THE ROLE OF THE AMYGDALA, RETROSPLENIAL CORTEX, AND MEDIAL PREFRONTAL CORTEX IN TRACE FEAR EXTINCTION AND RECONSOLIDATION.

by

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ABSTRACT
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Under the Supervision of Professor Fred J. Helmstetter

A wealth of research has outlined the neural circuits responsible for the consolidation, reconsolidation, and extinction of standard "delay" fear conditioning, in which awareness is not required for learning. Far less is understood about the neural circuit supporting more complex, explicit associations. "Trace" fear conditioning is considered to be a rodent model of explicit fear because it relies on the cortex and hippocampus and requires explicit contingency awareness in humans for successful acquisition. In the current set of studies, we aimed to better characterize the neural circuit supporting the consolidation, reconsolidation, and extinction of trace fear in order to better understand how explicit associations are stored and updated in the brain. We found that trace fear extinction relies on NMDA receptors in the retrosplenial cortex and prelimibic medial prefrontal cortex, rather than the amygdala, which is required for extinguishing delay fear. NMDA receptors in the infralimbic medial prefrontal cortex, on the other hand, were involved in the extinction of both delay and trace associations. These results suggest that trace fear is

consolidated and stored in a distributed cortical manner, relying in part on the retrosplenial and prelimbic cortices. The amygdala, on the other hand, is responsible for the long-term storage of delay, but not trace associations. Consistent with this, our next study demonstrated that protein synthesis in the retrosplenial cortex was required for the acquisition or early consolidation of trace, but not delay fear. Finally, we demonstrated that a reconsolidation-dependent updating procedure can be used to shift a relatively complex trace fear association to the more basic neural circuit that supports delay fear. Together, these results provide a more complete understanding of the neural circuit supporting trace fear, demonstrating that this type of complex association relies on multiple cortical structures for extinction, including the retrosplenial and prelimbic cortices, instead of the amygdala. Our results also demonstrate that updating procedures can reorganize the neural circuit supporting a complex trace fear memory so that the association relies on a simpler set of structures.

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LIST OF ABBREVIATIONS

4T	4-trial retrieval group
ACSF	artificial cerebrospinal fluid
AMPAR	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor
ANI	anisomycin
APV	D-2-amino-5-phosphonopentanoic acid
BA	basal nucleus of the amygdala
BLA	basolateral nucleus of the amygdala
CaN	calcineurin
CeA	central nucleus of the amygdala
CeL	lateral sector of the central nucleus of the amygdala
CeM	medial sector of the central nucleus of the amygdala
CNQX	6-cyano-7-nitroquinoxaline-2,3-dione
CPP	3-(2-carboxypiperazin-4-yl)propyl-1-phosphonic acid
CR	conditional response
CS	conditional stimulus
DFC	delay fear conditioning
DH	dorsal hippocampus
DMSO	dimethyl sulfoxide
ERK	extracellular signal-related protein kinase
EXT	extinction
GABA	gamma-aminobutyric acid
IL	infralimbic cortex
ITI	intertrial interval
ITC	intercalated cells
LA	lateral amygdala
LTD	long-term depression
LTP	long-term potentiation
MAPK	mitogen-activated protein kinase
mPFC	medial prefrontal cortex
mTOR	mammalian target of rapamycin
NE	no extinction
NMDAR	n-methyl-D-aspartic acid receptor
PAG	periaqueductal gray matter
pERK	phosphorylated ERK
PKA	protein kinase A
PKMζ	protein kinase Mzeta
PL	prelimbic cortex
PP2B	protein phosphatase 2B
RSC	retrosplenial cortex
TFC	trace fear conditioning
TI	trace interval
UCS	unconditional stimulus
UPS	ubiquitin-proteasome system
VEH	vehicle
vmPFC	ventromedial prefrontal cortex

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Pavlovian fear conditioning is a simple form of learning in which a neutral conditional stimulus (CS), such as a white noise cue, is paired with an aversive unconditional stimulus (UCS), typically a footshock, so that the CS comes to predict the UCS. After being exposed to only a few pairings of the CS and UCS, an animal will show a conditional fear response (CR) to the once-neutral CS. CRs include increased blood pressure, heart rate, respiration, and defecation, as well as freezing behavior, an innate defensive response characteristic of most rodents (LeDoux, 2000). The strength of a rodent's memory for the training session can be easily assessed by placing the animal in a novel environment and quantifying the amount of freezing behavior the CS elicits. Fear conditioning is an ideal paradigm for understanding the cellular and molecular components of memory formation and storage and has been widely used to identify the neural circuits that support learning (Johansen, Cain, Ostroff, & LeDoux, 2011; LeDoux, 2000).

There are two major training procedures used in fear conditioning with discrete stimuli: delay and trace. The standard form of fear conditioning, called "delay" fear conditioning (DFC), has historically received the most attention and, accordingly, is extremely well-characterized. In DFC, the presentation of the CS and UCS are contiguous, generally coterminating so that there is a brief overlap between the two stimuli. The circuitry supporting DFC acquisition, consolidation, storage, and retrieval has been outlined through a rich history of research (for review, see LeDoux, 2000). As the neural circuit supporting delay fear has been clearly identified, DFC serves as a good model learning paradigm for identifying the specific molecular and cellular mechanisms supporting learning.

Trace fear conditioning (TFC) differs from DFC by the inclusion of a stimulus-free period of time, called a trace interval, between the CS and UCS. The temporal separation of the two stimuli drastically changes the brain circuitry and attentional mechanisms required for acquisition. Whereas DFC can be acquired without awareness and relies largely on subcortical structures (particularly the amygdala), TFC requires awareness of the CS-UCS contingency and relies on hippocampal and cortical participation for acquisition (Gilmartin & Helmstetter, 2010; Han et al., 2003; Knight, Nguyen, & Bandettini, 2006; Quinn, Oommen, Morrison, & Fanselow, 2002). Trace fear conditioning is often considered to be an animal model of human declarative memory because of its complex and relational qualities, awareness requirement, and hippocampal involvement (Han et al., 2003; Squire, 1992). This makes TFC a particularly good paradigm for understanding how more complex, explicit associations are formed and stored in the brain. Despite the clear value of TFC as a model of memory, the circuitry that supports TFC is incompletely understood. In particular, it is unclear how the basic fear circuit changes as a result of cortical involvement in acquisition. This dissertation aimed to answer key questions about how the fear circuit changes to support trace fear acquisition, retrieval, and extinction.

Delay Fear Conditioning Circuitry

The acquisition and storage of DFC relies on a well-characterized brain circuit. Information about the auditory CS is processed in the auditory thalamus and auditory cortex, both of which project to the amygdala (LeDoux, 2000; LeDoux, Farb, & Ruggiero, 1990). Information about the training context is processed in the hippocampus,

which is believed to compile the distinct elements of the training chamber (such as the lighting, shape, color, and texture of the training chamber) into a single configural representation of the context (Matus-Amat, Higgins, Barrientos, & Rudy, 2004; O'Reilly & Rudy, 2001; Rudy & O'Reilly, 1999). This representation is then projected to the amygdala. Information about the UCS also enters the amygdala, through parallel thalamic and cortical pathways (Maren, 2001b; Shi & Davis, 1999). The amygdala, which receives information about both the CS and UCS serves as a site of synaptic convergence that allows association of the stimuli. Accordingly, lesioning or temporarily inactivating the amygdala prevents normal DFC acquisition (Blanchard & Blanchard, 1972; Helmstetter, 1992; Helmstetter & Bellgowan, 1994; Helmstetter, Parsons, & Gafford, 2008). Further, DFC training produces altered gene expression in amygdala neurons (Levenson et al., 2004; Ressler, Paschall, Zhou, & Davis, 2002), the induction of long-term potentiation (LTP) at local synapses (Rogan & LeDoux, 1995), and the activation of intracellular signaling pathways involved in long-term synaptic modification (Parsons, Gafford, & Helmstetter, 2006). If amygdala neurons are prevented from synthesizing new mRNA or protein during the period immediately after training, when the newly formed memory is labile, no new memories are formed (Bailey, Kim, Sun, Thompson, & Helmstetter, 1999; Parsons, Gafford, & Helmstetter, 2006; Schafe & LeDoux, 2000). Similarly, inactivation of the amygdala disrupts the fear response itself, indicating that the amygdala is also necessary for fear expression (Anglada-Figueroa & Quirk, 2005; Bellgowan & Helmstetter, 1996; Duvarci, Popa, & Pare, 2011; Helmstetter, 1992; Pare & Duvarci, 2012). Thus, the amygdala is generally acknowledged to be a crucial site in the brain network required for DFC.

The amygdala is also a major site of storage for DFC memory. The amygdala appears to be permanently required for the expression of learned fear, as lesions one month after conditioning disrupt both CS and context freezing (Maren, Aharonov, & Fanselow, 1996). Further, post-training lesions of the amygdala appear to abolish the memory of DFC training so that no evidence of savings are observed (Maren, 2001a). In an elegantly designed experiment, Gale and colleagues (2004) demonstrated that the amygdala appears to store DFC associations across a rat's adult lifespan. In this study, animals were exposed to DFC training at a recent (24 hours) and remote (16 months) timepoint before receiving excitotoxic amygdala lesions. Lesioned animals showed disrupted fear to both the recent and remote CS and context, demonstrating that there is no temporal gradient of amygdala involvement for either tone or context fear. They then demonstrated that the amygdala-lesioned rats did not show hyperactivity in an open field test and were able to acquire a normal freezing response through overtraining, indicating that the animals are capable of showing freezing expression (Gale et al., 2004). Recent studies examining the memory maintenance molecule protein kinase Mzeta (PKMζ) have provided further support for the idea that the amygdala is the site of long-term DFC storage; inhibiting PKMζ in the amygdala 1d after DFC disrupts both CS and context memory (Kwapis, Jarome, Gilmartin, & Helmstetter, 2012; Kwapis, Jarome, Lonergan, & Helmstetter, 2009; Serrano et al., 2008). Together, these studies suggest that critical aspects of DFC memory may be permanently stored in the amygdala.

The amygdala is composed of several subnuclei that play distinct roles in the conditioning process. The lateral nucleus (LA) is the site of convergence between sensory CS and UCS information and is the major site of synaptic plasticity supporting the

association between the auditory CS and the somatosensory footshock UCS in DFC (for review, see Pape & Pare, 2010). Neurons in the dorsal LA show increased response to a tone CS following DFC (Maren, 2000; Quirk, Repa, & LeDoux, 1995; Repa et al., 2001; Rogan, Staubli, & LeDoux, 1997), consistent with the idea that associative plasticity in LA strengthens weak CS inputs that are paired with robust UCS inputs to the same area. Further, lesions restricted to the LA disrupt both the acquisition and expression of DFC, suggesting that the LA is necessary for the CS-UCS association (Amorapanth, LeDoux, & Nader, 2000; Blair, Schafe, Bauer, Rodrigues, & LeDoux, 2001; Goosens & Maren, 2001; LeDoux, Cicchetti, Xagoraris, & Romanski, 1990; Nader, Majidishad, Amorapanth, & LeDoux, 2001). More recent work has demonstrated that fear conditioning also occludes future LTP in amygdala slices (Schroeder & Shinnick-Gallagher, 2004, 2005; Tsvetkov, Carlezon, Benes, Kandel, & Bolshakov, 2002) and activates multiple signaling pathways in the LA (Johansen et al., 2011). These studies provide compelling evidence that the LA is a key locus of plasticity supporting the acquisition and storage of the DFC association.

Although the LA serves as the site of CS-UCS convergence, other parts of the neural circuit also contribute to DFC memory. The basal nucleus of the amygdala (BA) receives robust projections from the hippocampus and, accordingly, appears to primarily support context fear conditioning (Canteras & Swanson, 1992; LeDoux, 2000; McDonald, 1998). Temporary inactivation of the BA with lidocaine selectively disrupts expression of context, but not auditory, conditioning (Pitkanen, Pikkarainen, Nurminen, & Ylinen, 2000; Yaniv, Desmedt, Jaffard, & Richter-Levin, 2004). Similarly, selective excitotoxic BA lesions (given before DFC) disrupted the acquisition of context, but not

auditory fear (Onishi & Xavier, 2010), suggesting that the BA plays a context-specific role in DFC acquisition.

The BA also serves as an important relay between the LA, where the tone-shock association occurs, and the central nucleus of the amygdala (CeA), the main output structure of the amygdala (Amano, Duvarci, Popa, & Pare, 2011). The CeA can be functionally dissociated into a medial sector (CeM) and a lateral sector (CeL). The CeM serves as the amygdala's sole output to brainstem areas controlling the fear CR, including connections to the periaqueductal gray matter (PAG), which controls freezing behavior, and other brainstem nuclei that control the cardiovascular response to fear (Cassell, Gray, & Kiss, 1986; Gray & Magnuson, 1987; Rizvi, Ennis, Behbehani, & Shipley, 1991; Veening, Swanson, & Sawchenko, 1984). Despite its central role in auditory CS-UCS association, the LA has no direct connections to the CeM (Krettek & Price, 1978; Pitkanen, Savander, & LeDoux, 1997). Instead, it appears to connect to the CeM indirectly through the BA or CeL to produce the fear response (Amano et al., 2011; Anglada-Figueroa & Quirk, 2005). Thus, the BA is important for both its role in context fear conditioning and for its ability to propagate the associative CS-UCS signal from the LA to the output center of the CeA.

The CeA has historically been viewed as an almost passive "output center" of the amygdala due to its connections to hypothalamic and brainstem structures that mediate the conditioned fear response. More recent work, however, indicates that the CeA may play an active role in acquiring the DFC association. Wilensky and colleagues (2006) used localized injections of the $GABA_A$ agonist muscimol and the protein synthesis inhibitor anisomycin to demonstrate that the CeA is necessary for the acquisition and

consolidation of DFC, in addition to its known role in the expression of fear (Wilensky, Schafe, Kristensen, & LeDoux, 2006). This indicates that plasticity supporting DFC may be more distributed throughout the amygdala than once thought.

The circuitry supporting DFC is thus well-understood and largely amygdala-centric. Although plasticity in the upstream pathway (supporting the auditory CS in the medial geniculate nucleus of the thalamus and the contextual representation in the hippocampus) and downstream in fear output areas (such as the PAG) has been demonstrated to be crucial for DFC, the amygdala appears to be the main site of associative plasticity supporting the implicit DFC memory.

Trace fear conditioning circuitry

The neural circuit supporting TFC acquisition, consolidation, and storage is less clearly outlined than that of DFC. Most of the studies on TFC have focused on the hippocampus and prefrontal cortex, as the involvement of these structures is necessary for trace, but not delay, conditioning. Early studies on the hippocampus used trace eyeblink conditioning in the rabbit, in which the CS (usually a tone) is separated from the UCS (commonly an air puff or shock to the eyelid) by a brief trace interval (TI) of 300-500 ms (Kim, Clark, & Thompson, 1995; Moyer, Deyo, & Disterhoft, 1990; Solomon, Vander Schaaf, Thompson, & Weisz, 1986). These studies demonstrated that an intact hippocampus is necessary for the acquisition and consolidation of trace eyeblink conditioning in addition to the basic cerebellar circuitry necessary for delay eyeblink conditioning (Kim et al., 1995; Moyer et al., 1990). More recently, the hippocampus was demonstrated to be important for the acquisition and consolidation of TFC (McEchron,

Bouwmeester, Tseng, Weiss, & Disterhoft, 1998). Lesioning the inputs to the hippocampus (McAlonan, Wilkinson, Robbins, & Everitt, 1995; Tsaltas, Preston, & Gray, 1983) or the hippocampus itself (McEchron et al., 1998; McEchron, Tseng, & Disterhoft, 2000) before conditioning is sufficient to disrupt TFC, but not DFC, acquisition. Similarly, neurotoxic hippocampal lesions produced after TFC disrupt trace fear consolidation or expression (Quinn et al., 2002). Plasticity in the hippocampus appears to be important for acquiring TFC, as inhibiting hippocampal NMDA receptors before conditioning blocks TFC learning (Misane et al., 2005; Quinn, Loya, Ma, & Fanselow, 2005). Hippocampal neurons also show learning-related increases in firing during TFC (Gilmartin & McEchron, 2005a; McEchron, Tseng, & Disterhoft, 2003). These neurons may encode the timing of the expected UCS presentation following the trace interval; after TFC, a subset of hippocampal neurons showed increased firing to the CS onset that matched the timing of the trace interval used in training (McEchron et al., 2003). Together, these studies show that the hippocampus is an important component of the circuitry supporting TFC.

The hippocampus seems to support the temporal or explicit components of the trace fear association, rather than serving as the permanent site of storage (Bangasser, Waxler, Santollo, & Shors, 2006; Clark & Squire, 1998, 2004). Lesions of the hippocampus at a remote time point (200d post-training) have no effect on the TFC memory (Quinn, Ma, Tinsley, Koch, & Fanselow, 2008). One theory suggests the hippocampus helps to bridge the temporal gap between the CS and UCS either by using contextual information (Quinn et al., 2002) or by producing persistent neural firing

during the TI (Rodriguez & Levy, 2001) in order to indirectly represent the CS during the empty period separating the two stimuli (Bangasser et al., 2006).

The medial prefrontal cortex (mPFC) is another important component of the TFC acquisition circuitry. The mPFC is known to participate in a variety of complex tasks, including working memory, attention, inhibition of learned responses, and fear expression (Fuster, 2001; Miller, 2000; Santini, Muller, & Quirk, 2001; Sotres-Bayon & Quirk, 2010). More recently, the mPFC has been demonstrated to be necessary for some forms of memory acquisition, generally those that have a temporal or attentional component, like TFC (Carter, Hofstotter, Tsuchiya, & Koch, 2003; Fuster, 2001; Gilmartin & Helmstetter, 2010; Han et al., 2003).

The mPFC can be dissociated into two functionally distinct subregions: the prelimbic cortex (PL), located in the dorsal portion of the mPFC and the infralimbic cortex (IL), immediately ventral to the PL. Both regions have reciprocal connections to the amygdala, but they appear to play opposite roles in the expression of fear. The PL has been shown to be important for the expression of fear (Sotres-Bayon & Quirk, 2010) and for the acquisition of TFC (Gilmartin & Helmstetter, 2010; Gilmartin, Kwapis, & Helmstetter, 2012; Gilmartin & McEchron, 2005b; Runyan, Moore, & Dash, 2004) whereas the IL is implicated in the consolidation and expression of delay fear extinction (Burgos-Robles, Vidal-Gonzalez, Santini, & Quirk, 2007; Laurent & Westbrook, 2009; Sotres-Bayon, Diaz-Mataix, Bush, & LeDoux, 2009). Indeed, recording studies have demonstrated opposite patterns of neural activity in the PL and IL (Gilmartin & McEchron, 2005b), with PL neurons showing increased activity to the CS and UCS following TFC and IL neurons showing decreased activity to these stimuli. Further,

temporary inactivation of the two regions produces opposite behavioral patterns. Specifically, inactivation of the PL produces impairment of freezing expression while inactivation of the IL produces persistent freezing in response to a CS that no longer predicts a shock (Sierra-Mercado, Padilla-Coreano, & Quirk, 2011). The IL and PL appear to project to distinct subregions of the amygdala in order to produce different behavioral responses to the CS; the IL projects to a collection of inhibitory interneurons called the intercalated cell masses (ITC), and to the CeL to inhibit fear output through the CeM whereas the PL connects directly to the BA to drive fear output (Gabbott, Warner, Jays, Salway, & Busby, 2005; Sotres-Bayon & Quirk, 2010; Vertes, 2004). The PL, which plays a role in fear expression and in the acquisition of complex fear associations, is therefore the target of studies investigating the role of the mPFC in TFC acquisition and consolidation.

Numerous studies have demonstrated that the PL is a crucial component of the TFC circuitry (Baeg et al., 2001; Gilmartin & Helmstetter, 2010; Gilmartin et al., 2012; Gilmartin & McEchron, 2005b; Runyan & Dash, 2004; Runyan et al., 2004). Temporarily inactivating the mPFC before conditioning prevents the acquisition TFC (Gilmartin & Helmstetter, 2010; Gilmartin et al., 2012). Further, lesions of the mPFC after TFC consolidation disrupt CS freezing at 2, 30, or 200d post-acquisition (Quinn et al., 2008; Runyan et al., 2004). Recently, our lab has demonstrated that the PL is necessary for TFC, but not DFC. Temporarily inactivating the PL or inhibiting NMDA receptors immediately before training disrupts the formation of trace and context associations without affecting delay fear (Gilmartin & Helmstetter, 2010). Further, bilateral inactivation of the mPFC is required to disrupt TFC; unilateral inactivation is

insufficient (Gilmartin et al., 2012). Together, these studies implicate the PL as a key structure in trace, but not delay fear conditioning.

The exact role of the PL in TFC is currently unclear. One hypothesis is that the PL serves to connect the temporally separated CS and UCS by sustaining neural activity during the trace interval. According to this hypothesis, the PL maintains a working CS representation during the empty period of time between the CS offset and the UCS onset so that the two stimuli can undergo associative plasticity in the amygdala. Support for this hypothesis comes from recording studies, which have demonstrated sustained neural activity in the PL during the trace interval period (Baeg et al., 2001; Gilmartin & McEchron, 2005b), and from a recent optogenetic study that demonstrated that selectively silencing PL neurons during the trace interval period prevents TFC memory formation (Gilmartin, Miyawaki, Helmstetter, & Diba, 2013). Thus, persistently firing neurons in the PL may sustain the CS signal to allow for association with the UCS.

The mPFC may also serve as the permanent site of storage for the TFC association (Runyan et al., 2004). Although the systems consolidation hypothesis posits that the mPFC and other cortical areas are not involved in hippocampus-dependent memory storage until remote timepoints, when the hippocampus "transfers" the memory to longer-term cortical storage areas (Teyler & DiScenna, 1986), there is evidence that TFC associations are stored in the mPFC immediately after acquisition. Runyan and Dash (2004) demonstrated support for this idea by measuring extracellular signal-related kinase (ERK, also known as MAPK) phosphorylation in the mPFC and hippocampus following TFC acquisition. ERK, a key component of the signaling cascade promoting neural plasticity, is upregulated following learning in a number of structures that support

memory storage, including the insular cortex (Berman, Hazvi, Rosenblum, Seger, & Dudai, 1998), the hippocampus (Blum, Moore, Adams, & Dash, 1999), the entorhinal cortex (Hebert & Dash, 2002) and the amygdala (Schafe et al., 2000). Runyan and colleagues (2004) showed that ERK phosphorylation was increased in the mPFC immediately following TFC. When this increase was blocked pharmacologically with U0126, the animals showed memory impairments for TFC at both 48 and 72h post-acquisition. This indicates that the mPFC serves as a storage site for TFC associations shortly, if not immediately, after TFC acquisition. Similarly, inhibiting dopamine D1 receptors in the mPFC produces similar disruptions in TFC memory without disrupting TFC acquisition (Runyan & Dash, 2004). Interestingly, upregulation of phosphorylated ERK was observed in the hippocampus on a slower timecourse than in the mPFC. For TFC, therefore, the mPFC appears to participate in storing the TFC association *before* the hippocampus is involved, contrary to the timecourse for most hippocampal-dependent associations, which do not require cortical areas until remote timepoints. TFC associations, therefore appear to produce plastic changes in the mPFC as a direct result of learning. Subsequent research has shown that temporary inactivation of mPFC neurons at both recent (2d) and remote (30d) timepoints following TFC disrupts recall of TFC associations (Blum, Hebert, & Dash, 2006), further supporting the hypothesis that the mPFC stores TFC associations from the time of training.

Not all research agrees with the idea that the mPFC serves as the storage site of trace associations immediately after conditioning, however. Quinn and colleagues (2008) found that mPFC lesions (including both the PL and IL) affected remote, but not recent trace fear memory, whereas hippocampal lesions disrupted recent, but not remote trace

associations. These results suggest that TFC undergoes the same type of systems consolidation as other hippocampus-dependent associations; the hippocampus is required to store the association for a temporary period of time before the association is transferred into cortical areas for permanent storage. The mPFC therefore appears to be important for storing TFC associations, but whether the mPFC is required for immediate storage in addition to remote storage is unclear.

It is apparent that normal learning in TFC requires a more distributed circuit than for DFC, which can be formed without cortical or hippocampal participation. It has been widely assumed that trace fear requires the same basic amygdala circuitry that supports the basic delay fear association (Selden, Everitt, Jarrard, & Robbins, 1991), yet until recently, this idea remained largely untested. While it is likely that the hippocampus and mPFC play trace-specific roles that supplement the basic association that depends on the amygdala, another possibility is that the involvement of these forebrain structures fundamentally changes the role of the amygdala in the formation of a new memory. Specifically, the amygdala may no longer be required to acquire, consolidate, or store the fear association in TFC; the cortex may take over some of these roles. Recently, our lab was the first to clearly demonstrate that the amygdala is required for the consolidation of both trace and delay fear (Kwapis, Jarome, Schiff, & Helmstetter, 2011). In this study, animals were first trained with four-trial delay fear conditioning (DFC), four-trial trace fear conditioning (TFC 4), or six-trial trace fear conditioning (TFC 6). TFC 4 training was designed to match the number of trials and total session length used in the DFC group whereas TFC 6 training aimed to produce stronger trace conditioning with approximately the same memory strength as the DFC group. Immediately after training,

the animals received intra-amygdala infusions of the protein synthesis inhibitor anisomycin or vehicle and were tested the following day (Fig. 1a). All three groups showed significant freezing impairments during both the CS and ITI periods of the CS test (Fig. 1b and 1c) and to the training context (Fig. 1d). Since this initial study, additional studies by our lab (Gilmartin et al., 2012) and others (Guimarais, Gregorio, Cruz, Guyon, & Moita, 2011) have demonstrated that temporary inactivation of the amygdala before conditioning is sufficient to disrupt TFC acquisition (but see Raybuck & Lattal, 2011) . Together, these studies strongly suggest that the amygdala is a crucial component of the circuitry supporting TFC acquisition and consolidation.

Despite these recent advances in understanding the circuitry of TFC, a number of significant questions remain. No research to date has examined whether the amygdala is required for the retrieval or extinction of TFC. Additionally, it is still unclear whether the amygdala's role in TFC is identical to its role in

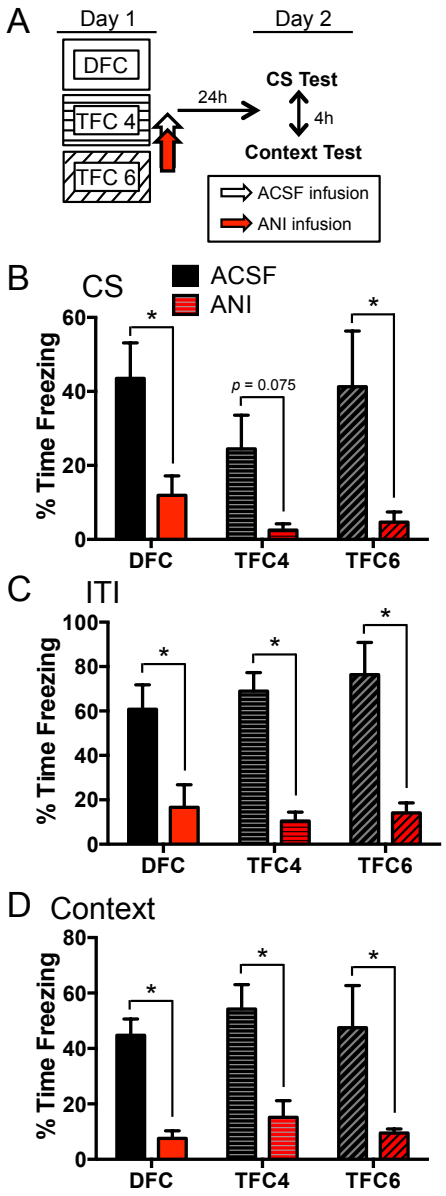

Figure 1. Both delay and trace fear conditioning are disrupted by intra-amygdala infusion of anisomycin. (A) The experimental timeline. (B) Mean percent time freezing during the first three CS presentations of the auditory CS test for animals given DFC (ACSF *n* = 8; ANI *n* = 7), TFC4 (ACSF *n* = 7; ANI *n* = 6), or TFC6 (ACSF *n* = 6; ANI *n* = 6) (C) Mean percent time freezing during the first three ITI periods of the CS test. (D) Mean percent time freezing during the context test. * Indicates that $p < 0.05$.

DFC; it is possible that the involvement of cortical structures and the requirement for awareness in TFC changes how the amygdala participates in the memory encoding. Further, it is unclear whether TFC associations are stored in the amygdala, like DFC associations, or whether they are stored in the mPFC as suggested by some research (Quinn et al., 2008; Runyan et al., 2004). This project aims to answer these basic questions about the circuitry underlying trace fear conditioning.

Extinction

Fear memory can be extinguished by repeatedly presenting the CS without the UCS. As the CS loses its predictive value, an animal's CR to the stimulus will gradually diminish. Fear extinction has received increasing attention in recent literature due to its clinical relevance as a translational model for understanding how exposure-based therapies work to treat a number of different anxiety disorders (Barad, Gean, & Lutz, 2006; Davis, 2002; Herry et al., 2010). Accordingly, the mechanisms supporting DFC extinction have been largely identified. Interestingly, to date, no research has systematically investigated whether TFC extinction occurs through the same mechanisms and circuitry as DFC extinction.

It is generally accepted that DFC extinction is new learning of a CS-no UCS relationship rather than degradation of the original CS-UCS memory trace. The new association acquired during extinction serves to inhibit the original CS-UCS association, which remains largely intact, but dormant. Evidence for this idea comes largely from three behavioral phenomena observed following extinction that suggest that the original memory persists: spontaneous recovery, reinstatement, and renewal (see Myers & Davis,

2007). Spontaneous recovery is observed when an animal begins to produce CRs in response to the extinguished CS following a period of rest. This recovery of the once-extinguished fear response suggests at least some of the original memory persists and is able to compete with the more recently acquired extinction association. Recovery of the fear response is also observed following reinstatement, in which the animal is given a single, unsignaled exposure to the UCS following extinction training. Reinstatement appears to be context-dependent, as an unsignaled UCS must be presented in the testing chamber in order for recovery of the CR to occur (Bouton & Bolles, 1979; Bouton & King, 1983). Finally, CR recovery is also observed in renewal, in which an extinguished animal is placed in a new chamber (any context other than the context in which extinction occurred) and the CS is presented. The pattern of conditional responding observed following renewal suggests that extinction is a context-specific association. Specifically, it appears that the context serves as an occasion-setter so that the animal learns that the CS is no longer fearful only in the specific chamber in which extinction occurred (Bouton, 1993). Together, these findings demonstrate that the original CS-UCS memory remains intact following extinction, suggesting extinction must be new learning that inhibits the original association.

Although extinction clearly involves new learning that the CS is no longer fearful, it may also involve partial degradation of the initial association. One important observation is that conditional responding only recovers partially following spontaneous recovery, renewal, or reinstatement (Delamater, 2004). Very rarely does an animal show recovered responding to the CS that is as robust as its response before extinction training. This suggests that the original memory persists in a weakened state following extinction.

Lin and colleagues (Lin, Lee, & Gean, 2003; Lin, Yeh, Leu, et al., 2003; Lin, Yeh, Lu, & Gean, 2003) have provided compelling evidence that the original memory is weakened following extinction. Specifically, they observed that the protein phosphatase calcineurin (CaN, also known as PP2B) is upregulated in the amygdala following extinction training. CaN appears to play opposing roles in the formation of fear and extinction memory; inhibiting CaN disrupts extinction memory but enhances fear memory formation whereas enhancing CaN expression facilitates extinction memory and inhibits fear memory formation (Baumgartel et al., 2008). Thus, some of the molecular changes that support extinction in the amygdala appear to oppose changes required for fear memory formation in that same structure. Additionally, the synaptic changes that occur during extinction resemble changes that occur during depotentiation (Lin, Lee, et al., 2003; Lin, Yeh, Lu, et al., 2003). If the synapses that were strengthened during the initial learning are depotentiated as a result of extinction, one would expect the memory engram to be severely degraded, if not erased, as a result of extinction (Barad, 2006). In reality, it seems most likely that a combination of degradation and new learning underlies the extinction memory; the initial memory trace may undergo some weakening through phosphatase activation and depotentiation of synapses supporting the original memory at the same time as a new inhibitory association is formed to inhibit expression of the initial CS-UCS association. Together, these mechanisms would produce reduced freezing to the CS following extinction that would also experience the phenomena of spontaneous recovery, renewal, and reinstatement.

A number of brain structures have been implicated in the general inhibition or extinction of fear, including the sensory cortex, the periaqueductal gray matter, the

inferior colliculus, the bed nucleus of the stria terminalis, and the striatum (Falls & Davis, 1993; McNally, 2005; Myers & Davis, 2007; Quirk, Armony, & LeDoux, 1997; Teich et al., 1989; Waddell, Morris, & Bouton, 2006; Yadin & Thomas, 1981, 1996). Relatively little information is available about how these structures contribute to extinction learning, however. A large majority of the research on fear extinction has been conducted on three principle structures: the hippocampus, the IL, and the amygdala. Accordingly, the current project will largely focus on these three structures.

Extinction Circuitry: The hippocampus

The hippocampus' role in extinction appears to be largely "context-centric." It appears that the hippocampus is responsible for driving the context-specificity of extinction. Pre-training lesions of the hippocampus or the fibria/fornix severely disrupt reinstatement, so that animals fail to show recovery of CS fear after UCS re-exposure in the testing context (Frohardt, Guarraci, & Bouton, 2000; Wilson, Brooks, & Bouton, 1995). The context-UCS association seems to play a key role in promoting the return of CS fear in reinstatement, so the hippocampus is likely crucial for formation of the contextual representation in this phenomenon. Similarly, renewal, in which the context serves as an occasion-setter to signal whether the CS will be followed by the UCS, is also disrupted by permanent hippocampal lesions, although these results are less consistent (Frohardt et al., 2000; Ji & Maren, 2005; Wilson et al., 1995). Hippocampal inactivation produces a curious pattern of behavioral results; inactivating the hippocampus before testing in a novel environment (AAB or ABC) prevents renewal. Inactivating the hippocampus has no effect on renewal, however, if the animal is tested in the training

context (ABA). Further, if the hippocampus is inactivated specifically during the extinction training session, the animal will show high levels of fear in any context, including the extinction context (Corcoran, Desmond, Frey, & Maren, 2005). Turning off the hippocampus during extinction may impair the animal's ability to recognize the extinction context as safe, causing renewal to occur. Generally, lesioning or inactivating the hippocampus before extinction training does not impair an animal's ability to acquire within-session extinction, (Frohardt et al., 2000; Ji & Maren, 2005; Wilson et al., 1995; but see Corcoran et al., 2005), however, which suggests that the hippocampus may not be necessary for learning extinction, per say, but instead controls the contextual gating of extinction.

Extinction Circuitry: The infralimbic cortex

The IL region of the mPFC has emerged as a central player in the circuitry supporting the consolidation of delay fear extinction memory. Early work by Morgan and colleagues (1993) demonstrated that lesions of the ventral portion of the mPFC, including the infralimbic area, had no effect on the acquisition of DFC but prevented the retention of extinction across days. Quirk and colleagues (2000) confirmed that the IL mPFC was crucial for this effect; using lesions restricted to the IL, but not the PL, these researchers demonstrated that within-session extinction occurred normally without the IL, but animals showed impaired extinction retention between sessions. Not all vmPFC lesion studies are consistent with this effect, however (see Quirk & Mueller, 2008 for a comprehensive listing of studies). Work by Davis and colleagues failed to show extinction deficits with pre-conditioning vmPFC lesions using a fear startle paradigm

(Falls & Davis, 1993; Gewirtz, Falls, & Davis, 1997). Garcia and colleagues (2006) also failed to find an effect of vmPFC lesions on DFC extinction when lesions were made either seven days before or immediately after conditioning. Even when lesions of the vmPFC are effective in disrupting between-session extinction, rats are eventually able to recall extinction memory with further training (Lebron, Milad, & Quirk, 2004).

It is possible that differences in the procedures used (i.e. fear potentiated startle vs. fear conditioning) or differences in the extent of the lesions can account for the discrepant results observed in the permanent lesion studies. Another alternative, however, is that the lesions themselves contributed to the inconsistent results; compensatory mechanisms provided by other brain structures may be activated by lesions under some circumstances, making it difficult to identify the functions normally supported by the lesioned structure (Anglada-Figueroa & Quirk, 2005). Recent studies using more specific or time-limited manipulations have convincingly demonstrated that the IL is a key player in extinction consolidation. Recording studies, for example, have shown that IL neurons fire during the retrieval phase of extinction, but not during the acquisition or extinction phases of fear conditioning (Milad & Quirk, 2002). The firing of these neurons inversely correlated with the amount of freezing observed – an animal that froze at a lower rate tended to have higher firing magnitude of these IL neurons. cFos, a marker of neuronal activity, has been shown to be upregulated in the IL in rats with good extinction learning (and low freezing during test) whereas cFos levels are lower in rats with poor extinction learning (and high freezing), consistent with the idea that IL activity correlates with extinction learning (Hefner et al., 2008; Knapska & Maren, 2009). Importantly, if DFC-trained animals are given IL stimulation during CS presentations (to mimic the IL firing

patterns observed following extinction), fear is decreased and extinction is enhanced (Milad & Quirk, 2002). Potentiation of hippocampal and thalamic inputs to the vmPFC has also been observed following extinction (Herry & Garcia, 2003), and stimulating these inputs to vmPFC can enhance or impair extinction memory, depending on whether LTP- or LTD-inducing stimuli are used. A recent study by the Quirk lab used small, spatially restricted infusions of the $GABA_A$ agonist muscimol to temporarily inactivate either the PL or the IL during extinction training (Sierra-Mercado et al., 2011). Their results demonstrate that the IL, but not the PL is necessary for extinction memory the following day. Specifically, they showed that inactivation of the PL disrupted freezing behavior during the extinction session without affecting extinction memory formation whereas IL inactivation had no effect on freezing behavior during the extinction session but severely impaired extinction memory the following day. Additionally, infusion of an NMDA receptor antagonist (Burgos-Robles et al., 2007; Sotres-Bayon, Bush, & LeDoux, 2007) or an ERK inhibitor (Hugues, Deschaux, & Garcia, 2004) into the vmPFC immediately after the extinction session is sufficient to block the consolidation of extinction memory. Together, these studies demonstrate that plastic changes in the IL mPFC are necessary for the consolidation and retrieval of extinction memory.

It is generally recognized that the primary role of the IL in DFC extinction is to inhibit those circuits that were originally potentiated to produce learning of the CS-UCS association. As previously discussed, the IL has robust connections to the ITC layer of the amygdala and the CeL (Pare, Quirk, & Ledoux, 2004), both of which send inhibitory projections to the CeM, the main output of the amygdala. From this perspective, IL plasticity during extinction strengthens projections to inhibitory regions of the amygdala

to block the fear output normally triggered by the CS response. Indeed, activation of the IL produces inhibition of the CeM (Quirk, Likhtik, Pelletier, & Pare, 2003). Further, specific deletion of ITC neurons in the amygdala after extinction training produces spontaneous recovery of the fear response and impairs the expression of the extinction memory (Likhtik, Popa, Apergis-Schoute, Fidacaro, & Pare, 2008), suggesting that ITC neurons are an integral part of circuit supporting the expression of extinction. The mPFC also projects directly to the targets of the CeM, including the hypothalamus and brain stem (Fisk & Wyss, 2000; Floyd, Price, Ferry, Keay, & Bandler, 2000) and could directly impose on the fear output structures, bypassing the amygdala entirely (Pare et al., 2004). It is currently unclear whether extinction produces plasticity in IL-ITC synapses in the amygdala, in the direct connections between IL and hypothalamic/brain stem areas, or whether the plasticity observed in the IL during extinction simply drives more IL input to inhibitory areas of the amygdala (ITCs and CeL) to dampen the fear output.

Extinction Circuitry: The amygdala

The amygdala also plays a crucial role in the extinction of DFC. In fact, the amygdala appears to be the central site in determining whether the extinction memory is expressed; mPFC neurons may integrate information from a number of sources, including the hippocampus, the brainstem, the thalamus, and the prefrontal cortex, in order to determine whether to drive excitation or inhibition of the amygdala based on the history of the stimulus, temporal and contextual cues, the internal state of the organism, and other cognitive information (Sotres-Bayon & Quirk, 2010).

Because lesions of the amygdala block freezing behavior, the amygdala's role in the acquisition and consolidation of extinction has been investigated largely with pharmacological methods. In one landmark study, Falls and colleagues (1992) demonstrated that NMDA receptors in the amygdala are critical for DFC extinction. Inhibiting NMDA receptors with APV before extinction disrupted extinction memory retention when tested the following day. As NMDA receptors are upstream of a number of the signaling cascades implicated in learning and memory, this finding strongly suggests that plasticity in the amygdala is crucial for DFC extinction memory. Studies have since built on this finding, showing that blocking the ERK pathway in the amygdala similarly impairs extinction of delay fear (Lin, Yeh, Lu, et al., 2003; Lu, Walker, & Davis, 2001) whereas infusions of D-cycloserine, an NMDAR partial agonist, enhances extinction (Mao, Lin, & Gean, 2008). All of these studies relied on the fear-potentiated startle paradigm, however, which does not allow the experimenter to distinguish between the acquisition and consolidation phases of extinction. More contemporary research has repeated these experiments in a traditional DFC extinction paradigm to demonstrate that blocking NMDA receptors (Sotres-Bayon et al., 2007), metabotropic glutamate receptors (Kim et al., 2007) or ERK activation (Herry, Trifilieff, Micheau, Luthi, & Mons, 2006) is sufficient to impair the acquisition of extinction.

The consolidation of extinction memory also requires the amygdala. Inhibiting various plasticity signaling molecules required for memory consolidation, including ERK, PI-3 kinase, cFos, and Zif268, disrupts extinction memory (Herry & Mons, 2004; Herry et al., 2006; Lin, Yeh, Lu, et al., 2003; Lu et al., 2001). On the other hand, enhancing BLA activity immediately after extinction with the $GABA_A$ antagonist

bicuculline strengthens extinction memory (Berlau & McGaugh, 2006). Lin and colleagues have also demonstrated that protein synthesis in the amygdala is required for extinction (Lin, Yeh, Lu, et al., 2003). This finding suggests that the general mechanisms that support amygdala-dependent DFC consolidation are also required for the consolidation of extinction memory in the amygdala.

Although it is clear that amygdala plasticity is necessary for DFC extinction, it is not currently known which synapses undergo plasticity to support extinction. One possibility is that IL-ITC synapses are potentiated. Consistent with this hypothesis, Royer and Paré (2002) demonstrated that stimulating afferent BLA neurons with either high- or low-frequency stimulation causes LTP or LTD, respectively, in ITC neurons in an NMDA receptor-dependent fashion. From this perspective, the plasticity observed in the amygdala during extinction results from strengthened connections onto ITC neurons, which would then inhibit amygdalar output through the CeA. Evidence that directly demonstrates that LTP or LTD occurs in ITC cells as a result of extinction is currently lacking, however. ITC-related plasticity could either occur in the IL, so that additional inputs project to the ITC of the amygdala to inhibit output, or in the ITC cell layer of the amygdala, so that ITC neurons are more likely to fire based on IL input. The latter case would presumably require NMDA receptor-dependent potentiation in the amygdala itself. Another possibility is that the neurons supporting the original DFC memory require plastic changes to incorporate the new information about the CS no longer predicting the UCS. It is possible that changes within the amygdala, which stores the original DFC association, are required to incorporate the new information acquired during the extinction session. Here, plasticity in the amygdala would be required to support the new

CS-no UCS association learned during extinction. Realistically, it is likely that multiple changes support the new DFC extinction association. IL inputs to the ITC may undergo plastic changes that support increased inhibition in the output circuit of the amygdala while plasticity within the BLA occurs to modify the existing memory trace in order to incorporate new information learned during extinction.

Extinction of TFC

No published studies to date have investigated how the basic fear extinction circuitry changes with the extinction of *trace* fear conditioning. It is possible that TFC associations are stored in different brain areas than DFC associations, which may change the structures that participate in TFC extinction. The mPFC, for example, has been implicated as a storage site for TFC memory (Runyan et al., 2004). If the TFC association is stored in the cortex immediately after conditioning, one might expect cortical involvement in the extinction process, both to weaken the original association and to incorporate new information about the CS-UCS relationship. From this perspective, the amygdala would still play a crucial role in the gating of the fear response, but plasticity in the amygdala would not necessarily be required for extinction. We recently tested whether the amygdala is required for TFC extinction by infusing either an AMPA or NMDA receptor antagonist directly into the BLA immediately before extinction training. Here, animals were trained with DFC or TFC 6 conditioning to produce approximately strength-matched fear learning between the two training types. The following day, animals were given intra-amygdala infusions of the AMPAR inhibitor CNQX, the NMDAR inhibitor APV, or the drugs' respective vehicles (DMSO or ACSF), about five

minutes before extinction training (Fig. 2a). Animals were then tested the following day to assess extinction memory recall. As shown in Figure 2, CNQX disrupted the expression of extinction (Fig. 2b) for both DFC- and TFC-trained animals. When tested the following day, however, only DFC animals given CNQX showed impaired extinction retention; TFC animals given intra-amygdala CNQX show good extinction retention despite AMPAR antagonism the previous day (Fig. 2c). This suggests that AMPARs in the amygdala are not necessary for trace fear extinction.

Similarly, we observed that NMDARs in the amygdala are necessary for DFC, but not TFC, extinction (Fig. 2d-e). During the extinction training session, we observed some decrease in freezing expression in the APV-infused groups (Fig. 2d), although this reduced freezing was minor compared to the near-complete reduction in freezing observed with CNQX. When tested the following day, the behavioral pattern for APV was identical to that of CNQX; we observed impaired extinction in the DFC animals only (Fig. 2e). TFC animals learned extinction despite intra-amygdala NMDAR antagonism during the extinction session. Together, these results indicate that glutamatergic signaling in the amygdala is required for DFC extinction but not for TFC extinction. The amygdala, therefore, may not be a crucial component in the TFC extinction circuit.

This study demonstrated that animals trained with TFC learned extinction normally despite AMPAR or NMDAR blockwwade in the amygdala. This suggests that other structures mediate the animals' ability to learn trace extinction. If the amygdala is not required for this new learning, which structures are? To answer this question, we performed a western blot study to measure ERK phosphorylation (pERK) in a number of candidate structures following the extinction of DFC or TFC. We chose pERK as our

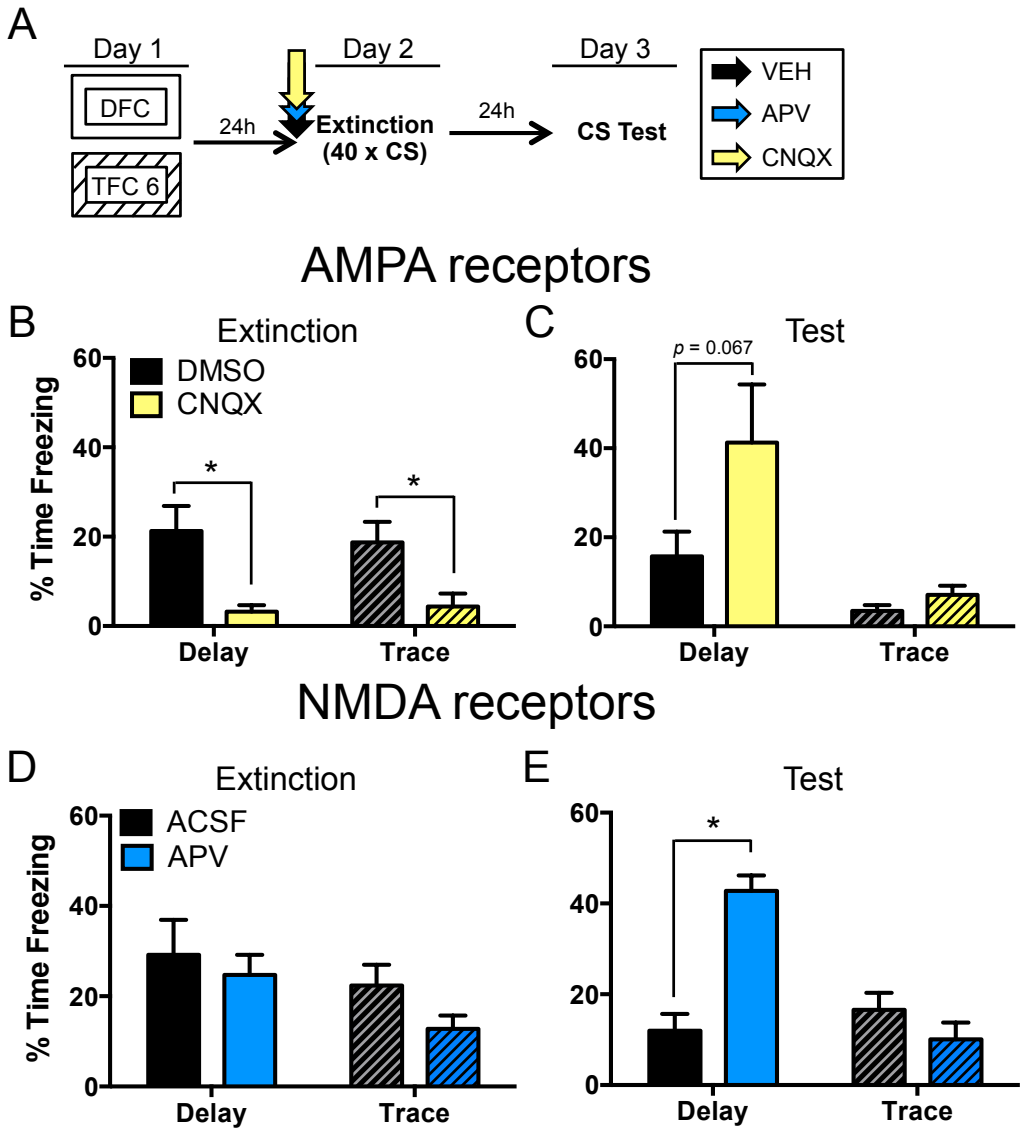

Figure 2. AMPAR and NMDAR antagonism in the amygdala disrupts the retention of DFC, but not TFC, extinction. (A) The experimental timeline. Animals were given an intra-amygdala infusion of an AMPAR antagonist (CNQX; B-E) or an NMDAR antagonist (APV; F-I) before extinction training. (B) Mean percent time freezing during the first 12 CS presentations of the extinction training session for CNQX-infused animals. Rats were given intra-amygdala infusions of either DMSO (DFC *n*= 6; TFC *n* = 6) or CNQX (DFC *n* = 6; TFC *n* = 6) before the extinction session. (C) Mean percent time freezing during all 8 CS presentations of the testing session. (D) Mean percent time freezing during the first 12 CS presentations of the extinction training session for APV-infused animals. Rats were given intra-amygdala infusions of either ACSF (DFC *n* = 7; TFC *n* = 8) or APV (DFC *n* = 7; TFC *n* = 7). (E) Average freezing during the CS presentations of the test session. * indicates that $p < 0.05$.

marker of extinction-related plasticity based on a wealth of studies demonstrating involvement of pERK in a number of learning paradigms, including auditory fear conditioning (Berman et al., 1998; Blum et al., 1999; Duvarci, Nader, & LeDoux, 2005; Kelly, Laroche, & Davis, 2003; Schafe et al., 2000; Zhang, Okutani, Inoue, & Kaba,

2003). Importantly, ERK phosphorylation has been shown to be upregulated in both the amygdala and the mPFC following DFC extinction training (Herry et al., 2006; Hugues et al., 2004; Kim, Hamlin, & Richardson, 2009; Parsons, Gafford, & Helmstetter, 2010). Increased rates of ERK phosphorylation therefore indicate that a structure is undergoing plastic changes characteristic of extinction learning.

In this study, we trained animals with DFC or TFC 6, followed by one of three protocols the following day (Fig 3a). Animals were not extinguished (NE), given a 4-trial retrieval session (4T), or given the full 40-trial extinction session (EXT). Thirty minutes after the end of each session, half the animals were killed for western blots and half of the animals were given an extinction retention test on day 3 (Figures 3a and 4a). We first demonstrated that our protocols (NE, 4T and EXT) were effective in producing the appropriate behavior (Fig. 3). The animals given the 40-trial extinction session showed significantly reduced freezing at test relative to the NE and 4T groups for both the delay- and trace-conditioned animals (Fig. 3b-c). This indicates that the 40T animals successfully acquired extinction. Further, we observed that the 4T group was not significantly different than the NE group during the test session for either delay or trace animals (Fig. 3b-c), indicating that the 4-trial retrieval procedure did not produce extinction. Importantly, the animals showed no difference in freezing during the first 4 CS presentations of the extinction session, indicating that they showed similar fear at the beginning of the day 2 session. Together, these results confirm that our NE, 4T, and 40T protocols produced the desired behavior, with only the 40T protocol producing reduced freezing characteristic of extinction.

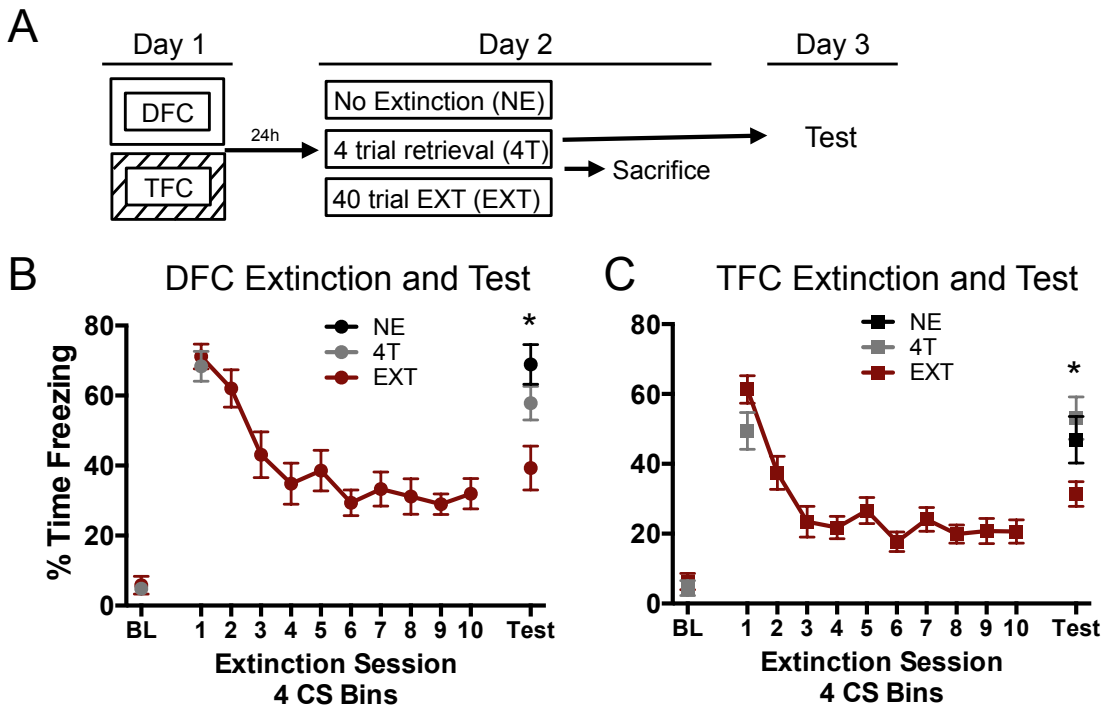

Figure 3. The 40-trial EXT protocol produces extinction whereas the 4T protocol does not. (A) Experimental timeline. Animals were trained with DFC or TFC on day 1 followed by either no extinction (NE; DFC *n* = 23; TFC *n* = 23), 4-trial retrieval (4T; DFC *n* = 22; TFC *n* = 24), or 40-trial extinction (EXT; DFC *n* = 23; TFC *n* = 22) on day 2. Half of the animals were sacrificed 30 minutes later for western blots and the other half were tested the following day. (B-C) Extinction and test data for delay animals (B) and trace animals (C). No group differences were observed in the amount of freezing during the first 4 CS presentations of the extinction session for either delay (B) or trace (C) animals. At test, EXT animals showed significantly less freezing than NE and 4T animals for both delay (B) and trace (C) conditioning. * indicates that $p < 0.05$.

Once we confirmed that only the EXT protocol produced extinction, we ran western blots on tissue samples from the amygdala, dorsal hippocampus (DH), prelimbic medial prefrontal cortex (PL), the infralimbic cortex (IL), and the retrosplenial cortex (RSC) and blotted for pERK and total ERK expression to identify structures that undergo TFC extinction-related plasticity. As shown in Figure 4b, in the amygdala, we found that ERK phosphorylation was upregulated following DFC, but not TFC, extinction. Thus, consistent with what we observed behaviorally, we found that extinction-related plasticity in the amygdala did not occur following TFC extinction but did occur following DFC extinction. Interestingly, we also observed upregulation of pERK in the amygdala

following the DFC 4-trial retrieval session, but not the TFC 4T session. This suggests that retrieval of the DFC memory may require upregulation of pERK in the amygdala whereas retrieval of the trace association does not.

In the mPFC, we ran western blots on the PL and IL separately to determine whether either structure is involved in DFC or TFC extinction. In the PL, we observed a nonsignificant increase in ERK phosphorylation following TFC, but not DFC extinction (Fig 4c). This effect, which did not meet the requirements for statistical significance, suggests that a small amount of pERK-related plasticity might occur in the PL to support TFC, but not DFC, extinction. Importantly, we did not observe upregulation of ERK phosphorylation following the 4T protocol in either DFC or TFC animals, suggesting that upregulation of ERK phosphorylation in the PL may be specific to EXT, rather than being induced by either extinction or retrieval.

Our pattern of ERK phosphorylation in the IL is consistent with its known role in extinction and inhibition of the fear-related output in the amygdala (Fig. 4d). We observed increases in ERK phosphorylation following both DFC and TFC extinction, suggesting that plasticity is induced in the IL during fear extinction regardless of the specific training type. This is consistent with the hypothesis that the IL undergoes plasticity to produce more inputs to the ITC layer in the amygdala, which inhibits the fear response, producing low freezing levels characteristic of extinction. Interestingly, we observed increased ERK phosphorylation in the IL following TFC retrieval, as well. It is possible that the IL plays a (currently undefined) role in the retrieval of trace fear.

We also measured ERK phosphorylation levels in the DH. Here, we observed steady levels of pERK following extinction and retrieval of both DFC and TFC associations (Fig. 4e). This suggests that the hippocampus, which plays a role in contextual gating of extinction, does not appear to undergo pERK-related plasticity following either trace or delay fear extinction. This is consistent with the idea that the

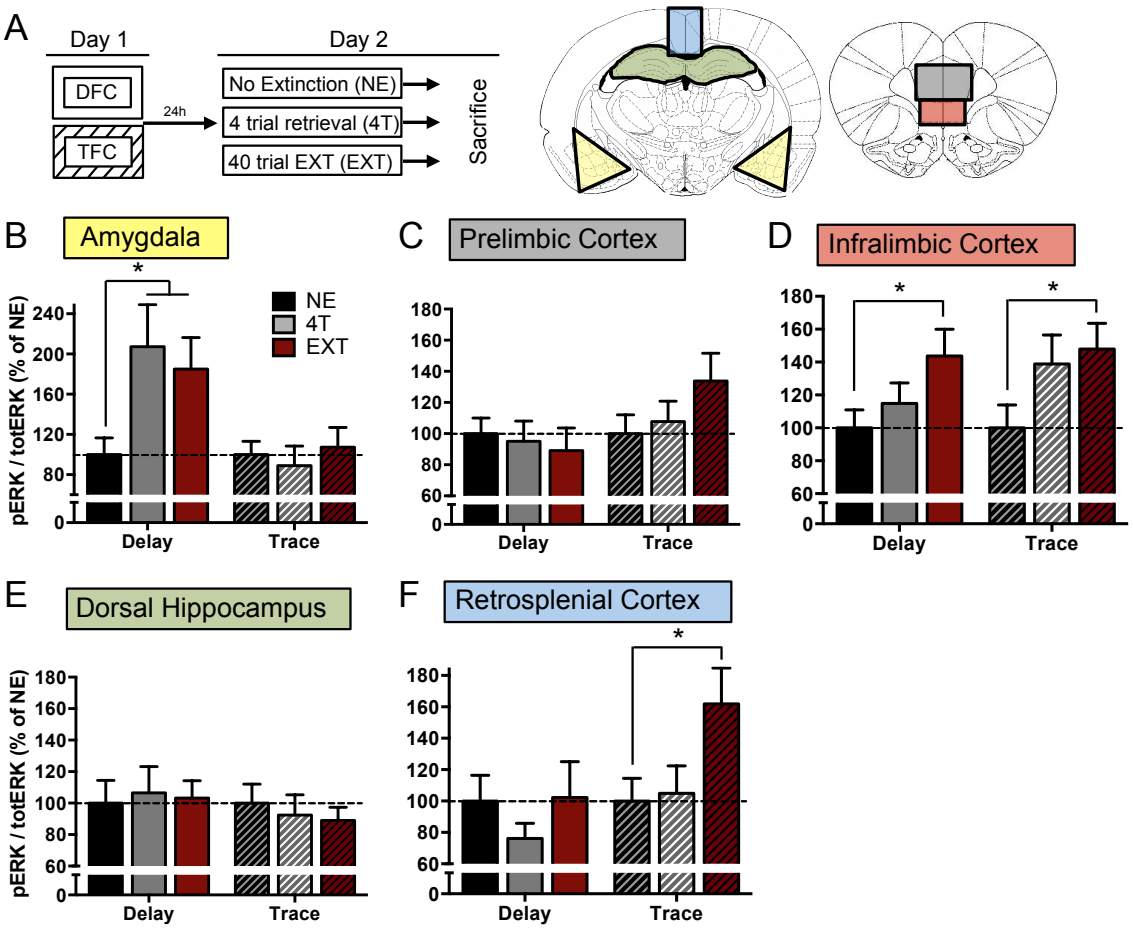

Figure 4. pERK is increased in the amygdala after DFC extinction and in the RSC after TFC extinction. (A) Experimental timeline (left) and images of the dissected brain regions (right). Figure adapted from Paxinos & Watson, 2007. After acquisition, animals were either not extinguished (NE; DFC *n* = 10; TFC *n* = 9); given a 4-trial retrieval (4T; DFC *n* = 9; TFC *n* = 10), or given extinction (EXT; DFC *n* = 10; TFC *n* = 9) (B) pERK expression in the amygdala was increased following DFC extinction and retrieval. No change was observed following TFC extinction. (C) In the PL, a nonsignificant increase in ERK phosphorylation was observed following TFC, but not DFC extinction. (D) In the IL, ERK phosphorylation was upregulated following both DFC and TFC extinction. (E) We observed no significant changes in ERK phosphorylation levels in the DH. (F) ERK phosphorylation in the RSC was significantly increased following TFC, but not DFC extinction. * indicates that $p < 0.05$.

hippocampus modulates the extinction memory but does not store the extinction association itself.

Finally, we investigated ERK phosphorylation levels in the retrosplenial cortex (RSC) following DFC or TFC extinction. The RSC is a relatively under-explored brain structure in the rat; only a few studies have investigated whether it plays a role in learning and memory. In humans, the RSC is activated by autobiographical memory recall (Maddock, 1999; Maguire, 2001; Steinvorth, Corkin, & Halgren, 2006; Svoboda, McKinnon, & Levine, 2006) and remote spatial memory (Rosenbaum, Ziegler, Winocur, Grady, & Moscovitch, 2004). The RSC appears to support spatial and contextual memory in rodents, as well (Aggleton, 2010; Cooper, Manka, & Mizumori, 2001; Haijima & Ichitani, 2008; Keene & Bucci, 2008a, 2008b; Robinson, Keene, Iaccarino, Duan, & Bucci, 2011). In one recent study, Corcoran and colleagues (2011) demonstrated that the RSC requires NR2A-containing NMDA receptors to retrieve both recent and remote context fear memories. DFC CS fear was not affected by NMDA receptor blockade in the RSC, however, suggesting that the RSC is selectively required for context fear retrieval. The involvement of the RSC in complex, explicit, contextual, and relational associations makes it a good candidate for participating in TFC memory storage and extinction. Consistent with this, we observed increases in pERK in the RSC following trace, but not delay, fear extinction (Fig. 4f), suggesting that the RSC may support TFC, but not DFC, extinction. Further, the retrieval control (4T) did not show increased ERK phosphorylation in the RSC for either the DFC or TFC group, suggesting that the upregulation of ERK phosphorylation is specific to extinction. Plasticity in the RSC, therefore, may be required for the extinction of TFC, but not DFC, associations.

Our blot study demonstrated three key points. First, it supported our behavioral study's conclusion that plasticity in the amygdala is important for extinction of DFC, but not TFC. Manipulations of AMPA or NMDA receptors in the amygdala did not prevent TFC-trained animals from learning extinction. Similarly, we did not observe increases in pERK, our plasticity marker, following TFC extinction despite DFC extinction-induced increases in ERK phosphorylation. Secondly, we observed increased ERK phosphorylation in the IL following both DFC and TFC extinction. This suggests that the IL plays a key role in extinction learning regardless of the specific type of fear conditioning used. Finally, we identified two candidate structures that might support extinction learning or memory in TFC animals: the PL and the RSC. The PL showed slight, nonsignificant increases in ERK phosphorylation following TFC extinction whereas the RSC showed a large, statistically significant increase in ERK phosphorylation. Neither of these structures showed increased ERK phosphorylation following DFC extinction. These cortical structures, therefore, may specifically participate in trace fear extinction.

Trace fear extinction: Hypothesized circuitry

Our research suggests that the amygdala is required for the acquisition of trace fear, as with delay fear, but that the extinction of TFC occurs independent of the amygdala. It is possible that the TFC association is stored in different structures than the DFC association, causing different structures to participate in the extinction process (see Figure 4). In DFC, the CS and UCS converge in the amygdala and the association is stored at amygdala synapses. Following DFC, plasticity in the BLA supports the

potentiated fear response observed upon future presentation of the CS, activating neurons in the CeA, which promote freezing through downstream thalamic and brainstem structures. When DFC extinction occurs, plasticity in the BLA may occur to incorporate the new, extinction-relevant information about the CS in the same population of neurons that supports the original CS-UCS association. Simultaneously, the IL may experience extinction-related potentiation that produces increased IL projections to the ITC layer of the amygdala. These inhibitory ITC neurons, as previously discussed, would inhibit output from the CeA, effectively preventing activation of downstream structures and inhibiting the fear response. Thus, when extinction occurs in DFC, a combination of storage-related plasticity in the amygdala and inhibitory plasticity in the IL might support the decreased fear response (Fig. 5a).

It is still unclear where the TFC memory is stored. It is possible that the TFC association is stored in cortical areas, rather than in the amygdala, leading to a requirement for plasticity in the cortex, but not in the amygdala, during extinction (see Fig. 5b). Trace fear learning, which requires awareness in humans and is a model for explicit memory, is known to require the prefrontal cortex for acquisition. Indeed, some evidence suggests that the mPFC *stores* the TFC association (Runyan et al., 2004). From this perspective, the original TFC association may occur largely in cortical areas, including the PL mPFC and possibly RSC (based on our western blot results). Plasticity in the amygdala would be required during the initial learning of TFC, as amygdala outputs from the CeA would need to be strengthened to produce the fear response upon future CS presentation. The storage of the TFC memory itself, however, would be supported by plasticity in distributed cortical areas, including the mPFC and RSC.

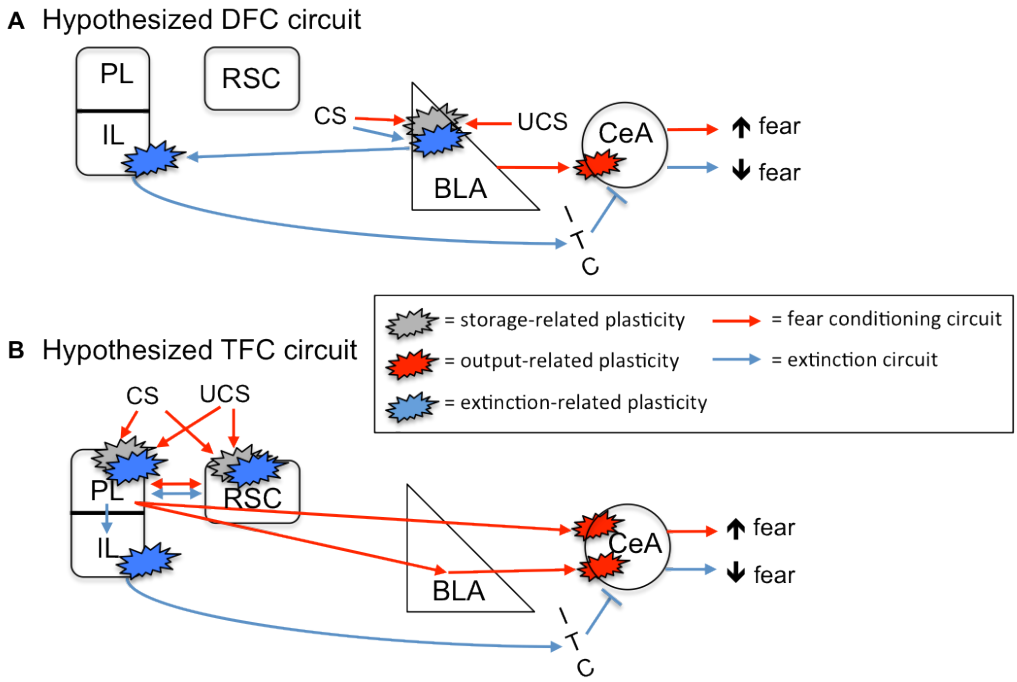

Figure 5. Hypothesized circuitry for delay and trace fear acquisition and extinction. (A) Delay fear acquisition and extinction circuit. During conditioning (red arrows), the CS and UCS information converges in the amygdala and promotes storage-related plasticity in the BLA. Plasticity in the CeA produces potentiated connections to "output" areas (hypothalamic and brainstem regions) that support the fear response. During extinction (blue arrows), plasticity occurs in the BLA and the IL. In the BLA, extinction-related plasticity is hypothesized to incorporate the new information about the CS learned during extinction. In the IL, potentiation is hypothesized to support increased input to the ITC layer of the amgydala, which promotes inhibition of the CeA to "switch off" the fear response. (B) Proposed TFC acquisition and extinction circuit. Here, the CS and UCS converge in distributed cortical areas (including the PL and RSC), which undergo plasticity to support the storage of TFC. Plasticity is also required in the amygdala for TFC, as connections to the CeA are potentiated to drive the fear response upon future CS presentation. Extinction of TFC requires plasticity in the cortical areas that store the memory, to allow for the new information about the CS to be incorporated. Extinction would also produce plasticity in the IL, as with DFC, to drive inputs to the ITC layer of the amygdala. No plasticity in the amygdala would be required according to this circuit because the memory is stored elsewhere.

According to this hypothesis, when extinction occurs, plasticity would be required in the cortex (which stores the TFC association) in order to incorporate new information about the CS-UCS relationship. Extinction-related plasticity would also occur in the IL, to produce strengthened IL-ITC connections that would inhibit the facilitated amygdala outputs acquired during TFC acquisition. This circuitry would not require plasticity in the amygdala during the extinction session; if the amygdala does not store the association, it would not need to undergo plastic changes during extinction.

Although this proposed circuitry fits the pattern of results we have so far observed, a number of crucial pieces of information are currently missing. First, it is not known whether the RSC participates in the acquisition, storage, or extinction of TFC associations. Further, it is not known whether the mPFC and other cortical areas store the TFC association immediately after conditioning, as predicted. Finally, it is not certain whether the plasticity observed in the amygdala during TFC consolidation is the result of strengthening output-specific synapses, rather than strengthening synapses that may store the TFC association. The goal of this dissertation was to clarify this circuitry in order to better understand how the TFC memory is stored, retrieved, updated, and extinguished.

Reconsolidation

After the consolidation process, memory is stored in a relatively stable form that is resistant to disruption with traditional amnesic agents. This phase, called the maintenance, or storage, phase, is believed to persist through the constitutive actions of the memory maintenance molecule, protein kinase Mzeta (Kwapis et al., 2012; Kwapis et al., 2009; Parsons & Davis, 2011; Sacktor, 2008; Serrano et al., 2008). A memory can be removed from this stable maintenance phase with a single retrieval trial, making the memory once again susceptible to disruption by protein synthesis inhibitors (Nader, Schafe, & Le Doux, 2000; Parsons, Gafford, Baruch, Riedner, & Helmstetter, 2006) and other manipulations, including behavioral manipulations (Alberini, Milekic, & Tronel, 2006; Dudai, 2004; Monfils, Cowansage, Klann, & LeDoux, 2009; Nader & Hardt, 2009; Sara, 2000; Schiller et al., 2010; Tronson & Taylor, 2007).

This process, called reconsolidation, may update the original memory so that new information can be incorporated into the original engram (Johansen et al., 2011). Consistent with this idea, synapses appear to undergo two phases during the reconsolidation process: destabilization and restabilization (Nader & Hardt, 2009). Following memory retrieval, the synapses storing the engram are destabilized, characterized by a period of deconstruction and protein degradation (Jarome, Werner, Kwapis, & Helmstetter, 2011; Lee, 2008; Lee et al., 2008). This would serve to weaken the stored memory, in a sense making the synapses malleable in order to incorporate new information about the CS. The second phase of reconsolidation is restabilization, the period of new protein synthesis that serves to re-solidify the synapses. Restabilization is similar to the initial consolidation process and ultimately results in the memory being stabilized and stored. In effect, this process reorganizes synapses and allows them to integrate new information into the existing memory.

Most of the work on reconsolidation to date has investigated DFC associations in the restabilization phase. Initially, studies showed that infusion of the protein synthesis inhibitor anisomycin into the amygdala immediately after DFC retrieval disrupted long-term memory when tested the following day (Nader et al., 2000), a finding that has been replicated numerous times (e.g. Jarome et al., 2012; Parsons, Gafford, Baruch, et al., 2006). Specific intracellular signaling pathways have been identified as important for reconsolidation, as well, including the ERK (Duvarci et al., 2005) and PKA (Tronson, Wiseman, Olausson, & Taylor, 2006) pathways. Blockade of either molecule in the amygdala disrupts fear memory reconsolidation, in the same manner as protein synthesis inhibition. Blocking other memory-related molecules, like Arc, Npas4, and Egr-1

similarly reduces the reconsolidation of memory (Maddox & Schafe, 2011a, 2011b; Ploski, Monsey, Nguyen, DiLeone, & Schafe, 2011). Finally, disruption of the mTOR pathway, a major regulator of translation, is also crucial to memory reconsolidation (Parsons, Gafford, & Helmstetter, 2006). Together, these molecular pathways seem to restabilize the synapse following retrieval.

The destabilization phase is relatively less well-studied. It appears that NMDA receptors, activated by memory retrieval, trigger protein degradation through the ubiquitin-proteasome system (UPS), ultimately destabilizing the initial memory trace to allow new information to be incorporated (Jarome et al., 2011). Accordingly, inhibiting NMDA receptors in the amygdala before retrieval prevents the effects of post-retrieval anisomycin (Ben Mamou, Gamache, & Nader, 2006; Jarome et al., 2011). NMDA receptors, therefore, seem to play a key role in converting a memory from a stable to a labile condition; if NMDA receptors are inhibited before retrieval, the destabilization phase of reconsolidation is prevented. Blocking UPS-mediated protein degradation also prevents memory destabilization and updating in the hippocampus (Lee, 2008; Lee et al., 2008) and amygdala (Jarome et al., 2011). NMDA receptors appear to be necessary to activate retrieval-induced degradation, suggesting that NMDA receptors are upstream of both degradation and destabilization of the memory trace (Jarome et al., 2011). Inhibiting either NMDA receptors or protein degradation is sufficient to prevent the destabilization of memory, which negates the post-retrieval deficits of anisomycin, as the memory trace is never converted to a labile state. Blocking destabilization of the memory trace, however, prevents new information from being integrated into the engram (Lee, 2008, 2010), as the synapses are effectively prevented from becoming malleable. Consistent

with this hypothesis, Lee (2010) demonstrated that inhibiting degradation prevented the destabilization of a context memory so that animals were unable to associate a previously acquired context representation with a shock given during retrieval. Inhibiting the restabilization of the context-shock association by knocking down Zif268 expression before retrieval disrupted the original context representation, as the memory destabilizd but failed to properly reconsolidate in the absence of Zif268. These results support the hypothesis that NMDAR-mediated protein degradation is required for destabilization of the memory trace (to allow the incorporation of new information) whereas restabilization through protein synthesis is required to maintain the original memory along with any new information provided during the retrieval trial.

Reconsolidation may only be required when a memory is updated during retrieval. Specifically, Diaz-Mataix and colleagues (2013) have shown that animals are not affected by post-retrieval anisomycin when the retrieval trial consists of the same CS and UCS timing used in training. If the retrieval trial included an interval shift between the CS and UCS (with the UCS presented either earlier or later after CS onset), intra-amygdala infusion of anisomycin was sufficient to disrupt the memory. This suggests that memories only destabilize when new information is provided during the retrieval event; if the retrieval trial is identical to the original training, there is no need to incorporate additional information and the memory does not destabilize. According to this hypothesis, the standard retrieval trials currently used to study reconsolidation, which consist of a single unreinforced CS presentation in a novel context, trigger destabilization of the memory trace because they differ from the initial training trials in that no UCS is presented and the context is novel.

This line of research, called memory updating, is still in its infancy. A number of predictions can be made based on this research, however. Blocking NMDA receptors before reconsolidation should prevent memory updating, as this manipulation should block the destabilization of synapses and thus prevent incorporation of new information. An animal with NMDAR blockade will therefore not learn any new information about the CS-UCS timing presented during retrieval. Infusing anisomycin after a retrieval trial should disrupt the restabilization phase, so that the destabilized memory fails to be re-stored and is lost, as has been observed following nonreinforced retrieval trials in a number of experiments (e.g. Jarome et al., 2012; Nader et al., 2000; Parsons, Gafford, Baruch, et al., 2006). This would not only prevent the animal from learning new information during the retrieval phase, but it should disrupt the animal's memory for the original training, as well, which was destabilized during retrieval. It is also possible that changing the CS-UCS relationship during the retrieval trial could alter how, and possibly where, the original memory is stored. If a relatively complex memory, like trace fear conditioning, is updated with a single retrieval trial of a simpler form of learning, like delay fear conditioning, it is possible that the association will now rely on the simpler, more well-defined circuitry of DFC.

To date, few studies have investigated the circuitry of TFC reconsolidation. The two studies that have investigated trace reconsolidation have focused on the mPFC and hippocampus; no studies have been published on the role of the amygdala. Work on TFC reconsolidation comes primarily from researchers in the Dash laboratory, who have demonstrated that *de novo* protein synthesis is required in the hippocampus (Runyan & Dash, 2005) but not in the mPFC (Blum, Runyan, & Dash, 2006) for TFC

reconsolidation. These results suggest that some, but not all, of the structures that support TFC acquisition undergo reconsolidation upon subsequent memory retrieval.

We recently tested whether the amygdala is required for the reconsolidation of TFC memory. We have previously demonstrated that the consolidation of TFC requires new protein synthesis in the amygdala (Fig. 1) while the extinction of TFC appears not to require processes in the amygdala, including NMDA receptors, AMPA receptors, and increased pERK expression (Fig. 2-4). It is unknown whether the amygdala is required for TFC reconsolidation. On one hand, if the amygdala does not store the memory for trace fear, it is possible that destabilization and restabilization of the stored memory trace (possibly in cortical areas) is required for reconsolidation and no updating of amygdala synapses is necessary. On the other hand, plastic changes in the amygdala are necessary for learning the original association. Even if these changes represent output-related synaptic potentiation, it is possible that these synapses would need to be deconstructed, updated, and restabilized during the reconsolidation process. In order to determine whether the amygdala participates in the reconsolidation of TFC, we trained animals with DFC or TFC 6 followed by a single retrieval trial the following day. Immediately after retrieval, animals were infused into the amygdala with vehicle or the protein synthesis inhibitor anisomycin to disrupt the restabilization process. Animals were then tested the following day to determine whether this infusion had any effect on memory.

As shown in Figure 6, both DFC and TFC animals given anisomycin showed a significant decrease in CS freezing during the testing session. This demonstrates that protein synthesis in the amygdala is required for restabilization of TFC, as well as DFC. Thus, the amygdala undergoes plastic changes during both consolidation and

reconsolidation of TFC associations. The extinction of TFC is therefore unique in that it does not appear to require the amygdala.

One outstanding question in the memory updating field is whether a retrieval trial can update a complicated memory into a simpler form. TFC, for example, is a relatively complex memory that requires multiple forebrain structures for acquisition and appears to rely on a complicated, currently undefined set of structures for extinction. It would be difficult to manipulate TFC memory in a clinical setting, as the circuit is more distributed than that of DFC. It would be of use, therefore, if a retrieval trial could be used to convert a TFC memory into the relatively simpler DFC form that requires the participation of fewer structures to support storage and extinction. If memories are updated through retrieval, perhaps presenting a TFC-trained animal with a single DFC trial could update the association so that it now relies on the well-defined DFC circuitry. One of the goals of this project was to determine whether this type of updating is possible.

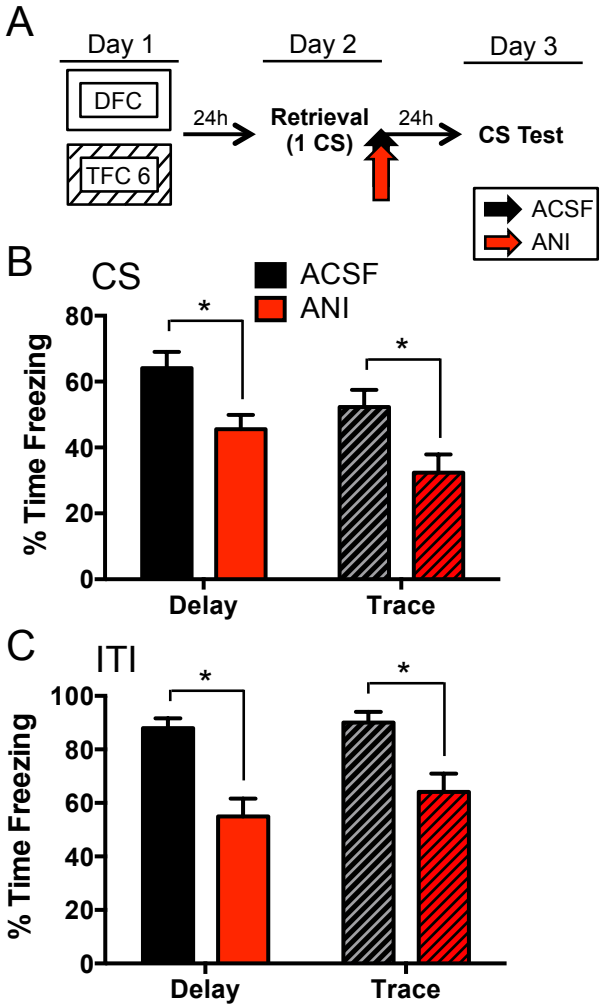

Figure 6. Both DFC and TFC require protein synthesis in the amygdala for reconsolidation. (A) Experimental timeline. Animals were infused in the amygdala with anisomycin (ANI; DFC *n* = 10; TFC *n* = 11) or vehicle (ACSF; DFC *n* = 16; TFC *n* = 14) following retrieval. (B) Mean percent time freezing during the first three CS presentations during the CS test. (C) Mean percent time freezing during the first three ITI periods of the CS test. * indicates $p < 0.05$.

Together, our preliminary results suggested that the neural circuits supporting trace and delay fear conditioning are drastically different, with a distributed set of cortical regions selectively participating in trace fear. While the amygdala is a crucial component of the delay extinction circuit, it does not seem to be required for trace extinction. On the other hand, ERK phosphorylation was increased in multiple cortical areas following trace, but not delay extinction, suggesting that trace fear extinction may be shifted from subcortical to cortical regions. The goal of the present project was to further clarify how the trace and delay fear circuits differ in order to better understand how complex fear is stored and updated in the brain. Additionally, we aimed to test whether a single update trial can be used to shift an association from the relatively complex trace fear circuit to that of a simpler delay association. To this end, we first identified the neural circuit supporting trace fear by systematically testing the roles of the retrosplenial, prelimbic, and infralimbic cortices in delay and trace fear extinction. Our results identified the retrosplenial and prelimbic cortices as being involved in trace, but not delay fear extinction, consistent with our preliminary data. We then tested whether the retrosplenial cortex, a poorly understood brain region, might also participate in the storage of trace fear. Here, our results demonstrated that the retrosplenial cortex is required for the consolidation of trace, but not delay fear conditioning. Finally, we tested whether a single update trial can be used to shift the neural circuit supporting an association so that a complex trace fear association can be shifted to the simpler delay fear circuit. Indeed, we found that when a trace association is updated with a single delay trial, the memory appears to rely on the basic delay circuit, rather than the complex neural circuit supporting trace fear. Together, our results provide a more complete understanding of the

neural circuit supporting trace fear and demonstrate, for the first time, that a single update trial can be used to change the neural circuit supporting an association.

Methods

Subjects

The subjects were 285 male Long-Evans rats (300-375g) obtained from Harlan (Madison, WI). The rats were housed individually in shoebox cages with free access to water and rat chow. The colony room was maintained under a 14:10-h light/dark cycle and all behavioral tests were conducted during the light portion of this cycle. All procedures were approved by the Institutional Animal Care and Use Committee and conducted within the ethical guidelines of the National Institutes of Health.

Surgery and Histology

Animals were adapted to handling for three days before surgery. On the day of surgery, animals were anesthetized with 2-4% isoflurane in 100% O_2 and implanted with bilateral stainless steel 26-gauge cannulae aimed at the basolateral amygdala (AP = -2.9 mm, ML ±0.5 mm, DV -7.2mm), anterior retrosplenial cortex (AP = -3.5 mm, ML = ±0.5mm, DV = -1.8 mm, using double cannulae with a 1.0 mm center-to-center distance), prelimbic cortex (AP +3.0 mm, ML ±1.6 mm, DV -3.0 mm, angled at 15°), or infralimbic cortex (straight coordinates: AP + 2.8 mm, ML ±0.6 mm, DV -4.4 mm, using double cannulae with a 1.2 mm center-to-center distance; angled coordinates: AP + 2.8 mm, ML ±3.1 mm, DV -4.2 mm, angled at 30°). All of the stereotaxic coordinates are relative to Bregma and were based on a rat brain atlas (Paxinos & Watson, 2007). Cannulae were secured to the skull with stainless steel screws, superglue, and dental cement, and stainless steel obdurators were inserted into the guide cannulae to prevent occlusion.

Each rat was given a recovery period of at least seven days before behavioral testing began.

When behavioral testing was complete, all cannulated animals were killed with an overdose of isoflurane and transcardially perfused with saline followed by a 10% buffered formalin solution. Heads were removed and submerged in buffered formalin for at least 24h. Brains were then removed and soaked in a 30% sucrose formalin solution until the brains sank to cryoprotect the tissue. Frozen 40 μm sections were collected throughout the cannulated region, mounted on slides, and stained with cresyl violet. Injection sites were determined with the aid of a rat brain atlas (Paxinos & Watson, 2007). Animals with injection sites outside of the target regions were not included in analyses.

A subset of animals from the first three experiments were injected with either a fluorescent antibody (Experiments 1 and 3) or a methylene blue stain (Experiment 2) to better visualize the region targeted by our infusions. For these animals, anti-mouse Alexa 488 (Experiment 3, straight IL cannulae), anti-rabbit Alexa 594 (Experiment 1 RSC cannulae and Experiment 3 angled IL cannulae), or meythlene blue (4% in DDH2O; Experiment 2 PL cannulae) was infused at the same volume as the drug used in each experiment (0.3 μl/side for PL and IL, 0.5μl/side for RSC). Approximately 10 minutes after infusion, these animals were perfused as described above and brains were immediately removed and placed in sucrose formalin for 2-4 days in a dark container. The brains were sliced at 40 μm in the dark, mounted on slides, and imaged with a fluorescent microscope (Nikon Eclipse) running NIS-Elements software. Alternate slices

from each of these brains were mounted and stained with cresyl violet as described above.

Apparatus

Fear conditioning was conducted in a set of four Plexiglas and stainless steel chambers housed within sound-attenuating boxes (Context A). The floor of these chambers was composed of stainless steel rods spaced 1.2 cm apart through which footshocks were delivered. Each chamber was illuminated by an overhead 7.5-W bulb and was connected to its own shock generator-scrambler (Grason-Stadler, West Concord, MA). Ventilation fans provided constant background noise (approximately 62-64 dB). Chambers were cleaned with a solution of 5% ammonium hydroxide between animals to provide Context A with a distinct scent. Speakers were mounted on the center of the right side panel of each of the four chambers in this context.

A separate set of chambers (Context B) was used for extinction training and for testing. Context B was located in a different room and had a number of distinct features designed to minimize generalization of contextual fear so that cued fear could be dissociated from context fear. Context B had a solid and opaque textured floor panel that was distinctly different from the steel rods used in Context A. Infrared lighting was used to illuminate Context B and the background noise was approximately 58 dB. Context B was cleaned with a 5% acetic acid solution between each testing or extinction session. The speakers in Context B were mounted above each chamber.

Drug Preparation and Infusion Procedure

Rats received bilateral infusions into the BLA (0.5μl/side), the RSC (0.5μl/side) the PL (0.3μl/side), or the IL (0.3μl/side), depending on the experiment. All infusions were given at a rate of 0.5μl/minute and the injection cannulae remained in place for an additional 90s to ensure proper diffusion and to minimize dragging of the drug back up the cannula. Injection cannulae were cut to extend approximately 0.5-0.7 mm beyond the guide cannulae.

Animals were infused with one of two drugs: the protein synthesis inhibitor anisomycin (ANI) or the NMDA receptor inhibitor $_{D}$-2-amino-5-phosphonopentanoic acid (APV). Anisomycin (125μg/μl; Sigma-Aldrich and R&D Systems) was dissolved in HCl (~90ul/25mg ANI) and diluted to the proper concentration of 125μg/μl with artificial cerebrospinal fluid (ACSF). Previous work with anisomycin has demonstrated that this solution should have a pH of approximately 7.2-7.4 and that this concentration is effective in disrupting consolidation (see Fig 1; Kwapis et al., 2011) and reconsolidation (see Fig. 6) in the amygdala. APV (10μg/μl; Sigma-Aldrich) was diluted in ACSF to the final concentration. Previous work with APV in our lab has demonstrated that this concentration is effective in disrupting DFC extinction in the amygdala (see Figure. 2) and the acquisition of TFC in the prefrontal cortex (Gilmartin & Helmstetter, 2010).

General behavioral procedures

Following recovery from surgery, all animals were acclimated to the transport, restraint, and injection procedures for 3 days. Each day, animals were transported to the laboratory on the transport cart, wrapped in a towel, and gently restrained by hand for

several minutes. During this time, the obdurators were removed and the infusion pump was activated to allow animals to habituate to its sound.

Two types of training were used during acquisition: 4-trial delay fear conditioning (DFC) and 6-trial trace fear conditioning (TFC). For each type of training, a 10s white noise CS (72dB) was paired with a 1s UCS footshock (1.0mA). DFC animals were given 4 pairings of delay conditioning after a 6-minute baseline period. Here, the UCS was delivered at the moment of CS offset and a variable intertrial interval (ITI) of 110 ±20s separated the four CS-UCS pairings. TFC animals were given 6 trials of the CS and UCS following a 6-minute baseline, but the two stimuli were separated by a 20s trace interval (TI). In order to boost the CS-UCS association in trace animals, the ITI was lengthened to 240 ±20s, based on previous research demonstrating that increasing the time between conditioning trials enhances learning of the CS-UCS relationship in TFC (e.g. Detert, Kampa, & Moyer, 2008). All animals were removed from the conditioning chambers four minutes after the final UCS. These protocols have previously been used in our lab to produce approximately equivalent freezing strength for both groups when tested 24h post-acquisition (Kwapis et al., 2011).

Some animals also received an extinction session. The day after acquisition or retrieval, these animals were placed in Context B (a novel environment) and, after a 1-min baseline, presented with 40 unreinforced white noise cues (30s each, 72dB) separated by 60s. The entire extinction session was 60 minutes long. Extinguished animals were tested 24h after the extinction session.

Animals were also tested to the white noise CS and, in some cases, to the context in which conditioning occurred (Context A). The white noise testing session always took

place in Context B and consisted of 8 white noise presentations, with a total session length of 12 minutes. When the test occurred after extinction, it was designed to assess the animals' recall of the extinction session (i.e. low freezing levels indicate strong memory for extinction). When the test took place after training or retrieval sessions, the test was intended to assess the animal's memory for the training session (i.e. high freezing levels indicate good memory for training). Animals given a context test were placed back into Context A (the training context) for 12 minutes.

Animals in Experiments 7 and 8 received slightly different treatment (see figures 7a and 8a). First, these animals were given two consecutive days of context pre-exposure before training to attenuate the context-shock association. During both pre-exposure sessions, the animals were each placed in the training context for five minutes. No stimuli were presented during this pre-exposure session. Animals in Experiments 7 and 8 were also given a single reinforced retrieval trial of either TFC or DFC one day after training. For this updating trial, it was imperative that the timing is identical to that used in training; our hypothesis was that new information causes memory to update and undergo reconsolidation whereas an identical trial, which does not provide new information should not trigger updating or reconsolidation. These animals were each given a single update trial in Context A consisting of the same baseline period (6 minutes), the same 10s white noise presentation (72dB), and the same shock (1s, 1.0mA) used in training. Animals given a single DFC retrieval trial received the UCS presentation immediately after the CS offset. Animals given a TFC retrieval trial had a 20s TI separating the CS offset and the UCS onset. All animals were removed 60s after the UCS presentation. These animals were then either tested the following day or extinguished the following

day in Context B. The testing and extinction procedures for these experiments were identical to the procedures described above.

Procedure, Experiments 1-3

In Experiments 1-3, we tested whether the RSC (Experiment 1; n=52), PL (Experiment 2; n=30), and IL (Experiment 3; n=56) are necessary for learning TFC or DFC extinction. In each of these experiments, animals were trained with DFC or TFC on Day 1. The following day, these rats received an infusion of the NMDAR antagonist APV or vehicle (ACSF) into the appropriate structure approximately 5 minutes before extinction training (see Fig. 7a, 8a, 9a). The animals were given a retention test the following day to determine the strength of their extinction memory. For each of the three brain structures, there were four experimental groups: two types of training (DFC or TFC) and two drug conditions (APV or VEH), with 12-14 animals per group in Experiment 1, 7-8 per group in Experiment 2, and 13-15 animals per group in Experiment 3.

Procedure, Experiment 4

Experiment 4 was designed to test whether protein synthesis in the RSC is necessary for the consolidation of DFC or TFC, as our preliminary results suggested that the RSC may play a role in the storage and extinction of trace fear memory. To this end, we trained animals in DFC (n=15) or TFC (n=16). Immediately after acquisition, animals were infused with the protein synthesis inhibitor anisomycin (ANI; DFC n=7; TFC n=8) or vehicle (VEH; DFC n=8; TFC n=8) directly into the RSC and tested to both the white noise and context the following day in a counterbalanced manner (see Fig. 11a).

Procedure, Experiment 5

Experiment 5 was a direct follow-up to the fourth experiment, which failed to show an effect for pre-training protein synthesis inhibition in the RSC on trace or delay consolidation. In order to rule out the possibility that our lack of effect was due to an improper infusion procedure, we subsequently ran a positive control to demonstrate that our intra-RSC infusion procedure was working properly. We have previously shown that intra-RSC infusion of APV can prevent normal memory recall in TFC animals while the drug is present (see figure 7e). Here, we used this finding to ensure that our infusion procedure was working correctly. To this end, the animals from Experiment 4 were regrouped into new drug conditions so that animals showed equivalent freezing before drug infusion (DFC: $t(13) = 0.86$; $p = 0.40$; TFC: $t(14) = 0.94$; $p = 0.36$; data not shown). Animals were then infused into the RSC with APV (DFC n=8; TFC n=8) or vehicle (DFC n=7; TFC n=8) five minutes before they were given a CS test in Context B. These animals were also given a second CS test the following day, after the drug had been metabolized, to determine if any effects were long-lasting or transient (Fig. 12a).

Procedure, Experiment 6

Experiment 6 aimed to test whether protein synthesis in the RSC is necessary for the acquisition or early consolidation of DFC or TFC. We observed no effect when we inhibited protein synthesis immediately after training (Experiment 4), but it is possible that an earlier wave of protein synthesis, either during or immediately after training, in the RSC is necessary for trace consolidation. To test this possibility, we infused animals

with the protein synthesis anisomycin (ANI; DFC n=14; TFC n=13) or vehicle (VEH; DFC n=15; TFC n=16) directly into the RSC 15 minutes before training in trace or delay fear conditioning. These animals were then tested the following day to both the white noise CS and the context in a counterbalanced manner (see Fig. 13a).

Procedure, Experiment 7

Experiments 7 and 8 tested whether a single delay trial can be used to update a trace fear memory and change the neural circuit responsible for supporting that association. In Experiment 7, we tested whether TFC memory undergoes updating in the amygdala following exposure to a single DFC update trial. Here, we first pre-exposed animals to the training context for two days to reduce association of the context with the UCS (Fig. 14a). The animals were then trained with TFC on day 3 followed by an update session on day 4 in the training context. During the update session, half of the animals were given a single trial of DFC to update the association (shift group; n=14) and half were given a trial of TFC that was identical to that used during training (no shift group; n=15). Immediately after the update session, animals were given an intra-amygdala infusion of anisomycin (DFC n=7, TFC n=7) or vehicle (DFC n=7; TFC n=8) to disrupt restabilization of the association. Animals were then given a CS test in Context B on day 5 to determine whether the memory was intact or disrupted. Impaired memory at the day 5 CS test indicates that the memory was destabilized to allow updating and blocking protein synthesis impaired restabilization. Intact memory, on the other hand, indicates that the association did not destabilize and therefore did not require protein synthesis for restabilization.

Procedure, Experiment 8

Once we successfully demonstrated that TFC memory is updated following a single DFC trial, we tested whether this also changes the neural circuit that supports the association. We have previously shown that DFC extinction requires the amygdala whereas TFC extinction does not (Fig. 2). We can use this distinction to test which neural circuit the updated association relies on. If the updated memory does not require the amygdala for extinction, it is likely supported by the distributed trace fear circuit. If, however, the updated association requires the amygdala for extinction, the update has changed the neural circuit that supports that association, shifting it to a more basic delay fear circuit that requires the amygdala for extinction.

In Experiment 8, we first pre-exposed animals to the training context to reduce association of the context with the UCS (Fig 15a). The animals were then trained with TFC on day 3 followed by an update session on day 4 in the training context. During the update session, half of the animals were given a single trial of DFC to update the association (n=15) and half were given a trial of TFC that was identical to that used during training (n=14). On day 5, animals were given an intra-amygdala infusion of APV (DFC n=7; TFC n=6) or ACSF (DFC n=8; TFC n=7) approximately 5 minutes before extinction training in context B. We have previously shown that this infusion, which blocks NMDA receptors in the amygdala, impairs delay, but not trace fear extinction (see Fig. 2d-e). On day 6, the animals were given a CS retention test in Context B to determine extinction learning was affected by NMDA receptor blockade in the amygdala. Impaired extinction would reflect a change in the circuit, as trace extinction does not normally require the amygdala (see Fig. 2).

Statistical Analyses

For all experiments, memory strength was assessed by measuring the percentage of time animals spent engaged in freezing behavior. Each rat's behavior was recorded on digital video and scored in real-time to assess freezing behavior using the FreezeScan 1.0 software (CleverSys., Inc., Reston, VA). The automatic scoring parameters were chosen to closely match hand-scoring methods previously used in our lab to measure freezing. Acquisition data were analyzed on a minute-by-minute basis. For the extinction and test sessions in Experiments 1-3 and 8, the data were handscored by a trained observer who was blind to the group assignments to correct for resting behavior; rats would commonly lie down during the long extinction training session, which would automatically be scored as freezing behavior by the computer. Otherwise, the freezing data collected by FreezeScan was used. For both the extinction (Experiments 1-3 and 8) and CS test sessions (Experiments 1-8), the percent time spent freezing during the 30s discrete CS presentation was calculated and *t*-tests were used to identify drug effects for the average of the first 8 or 12 trials of the extinction session and the first 3 or all 8 trials of the test session. For the context test (Experiments 4 and 6), we calculated the average percent time freezing during the first 4 minutes of the session. For the update sessions (Experiments 7 and 8), we calculated the average freezing during each period of the update session (baseline, CS, trace interval, and post-shock periods). Differences between groups were tested using two-way ANOVAs (factors: training type and drug), Fisher Least Significant Difference (LSD) post hoc tests, and student's *t*-tests where appropriate. A value was determined to be an outlier and removed if the value was greater than two

standard deviations from the group mean. In all analyses, an α value of 0.05 was required for significance.

Hypotheses

Hypothesis 1. NMDA receptors in the retrosplenial cortex are required for trace, but not delay extinction. We tested this hypothesis in Experiment 1 by blocking NMDA receptors in the RSC before delay and trace fear extinction.

Hypothesis 2. NMDA receptors in the prelimbic cortex are required for trace, but not delay extinction. We tested this hypothesis in Experiment 2 by blocking NMDA receptors in the PL mPFC before delay and trace fear extinction.

Hypothesis 3. NMDA receptors in the infralimbic cortex are required for both delay and trace extinction. We tested this hypothesis in Experiment 3 by blocking NMDA receptors in the IL mPFC before delay and trace fear extinction.

Hypothesis 4. Protein synthesis in the RSC is necessary for trace, but not delay fear consolidation. Our results suggest that the RSC is involved in trace, but not delay fear extinction, which may occur because the RSC participates in the storage of trace associations. In order to test whether the RSC is involved in trace fear consolidation, in Experiment 4, we infused the protein synthesis inhibitor anisomycin immediately after training rats with trace or delay fear conditioning.

Hypothesis 5. NMDA receptors in the RSC are necessary for trace, but not delay fear memory retrieval. In order to determine whether our null effect in Experiment 4 was due to an ineffective infusion procedure, Experiment 5 tested whether infusion of APV into the RSC is sufficient to impair memory retrieval for trace, but not delay fear, as we have previously demonstrated. We test this hypothesis by infusing APV into the RSC immediately before a CS test. A second test was included the following day to test whether the effect is transient or permanent.

Hypothesis 6. Protein synthesis in the RSC is necessary for the acquisition or early consolidation of trace, but not delay fear. To test whether protein synthesis is required during or immediately after training, we infused the protein synthesis inhibitor anisomycin into the RSC before DFC or TFC training in Experiment 6.

Hypothesis 7. Memory only undergoes updating and reconsolidation when new information about the CS-UCS relationship is presented during the update session. Specifically, we expect that presenting a single DFC trial to TFC-trained animals will trigger updating whereas presenting a TFC trial to TFC-trained animals will not. We tested this in Experiment 7 by infusing the protein synthesis inhibitor anisomycin into the amygdala immediately after updating a TFC memory with a single DFC or TFC trial.

Hypothesis 8. Updating a memory can change not only the content of that association, but also where it is stored. Specifically, we hypothesize that updating a TFC memory with a single DFC trial will change the neural circuit that supports the association,

shifting the memory from the cortical trace fear circuit to the simpler, subcortical delay fear circuit. To test this, we used our previous finding that delay, but not trace fear requires NMDA receptors in the amygdala for extinction (Fig. 2). In Experiment 8, TFC-trained animals were updated with a single delay or trace retrieval trial. These animals then received an infusion of APV or vehicle to test whether NMDA receptors in the amygdala are required for extinction.

Results

Experiments 1-3: How does the neural circuit supporting trace fear extinction differ from that of delay fear extinction?

Our preliminary results suggested that the neural circuits supporting trace and delay fear extinction differ, as the amygdala appears to be involved in delay, but not trace extinction (Fig. 2). Our western blot study further suggested that extinction-related plasticity occurs in cortical, rather than subcortical areas for trace fear extinction (Fig. 4); we observed increased ERK phosphorylation in the retrosplenial cortex and in the prelimbic cortex (although the increase in PL was not significant), suggesting that a distributed cortical network is responsible for trace fear extinction. Finally, ERK phosphorylation was increased in the IL following both delay and trace fear extinction, suggesting that this structure plays a role in both types of extinction. In Experiments 1-3, we systematically tested whether NMDA receptor-dependent plasticity in each of these structures is necessary for delay or trace fear extinction. The ultimate goal of these experiments was to identify how the neural circuit for trace fear extinction differs from that of delay.

Experiment 1. NMDA receptors in the RSC are necessary for the retrieval and extinction of trace, but not delay fear conditioning.

In our first experiment, we tested whether NMDA receptor-dependent plasticity in the retrosplenial cortex is required for trace or delay fear extinction (Fig. 7a). To this end, animals were first implanted with RSC cannulae. In order to ensure that our cannulae placement correctly targeted the RSC, we injected a subset of the animals (n=3) with a

fluorescent secondary antibody after the experiment and visualized its spread. The injection covered both the granular and dysgranular layers of the RSC and the antibody showed no spread into the hippocampus. (Fig 7b). After recovering from surgery, animals were trained with strength-matched DFC or TFC on day 1. During training, all animals acquired fear normally (Fig. 7c-d) and no differences were observed between groups during the postshock period for either delay ($t_{(25)} = 0.458$, $p = 0.651$; Fig. 7c) or trace ($t_{(23)} = 0.002$, $p = 0.998$; Fig 7d) animals.

On day 2, animals were infused into the anterior RSC with the NMDA receptor antagonist APV or vehicle (ACSF) before extinction. The first 8 trials of the extinction session were analyzed in order to determine whether the retrieval or expression of the fear memory was affected by this infusion (Fig. 7e). Delay animals showed normal levels of freezing at the beginning of the extinction session; APV animals and ACSF animals froze at similar levels during the first 8 CS presentations ($t_{(25)} = 1.372$, $p = 0.181$). Trace animals given APV, on the other hand, showed significantly reduced freezing compared to the ACSF group during the first 8 CS presentations of the extinction session ($t_{(23)} = 2.752$, $p = 0.011$). Thus, blocking NMDA receptors in the RSC only disrupted freezing for the trace animals. As delay animals were able to express normal levels of freezing in the presence of the same manipulation, this finding suggests that inhibiting NMDA receptors in the RSC does not generally impair freezing behavior, but may more selectively block the retrieval of trace fear memory.

On day 3, animals were given a drug-free extinction retention test in order to assess their memory for the extinction session they received in the presence of APV or vehicle (Fig. 7f). APV infusions into the RSC impaired trace extinction memory without

affecting extinction memory retention in the delay group. No differences in freezing were observed during the during the pre-CS baseline period of the test session for either delay ($t_{(25)}$ = 0.776, p = 0.585) or trace ($t_{(23)}$ = -0.685, p = 0.500) animals (data not shown).

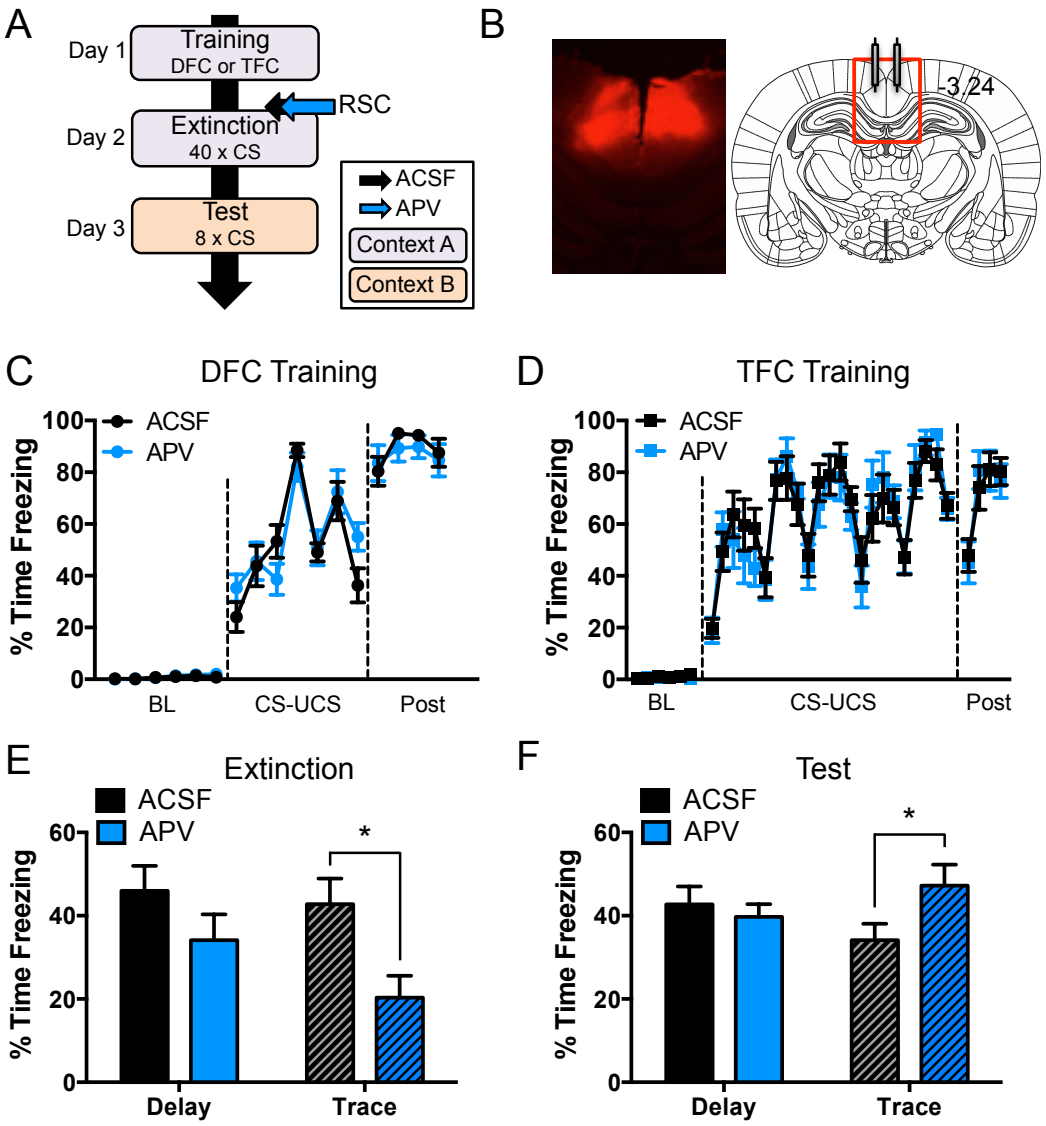

Figure 7. Blocking NMDA receptors in the retrosplenial cortex disrupts the retrieval and extinction of trace, but not delay fear conditioning. (A) The experimental timeline. Before extinction, delay- or trace- trained animals were given intra-RSC infusions of ACSF (DFC n = 14; TFC n = 13) or APV (DFC n = 13; TFC n = 12). (B) Sample image illustrating the infusion site with an intra-RSC infusion of Alexa 594 secondary antibody. (C-D) Mean percent time freezing during each minute of the training session for delay (C) and trace (D) animals. (E) Mean percent time freezing during the first 8 CS presentations of the extinction training session. Trace animals showed impaired memory retrieval in the presence of APV. (F) Mean percent time freezing during all 8 CS presentations of the test session. Trace animals given APV showed impaired extinction retention relative to trace vehicle animals. *indicates that $p < 0.05$

During the CS, no differences in freezing levels were observed for delay animals ($t_{(25)}$ = 0.554, p = 0.585). For the trace group, however, an average of all 8 CS trials during the test session revealed significantly higher freezing for APV-infused animals compared to vehicle controls animals ($t_{(23)}$ = 2.464, p = 0.022). These results demonstrate that NMDA receptors in the RSC are important for both the retrieval of trace fear memory (Fig. 7e) and the extinction of trace fear (Fig. 7f). The RSC does not appear to play a key role in either the retrieval or the extinction of delay fear associations, however.

Experiment 2. NMDA receptors in the PL mPFC are necessary for the extinction of trace, but not delay fear conditioning.

In Experiment 2, we tested whether NMDA receptor-dependent plasticity in the prelimbic medial prefrontal cortex is required for trace or delay fear extinction. To test this, animals were first implanted with PL cannulae. In order to ensure that our cannulae placement correctly targeted the RSC, we injected a subset of the animals (n=3) with a methylene blue stain after the experiment and visualized its spread (Fig. 8b). The focal point of the injection was in the middle of the PL, with the spread largely contained to the PL region. After recovering from surgery, animals were trained with strength-matched DFC or TFC on day 1. During training, all animals acquired fear normally (Fig 8c-d) and no group differences were observed in the levels of postshock freezing for either delay ($t_{(12)}$ = 1.049, p = 0.315; Fig. 8c) or trace ($t_{(14)}$ = -0.594, p = 0.562; Fig. 8d) animals.

On day 2, animals were infused into the PL with APV or vehicle (ACSF) before extinction (Fig. 8a). The first 8 trials of the extinction session were analyzed to determine whether the retrieval or expression of the fear memory was affected by drug infusion.

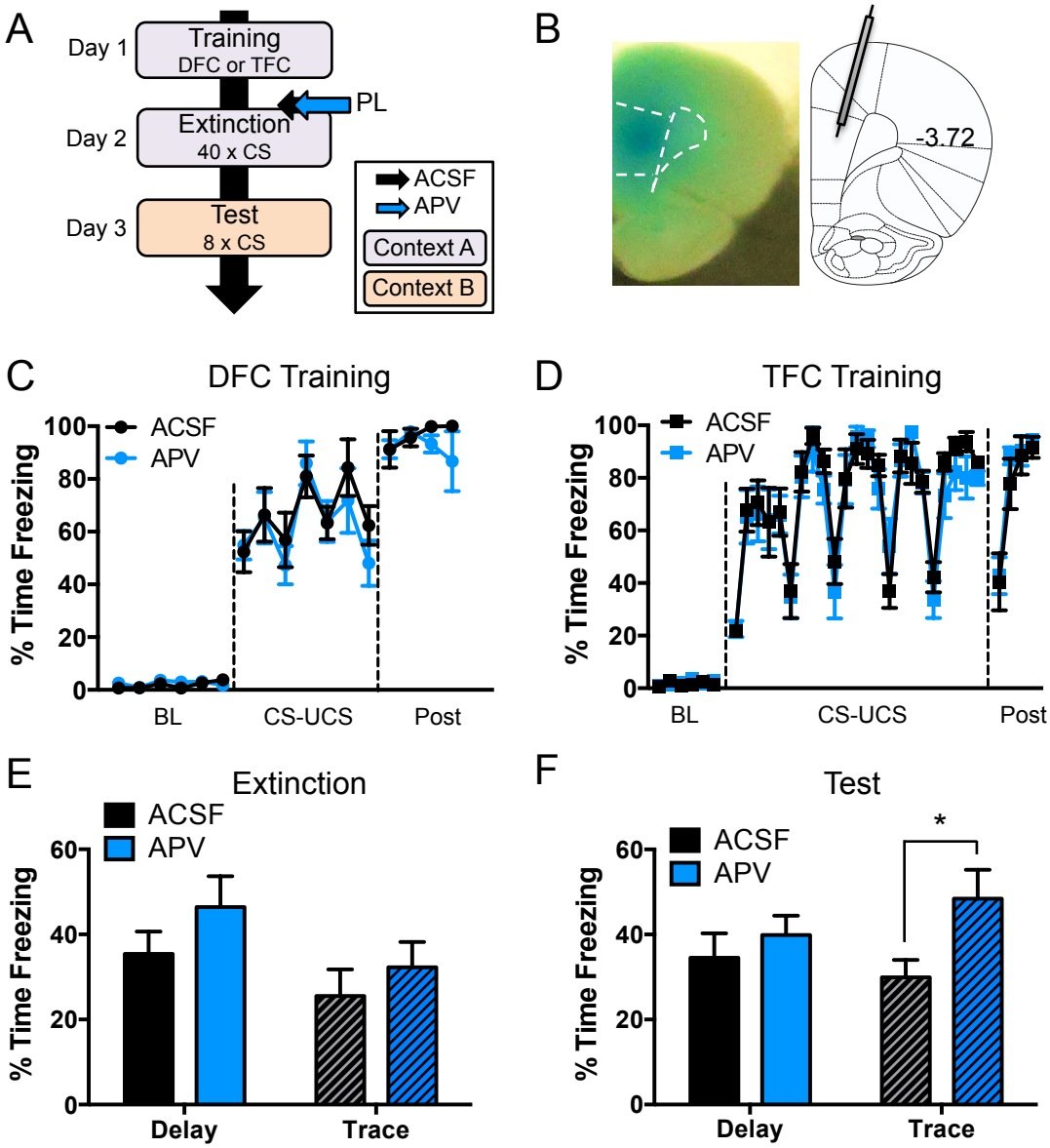

Figure 8. Blocking NMDA receptors in the prelimbic cortex disrupts the retention of delay but not trace fear extinction. (A) The experimental timeline. Before extinction, delay- or trace- trained animals were given intra-PL infusions of ACSF (DFC *n* = 7; TFC *n* = 8) or APV (DFC *n* = 7; TFC *n* = 8). (B) Sample image illustrating the infusion site with an intra-PL infusion of methylene blue. (C-D) Mean percent time freezing during each minute of the training session for delay (C) and trace (D) animals. (E) Mean percent time freezing during the first 8 CS presentations of the extinction training session. APV infusion did not affect freezing for either delay or trace animals during the extinction session. (F) Mean percent time freezing during all 8 CS presentations of the test session. Trace APV animals show impaired extinction relative to trace vehicle animals. *indicates that $p < 0.05$

(8e). There was no effect of APV infusion on freezing levels during the first 8 CS presentations of the extinction training session for either delay ($t_{(12)} = -1.230$, $p = 0.242$) or trace ($t_{(14)} = -0.787$, $p = 0.445$) animals. This suggests that NMDA receptor blockade

in the PL does not disrupt freezing expression or memory retrieval for either delay or trace animals.

On day 3, animals were given a drug-free extinction retention test (Fig. 8f). APV infusion into the PL impaired retention of trace fear extinction without affecting delay extinction memory. No differences in freezing were observed during the pre-CS baseline period of the test session for either delay ($t_{(12)} = 0.000$, $p = 1.000$) or trace ($t_{(14)} = -1.033$, $p = 0.319$) animals (data not shown). For delay animals, freezing during an average of all 8 CS trials revealed no significant difference between vehicle and APV animals ($t_{(12)} = -0.732$, $p = 0.478$), indicating that the drug did not impair extinction memory. For trace animals, on the other hand, APV animals showed significantly higher freezing to the CS than vehicle controls ($t_{(25)} = 0.458$, $p = 0.651$). Together, these findings suggest that NMDA receptors in the PL are necessary for trace fear extinction but are not required for the extinction of delay fear.

Experiment 3. NMDA receptors in the IL mPFC are involved in both delay and trace fear extinction.

In our third experiment, we tested whether NMDA receptor-dependent plasticity in the infralimbic medial prefrontal cortex is required for trace or delay fear extinction. In this study, we used two types of cannulae placements to target the IL: straight cannulae inserted directly above the target region and cannulae angled towards the midline at 30 degrees to avoid damaging the PL region just dorsal to the IL. In order to ensure that both cannulae types appropriately targeted the IL, we infused a fluorescent secondary antibody into the IL in a subset of these animals (straight n=3; angled n=3) and visualized its

spread. Infusions through both straight and angled cannulae covered the IL region appropriately (Fig. 9b).

These animals were trained with strength-matched delay or trace fear conditioning on day 1 (Fig. 9a-b). All animals showed normal acquisition (Fig. 9c-d) and no group differences were observed in the levels of postshock freezing for either delay ($t_{(27)}$ = -0.189, p = 0.852; Fig 9c) or trace ($t_{(25)}$ = -0.622, p = 0.540; Fig. 9d) animals.

On day 2, animals were infused into the IL mPFC with either APV or vehicle (ACSF) before extinction (Fig. 9b). The first 8 trials of extinction were analyzed to determine whether the retrieval or expression of the fear memory was affected by this infusion (Fig. 9e). Both delay and trace APV animals showed reduced freezing compared to vehicle controls during the extinction session. Delay animals given APV showed significantly lower freezing than vehicle animals during the first 8 CS presentations ($t_{(27)}$ = 6.831, $p < 0.001$). Similarly, trace APV animals also showed significantly lower freezing than vehicle controls during the first 8 CS presentations ($t_{(25)}$ = 6.077, $p < 0.001$). Blocking NMDA receptors, therefore, impaired freezing expression for both delay and trace animals during the extinction session.

On day 3, animals were given a drug-free extinction retention test (Fig. 9f). Intra-IL infusion of APV enhanced extinction for both delay and trace animals. No differences in baseline freezing were observed before the first CS presentation for either delay ($t_{(27)}$ = 0.830, p = 0.414) or trace ($t_{(25)}$ = 1.066, p = 0.297) animals (data not shown). An average of all 8 CS trials, however, revealed significantly reduced freezing in delay animals given APV relative delay ACSF animals ($t_{(27)}$ = 3.342, p = 0.002). Similarly, trace animals given APV also showed significantly lower freezing during the CS compared to trace

vehicle animals ($t_{(25)} = 2.973$, $p = 0.007$). This suggests that inhibiting NMDA receptors in the IL enhances extinction for both delay and trace associations.

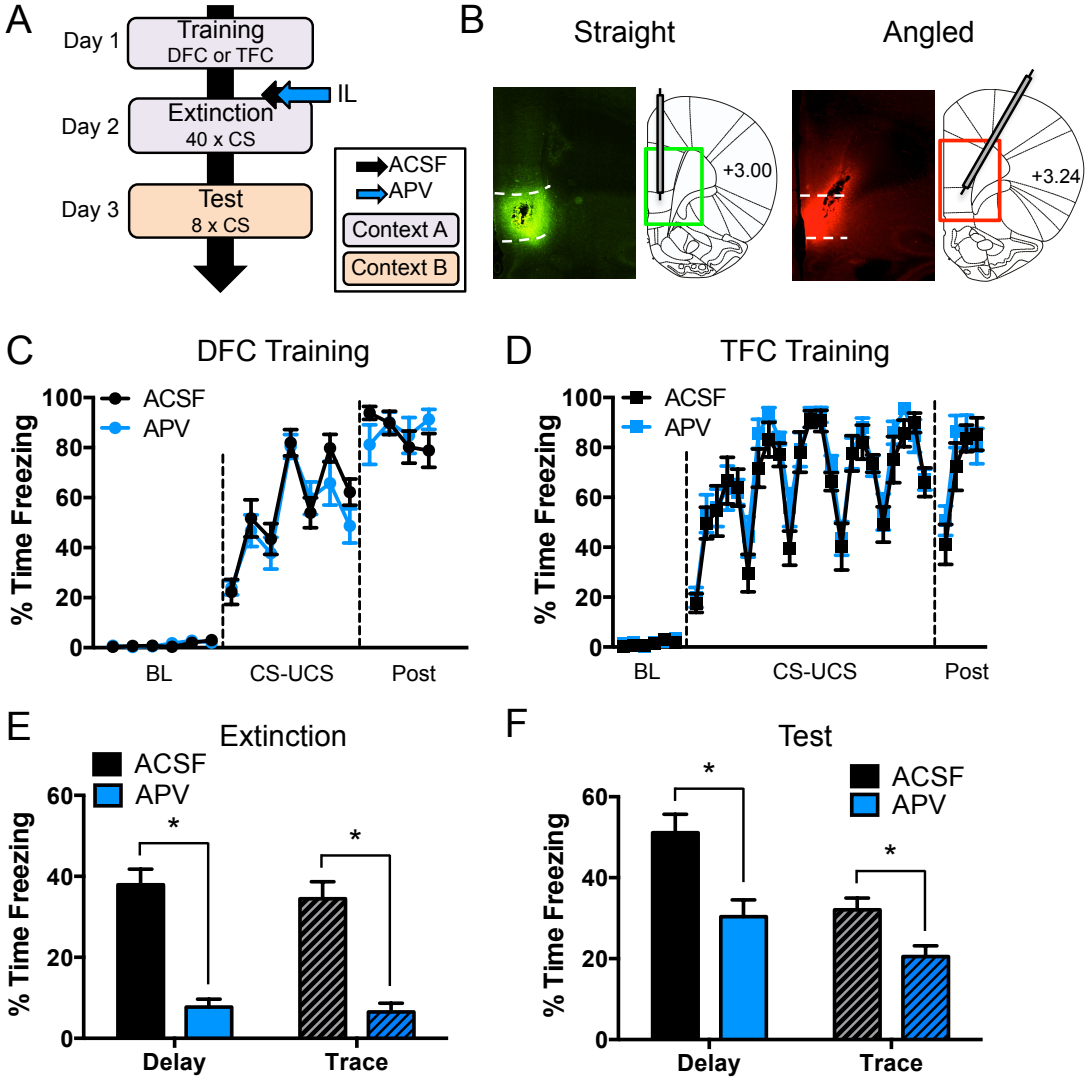

Figure 9. Blocking NMDA receptors in the infralimbic cortex disrupts the expression and enhances the extinction of both delay and trace fear conditioning. (A) The experimental timeline. Before extinction, delay- or trace- trained animals were given intra-IL infusions of ACSF (DFC n = 15; TFC n = 13) or APV (DFC n = 14; TFC n = 14). (B) Sample images illustrating the infusion site with an intra-IL infusion of Alexa 488 or 594 secondary antibodies. The image on the left shows a sample infusion for the straight cannulae and the image on the right shows a sample infusion for the angled cannulae. (C-D) Mean percent time freezing during each minute of the training session for delay (C) and trace (D) animals. (E) Mean percent time freezing during the first 8 CS presentations of the extinction training session. Both delay and trace animals show impaired freezing in the presence of APV in the IL. (F) Mean percent time freezing during all 8 CS presentations of the test session. Both delay and trace animals show enhanced extinction relative to their vehicle controls. *indicates that $p < 0.05$

As previously mentioned, we used two types of cannulae placements to target the IL: straight cannuale inserted directly above the target region and cannulae angled toward the midline at 30 degrees to avoid damaging the PL region just dorsal to the IL. APV infusion into both types of cannulae produced the same general behavior pattern (Fig. 10). During the extinction session, we observed significant decreases in freezing for both delay and trace animals given APV through both straight (Delay: $t_{(25)} = 6.077$, $p < 0.001$; Trace: $t_{(25)} = 6.077$, $p < 0.001$) and angled (Delay: $t_{(25)} = 6.077$, $p < 0.001$; Trace: $t_{(25)} =$

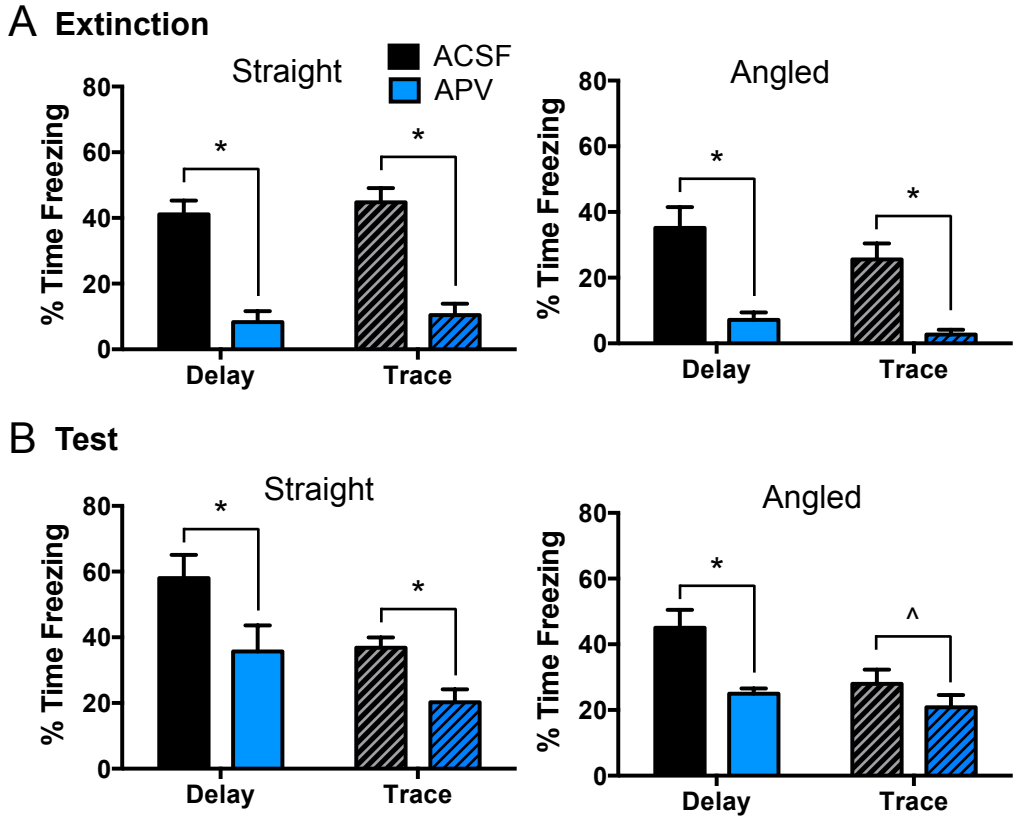

Figure 10. Similar behavioral results are obtained using straight and angled cannulae to infuse APV into the IL. (A) Average freezing during the first 8 CS presentations of the extinction session broken down by cannulae type. The left panel shows animals given APV (DFC n = 7; TFC n = 6) or ACSF (DFC n = 7; TFC n = 7) infusions through straight IL cannulae. The right panel shows animals infused with ACSF (DFC n = 8; TFC n = 7) or APV (DFC n = 7; TFC n = 7) through angled IL cannulae. For both cannulae types, APV impairs freezing expression during the extinction session. (B) Average freezing during all 8 CS presentations in the test session, broken down by cannulae type. The left panel shows animals infused with APV through straight IL cannulae. The right panel shows animals infused through angled IL cannulae. For both cannulae types, APV enhances extinction for both delay and trace animals (although this effect is not significant for trace animals infused through angled cannulae). *indicates that $p < 0.05$. ^ indicates that $p = 0.236$, not significant.

6.077, $p < 0.001$) cannulae (Fig. 10a). During the extinction retention test, we observed decreased freezing in delay and trace APV animals for both cannulae types. This effect was significant for both delay ($t_{(25)} = 6.077$, $p < 0.001$) and trace ($t_{(25)} = 6.077$, $p < 0.001$) animals infused through straight cannulae (Fig. 10b, left). For angled cannulae (Fig 10b, right), delay animals showed significantly reduced freezing relative to ACSF controls ($t_{(25)} = 6.077$, $p < 0.001$) while trace animals given APV showed a nonsignificant decrease in freezing ($t_{(25)} = 6.077$, $p < 0.001$). Trace ACSF animals had low freezing levels at test, making it difficult to detect any enhanced extinction in the APV animals. Regardless, both types of cannulas produced similar behavior patterns, indicating that APV infusion in the IL enhanced extinction retention regardless of damage to the PL. Together, our results demonstrate that NMDA receptor blockade in the IL affects delay and trace extinction in the same manner: it impairs recall of both delay and trace fear and enhances extinction learning for both types of conditioning.

Experiments 4-6: Is the retrosplenial cortex involved in the consolidation of trace or delay fear?

Our research indicates that trace and delay fear extinction rely on different neural circuits. While the amygdala is important for delay fear extinction, the retrosplenial and prelimbic cortices seem to be important for trace extinction. Why are different circuits required for the extinction of trace and delay fear? One possible explanation is that the TFC association is stored in different structures than the DFC association, causing different structures to participate in the extinction process (see Fig. 5). In other words, if the TFC association is stored in a distributed network of cortical areas, including the RSC

and PL, rather than the amygdala, one might expect these cortical areas, and not the amygdala, to be required for that association's extinction. Evidence already exists to support the participation of the mPFC in storing the trace fear association (Blum, Runyan, et al., 2006; Runyan & Dash, 2004; Runyan et al., 2004), yet no one has tested whether the RSC is involved in storing trace fear conditioning.

In order to determine whether the RSC plays a role in storing TFC, we ran Experiments 4-6 to test whether blocking protein synthesis in the RSC could impair consolidation of trace fear. If the RSC is involved in TFC memory storage, it should participate in the consolidation process so that blocking protein synthesis in this region should impair the storage of TFC.

Experiment 4. Blocking protein synthesis in the RSC immediately after training has no effect on DFC or TFC memory consolidation.

In Experiment 4, we tested whether blocking protein synthesis in the retrosplenial cortex immediately after DFC or TFC acquisition would block memory consolidation. To this end, animals implanted with RSC cannulae were trained with strength-matched DFC or TFC on day 1 followed immediately by an injection of anisomycin or vehicle (ACSF) into the RSC (Fig. 11a-b). All animals showed normal acquisition (Fig. 11c-d) and no group differences were observed in the levels of postshock freezing for either delay ($t_{(13)}$ = -0.873, p = 0.398; Fig. 11c) or trace ($t_{(14)}$ = 0.774, p = 0.774; Fig. 11d) animals.

Testing on day 2 revealed no significant drug effects for either delay or trace animals (Fig. 11e-f). During the CS test, an average of the first 4 CS trials revealed no significant difference between vehicle and anisomycin animals for either the DFC ($t_{(13)}$ =

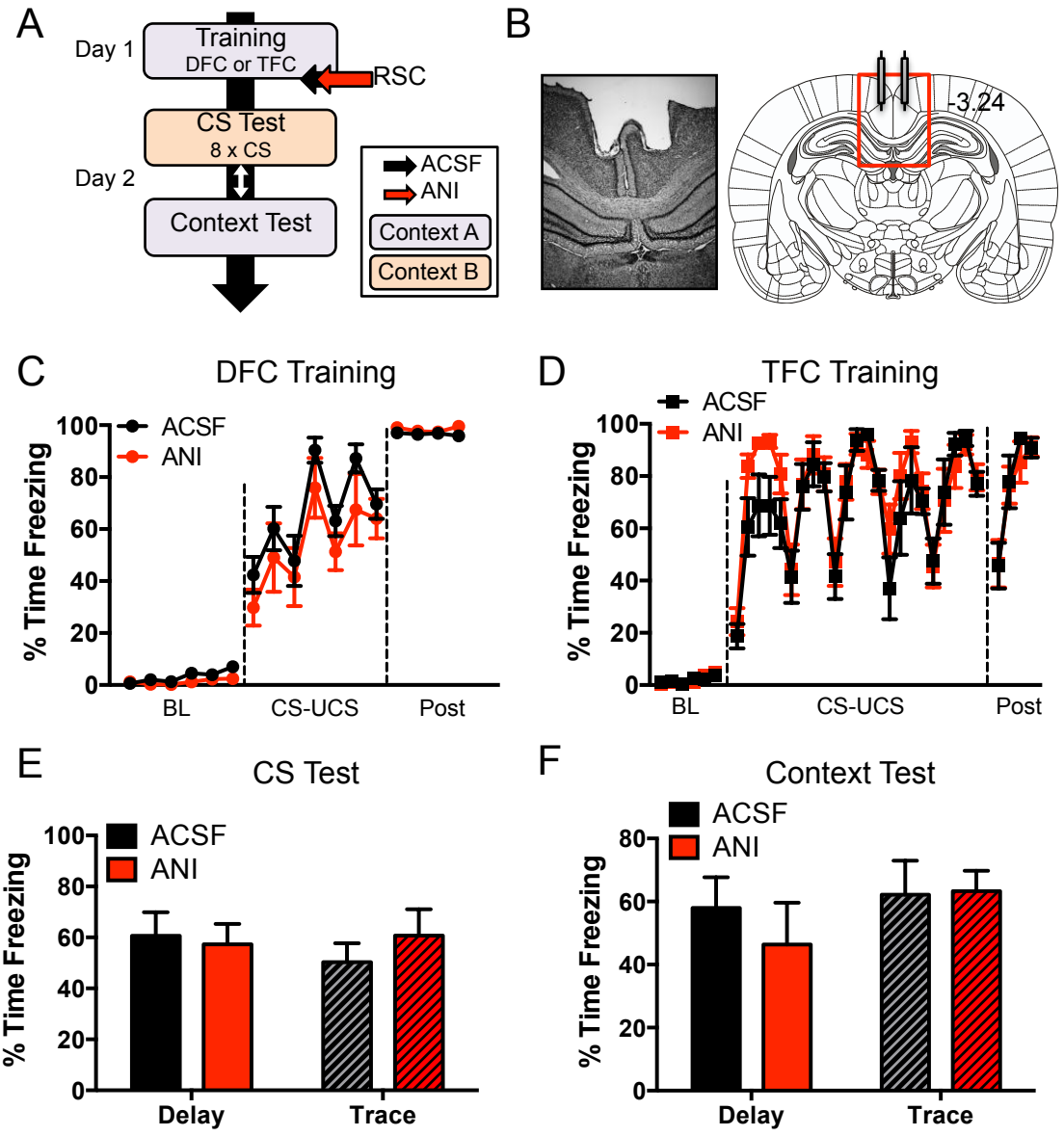

Figure 11. Blocking protein synthesis in the retrosplenial cortex immediately after training does not affect consolidation of either trace or delay fear conditioning. (A) The experimental timeline. Immediately after delay or trace conditioning, animals were given intra-RSC infusions of either ACSF (DFC *n* = 8; TFC *n* = 8) or ANI (DFC *n* = 7; TFC *n* = 8). (B) Sample Nissl stained image illustrating the infusion site. (C-D) Mean percent time freezing during each minute of the training session for delay (C) and trace (D) animals. (E) Mean percent time freezing during the first 4 CS presentations of the white noise CS test session. APV did not affect freezing levels for either trace or delay animals. (F) Mean percent time freezing during the first 4 minutes of the context test. APV did not affect context freezing for either delay or trace animals.

0.270, $p = 0.792$) or TFC ($t_{(14)} = -0.816$, $p = 0.428$) groups (Fig. 11e). Similarly, no differences were observed between drug conditions during the context test, as assessed by comparing the average freezing during the first 4 minutes (Fig. 11f; DFC: $t_{(13)} = 0.717$, p

= 0.486; TFC: $t_{(14)}$ = -0.089, p = 0.930). This suggests that protein synthesis in the RSC after conditioning may not be required for TFC or DFC consolidation.

Experiment 5. NMDA receptors in the RSC are required for the retrieval of trace, but not delay fear memory.

While it is possible that protein synthesis in the RSC is not required for trace or delay fear extinction, it is also possible that our infusion procedure was ineffective or timed incorrectly. In order to rule out the possibility that our cannulae placement and infusion procedures were effective, we ran a follow-up study using the animals from Experiment 4. Four days after the completion of Experiment 4, the animals were regrouped and infused with APV or vehicle before a CS test (Fig 12a). We have previously shown that NMDA receptor blockade during a CS test impairs memory retrieval for trace, but not delay fear (Fig. 7e), so if our infusion procedure is effective, we should observe impaired freezing at test while APV is present.

We ensured that the new ACSF and APV groups showed similar CS freezing before we began this experiment. There were no significant difference in freezing levels during the initial CS test for either delay ($t_{(13)}$ = -0.861, p = 0.405) or trace ($t_{(14)}$ = 0.937, p = 0.364) animals before drug injection. These animals were infused with either APV or ACSF approximately 5 minutes before being given a CS test (the "retrieval" test). During the retrieval test, we observed no significant differences in freezing during the pre-CS baseline period for either delay ($t_{(13)}$ = 0.646, p = 0.530) or trace ($t_{(14)}$ = 0.983, p = 0.342) animals (data not shown). For delay animals, freezing during an average of the first 4 CS presentations revealed no significant difference between vehicle and APV animals ($t_{(13)}$ =

-0.120, $p = 0.906$), indicating that the drug did not impair memory retrieval (Fig. 12b). For trace animals, on the other hand, APV animals showed significantly reduced freezing to the CS compared to vehicle controls (Fig. 4b; $t_{(14)} = 2.653$, $p = 0.019$). When tested drug-free the following day (i.e. the "recovery" test), no drug effects were observed for either trace ($t_{(14)} = -0.145$, $p = 0.887$) or delay ($t_{(13)} = 0.061$, $p = 0.952$) animals (Fig. 12c). These results support our previous finding that NMDA receptors in the RSC play a key role in memory retrieval for trace, but not delay fear. Further, they indicate that this effect is transient and the memory fully recovers the following day. Finally, as we observed a positive effect in these animals, we can rule out the possibility that our null effect from Experiment 4 was due to an ineffective infusion volume or cannulae placement.

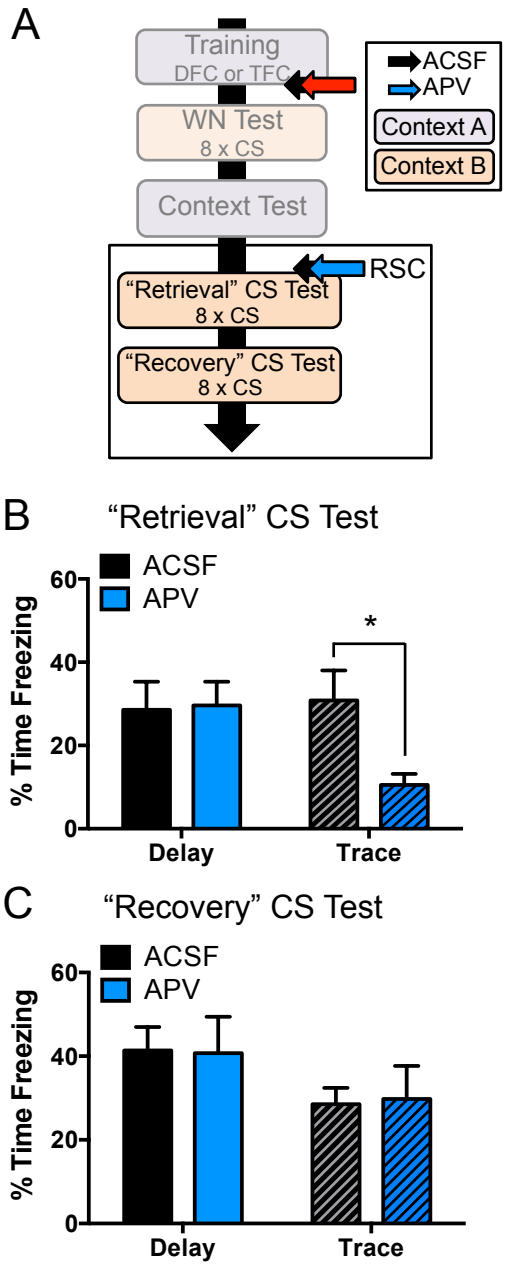

Figure 12. Blocking NMDA receptors in the retrosplenial cortex transiently disrupts the retrieval of trace, but not delay fear. (A) The experimental timeline. Animals were given intra-RSC infusions of ACSF (DFC $n = 7$; TFC $n = 8$) or APV (DFC $n = 8$; TFC $n = 8$) five minutes before the "retrieval" CS test. (B) Mean percent time freezing during the first 4 CS presentations of the "retrieval" white noise test. Only trace animals showed impaired memory retrieval in the presence of intra-RSC APV. (C) Mean percent time freezing during the first 4 CS presentations of the "recovery" white noise test. Trace animals showed normal freezing after the drug was metabolized, indicating that blocking NMDA receptors in the RSC transiently impaired trace memory retrieval. *indicates that $p < 0.05$

Experiment 6: Blocking protein synthesis in the RSC before training impairs context and TFC fear

In this experiment, we tested whether blocking protein synthesis in the RSC before training would block the acquisition of trace or delay fear. We observed no effect when we inhibited protein synthesis immediately after training (Experiment 4), but it is possible that our infusion was too late to be effective. Specifically, it is possible that an earlier wave of protein synthesis in the RSC (before our post-training anisomycin infusion was able to block protein synthesis) was required for successful consolidation. We tested this possibility in Experiment 6. The procedure for this study was identical to that of Experiment 4, except that we gave the injection 15 minutes before, rather than immediately after training (Fig. 13a). All animals showed normal acquisition following drug infusion (Fig. 13b-c). Importantly, no group differences were observed in the levels of postshock freezing for either delay ($t_{(27)} = 0.691$, $p = 0.495$) or trace ($t_{(27)} = -0.609$, $p = 0.548$) animals, indicating that infusion of anisomycin did not impair freezing expression during training.

On day 2, animals were tested to both the white noise and the context in a counterbalanced manner. Infusing anisomycin before training reduced freezing to the CS in TFC animals and context freezing in both delay and trace animals (Fig 13d-e). For the CS test, we observed no differences in baseline freezing before the first CS presentation for either delay ($t_{(27)} = -0.059$, $p = 0.953$) or trace ($t_{(27)} = -1.189$, $p = 0.245$) animals (data not shown). An average of the first 4 CS trials revealed similar levels of freezing between both delay groups (Fig. 13d; $t_{(27)} = 1.353$, $p = 0.187$). For trace animals, we observed a significant reduction in freezing to the CS in trace animals given anisomycin relative to

their vehicle controls (Fig. 13d; $t_{(27)} = 2.753$, $p = 0.010$). Context freezing, on the other hand, was reduced by intra-RSC anisomycin for both delay and trace animals (Fig. 13e). An average of the first four mintues of the context test revealed that both delay ($t_{(27)} =$

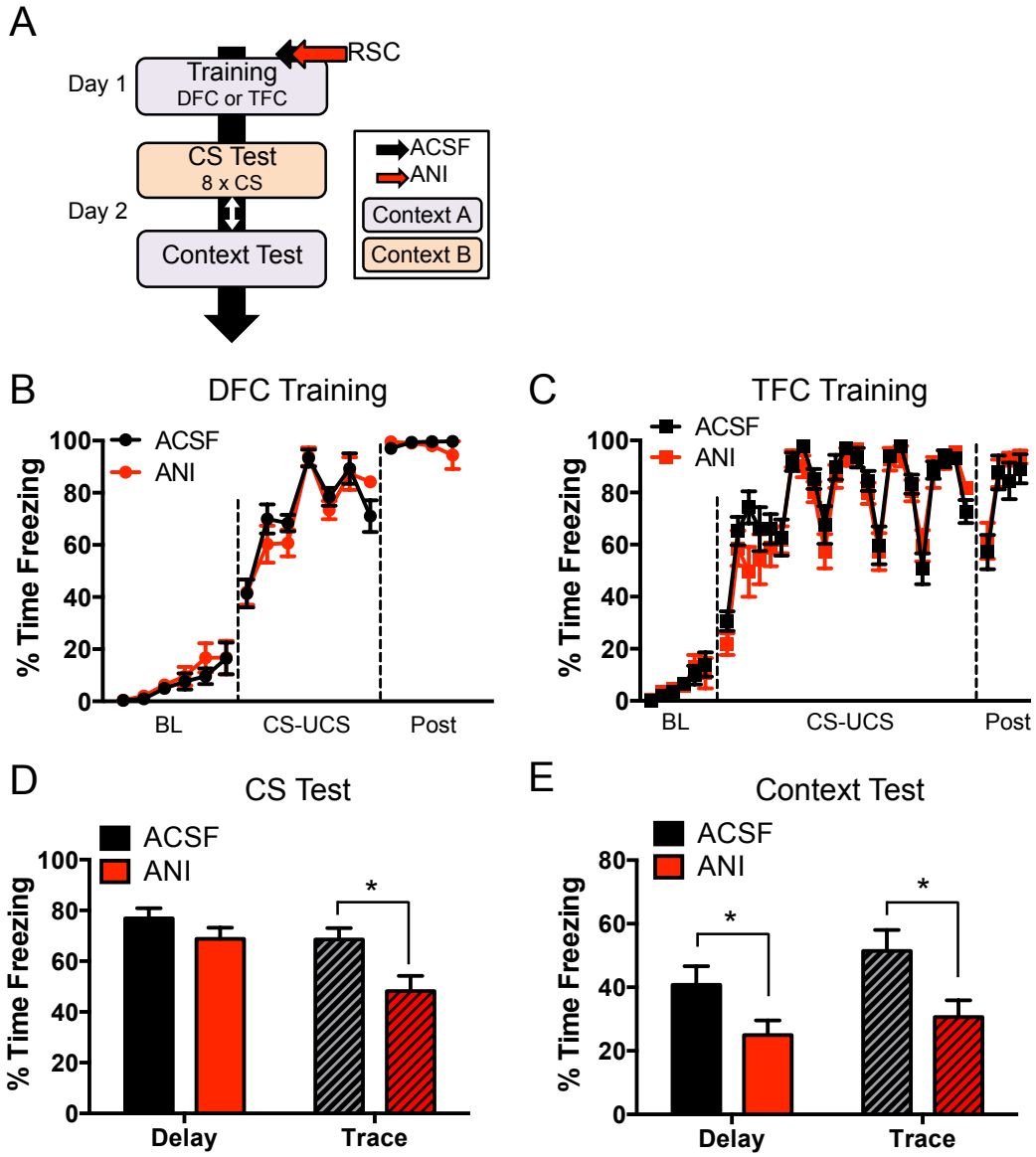

Figure 13. Blocking protein synthesis in the retrosplenial cortex before training impairs context and trace CS fear without affecting delay CS fear. (A) The experimental timeline. Fifteen minutes before delay or trace conditioning, animals were given intra-RSC infusions of either ACSF (DFC *n* = 15; TFC *n* = 16) or ANI (DFC *n* = 14; TFC *n* = 13). (B-C) Mean percent time freezing during each minute of the training session for delay (B) and trace (C) animals. (D) Mean percent time freezing during the first 4 CS presentations of the white noise CS test session. Intra-RSC anisomycin infused before acquisition impaired CS freezing for trace, but not delay animals (E) Mean percent time freezing during the first 4 minutes of the context test session. Anisomycin impaired context freezing for both delay and trace animals. *indicates that $p < 0.05$

2.099, $p = 0.045$) and trace ($t_{(27)} = 2.389$, $p = 0.024$) animals given anisomycin showed a significant reduction in freezing compared to vehicle animals. These results demonstrate that protein synthesis is required in the RSC for the consolidation of TFC CS fear and context fear for both delay and trace conditioning. Together with Experiments 4 and 5, our results indicate that protein synthesis in the RSC is required either during or immediately after conditioning for the proper consolidation of TFC CS fear and context fear for both DFC and TFC. Delay CS fear, on the other hand, was not affected by intra-RSC anisomycin infusion.

Experiments 7-8: Can a single delay trial be used to update a complex trace fear memory and change the neural circuitry supporting that association?

In Experiments 7 and 8, we tested whether a single trial of delay fear can be used to update a relatively complex trace fear association to a simpler circuit. We have previously shown that the circuits supporting trace and delay fear are distinct; extinction of trace fear requires the participation of the retrosplenial and prelimbic cortices (Figures 7-8) instead of the amygdala, which is required for delay fear extinction (Fig. 2). This distinction can be leveraged to test which circuit a fear memory relies on: if the amygdala is required for extinction, the memory relies on the delay circuit whereas if the amygdala is not required, the trace circuit is supporting the memory. In Experiments 7 and 8, we test whether a single DFC trial is sufficient to trigger reconsolidation and updating in TFC-trained animals and whether this updating process changes the neural circuit that supports the association.

Experiment 7: Exposing TFC-conditioned animals to a single trial of DFC triggers updating in the amygdala

In Experiment 7, we tested whether a single trial of DFC would be sufficient to trigger updating in the amygdala of animals trained with TFC. Diaz-Mataix and colleauges (2013) previously demonstrated that protein synthesis-dependent updating only occurs when new information is presented during the retrieval/update session, so presenting a trace-trained animal with a delay trial should trigger updating whereas presenting the same animal with another presentation of trace conditioning should not. Here, amygdala-cannulated animals were first pre-exposed to the training context over two days to familiarize animals to the context before training (Fig. 14a). No differences were observed between groups on average during the pre-exposure sessions, (DFC update: $t_{(12)} = -0.718$, $p = 0.487$; TFC update: $t_{(13)} = -1.265$, $p = 0.228$). On day 3, all animals were trained with our 6-trial TFC procedure (Fig. 14b-c). All of the animals acquired fear normally and no group differences were observed in the levels of postshock freezing for either DFC update (Fig. 14b; $t_{(12)} = -0.281$, $p = 0.783$) or TFC update (Fig. 14c; $t_{(13)} = -0.484$, $p = 0.636$) animals.

On day 4, animals were given an update session in which they were presented with either a single trial of DFC (shift group; Fig. 14d) or TFC (no shift group; Fig. 14e). During this update, we observed no significant differences in freezing levels during the baseline period (DFC update: $t_{(12)} = 0.350$, $p = 0.732$; TFC update: $t_{(13)} = -0.312$, $p = 0.760$), during the CS period (DFC update: $t_{(12)} = 0.435$, $p = 0.671$; TFC update: $t_{(13)} = 1.203$, $p = 0.250$), the post-shock period (DFC update: $t_{(12)} = -0.529$, $p = 0.607$; TFC update: $t_{(13)} = 0.590$, $p = 0.565$) or during the trace interval period of the TFC update ($t_{(13)}$

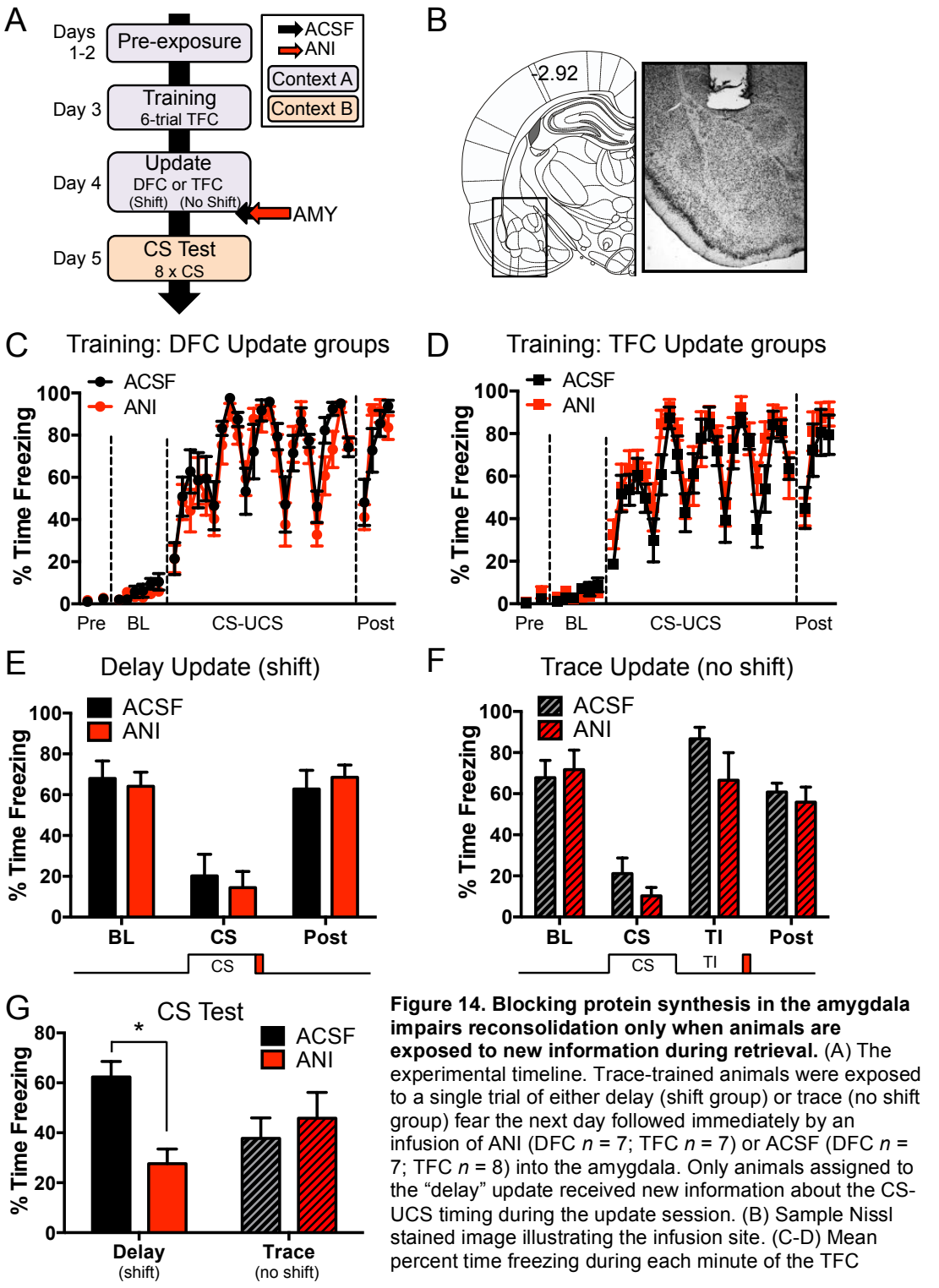

Figure 14. Blocking protein synthesis in the amygdala impairs reconsolidation only when animals are exposed to new information during retrieval. (A) The experimental timeline. Trace-trained animals were exposed to a single trial of either delay (shift group) or trace (no shift group) fear the next day followed immediately by an infusion of ANI (DFC *n* = 7; TFC *n* = 7) or ACSF (DFC *n* = 7; TFC *n* = 8) into the amygdala. Only animals assigned to the "delay" update received new information about the CS-UCS timing during the update session. (B) Sample Nissl stained image illustrating the infusion site. (C-D) Mean percent time freezing during each minute of the TFC training session for animals assigned to the delay update (C) and trace update (D) conditions. (D-E) Mean percent time freezing during each period of the update session. (E) Average freezing during the baseline, CS, and post-shock period of the delay (shift) update session. No difference in freezing were observed between drug conditions. (F) Average freezing during the baseline, CS, trace interval, or post-shock period of the trace update session. No differences were observed between drug conditions. (G) Average freezing during the CS test. Anisomycin impaired freezing in animals given the shifted delay update but had no effect in animals given the non-shifted trace update. *indicates that $p < 0.05$

= 1.458, $p = 0.169$). Our groups therefore showed similar levels of freezing during the update session, before drug infusion. Immediately after this update session, animals were infused into the amygdala with either anisomycin (to block any reconsolidation triggered in the amygdala by this update procedure) or vehicle.

All animals were given a CS test on day 5 (Fig. 14f). Anisomycin infusion impaired memory in animals updated with a shifted DFC trial but had no effect on animals given a trace trial identical to that used in training. We observed no significant difference in freezing levels during the baseline period of the CS test for either DFC-updated ($t_{(12)} = -0.179$, $p = 0.861$) or TFC-updated ($t_{(13)} = 0.192$, $p = 0.851$) animals. An average of the first 3 CS presentations revealed a significant drug effect for DFC-update (shift) animals, with anisomycin animals freezing at significantly lower levels than their vehicle counterparts ($t_{(12)} = 4.087$, $p = 0.002$). TFC-updated (no shift) animals, on the other hand showed no effect of anisomycin ($t_{(13)} = -0.619$, $p = 0.547$). These results indicate that trace fear can be updated in the amygdala with a single trial of delay fear conditioning. Further, our results indicate that this updating process is not triggered if the retrieval trial is identical to that presented during the initial training session.

Experiment 8: Updating TFC-trained animals with a single trial of DFC changes the neural circuit required for extinction.

Our final experiment tested the hypothesis that updating a TFC memory with a single trial of DFC can change the neural circuit that supports the association, shifting the memory from the cortical trace fear circuit to the simpler, subcortical delay fear circuit. In order to identify which neural circuit supports the association after updating, we tested

whether the amygdala is involved in extinction. Our research demonstrates that trace and delay fear associations rely on distinct neural circuits for extinction; extinction of delay fear requires the participation of the amygdala whereas trace fear extinction does not (Fig. 2). Therefore, by testing whether the amygdala is required for extinction, we can determine whether the trace or delay fear circuit is involved: if the amygdala is not required, the memory is supported by the trace fear circuit despite the update. If, however, the amygdala is now required for extinction, this will suggest that the memory has been shifted to the delay fear circuit.

Here, animals were first trained and updated as in Experiment 7 (Fig. 15a). All animals were pre-exposed to the context on days 1 and 2. No group differences in freezing were observed on average during the pre-exposure sessions (DFC update: $t_{(13)} = 1.178$, $p = 0.260$; TFC update: $t_{(12)} = -0.483$, $p = 0.638$). On day 3, all animals were trained with 6-trial TFC. The animals all acquired fear normally and we observed no group differences in the level of postshock freezing for either the DFC update group (Fig. 15b; $t_{(13)} = -0.627$, $p = 0.542$) or the TFC update group (Fig. 15c; $t_{(12)} = 0.477$, $p = 0.642$).

On day 4, animals were updated as before with a single trial of either DFC (shift group; Fig. 15d) or TFC (no shift group; Fig. 15e). During this update, we observed no significant differences in freezing between drug groups during the baseline period (DFC update:$t_{(13)} = 0.45$, $p = 0.965$; TFC update: $t_{(12)} = 1.421$, $p = 0.181$), the CS period (DFC update: $t_{(13)} = 0.451$, $p = 0.660$; TFC update: $t_{(12)} = 0.564$, $p = 0.583$), the post-shock period (DFC update: $t_{(13)} = -2.065$, $p = 0.059$; TFC update: $t_{(12)} = 0.517$, $p = 0.615$) or during the trace interval period of the TFC update ($t_{(12)} = 1.669$, $p = 0.121$). Therefore, animals in all conditions showed similar freezing during the update session.

The following day, animals were infused into the amygdala with either APV or ACSF before extinction training. We have previously shown that blocking NMDA receptors in the amygdala with APV impairs extinction memory retention for delay, but not trace fear memories. During the extinction session (Fig. 15f), an average of the first 8 CS presentations revealed no significant differences in freezing levels between ACSF- and APV-infused animals for either the delay update group ($t(13) = -0.888$, $p = 0.391$) or the trace update group ($t_{(12)} = -0.028$, $p = 0.978$). During the extinction session, therefore, the animals displayed similar levels of freezing within each update group.

Finally, on day 6, animals were given a CS test to determine whether the blockade of NMDA receptors in the amygdala impacted their ability to remember the extinction session. We found that APV only impaired extinction memory in animals given the delay fear update session (Fig. 15g). We observed no differences in freezing levels during the baseline period of the CS test for either delay-updated ($t_{(13)} = 0.739$, $p = 0.473$) or trace-updated ($t_{(12)} = 0.801$, $p = 0.439$) animals. For animals given the delay update (shift group), averaging the first 3 CS test trials revealed that APV animals showed significantly higher freezing ($t_{(13)} = -2.852$, $p = 0.014$), indicating that their memory for extinction was disrupted by intra-amygdala NMDA receptor blockade. Animals given the trace update (no shift group) were not affected by the intra-amygdala APV infusion; there was no significant difference in freezing between ACSF and APV animals during the first 3 CS test trials for the trace update group ($t_{(12)} = -0.693$, $p = 0.502$). The delay update, therefore, changed the neural circuit required for extinction; whereas the amygdala was not required for animals in the trace-trace group, the amygdala was required for extinction in the trace-delay update group, even though these animals were initially

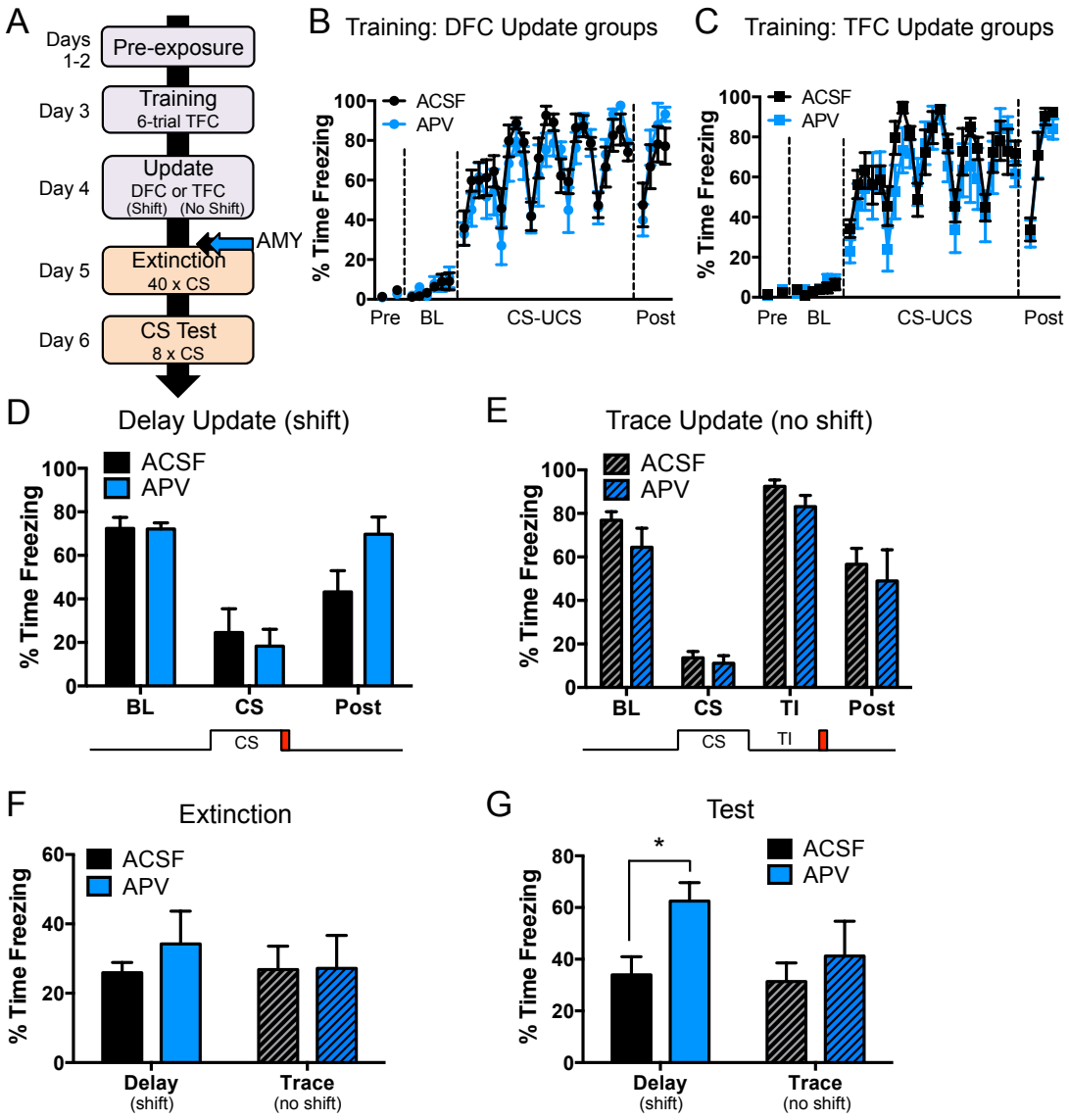

Figure 15. Blocking NMDA receptors in the amygdala impairs extinction in trace animals that are updated with a single trial of delay fear. (A) The experimental timeline. Trace-trained animals were updated with a single trial of delay (shift group) or trace (no shift group) fear as before. Here, the following day, the animals were extinguished in the presence of intra-amygdala APV (DFC n = 7; TFC n = 6) or vehicle (DFC n = 8; TFC n = 8) and tested the following day. (B-C) Mean percent time freezing during each minute of the TFC training session for animals assigned to the delay update (B) and trace update (C) conditions. (D-E) Mean percent time freezing during each period of the update session. (D) Average freezing during the baseline, CS, and post-shock period of the delay (shift) update session. No difference in freezing were observed between drug conditions. (E) Average freezing during the baseline, CS, trace interval, or post-shock period of the trace (no shift) update session. No differences were observed between drug conditions. (F) Average freezing during the first 8 CS presentations of the extinction session. No differences were observed in freezing between the drug groups. (G) Average freezing during the CS test. APV impaired extinction in animals given the shifted delay update but had no effect in animals given the non-shifted trace update. *indicates that $p < 0.05$

trained with trace fear conditioning. These results demonstrate that memory updating can be used to change not only the content of a memory but also the neural circuit responsible for supporting that association.

Discussion

The overall goal of this project was to provide a more complete understanding of the neural circuit supporting trace fear and to determine whether this circuit can be altered through the reconsolidation-dependent updating process. We have demonstrated that 1) the trace extinction circuit differs from that of delay, with the participation of the retrosplenial and prelimbic cortices instead of the amygdala 2) the infralimbic cortex plays a similar role in both the trace and delay extinction circuits, 3) the retrosplenial cortex is involved in the consolidation of trace and context, but not delay fear, and 4) the neural circuit supporting a complex trace fear memory can be updated and simplified by exposing the animals to a single presentation of a delay fear trial. Together, our results provide new information about how trace fear is stored, extinguished, and updated in the brain.

The neural circuit supporting trace fear extinction is distinctly different from that of delay fear extinction.

Experiments 1-3 aimed to characterize the neural circuit supporting trace fear extinction and to identify how the trace extinction circuit differs from that of delay. In these experiments, we systematically tested whether the prelimbic, retrosplenial, and infralimbic cortices were required for trace or delay fear extinction by inhibiting NMDA receptors in each structure before the extinction session. We found that the trace extinction circuit differed drastically from that of delay. Whereas the amygdala was required for delay fear extinction (see Fig. 2), the retrosplenial and prelimbic cortices were instead required for trace fear extinction (Fig. 7-8). The infralimbic cortex was

involved in extinguishing both types of associations, indicating that this structure may be involved in extinction learning regardless of the type of initial learning. Together, these results demonstrate that trace fear extinction requires a distributed cortical circuit for extinction, consistent with our hypotheses (see Fig. 5).

Extinction circuit: Retrosplenial cortex

In the retrosplenial cortex, we found that NMDA receptor blockade impaired extinction for trace, but not delay animals. This supports our preliminary data showing that ERK phosphorylation increases in the RSC following trace, but not delay extinction. Plasticity in the RSC therefore appears to be crucial to extinction learning in trace, but not delay fear conditioning.

Interestingly, we observed that trace memory retrieval (assessed by averaging the first 8 trials of the day 2 extinction training session) was disrupted when APV was infused into the RSC, while delay memory retrieval was not affected by this infusion. RSC infusions do not simply prevent freezing expression, however, as freezing levels were normal in DFC animals. This suggests that inhibiting NMDA receptors in the RSC selectively prevented the retrieval of TFC memory. This finding is similar to the results of previous studies in which RSC manipulations (either lesions or NMDA receptor blockade) in animals trained with delay fear conditioning were shown to impair context memory retrieval without affecting fear to the discrete delay CS (Corcoran et al., 2011; Keene & Bucci, 2008a, 2008c). In particular, Keene & Bucci (Keene & Bucci, 2008a) demonstrated that lesions of the RSC one day after delay fear conditioning impaired freezing to the context without affecting fear to the auditory delay CS. It therefore

appears that retrieving fear to the discrete CS in trace conditioning relies on cortical structures that also participate in context fear retrieval. Delay fear, on the other hand, does not seem to require this complex circuit for retrieval. Together, these studies indicate that NMDARs in the RSC are required for the retrieval of complex contextual and trace fear associations but are not required for the retrieval of CS fear for delay fear conditioning.

Extinction circuit: Prelimbic cortex

In the prelimbic cortex, we found that blocking NMDA receptors disrupted extinction for trace, but not delay animals. This is consistent with our preliminary data showing that ERK phosphorylation increases in the PL following trace, but not delay extinction. Further, this effect is similar to what we observed in the RSC, suggesting that these regions play a similar, coordinated role in supporting trace fear extinction. Plasticity in the PL therefore appears to be important for trace, but not delay extinction.

During the extinction session, we observed no effect of intra-PL NMDA receptor inhibition on retrieval or expression of the fear memory for either trace or delay animals. This indicates that NMDA receptors in the PL are not involved in retrieving or expressing either trace or delay fear. The RSC and PL therefore play different roles in the retrieval process; whereas NMDA receptors in the RSC are necessary for both trace retrieval and extinction, NMDA receptors in the PL are specifically involved in the trace extinction process. Although both the RSC and PL are important for trace extinction, they may be playing different, though coordinated roles in the extinction process.

The prelimbic cortex is known to play a role in trace fear acquisition (Gilmartin & Helmstetter, 2010; Gilmartin & McEchron, 2005b; Runyan et al., 2004) and storage (Blum, Runyan, et al., 2006; Runyan & Dash, 2004; Runyan et al., 2004) but is not necessary to acquire basic delay fear conditioning (Gilmartin & Helmstetter, 2010). The PL is also involved in the acquisition of context fear in both trace and delay animals (Gilmartin & Helmstetter, 2010). Here, we demonstrate that NMDA receptors in the PL are also necessary for trace (but not delay) extinction in addition to its role in acquisition and storage. This contributes to a growing body of literature suggesting that the PL is a key structure in learning and storing complex and relational associations.

Extinction circuit: Infralimbic cortex

In Experiment 3, we tested whether NMDA receptors in the infralimbic cortex are necessary for trace and delay extinction. The IL has been identified as a crucial structure in extinguishing delay fear (Burgos-Robles et al., 2007; Hugues et al., 2004; Morgan et al., 1993; Quirk et al., 2000; Sierra-Mercado et al., 2011; Sotres-Bayon et al., 2007). IL neurons are believed to project to the intercalated cell layer of the amygdala, where they activate a group of GABAergic interneurons that inhibit projection neurons in the central nucleus of the amygdala, effectively shutting down amygdala output to prevent freezing behavior (Pare et al., 2004; Pare & Smith, 1993; Royer, Martina, & Pare, 1999; Sotres-Bayon & Quirk, 2010). We observed that inhibiting NMDA receptors in the IL disrupted freezing during the extinction session for both delay and trace animals. Further, NMDA receptor blockade augmented extinction, as both delay and trace animals given APV during extinction showed lower CS freezing the following day compared to ACSF

animals. Importantly, the IL appears to play a similar role in both types of extinction; both delay and trace animals showed reduced freezing during extinction training and during the extinction retention test. This suggests that the IL plays a key role in extinction regardless of the specific type of training used.

The enhanced extinction we saw following NMDA receptor blockade in the IL was somewhat unexpected. We anticipated that this manipulation would similarly affect delay and trace animals, as we observed, but we predicted that inhibiting intra-IL NMDA receptors would *impair* extinction retention, rather than enhance it. Although a number of previous studies have demonstrated that disrupting activity (Sierra-Mercado, Corcoran, Lebron-Milad, & Quirk, 2006; Sierra-Mercado et al., 2011) or signaling cascades (Burgos-Robles et al., 2007; Hugues et al., 2004; Mueller, Porter, & Quirk, 2008; Sotres-Bayon et al., 2007) in the IL impair extinction memory for delay fear conditioning, there are a number of notable exceptions that show either no effect (Akirav, Khatsrinov, et al., 2006; Sotres-Bayon et al., 2009) or enhanced extinction (Akirav, Raizel, & Maroun, 2006) with pre-extinction IL manipulations. One important note is that other studies that have inhibited NMDA receptors in the IL targeted the ventromedial portion of the medial prefrontal cortex with a single midline cannula, including placements in both infralimbic and prelimbic tissue (Burgos-Robles et al., 2007; Laurent & Westbrook, 2008; Sotres-Bayon et al., 2009). In the current study, we used a low infusion volume and dual cannulae in order to selectively target the drug to the IL region of the mPFC and dissociate it from the dorsal PL region. It is possible that our effect was due to the precise infusion procedure, as neurons in the IL and PL have opposing roles in freezing expression (Gilmartin & McEchron, 2005b; Sierra-Mercado et al., 2011; Vidal-Gonzalez,

Vidal-Gonzalez, Rauch, & Quirk, 2006). This would suggest that while inactivation of NMDA receptors in both PL and IL regions impairs extinction memory, selectively inhibiting NMDA receptors in the infralimbic cortex enhances extinction retention. Alternatively, it is possible that procedural differences (such as our ABB design vs. others' AAA design with context pre-exposure) or a difference in the specific NMDA receptor antagonist used (APV, used in the present study vs. CPP or ifenprodil used in others' studies) may explain these discrepant results. Nonetheless, our results were consistent; two different types of cannulae placements produced the same general behavioral results (Fig. 10) and demonstrated that both delay and trace fear extinction were enhanced with intra-IL blockade of NMDA receptors.

Extinction circuit: Conclusions

Together, our results indicate that the RSC and PL mPFC are only necessary for trace fear extinction whereas the IL plays a role in both delay and trace fear extinction. Along with our preliminary results (Fig. 2), this suggests that the neural circuits supporting trace and delay fear extinction are distinct. Delay fear requires both the amygdala and infralimbic cortex for proper extinction. Trace extinction, on the other hand, does not require plasticity in the amygdala but requires NMDA receptors in the RSC and the PL in addition to the IL.

Although it is currently unclear why delay and trace extinction rely on distinct neural circuits, one possible reason for this shift in circuitry is that trace and delay fear memories are stored in different brain regions, causing different structures to be involved in their extinction (see Fig. 5) It is well-established that delay fear memory relies on the

amygdala for storage (Gale et al., 2004; Kwapis et al., 2009; Maren, 2001a; Maren et al., 1996; Serrano et al., 2008). The plastic changes that occur in the amygdala during delay extinction may reflect updating of those synaptic connections that support the delay engram whereas IL plasticity appears to support the inhibitory connections that produce the extinction memory itself (Fig. 5a; Quirk & Mueller, 2008). In the amygdala, two populations of neurons are inversely regulated during extinction: "fear neurons" and "extinction neurons" (Herry et al., 2008; Repa et al., 2001). During extinction, "fear neurons" (characterized by increased responding to the CS during fear conditioning) are inhibited whereas "extinction neurons" (characterized by CS-evoked responding during extinction learning) are activated (Herry et al., 2008). This suggests that separate populations of neurons within the same brain structure distinctly encode the fear and extinction memories in a coordinated manner. Blocking plasticity in the amygdala during extinction would likely impair both the activation of "extinction neurons" and also the inhibition of "fear neurons" that encode original CS-UCS association for DFC (Herry et al., 2010). Together, these changes in the amygdala likely update the original association to reflect the new CS-no UCS information learned during extinction.

For trace fear, perhaps the memory is stored in distributed cortical circuits, rather than in the amygdala (Fig. 5b), making cortical plasticity (instead of amygdala plasticity) required for extinction. There is evidence that the medial prefrontal cortex participates in encoding and storing the trace memory engram (Blum, Runyan, et al., 2006; Runyan & Dash, 2004; Runyan et al., 2004). Plasticity in the amygdala that occurs during training may reflect strengthening of the amygdala output circuit that facilitates connections to downstream structures to drive freezing, rather than storing the trace fear engram itself. If

the prelimbic and retrosplenial cortices together store the trace association, rather than the amygdala, one would expect these structures, and not the amygdala, to undergo updating during extinction. Extinction-related changes (including both the shift from "fear neurons" to "extinction neurons" and the upregulation of plastic mechanisms to update the existing memory trace) would therefore occur in the retrosplenial and prelimbic cortices instead of the amygdala. Again, IL regions would participate in extinction to disable amygdala output, reducing the fear response. Although more work is necessary to test this hypothesized circuit (including the identification of "fear neurons" and "extinction neurons" in cortical regions), it could explain why the circuits for trace and delay extinction are different and why trace fear extinction requires cortical, rather than subcortical participation for extinction.

The retrosplenial cortex is involved in trace, but not delay fear memory consolidation

One key assumption made by this hypothesized circuitry is that the retrosplenial and prelimbic cortices participate in storing trace fear memories. While evidence exists to support our contention that the PL participates in trace fear memory storage (Runyan & Dash, 2004; Runyan et al., 2004), no study to date has tested whether the RSC is involved in consolidation or storage of trace fear. In Experiments 4-6, we tested whether protein synthesis in the retrosplenial cortex during trace or delay fear consolidation. As the retrosplenial cortex is selectively involved in the retrieval of contextual and not delay fear in rodents (Corcoran et al., 2011; Keene & Bucci, 2008a, 2008c) and we demonstrated in Experiment 1 that this structure is also involved in trace, but not delay extinction, we

hypothesized that the retrosplenial cortex would be involved in the consolidation of trace (but not delay) fear. Consistent with our hypothesis, we found that blocking protein synthesis in the retrosplenial cortex immediately before, but not after conditioning was sufficient to disrupt consolidation of context and trace fear without impairing delay CS fear. Further, Experiment 5 replicated our previous finding (see Fig. 7e) that NMDA receptors in the RSC are required for the retrieval of trace, but not delay associations and that this is a transient effect.

Post-training blockade of protein synthesis in the retrosplenial cortex

In Experiment 4, we tested whether the RSC is required for the consolidation of trace fear by blocking protein synthesis in this region immediately after training animals with trace or delay fear conditioning. Surprisingly, we observed no effect of anisomycin infusion on either white noise or context fear for either trace or delay animals. This suggested that the RSC may not be involved in trace or delay consolidation.

While our initial study indicated that the RSC might not be necessary for trace fear consolidation, contrary to our hypotheses, we could not yet rule out the possibility that the RSC was involved. First, as neither group was affected by anisomycin infusion, it was impossible to determine whether protein synthesis was not required or whether our cannulation and infusion procedure was ineffective. A second possibility was that our injection was timed incorrectly and protein synthesis was required before the inhibitor in the current experiment was effective. To rule out each of these possibilities, we ran two additional experiments.

Pre-retrieval blockade of NMDA receptors on trace and delay retrieval.

In our next experiment, we tested whether we could replicate a positive effect in the retrosplenial cortex using the animals from Experiment 4. Our first experiment found that blocking NMDA receptors in the RSC prevents the retrieval of trace fear (Fig. 7e). To test whether our infusion and cannulation procedure was working properly, we regrouped the animals from the previous experiment into new drug conditions and demonstrated that a pre-retrieval infusion of APV impaired memory retrieval for trace, but not delay animals, consistent with our previous findings. We also tested these animals again the following day, once the APV had been metabolized, to test whether this retrieval deficit was transient or long-lasting. We found that freezing in the trace APV group recovered when tested the following day, suggesting that memory for trace fear is not permanently disrupted, but retrieval of the association is blocked while the drug is active. Importantly, our ability to replicate this effect in the animals from Experiment 4 demonstrates that the cannula placement and infusion procedure were working properly.

Pre-training blockade of protein synthesis in the retrosplenial cortex

The other possible reason for our lack of effect in Experiment 4 was that we timed our injection incorrectly. Specifically, it is possible that protein synthesis in the RSC is a requirement for trace fear consolidation, but our post-training anisomycin infusion took effect too late to cause a disruption. Indeed, recent work has demonstrated that infusion of a protein synthesis inhibitor given 15 minutes before contextual inhibitory avoidance training was effective in disrupting memory for the task, indicating that an early wave of protein synthesis in the RSC during or immediately after learning may be required for

context memory consolidation (Katche, Dorman, Slipczuk, Cammarota, & Medina, 2013). In order to test whether an early wave of protein synthesis is similarly required for the consolidation of trace fear, we ran a group of animals with the same basic procedure as Experiment 4, except that we infused anisomycin or vehicle before training so that protein synthesis would be inhibited earlier, during and immediately after training. Here, we found that blocking protein synthesis in the RSC 15-min before training impaired consolidation of both context and trace CS fear without affecting delay CS fear. This suggests that the retrosplenial cortex plays a role in either the acquisition or the early consolidation of trace and context fear. Delay fear was not affected by this infusion, however, suggesting that delay fear consolidation does not require *de novo* protein synthesis in the retrosplenial cortex.

The retrosplenial cortex and consolidation: Conclusions

Together, our results demonstrate that protein synthesis in the retrosplenial cortex is required for the consolidation of context and trace, but not delay CS fear. Interestingly, our results indicate that protein synthesis is required relatively early, either during or immediately after acquisition. Further, this study replicated our previous finding that NMDA receptors in the retrosplenial cortex are required for the expression of trace, but not delay fear (Fig. 7e).

Our finding that the retrosplenial cortex participates in the consolidation of trace fear corresponds with our hypothesis that the RSC plays a key role in the storage of trace fear memory (see Fig. 5b). Along with evidence from other labs that the PL is also involved in trace fear consolidation (Runyan & Dash, 2004; Runyan et al., 2004), these

results indicate that multiple cortical areas participate in the consolidation of trace fear. If these areas also store the trace fear association, as is implied by their participation in consolidation, it is possible that their participation in trace fear extinction reflects an updating of those synaptic connections that store the trace fear memory. Thus, our results are consistent with the hypothesis that distributed cortical regions participate in storing trace fear.

Although it is unclear why blocking protein synthesis in the RSC before, but not after trace conditioning was effective in impairing the memory, there is some evidence to suggest that the RSC is similarly involved in the early consolidation of context fear in an inhibitory avoidance task (Katche et al., 2013). Further, other research has shown that cortical plasticity occurs immediately after trace conditioning; in the medial prefrontal cortex, ERK phosphorylation was increased immediately (but not at 1 or 4h) following trace fear conditioning (Runyan et al., 2004). If the RSC and mPFC work together to consolidate trace fear, as hypothesized, it is likely that ERK phosphorylation occurs along a similar timecourse in both of these cortical regions. Our post-training infusion of anisomycin, therefore, may not have fully blocked protein synthesis until after this early wave of plasticity was complete. Infusing anisomycin 15 minutes before training, on the other hand, would have been sufficient to block protein synthesis during this immediate post-training period of plasticity. Regardless, our results clearly show that pre- but not post-training protein synthesis blockade impairs trace and context fear.

The neural circuit supporting trace fear is updated and reorganized following exposure to a single (updating) trial of the delay fear procedure.

It was recently shown that memory only undergoes reconsolidation when new information is presented at retrieval. Diaz-Mataix and colleagues (2013) demonstrated that animals are not affected by post-retrieval anisomycin when the retrieval trial consists of the same CS-UCS timing as the training session trials. When the CS-UCS interval is changed during retrieval, however, anisomycin is sufficient to disrupt the memory. Presumably, the memory destabilizes when new information is presented, so that blocking protein synthesis following an updating trial prevents the restabilization of the association, disrupting the stored memory. If no new information is provided, the memory does not destabilize, making *de novo* protein synthesis unnecessary. In our final two experiments, we tested whether this type of updating would occur when a trace fear memory is updated with a single trial of delay fear. We also tested whether this updating process could reorganize the memory at a circuit level so that a complex trace memory could be made to rely on the more basic delay fear circuit. We first established that trace-trained animals undergo updating in the amygdala following exposure to a single delay trial. We then demonstrated that updating a trace association with a single trial of delay conditioning shifted the memory to the neural circuit that normally supports delay fear by showing that the amygdala required to extinguish the updated memory. Together, our results support the idea that retrieval can be used to update not only the content of memory, but also how that memory is stored.

Trace fear memory undergoes protein synthesis-dependent reconsolidation in the amygdala following an exposure to a single trial of delay conditioning.

In Experiment 7, we tested whether TFC memory undergoes updating in the amygdala following exposure to a single updating trial of DFC. In this study, animals trained with TFC were given a single presentation of TFC or DFC on day 2 followed immediately by an intra-amygdala infusion of anisomycin or vehicle. We found that when TFC-trained animals were presented with an updating trial of DFC, which provided new information about the CS-UCS timing, protein synthesis was required in the amygdala. When TFC-trained animals were exposed to another TFC trial during retrieval, however, protein synthesis was not required in the amygdala, indicating that the memory was not rendered labile. Thus, our findings demonstrate that a trace conditioning memory undergoes protein synthesis-dependent reconsolidation in the amygdala following exposure to a single trial of delay conditioning.

Updating a TFC memory with a single trial of DFC also shifts the memory to the simpler neural circuit responsible for delay fear

In our final experiment, we tested whether memory updating can also reorganize the circuit supporting that memory, so that a relatively complex trace fear association can be shifted to the more basic delay fear circuit. In order to identify which circuit the memory relies on, we tested whether the amygdala was required to extinguish the updated memory. As we have previously demonstrated, the amygdala is required to extinguish delay, but not trace fear associations. Thus, if the amygdala is required for extinction, the memory relies on the delay fear circuit. If the amygdala is not required for extinction, the trace fear circuit is supporting the association.

In Experiment 8, animals trained with trace conditioning were updated with a single presentation of DFC or TFC as in Experiment 7. The animals were then extinguished in the presence of either APV or vehicle in the amygdala to test whether intra-amygdala NMDA receptors were required for extinction. We found that animals given both trace training and trace retrieval showed normal extinction in the presence of intra-amygdala APV. This is consistent with our previous work showing that the amygdala is not required to extinguish trace fear associations (Fig. 2). When trace animals were given an updating trial of delay, however, intra-amygdala APV application impaired extinction. This suggests that updating a trace memory with a single trial of delay conditioning triggered reorganization of the neural circuit supporting the memory so that the amygdala is now required for extinction. As the amygdala is required for the extinction of delay, but not trace fear, this updating procedure seems to shift the memory from the complex trace fear circuit to the more basic delay fear circuit. These results indicate, for the first time, that updating can be used to change the neural circuit that supports an association.

Reconsolidation-dependent updating: Conclusions

Together, our results demonstrate that trace fear undergoes a protein synthesis-dependent updating process in the amygdala following exposure to a single trial of delay conditioning and that this updating process reorganizes the neural circuit that supports the association. This updating procedure appears to shift a complex trace fear memory to the more basic delay fear circuit, as the amygdala was required for extinction only when the delay updating trial was presented.

While our results suggest that the memory actually shifts neural circuits (from trace to delay) following this type of update, it is also possible that updating the memory instead recruits additional structures to help support the association. Following update, perhaps the memory requires elements of both the trace and delay neural circuits for storage, so that the amygdala is required for extinction even though the memory still involves a distributed cortical circuit for storage. Although we cannot rule out this possibility entirely, it seems unlikely that this is the case, as our initial studies suggest that extinction-related plasticity needs to occur at the site of memory storage. If the cortex is still involved in storing the trace-delay updated memory, plasticity should be required in the cortex, rather than the amygdala for extinction. In order to more conclusively exclude the possibility that the trace-to-delay updated memory relies on a hybrid of the trace and delay fear circuits, other elements of the circuit will need to be tested in future studies. For example, if the memory is simply shifted to the delay circuit, the prelimbic and retrosplenial cortices should not be required for extinction of the updated memory. Further, the retrosplenial cortex, which is only required to retrieve trace fear, should not be required for retrieval if the updated memory now relies on the delay circuit. Identifying the role of these structures following the updating process will more completely determine whether the memory is shifted, as hypothesized, or whether it relies on a hybrid of the trace and delay fear circuits. Either way, our results are the first to demonstrate that updating procedures can be used to alter the neural circuit that supports a memory.

Conclusions

Although the neural mechanisms supporting standard delay fear conditioning and extinction have been well-characterized, few studies have identified how these mechanisms and circuits change for a relatively complex association, like trace fear conditioning. Here, we identified how the neural circuit supporting trace fear extinction and consolidation differs from that of delay. Our results add to a growing body of literature that demonstrates that trace fear conditioning requires a more complex circuit that involves cortical participation. We first demonstrated that trace fear extinction relies on a different circuit than that of delay fear extinction. The amygdala, which is required for delay fear extinction, is not critical for extinguishing trace associations. On the other hand, our results show that both the retrosplenial and prelimbic cortices are required for trace fear extinction. The infralimbic cortex is involved in both delay and trace extinction. These results suggest that trace fear is consolidated and stored in a distributed cortical manner, in which the retrosplenial and prelimbic cortices support the storage of trace fear, rather than the amygdala, which is responsible for storing delay fear. Consistent with this, we found that *de novo* protein synthesis in the retrosplenial cortex was required for successful early consolidation of trace, but not delay fear. Finally, we demonstrated that updating procedures can be used to shift a relatively complex trace fear association to the more basic neural circuit that supports delay fear. Together, our results provide a more complete understanding of the complex neural circuit supporting trace fear and show, for the first time, that updating procedures can reorganize the neural circuit that supports an association.

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Publications

1. **Kwapis, J.L.**, Jarome, T.J., Ferrara, N.C., & Helmstetter, F.J. (In Preparation). Updating procedures can be used to reorganize the neural circuit supporting a memory.
2. **Kwapis, J.L.**, Jarome, T.J., Lee, J.L., & Helmstetter, F.J. (In Preparation). The retrosplenial cortex is involved in trace, but not delay fear acquisition.
3. **Kwapis, J.L.**, Jarome, T.J., & Helmstetter, F.J. (Submitted). The role of the medial prefrontal cortex in trace fear extinction. *Learning & Memory*
4. **Kwapis, J.L.**, & Helmstetter, F.J. (In Press). Does PKMζ maintain memory? *Brain Research Bulletin*.
5. **Kwapis, J.L.**, Jarome, T.J., Lee, J.L., Gilmartin, M.R., & Helmstetter, F.J. (In Press). Extinguishing trace fear engages the retrosplenial cortex rather than the amygdala. *Neurobiology of Learning and Memory*.
6. Jarome, T.J., **Kwapis, J.L.**, Hallengren, J.J., Wilson, S.M., & Helmstetter, F.J. (In Press). The ubiquitin-specific protease 14 (USP14) is a critical regulator of long-term memory formation. *Learning & Memory.*
7. Jarome, T.J., **Kwapis, J.L.**, Ruenzel, W.L., & Helmstetter, F.J. (2013). CaMKII, but not protein kinase A, regulates Rpt6 phosphorylation and proteasome activity during the formation of long-term memories. *Frontiers in Behavioral Neuroscience, 7*, 115.

8. Gilmartin, M.R., **Kwapis, J.L.**, & Helmstetter, F.J. (2013). NR2A- and NR2B-containing NMDA receptors in the prelimbic medial prefrontal cortex differentially mediate trace, delay, and contextual fear conditioning. *Learning & Memory, 20*, 290-294.
9. **Kwapis, J.L.**, Jarome, T.J., Gilmartin, M.R., & Helmstetter, F.J. (2012). Intra-amygdala infusion of the protein kinase Mzeta inhibitor ZIP disrupts foreground context fear memory. *Neurobiology of Learning and Memory, 98*, 148-153.
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12. **Kwapis, J.L.**, Jarome, T.J., Schiff, J.C., & Helmstetter, F.J. (2011). Memory consolidation in both trace and delay fear conditioning is disrupted by intra-amygdala infusion of the protein synthesis inhibitor anisomycin. *Learning & Memory, 18*, 728-732.
13. Jarome, T.J., Werner, C.T., **Kwapis, J.L.**, & Helmstetter, F.J. (2011). Activity-dependent protein degradation is critical for the formation and stability of fear memory in the amygdala. *PLoS ONE, 6*, e24349.

14. Jarome, T.J., **Kwapis, J.L.**, Nye, S.J., & Helmstetter, F.J. (2010). Introgression of Brown Norway chromosome 1 onto the Fawn Hooded hypertensive background rescues long-term fear memory deficits. *Behavior Genetics, 40*, 85-92.
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Poster Presentations/Abstracts

1. **Kwapis, J.L.**, Jarome, T.J., & Helmstetter, F.J. (2013). Updating a memory can change the way it is stored. Molecular and Cellular Cognition Society Annual Meeting, San Diego, CA.
2. **Kwapis, J.L.**, Jarome, T.J., Lee, J.L., & Helmstetter, F.J. (2013). The retrosplenial cortex is involved in the consolidation, retrieval, and extinction of trace, but not delay fear conditioniong. The Society for Neuroscience Annual Meeting, San Diego, CA.
3. Jarome, T.J., **Kwapis, J.L.**, & Helmstetter, F.J. (2013). Contextual novelty, but not prediction error, is the new information present at retrieval that controls the reconsolidation-dependent updating of an auditory fear memory. The Society for Neuroscience Annual Meeting, San Diego, CA.
4. **Kwapis, J.L.,** Jarome, T.J., Gilmartin, M.R., Lee, J.L., & Helmstetter, F.J. (2012). Dissociable roles of the amygdala and retrosplenial cortex in delay and trace fear extinction. The Society for Neuroscience Annual Meeting, New Orleans, LA.

5. **Kwapis, J.L.**, Jarome, T.J., Lee, J.L., Gilmartin, M.R., & Helmstetter, F.J. (2012). Extinction of trace fear conditioning requires the retosplenial cortex but not the amygdala. The Pavlovian Society Annual Meeting, Jersey City, NJ. ****Pavlovian Poster Award Winner****
6. Jarome, T.J., **Kwapis, J.L.**, Ruenzel, W.L., & Helmstetter, F.J. (2012). CaMKII regulates proteasome-dependent increases in GluR2 in the amygdala during fear memory reconsolidation. Poster presented at Pavlovian Society Annual Meeting, Jersey City, NJ.
7. **Kwapis, J.L.**, Jarome, T.J., Gilmartin, M.R., & Helmstetter, F.J. (2011). The basolateral amygdala may be required for delay, but not trace fear extinction. The Society for Neuroscience Annual Meeting, Washington, D.C.
8. Gilmartin, M.R., **Kwapis, J.L.**, & Helmstetter, F.J. (2011). Differential expression patterns of learning-related protein in the trace fear network following targeted inactivation of prelimbic medial prefrontal cortex, amygdala, or ventral hippocampus. The Society for Neuroscience Annual Meeting, Washington, D.C.
9. Jarome, T.J., Ruenzel, W.L., **Kwapis, J.L.**, & Helmstetter, F.J. (2011). Temporally graded increases in proteasome number and activity in the amygdala following fear conditioning. The Pavlovian Society Annual Meeting, Milwaukee, WI.
10. Gilmartin, M.R., **Kwapis, J.L.**, Segrin, P.K., Ruenzel, W.L., & Helmstetter, F.J. (2011). Network-level examination of MAPK signaling in trace and delay fear conditioning. The Pavlovian Society Annual Meeting, Milwaukee, WI.

11. **Kwapis, J.L.**, Schiff, J.C., Jarome, T.J., Lonergan, M.E., Gilmartin, M.R., & Helmstetter, F.J. (2010). The basolateral amygdala my use different neural mechanisms for delay and trace fear extinction. The Society for Neuroscience Annual Meeting, San Diego, CA.
12. Jarome, T.J., Werner, C.T., **Kwapis, J.L.**, Lonergan, M.E., & Helmstetter, F.J. (2010). Protein degradation controls protein synthesis and synaptic remodeling in the amygdala during the formation and stability of long-term fear memories. The Society for Neuroscience Annual Meeting, San Diego, CA.
13. Werner, C.T., Jarome, T.J., **Kwapis, J.L.**, Parsons, R.G., Gafford, G.M., & Helmstetter, F.J. (2010). The timing of multiple retrieval events can reverse synaptic GluR1 phosphorylation and alter the requirement for protein synthesis in fear memory reconsolidation. The Society for Neuroscience Annual Meeting, San Diego, CA.
14. Gilmartin, M.R., **Kwapis, J.L.**, & Helmstetter, F.J. (2010). Ventral hippocampus to medial prefrontal cortex pathway in trace fear conditioning. The Society for Neuroscience Annual Meeting, San Diego, CA.
15. Lonergan, M.E., Leidel, R.J., **Kwapis, J.L.**, & Helmstetter, F.J. (2010). ERK inhibition in the amygdala decreases mTOR signaling in the hippocampus during contextual fear conditioning. The Society for Neuroscience Annual Meeting, San Diego, CA.
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18. Jarome, T.J., **Kwapis, J.L.**, Parsons, R.G., Gafford, G.M., & Helmstetter, F.J. (2008). Altered gene expression and a change in the requirements for protein synthesis following multiple retrieval events in fear memory reconsolidation. The Society for Neuroscience Annual Meeting, Washington, D.C.
19. **Kwapis, J.L.**, Jarome, T.J., Parsons, R.G., & Helmstetter, F.J. (2008). Protein kinase Mzeta activity is required in the amygdala but not in the hippocampus to maintain context fear memory. The Pavlovian Society Annual Meeting, Weehawken, NJ.
20. **Karbowski-Kwapis, J.L.**, Jarome, T.J., Parsons, R.G., & Helmstetter, F.J. (2007). Protein kinase Mzeta and the maintenance of fear memory. The Pavlovian Society Annual Meeting, Austin, TX.
21. Jarome, T.J., **Karbowski-Kwapis, J.L.**, & Helmstetter, F.J. (2007). Multiple retrieval events can change the requirements for protein synthesis in fear memory reconsolidation. The Society for Neuroscience Annual Meeting, San Diego, CA.
22. Jarome, T.J., **Karbowski-Kwapis, J.L.**, & Helmstetter, F.J. (2007). The timing of multiple retrieval events can change the requirements for protein synthesis in fear memory reconsolidation. The Pavlovian Society Annual Meeting, Austin, TX.

Invited Talks

1. Illinois Wesleyan University (2013): Modifying memories: Using retrieval to change the neural circuit that stores memory. (Invited by Dr. Abigail Kerr).
2. University of California, Irvine (2013): Extinguishing complex fear: Retrosplenial and prefrontal circuitry. (Invited by Dr. Marcelo Wood).
3. New York University (2013): Extinguishing complex fear: Retrosplenial and prefrontal circuitry. (Invited by Dr. Eric Klann).

Colliquia and Symposia

1. The circuitry of trace fear extinction. Presentation as part of a Data Blitz for Dr. Jarrod Lewis-Peacock (Princeton University). January 2013.
2. The role of the amygdala in trace fear extinction. Presentation at the Annual Graduate Research Symposium. April 2012. ****Received 3rd place presentation award****
3. The role of the amygdala in trace fear conditioning. Presentation as part of a Data Blitz for Dr. Brad Postle (UW-Madison). March 2012.
4. The role of the amygdala in trace fear conditioning. Presentation as part of a Data Blitz for Dr. Neal Cohen (University of Illinois). October 2011.
5. The role of the amygdala in trace fear consolidation and extinction. Presentation as part of a Data Blitz for Dr. Bruce McEwen (Rockefeller University). May 2011.
6. The role of the amygdala in trace fear consolidation and extinction. Presentation as part of a Data Blitz for Dr. Gregory Quirk (Univ. of Puerto Rico). May 2011.

7. The role of the amygdala in trace fear conditioning. Presentation at the Neuroscience and Physiology brown bag seminar, University of Wisconsin-Milwaukee. April 2011.
8. Protein kinase Mzeta maintains fear memory in the amygdala but not in the hippocampus. Presentation as part of a Data Blitz for Dr. Tom Carew (UC-Irvine). March 2010.
9. Protein kinase Mzeta activity is necessary in the amygdala but not in the hippocampus to maintain a context fear memory. Presentation as part of a Data Blitz for Dr. Howard Eichenbaum (Boston University). October 2008.
10. Protein kinase Mzeta and the maintenance of fear memory. Presentation at the Annual Graduate Research Symposium. April 2008. ****Received 1st place presentation award****
11. Protein kinase Mzeta and the maintenance of fear memory. Presentation at the Neuroscience and Physiology brown bag seminar, University of Wisconsin-Milwaukee. November 2007.
12. The role of protein kinase Mzeta in the maintenance of fear memory. Presentation at the Neuroscience and Physiology brown bag seminar, University of Wisconsin-Milwaukee. March 2007.

Membership in Professional Associations

- Women in Learning 2011-present
  Luncheon Organizer (2011, 2012)
- Graduate Students in Behavioral Neuroscience (GSIN) 2009-present
  Secretary (2010-2011)
  President (2009-2010)
  Founding Member (2009)

- Molecular and Cellular Cognition Society 2008-present
- Society for Neuroscience 2007-present
- Pavlovian Society 2007-present
- Midwestern Psychological Association 2007-present
- Association of Graduate Students in Psychology 2006-present
- Psi Chi 2004-present
- Omicron Delta Kappa (Leadership Honor Society) 2005-present

Professional Positions

1. Postdoctoral Fellow, University of California, Irvine

   Mentor: Marcelo Wood, Ph.D.
   January 2014-present

2. National Institutes of Health

   Predoctoral Fellow (Mentor: Fred J. Helmstetter, Ph.D.)
   January 2011-December 2013

3. Guest Lecturer – University of Wisconsin-Milwaukee

   Proseminar in Biological Psychology, *Extinguishing complex fear: Retrosplenial and prefrontal circuitry* (Fall 2013)
   Research Methods, *Threats to Validity* (Spring 2010)
   Introduction to Psychology, *Memory Lectures (2)* (Spring 2007)
   Introduction to Psychology, *Conditioning Lecture* (Fall 2006)

4. Teaching Assistant – University of Wisconsin-Milwaukee

   Physiological Psychology (Dr. James Moyer, Jr.) Spring 2009
   Physiological Psychology (Dr. Fred Helmstetter) Fall 2008
   Research Methods in Psychology (Dr. Marcellus Merritt) Spring 2008, 2010
   TA Coordinator (Spring & Fall, 2010) Fall 2010
   Research Methods in Psychology (Dr. Susan Lima) Fall 2007, 2009
   Introduction to Psychology (Dr. Kristin Flora) Spring 2007
   Introduction to Psychology (Dr. Katie Mosack) Fall 2006

5. Research Assistant Positions – University of Wisconsin-Milwaukee
   Graduate Research Assistant (Dr. Fred Helmstetter) Summer, 2007-2013
   Part-time Research Assistant (PhysioGenix, Inc.) Summer 2007