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# Extinguishing trace fear engages the retrosplenial cortex rather than the amygdala

Janine L. Kwapis, Timothy J. Jarome, Jonathan L. Lee, Marieke R. Gilmartin, Fred J. Helmstetter *

*Department of Psychology, University of Wisconsin-Milwaukee, 2441 E. Hartford Ave., Milwaukee, WI 53211, USA*

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ABSTRACT

Extinction learning underlies the treatment for a variety of anxiety disorders. Most of what is known about the neurobiology of extinction is based on standard "delay" fear conditioning, in which awareness is not required for learning. Little is known about how complex, explicit associations extinguish, however. "Trace" conditioning is considered to be a rodent model of explicit fear because it relies on both the cortex and hippocampus and requires explicit contingency awareness in humans. Here, we explore the neural circuit supporting trace fear extinction in order to better understand how complex memories extinguish. We first show that the amygdala is selectively involved in delay fear extinction; blocking intra-amygdala glutamate receptors disrupted delay, but not trace extinction. Further, ERK phosphorylation was increased in the amygdala after delay, but not trace extinction. We then identify the retrosplenial cortex (RSC) as a key structure supporting trace extinction. ERK phosphorylation was selectively increased in the RSC following trace extinction and blocking intra-RSC NMDA receptors impaired trace, but not delay extinction. These findings indicate that delay and trace extinction require different neural circuits; delay extinction requires plasticity in the amygdala whereas trace extinction requires the RSC. Anxiety disorders linked to explicit memory may therefore depend on cortical processes that have not been traditionally targeted by extinction studies based on delay fear.



## 1. Introduction

The ability to accurately predict and respond to danger signals is critical for an animal's survival. Failure to react in the face of a cue that signals danger can result in harm or death. Conversely, it is also maladaptive for an animal to respond in a disproportionate manner to a nonthreatening cue. In humans, excessive responding to situations and cues that are poor predictors of danger may contribute to anxiety disorders (Davis, 2002; Rothbaum & Davis, 2003).

Anxiety disorders are often treated clinically through behavioral extinction in which the individual is exposed to the threatening stimulus in the absence of an aversive outcome (Barad, 2005; Foa, 2000; Rothbaum & Schwartz, 2002; Wolpe, 1969). Affective reactions to the stimulus are gradually reduced as the person learns that the cue does not predict danger. These exposure-based therapies can be modeled in rodents through fear conditioning and extinction training as a way to understand the neural mechanisms underlying anxiety reduction (Davis, 2002; Milad & Quirk, 2012).

To date, most of the research on the neural mechanisms of extinction learning comes from rodent studies that use delay fear conditioning to model anxiety (for review, see Milad & Quirk, 2012). In delay fear conditioning, an initially neutral conditional stimulus (CS), such as a white noise or tone, is presented contiguously with a naturally aversive unconditional stimulus (UCS), such as a foot shock. Delay fear can be acquired very rapidly and, in humans, can be learned and expressed without awareness of the stimulus relationship (Clark & Squire, 1998; Knight, Nguyen, & Bandettini, 2006) making it a good model for basic, implicit fear memories. Delay fear extinction requires three critical brain structures: the infralimbic medial prefrontal cortex (IL), the hippocampus, and the amygdala (Sierra-Mercado, Padilla-Coreano, & Quirk, 2011). The hippocampus' role in extinguishing fear to a discrete auditory CS is largely restricted to controlling the context specificity of extinction (Corcoran, Desmond, Frey, & Maren, 2005; Hobin, Ji, & Maren, 2006) although more recent evidence points to a central role of the hippocampus in extinction when the most salient predictor of shock is the training context (Fischer et al., 2007; Huh et al., 2009; Radulovic & Tronson, 2010; Schimanski, Wahlsten, & Nguyen, 2002; Tronson et al., 2009; Vianna, Szapiro, McGaugh, Medina, & Izquierdo, 2001). In contrast to the hippocampus, both the IL and amygdala undergo plastic changes during the extinction of an auditory CS previously used in delay fear conditioning. This plasticity in IL and amygdala regions is believed to support the formation of a new extinction memory (Herry et al., 2010; Quirk & Mueller, 2008). Blocking neural activity or general

* Corresponding author. Address: Department of Psychology, University of Wisconsin-Milwaukee, 224 Garland Hall, 2441 E. Hartford Ave., Milwaukee, WI 53211, USA. Fax: +1 414 229 5219.
*E-mail address:* fjh@uwm.edu (F.J. Helmstetter).

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plasticity in the IL (Burgos-Robles, Vidal-Gonzalez, Santini, & Quirk, 2007; Sierra-Mercado et al., 2011) or amygdala (Sierra-Mercado et al., 2011; Sotres-Bayon, Bush, & LeDoux, 2007) is sufficient to disrupt the formation of extinction memory, usually tested the following day.

While the neural mechanisms supporting delay fear extinction have received substantial recent attention, far less is understood about the extinction of more complex associations that may better relate to explicit memory in humans. This is important because anxiety disorders can involve both implicit and explicit associations (Brewin, 2001; Rothbaum & Davis, 2003). One way to investigate the neural basis of explicit memory extinction is to use trace fear conditioning. In trace fear conditioning, the CS and UCS are separated by an empty period of time, called the trace interval. Temporal separation of the two cues makes the association slightly more difficult to learn but significantly alters the circuitry and attentional mechanisms required for acquisition. Whereas delay fear can be acquired without awareness and relies largely on subcortical structures (particularly the amygdala), trace fear conditioning requires awareness of the CS–UCS contingency and relies on hippocampal and cortical participation for acquisition (Gilmartin & Helmstetter, 2010; Gilmartin, Kwapis, & Helmstetter, 2012; Han et al., 2003; Knight et al., 2006; Quinn, Oommen, Morrison, & Fanselow, 2002) in addition to the amygdala (Gilmartin et al., 2012; Kwapis, Jarome, Schiff, & Helmstetter, 2011). Trace conditioning shares a number of important characteristics with human declarative memory. First, as with explicit memory in humans, trace fear conditioning involves learning a relatively complex relationship between multiple stimuli. Second, explicit awareness of the CS–UCS contingency is necessary for human participants to learn trace fear (Knight et al., 2006; Weike, Schupp, & Hamm, 2007). Finally, trace fear conditioning involves structures known to participate in declarative memory, including the hippocampus and cortex (Gilmartin & Helmstetter, 2010; Gilmartin et al., 2012; Han et al., 2003; Quinn et al., 2002; Squire, 1992). Trace fear conditioning is therefore a particularly good paradigm for modeling explicit fear memory in rodents.

Despite the clear value of trace fear conditioning as a model of fear memory, few studies have investigated extinction after this training procedure (Abumaria et al., 2011; Kaczorowski, Davis, & Moyer, 2012). To date, no study has systematically investigated how the circuitry supporting trace extinction differs from the established circuit that supports delay fear extinction. Delay and trace conditioning rely on different structures for acquisition, however, so it is feasible that the circuits required for extinction are also distinct. Structures such as the PL, which is required for trace fear acquisition (Gilmartin & Helmstetter, 2010), and the retrosplenial cortex (RSC), which is involved in contextual and relational associations (Aggleton, 2010; Cooper, Manka, & Mizumori, 2001; Corcoran et al., 2011; Haijima & Ichitani, 2008; Katche, Dorman, Gonzalez, et al., 2013; Katche, Dorman, Slipczuk, Cammarota, & Medina, 2013; Keene & Bucci, 2008a, 2008b; Robinson, Keene, Iaccarino, Duan, & Bucci, 2011), for instance, may supplement or take over the roles of the amygdala, IL, and hippocampus in the extinction of trace fear. The RSC is particularly suitable for supporting explicit associations, as it plays a well-documented role in supporting autobiographical, relational, and spatial memory in humans (Maddock, 1999; Maguire, 2001; Rosenbaum, Ziegler, Winocur, Grady, & Moscovitch, 2004; Steinvorth, Corkin, & Halgren, 2006; Svoboda, McKinnon, & Levine, 2006). Whether the RSC plays a role in trace extinction, however, is unknown. Characterizing the neural circuit that underlies trace fear extinction is an important step towards a comprehensive understanding of anxiety reduction in humans.

Here, we tested whether the circuitry supporting trace fear extinction is the same or different from that of delay extinction. First, we tested whether the amygdala is necessary for trace extinction, as it is with delay. We then measured the phosphorylation of extracellular regulated kinase (pERK) in a number of candidate brain structures in order to identify regions that undergo extinction-related plasticity following trace fear extinction. One region of interest, the retrosplenial cortex, showed elevated pERK following trace, but not delay extinction, suggesting that this region is selectively involved in the extinction of trace fear. In our final study, we directly tested whether the RSC is required for trace, but not delay fear extinction. Together, our results demonstrate that trace fear extinction relies on a different neural circuit than delay extinction.

## 2. Materials and methods

### 2.1. Subjects

Subjects were 238 male Long-Evans rats obtained from Harlan (Madison, WI) weighing approximately 350 g. Rats were housed individually and allowed 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 animals were handled for 3 days before surgery and 3 days before training. For the western blot study, all animals were handled for 6 days: 3 days of standard handling in the animal room followed by 3 days of transport to another room in the lab (in order to acclimate animals to the transportation cart) followed by handling in that room. All procedures were approved by the university Animal Care and Use Committee and were in compliance with the National Institutes of Health guidelines.

### 2.2. Surgery

Animals were implanted with bilateral cannulae aimed at either the basolateral nucleus of the amygdala (BLA) or the anterior retrospenial cortex (RSC) (Kwapis, Jarome, Lonergan, & Helmstetter, 2009; Paxinos & Watson, 2007). Before surgery, each rat was anesthetized with 2–4% isoflurane in oxygen and implanted with bilateral stainless steel 26-guage cannulae aimed at the basolateral nucleus of the amygdala (BLA) or dual 26-guage cannulae (1 mm center-to-center) aimed at the anterior retrosplenial cortex (RSC). BLA coordinates were 3.0 mm posterior, ±5.0 mm lateral, 7.2 mm ventral relative to bregma. RSC coordinates were 3.5 mm posterior, ±0.5 mm lateral, 1.8 mm ventral relative to bregma (Paxinos & Watson, 2007). Cannulae were secured to the skull with stainless steel screws, superglue, and dental cement. Following surgery, the incision site was swabbed with a lidocaine and prilocaine solution (2.5%/2.5%) to minimize discomfort during the recovery period. Stainless steel obdurators remained in the cannulae when rats were not being injected to prevent occlusion. Rats were given a recovery period of at least 7 d before behavioral testing.

Following recovery from surgery, all animals were transported and handled for 3 days before behavioral testing began. During this handling period, animals were gently restrained with a towel while the infusion pump was activated in order to allow the animals to habituate to its noise. The obdurators were removed from the cannulae during this handling session and the surgical site was cleaned with a cotton swab.

### 2.3. Apparatus

Fear conditioning was conducted in a set of four identical chambers (Context A). The floor of Context A was composed of stainless steel rods 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,