plasticity throughout this network in a way that partially compensates for the disruption of CaN activity in the amygdala alone. In light of this, it is notable that all previous studies reporting a role for CaN in the long-term retention of fear extinction have used local micro-infusions (Merlo et al., 2014; de la Fuente et al., 2011). However, additional work to characterize activity-dependent differences between and interactions among brain structures in the presence of CaN inhibitors is required to reconcile these results.

Given the distinct pattern of behavioral effects following amygdala-targeted infusion of either FK506 or the CaN ODN observed in the second aim, the final set of experiments assessed the nature of CaN-dependent plasticity. Specifically, the third aim addressed whether CaN-mediated signaling during fear extinction was required for depotentiation-like molecular changes in the LA and whether substrate specificity could be detected among the pools of pre-existing and newly translated CaN. Because both homomeric GluR1/1 and heteromeric GluR1/2 AMPARs are upregulated 24 hours following auditory fear conditioning, changes in the GluR1 and GluR2 subunit were assessed (McCormack et al., 2006; Yeh et al, 2006; Kim et al., 2007).

Surprisingly, manipulation of CaN activity did not strongly affect synaptosomal expression or phosphorylation of GluR1. Under basal conditions, CaN has been shown to dephosphorylate serine 845 on GluR1, which limits the signal to recruit GluR1-containing AMPARs to the membrane (Sanderson et al., 2012). Thus, one possibility is that basal and activity-dependent regulation of serine 845 phosphorylation rely on different substrates. Another possibility would the induction of a compensatory mechanism in lieu of appropriate CaN signaling. Changes in PKA or protein phosphatase 1 activity are two potential candidates that could underlie either of these possibilities (Snyder et al., 2003). Regardless of the specific mechanism, there is a clear dissociation in the regulation of serine 845 phosphorylation during extinction and retrieval. Consistent with previous reports, phosphorylation of serine 845 is increased following retrieval, suggesting subsequent recruitment of AMPARs to the surface (Jarome et

al, 2012; Man et al., 2007). In contrast, there appears to be no change or a mild dephosphorylation of serine 845 following extinction. This suggests the absence of a strong signal for subsequent AMPAR recruitment, which is consistent with what is known about AMPAR dynamics during phenomena that reduce synaptic strength (Man et al., 2007; Beattie et al., 2000; Wang, 2008).

Differences in the regulation of AMPAR trafficking based on subunit composition may explain the apparent CaN-independence of GluR1 endocytosis (Lee, Simonetta, & Sheng, 2004; Wang, 2008). Given that manipulations of CaN activity disrupted GluR2 endocytosis (discussed further below), it may be that distinct regulatory signals control the trafficking of homomeric GluR1/1 receptors (Hanley, 2014; Soto et al., 2009). Not only has the decrease in GluR1 been found to be of lesser magnitude than GluR2 24 hours following fear extinction, but fear renewal following extinction has been reported to depend on an increase in AMPAR channel conductance induced by phosphorylation of serine 831 on GluR1 (Lee et al., 2013). Critically, phosphorylation at this site only regulates channel conductance in homomeric GluR1/1 AMPARs (Oh et al., 2005). However, this issue requires further investigation.

In contrast to GluR1, inhibition of CaN activity, but not CaN translation, prevented reductions in synaptosomal GluR2 expression during extinction. Notably, Kim and colleagues (2007) previously reported that blocking GluR2-containing AMPAR endocytosis disrupts within-session extinction, which is consistent with the pattern of behavioral and molecular data observed presently with FK506. Together with the lack of effect of the CaN-targeted ODN on within-session extinction and GluR2 trafficking, the results suggest that pre-existing CaN is the critical mediator of GluR2-containing AMPAR endocytosis during fear extinction.

Despite previous work demonstrating that GluR2 levels remain low even 24 hours after extinction, the present results, obtained immediately following the end of the extinction session, are not strictly indicative of a depotentiation-like phenomenon (Kim et al., 2007). Moreover, the CaN-mediated

reduction in GluR2 expression was not specific to extinction. Because expression of GluR2-containing AMPARs is linked with long-term memory stability, pre-existing CaN may be part of a more general destabilization process triggered under certain conditions of memory retrieval (i.e. those that induce extinction or reconsolidation) (Migues et al., 2010). Given that reconsolidation does not involve long-lasting reductions in synaptic strength, it is unlikely the pre-existing CaN provides an unambiguous signal for depotentiation.

In contrast to GluR2 trafficking, the present results reveal an extinction-specific, CaN-dependent reduction in PSD-95. Of great relevance here is that, during an initial learning event, PSD-95 is proposed to facilitate long-term increases in synaptic strength by creating "slots" at active synapses that will subsequently be filled AMPARs (Opazo et al., 2012). Once AMPARs are in place, PSD-95 functions as an anchor to maintain their synaptic localization (Chen et al., 2012; Yudowski et al., 2013). Two predictions derived from this are that 1) under memory retrieval conditions that destabilize the synapse but ultimately maintain synaptic strength, PSD-95 serves as a tag to appropriately guide the reinsertion of AMPARs into the membrane following endocytosis and 2) under memory retrieval conditions that destabilize the synapse and ultimately reduce synaptic strength, the removal of PSD-95 results in a loss of the signal for reinsertion. The extinction-specific reduction in PSD-95 observed here, together with previous data supporting long-term reductions in AMPAR expression following extinction, are consistent with these predictions. However, additional work is required to confirm this functional role of PSD-95 in retrieval and extinction.

Of particular interest is the finding that the reduction in PSD-95 expression is mediated by newly-translated CaN. Though not directly assessed here, it has been shown that the synthesis of CaN protein in the amygdala does not occur follow non-extinction inducing retrieval sessions (Merlo et al., 2014). This suggests that the coupling of CaN synthesis and PSD-95 degradation is an extinction-specific facet of signaling in the amygdala. Moreover, in contrast to AMPAR trafficking mediated by pre-existing

CaN, degradation of PSD-95 is more directly suggestive of a decrease in synaptic strength (Steiner et al., 2008; Opazo et al., 2012; Xu et al., 2008).

However, it must be noted that significant loss of PSD-95 in the lateral amygdala has previously been observed to occur only with strong extinction training (where the strength of extinction training is assessed by the level of spontaneous recovery over multiple testing days following the extinction session) (Mao et al., 2013). The low degree of CR recovery between the end of extinction training and the retention test observed in the current study provides some indication that our behavioral procedures induced a relatively strong form of extinction. That some degree of behavioral extinction may be retained in the absence of reduction in PSD-95 may partially explain why the inhibition of CaN translation resulted in qualitatively intermediate CR expression at the retention test. Nonetheless, translation of CaN in the amygdala has been observed following even relatively mild forms of extinction training (Merlo et al., 2014). Therefore, additional targets of newly translated CaN, and their specificity for extinction, should be addressed in future studies.

Collectively, the results provide specific links between changes in several proteins, including increased CaN activity, AMPAR endocytosis, and loss of PSD-95, that are consistent with depotentiation as an underlying mechanism of auditory fear extinction in the lateral amygdala (Lin et al., 2003a,b,c; Kim et al., 2007; Dalton et al., 2008). Although the contribution of at least two distinct pools of CaN is supported, signaling by pre-existing CaN may have a more general involvement in memory retrieval-induced plasticity, while translation of CaN may be an extinction-specific component of plasticity that is preferentially involved in reducing synaptic strength. Thus, identifying conditions that induce the translation of CaN are of particular interest. However, the development of these conditions are unlikely to be understood through the study of the lateral amygdala in isolation. Careful dissection of projections between the lateral amygdala and other regions implicated in extinction, including the infralimbic and prelimbic division of the mPFC, the auditory cortex and thalamus, and other subnuclei of the amygdala,

is required to properly situate the function of CaN-mediated depotentiation among the multitude of plastic changes induced by auditory fear extinction.

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