Published in final edited form as:

Behav Processes. 2014 January ; 0: 103–111. doi:10.1016/j.beproc.2013.08.016.

Bridging the interval: Theory and Neurobiology of Trace Conditioning

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Abstract

An early finding in the behavioral analysis of learning was that conditioned responding weakens as the conditioned stimulus (CS) and unconditioned stimulus (US) are separated in time. This “trace” conditioning effect has been the focus of years of research in associative learning. Theoretical accounts of trace conditioning have focused on mechanisms that allow associative learning to occur across long intervals between the CS and US. These accounts have emphasized degraded contingency effects, timing mechanisms, and inhibitory learning. More recently, study of the neurobiology of trace conditioning has shown that even a short interval between the CS and US alters the circuitry recruited for learning. Here, we review some of the theoretical and neurobiological mechanisms underlying trace conditioning with an emphasis on recent studies of trace fear conditioning. Findings across many studies have implications not just for how we think about time and conditioning, but also for how we conceptualize fear conditioning in general, suggesting that circuitry beyond the usual suspects needs to be incorporated into current thinking about fear, learning, and anxiety.

Keywords

fear; memory; consolidation; learning theory; neurobiology; amygdala; hippocampus

Introduction

A key feature of associative learning is its sensitivity to the temporal arrangement of stimuli. In Pavlovian conditioning procedures, research has focused on the temporal relation between the conditioned stimulus (CS) and the unconditioned stimulus (US). Pavlov (1927) noted that as the trace interval, the interval between CS offset and US onset, was increased, responding during the CS decreased. This pattern of results is a textbook finding that has been replicated across many different Pavlovian preparations (e.g., Ellison, 1964; Kamin, 1961). The study of trace conditioning has had a major impact on theories of learning and timing, and has revealed novel neurobiological mechanisms of learning and memory. In this review, we focus primarily on trace fear conditioning, where a subtle change in the interval between the CS and US results in the recruitment of distinct neurobiological circuitry.

Trace conditioning has been studied with multiple behavioral approaches that reveal common and unique characteristics. At a behavioral level, trace conditioning procedures

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generally slow the rate of acquisition of a behavioral response and lead to less behavior during subsequent tests, relative to delay conditioning procedures. As with delay procedures, however, trace conditioning often results in the emergence of response patterns consistent with precise timing of the CS-US relation (e.g., Balsam, 1984) and this timing is stimulus and contingency specific (e.g., Kehoe *et al.*, 2009; Woodruff-Pak & Disterhoft, 2008). There is a growing literature from neurobiological studies of trace fear conditioning suggesting that the neural, molecular, and biochemical mechanisms that support long-term learning and anxiety may differ in trace and delay conditioning (Raybuck & Lattal, 2011).

Theoretical Mechanisms of Trace Fear Conditioning

In trace fear conditioning, the CS and US are temporally discontinuous. Thus, CS offset and US onset are separated by a stimulus-free interval. During subsequent testing, responding is weaker compared to that of delay conditioned subjects, where the CS and US co-terminate, thus overlapping in presentation. This is a robust behavioral difference that occurs after relatively few or many trials (Ellison, 1964; Kamin, 1961; Pavlov, 1927). The difference between trace and delay conditioning has led to different theoretical accounts that have focused on three potential mechanisms. These mechanisms include differences in associative strength (which has been the theoretical focus of most neurobiological studies of trace fear conditioning), inhibitory learning, or temporal pattern of responding.

Weakened Associative Strength

One obvious interpretation of the behavioral differences between trace and delay conditioning is that increasing the trace interval weakens the relation between the CS and US, resulting in poorer associative learning compared to when there is no trace interval (Pavlov, 1927). Thus, according to this interpretation, the difference in conditioned freezing between delay and trace fear conditioning demonstrates a deficit in learning; the two groups differ in terms of the associative strength of the CS. This may occur because in delay conditioning, the CS is a better predictor of the US compared to trace conditioning, where the CS does not immediately predict the US. Indeed, the term “trace” originated in this way of thinking, with the idea being that residual activation of the CS center in the brain was what was paired with US delivery (Pavlov, 1927). In modern approaches, this “trace” is most associated with the idea of a memory trace that decays as a function of time, resulting in a weaker CS representation paired with the US. The primary evidence for this account comes from simple differences in behavior during the CS. When a different stimulus intervenes between CS and US, the associative linking of the CS and US may be strengthened (e.g., Bolles *et al.*, 1978; Rescorla, 1982). This bridging effect itself may occur through various mechanisms that include not just strengthened CS-US learning, but conditioned reinforcement and occasion setting (Rescorla, 1982; Thomas *et al.*, 1989; Williams, 1991). The challenge, of course, for a weakened associative strength account is to demonstrate that weakened conditioned responding in the presence of the CS reflects weakened associative learning about the CS-US contingency (e.g., Lockhart, 1966; Smith *et al.*, 2007). Experiments describe below suggest that weak behavioral responses after trace conditioning are not necessarily indicative of weak associative learning.

CS as Safety Signal

A second account of the difference in behavior induced by trace and delay conditioning focuses not on the effects on excitatory learning that occur during conditioning, but instead on the possibility that inhibitory learning causes reduced responding to the CS. According to this account, as the trace interval increases, the CS comes to signal the explicit absence of the US; i.e., the animal learns that the US will not occur when the CS is present (e.g., Kalat & Rozin, 1973; Moscovitch & LoLordo, 1968). Indeed, as the trace interval is lengthened,

the trace conditioning procedure effectively becomes an explicitly unpaired procedure, in which the CS and the US have no contiguous relation (Smith *et al.*, 2007). In these cases, there is more ongoing behavior in the absence of the CS, because the context alone better predicts the US (Marlin, 1981). Consistent with this idea, Huerta and colleagues (2000) found that a 30-s trace interval during conditioning resulted in a high level of freezing in the absence of the CS. This was followed by a depression of freezing with CS onset, followed by a resumption of freezing after CS termination. This suggests that the CS may act as a safety signal in trace fear conditioning, signaling the explicit absence of shock. However, the inhibitory nature of this learning, the time course over which this learning develops, and the procedural variations necessary to generate it remain to be determined.

Timing

A third theoretical account is that excitatory learning is maintained, even with increased trace intervals, but responses are timed to US presentation. Although the measured response in the presence of the CS is attenuated, the CS still retains the ability to signal to the animal when the US will occur. Thus, the weak responding in the presence of the CS after trace conditioning does not reflect what the animal learns, but rather, responding (or some other measure of learning) needs to be assessed at the time of US expectancy.

An examination of responding in fine temporal blocks sometimes reveals that responding begins low during the CS, but rises gradually to peak around the time of the previous US presentations (e.g., Drew *et al.*, 2005; Huerta *et al.*, 2000). With more conditioning, this peak becomes sharper, suggesting that the CS acquires temporally specific excitatory learning. Thus, responding is low during the CS not necessarily because of its poor excitatory or strong inhibitory association with the US. Instead, there is a strong excitatory association between the CS and the time of US presentation that is revealed by responding that peaks at the time of expected US presentation (Balsam, 1984).

The best evidence for timing of the conditioned response in trace conditioning comes from studies of eyeblink conditioning, which have revealed that as trace conditioning progresses, peak response shifts toward the time of US occurrence. This temporal learning is stimulus specific and shows selectivity in extinction (Joscelyne & Kehoe, 2007; Kehoe & Joscelyne, 2005). In fear conditioning, which involves much longer intervals, there is some evidence that rapidly learned CS-US associations result in a conditioned response being timed to coincide with the time at which the US is expected (e.g., Burman & Gewirtz, 2004; Drew *et al.*, 2005). To account for the difference in response strength between trace and delay fear conditioning, timing would have to develop rapidly, because differences in trace and delay fear conditioning often occur after a single trial. The evidence for timing, as measured as peak responding at the time of expected footshock during a post-conditioning test, is mixed (see Bevins & Ayres, 1995; Davis, *et al.* 1989; Lattal & Abel, 2001). Based on this examination of behavior during the CS, it appears that temporal response patterns do occur, but may require extensive training to develop (see also Delamater & Holland, 2008).

It is important to note, however, that the absence of a peak response around the expected time of the US does not mean that animals do not learn temporal contingencies early in conditioning. Theories of timing do not necessarily expect a pattern of behavior consistent with timed responses to be evident in behavior immediately (see Balsam, *et al.* 2002). Indeed, it is possible that the absence of timing in behavior reflects a performance deficit rather than a learning deficit; that is, the animals learn the temporal relations quickly and accurately, but it takes some time for this learning to be expressed in behavior. A series of experiments by Miller and colleagues has demonstrated that animals code temporal relations even in situations where responding in the presence of the CS does not show patterns of behavior associated with a timed response (Cole *et al.*, 1995; Molet *et al.*, 2012). An

implication of this temporal coding hypothesis is that animals form rapid temporal associations that can be revealed in behavior through ways other than direct responding to the stimulus that has been conditioned. Thus in trace conditioning, if the CS signals the time at which the US occurs, then subsequent pairings of that CS with a second stimulus should result in the greatest association when that second CS is presented at the expected time of US delivery.

Clear evidence for this comes from second-order conditioning studies. In the first phase of a second-order conditioning experiment, CS X is paired with a US. In the second phase, a new CS A is paired with CS X. As A-X pairings increase, conditioned responding emerges in the presence of CS A and persists when the stimulus is subsequently tested on its own. Cole et al. (1995) found that second-order conditioning to trace cues was greater when the second-order CS is presented immediately prior to the time of expected shock than when it was presented immediately prior to the first-order CS. This means that a backwards temporal arrangement between A and X produces more conditioning than does a forwards relation. This surprising finding has been extended to other temporal conditioning procedures (e.g., Urushihara & Miller, 2010) and strongly suggests that animals code the temporal arrangement of stimuli during trace conditioning. These findings are consistent with the idea that animals learn both an association between CS and US, as well as the temporal relation between them.

The challenge for theoretical accounts based on timing is to demonstrate that differences in single-trial learning between trace and delay groups are due to differences in US timing. This is an unresolved issue, as most studies of trace conditioning that show timing assess performance after a number of conditioning trials, suggesting that even if it is not apparent early, timing does emerge with training (e.g., Burman & Gewirtz, 2004). Other studies show that there is some degree of temporal learning after a single trial (Davis, et al. 1989), but it is not necessarily clear how an increase in behavioral response corresponds to the encoding of precise temporal information. The challenge in using the expression of the conditioned response as the key dependent variable to distinguish different accounts is that we do not know the relation between the learning of the temporal interval and the expression of that learning in freezing behavior (Balsam *et al.*, 2002; Balsam, 1984; Lockhart, 1966). It is possible that learning is expressed in other ways, particularly early in conditioning, and that null effects on performance do not necessarily correspond to null effects on temporal learning. It also is possible that animals do begin timing immediately, even when no anticipatory responses are evident in behavior (Balsam *et al.*, 2002; Drew *et al.*, 2005). Indeed, experimental approaches that assess learning independent of the response evoked by a CS have revealed a key role for time in associative learning (see, Molet & Miller, 2013).

These different theoretical accounts paint a complicated picture of the differences between trace and delay fear conditioning. In many experiments, differences between trace and delay fear conditioned groups emerge quickly, often after a single trial. It is possible for any of the theoretical accounts described above (weakened excitation, strengthened inhibition, and temporal coding) to account for the trace-delay difference. An emerging literature on the neurobiology of trace fear conditioning demonstrates that these rapidly developed differences in behavior correspond to recruitment of different neurobiological circuitry, even after a single conditioning trial. This literature suggests that insertion of a trace interval results in immediate systems-level differences that may shed light on theoretical processes.

Neurobiology of Trace Fear Conditioning

Just as there are multiple theoretical accounts for the learning that underlies trace fear conditioning, there are multiple neurobiological circuits that support it. Much of what is

known about the circuits of fear conditioning come from the study of delay fear conditioning (Fanselow & Gale, 2003). While trace fear conditioning has been influential in theoretical approaches to learning, remarkably little is known about its neurobiology, though a great deal is known about neurobiology trace conditioning in other preparations, such as eyeblink conditioning (Woodruff-Pak & Disterhoft, 2008).

Perhaps the most consistent finding in the neurobiology of learning and memory is that the amygdala is a key structure involved in learning during delay fear conditioning (Fanselow & LeDoux, 1999; Phillips & LeDoux, 1992). Recent research on amygdala function has revealed that substructures within the amygdala can support different aspects of acquisition, consolidation, and expression of conditioned fear (Holmes & Singewald, 2013; Kim & Jung, 2006; Pape & Pare, 2010). When delay fear conditioning is arranged experimentally, the animal learns not just associations between the CS and US, but also associations between the background context and the US (Marlin, 1981). This contextual learning recruits the hippocampus for the encoding of complex multi-modal features (Rudy *et al.*, 2004). Other regions play important roles in relaying stimulus inputs and response outputs, but the amygdala has been the key intermediary between these various structures.

An emerging literature shows that the insertion of a trace interval between CS and US presentations alters the circuitry that mediates fear learning. Although our understanding of the neurobiology of trace fear conditioning is still in its infancy, distinctions between trace and delay conditioning and the variety of brain regions supporting trace conditioning make it a powerful assay for study of the neurobiology, molecular biology, and biochemistry of cognition and learning. Indeed, trace conditioning is often used as an assay for disruption of hippocampal learning in mouse genetic knock-down models (Brzózka & Rossner, 2013; Mao *et al.*, 2009), fragile X syndrome (Hayashi *et al.*, 2007; Zhao *et al.*, 2005), and Alzheimer's disease (Kaczorowski *et al.*, 2011; Ohno *et al.*, 2006), although it should be noted that hippocampal dependence is clearly dependent on length of the trace interval (Chowdhury *et al.*, 2005; Misane *et al.*, 2005). Much of the focus of the neurobiological literature on trace fear conditioning is directed at understanding the mechanisms of association formation with degraded CS-US contingencies. As a consequence, the idea that trace fear conditioning results in a weak CS-US association is the dominant theoretical perspective in modern neurobiological studies. Nonetheless, a close examination of the structures that support trace fear conditioning reveals how different systems may support some of the different theoretical processes described above. We focus our neurobiological review on the hippocampus, medial prefrontal cortex, entorhinal and perirhinal cortices, and amygdala.

Hippocampus

Much evidence demonstrates involvement of the hippocampus in trace fear conditioning, but the specific role of the hippocampus remains unclear. Studies have shown that lesions to the hippocampus disrupt trace fear learning (Bangasser *et al.*, 2006; Burman *et al.*, 2006; McEchron *et al.*, 1998). Of note is a computational model accounting for hippocampal involvement in some forms of conditioning (Moustafa *et al.*, 2013), although this model primarily accounts for the emergence of trace-cued responses over many more trials than those used in fear conditioning. The roles of different hippocampal subregions in this task are less clear with reports of conflicting roles for dorsal and ventral poles of the hippocampus (Cox *et al.*, 2013; Czerniawski *et al.*, 2009; Czerniawski *et al.*, 2012; Esclassan *et al.*, 2009b; Trivedi & Coover, 2006; Yoon & Otto, 2007).

Electrolytic and NMDA induced lesions of the dorsal hippocampus (DH) disrupt acquisition of trace fear conditioning (Burman *et al.*, 2006; Chowdhury *et al.*, 2005; Desmedt *et al.*, 2003; Fendt *et al.*, 2005; McEchron *et al.*, 2000; Quinn *et al.*, 2002; Quinn *et al.*, 2008;

Trivedi & Coover, 2006). Additionally, inactivation of the DH with muscimol disrupts both acquisition and expression of trace conditioning (Esclassan *et al.*, 2009b; Guimarães *et al.*, 2011; Raybuck & Lattal, 2011). Similarly, NMDA receptor antagonism (Misane *et al.*, 2005; Quinn *et al.*, 2005; Seo *et al.*, 2008; Wanisch *et al.*, 2005), disruption of cell signaling cascades during training or testing (Huang *et al.*, 2010; Peters *et al.*, 2009), inhibition of protein synthesis following conditioning (Runyan & Dash, 2005; Wanisch *et al.*, 2005), and inhibition of the micro RNA mir-123 (Wang *et al.*, 2013) all impair trace fear conditioning. For example, CREB phosphorylation in the DH is enhanced following expression of trace fear conditioning and suppression of CREB activity with RNAi infusion produces significant deficits in trace conditioning (Peters *et al.*, 2009). Importantly, a number of these reports demonstrate that disruption of DH processes affect trace conditioning without affecting delay conditioning (Beeman *et al.*, 2013; Burman *et al.*, 2006; Chowdhury *et al.*, 2005; Esclassan *et al.*, 2009b; Guimarães *et al.*, 2011; McEchron *et al.*, 2000; Misane *et al.*, 2005; Quinn *et al.*, 2002; Raybuck & Lattal, 2011). For instance, we have shown that DH inactivation with muscimol disrupts acquisition of trace conditioning, but does not affect delay conditioning (Raybuck & Lattal, 2011). Collectively these findings suggest that the DH is involved selectively in trace conditioning rather than generally involved in fear learning or expression.

A number of studies suggest that the ventral hippocampus (VH) also plays a critical role in trace fear learning. For instance, inactivation of the VH produces deficits in trace fear conditioning (Cox *et al.*, 2013; Czerniawski *et al.*, 2009; Gilmartin *et al.*, 2012). Similarly, lesions to the VH produce deficits in trace conditioning (Yoon & Otto, 2007). Although a number of investigations of the DH in trace fear learning demonstrate selective effects on trace conditioning (i.e., no effects on delay conditioning; see Burman *et al.*, 2006; Chowdhury *et al.*, 2005; Esclassan *et al.*, 2009b; Guimarães *et al.*, 2011; McEchron *et al.*, 2000; Misane *et al.*, 2005; Quinn *et al.*, 2002; Raybuck & Lattal, 2011), few studies have examined whether the effects of VH manipulations are unique to trace conditioning. However, some reports suggest that manipulations in the VH have greater effects on trace conditioning compared to DH manipulations (Cox *et al.*, 2013; Czerniawski *et al.*, 2009; Yoon & Otto, 2007). Yoon and colleagues (2007) reported that pre- or post-training NMDA induced excitotoxic lesions to the VH produce deficits in trace conditioning, whereas in the DH only post-training lesions produced deficits. Similarly, Czerniawski *et al.* (2009) reported that inactivation of the VH with muscimol during either training or testing produced deficits in trace conditioning, while inactivation of the DH was without effect (Czerniawski *et al.*, 2009). Further, Cox *et al.* (2013) showed that VH inactivation had robust effects across multiple measures fear expression, but that DH inactivation also decreased trace cued-freezing. These interesting effects, though in contrast to other findings of similar manipulations (Esclassan *et al.*, 2009b), may suggest that under certain conditions the VH takes control of learning of trace conditioning.

Equally of note are studies that have examined contributions of DH and VH to both trace and delay conditioning. Lesion or inactivation of the DH selectively affects trace conditioning, whereas lesion or inactivation of the VH affects both trace and delay fear conditioning (Esclassan *et al.*, 2009b; Fanselow & Dong, 2010; Sierra-Mercado *et al.*, 2011; Trivedi & Coover, 2006), suggesting that while the DH may play a selective role in trace fear learning the role of the VH may extend to fear learning in general or anxiety expression. The roles of the DH and VH in fear remain complicated, however, because a recent study found that ventral hippocampus inactivation affects expression of trace but not delay fear conditioning (Cox *et al.*, 2013).

Hippocampal manipulations have axis dependent effects on anxiety, with greater effects in VH than in DH (Bannerman *et al.*, 2003; Bannerman *et al.*, 2004; Trivedi & Coover, 2004).

This relation may suggest that hippocampal support for trace conditioning is dependent on anxiety state. Indeed, long-term potentiation (LTP), a cellular model of learning, is differentially regulated along the septo-temporal axis of the hippocampus, and exposure to stress can decrease LTP in DH and increase it in the VH (Maggio & Segal, 2007). Thus, more extensive training (entailing more aversive US exposures) or more stressful manipulations (such as multiple behavioral assays in individual subjects or stressful handling procedures) may shift support for acquisition or expression of trace fear conditioning from the DH to the VH. Though there have not yet been any reports explicitly examining the effects of stress or training intensity on DH and VH involvement in trace fear conditioning, such effects would explain many discrepant findings about the roles of these subregions in learning, as well as have clear implications for the involvement of different hippocampal regions in human anxiety disorders.

The hippocampus is sub-divided into multiple subregions (CA1, CA3, & DG) that may differentially contribute to trace fear conditioning. Some studies have provided evidence for differential roles of these sub-regions in trace fear conditioning (Gilmartin & McEchron, 2005a; Huerta *et al.*, 2000; Rogers *et al.*, 2006; Weitemier & Ryabinin, 2004), with independent evidence supporting an interaction between the DG and CA1 in acquisition and retrieval of trace conditioning. However, considering the multitude of hippocampal mechanisms that may support trace conditioning and the limited studies showing activity or effects of disruption of these regions in trace conditioning, it may be premature to suppose clear roles for these regions. Recent advances, such as optogenetics, may allow more targeted manipulations that can address the distinct roles for CA1, CA3, and DG in trace conditioning.

Medial prefrontal cortex

Much evidence implicates structures of the medial prefrontal cortex (mPFC) in trace fear conditioning. However, the mPFC comprises multiple subregions with distinct functions. Prelimbic cortex and infralimbic cortices are both involved in expression and inhibition of fear responses (Peters *et al.*, 2009; Santini *et al.*, 2008; Stafford *et al.*, 2013) and the anterior cingulate cortex is involved in attentional processes (Han *et al.*, 2003; Perry *et al.*, 2011; Sarter *et al.*, 2006; Vertes, 2006). Work suggests that distinct contributions of the prelimbic cortex and anterior cingulate contribute to trace fear conditioning.

Lesion and inactivation studies demonstrate that the prelimbic (PrL) cortex critically contributes to trace fear conditioning. Indeed, NMDA antagonism in the mPFC produces deficits in acquisition of trace conditioning (Gilmartin & Helmstetter, 2010; Gilmartin *et al.*, 2013a) and perturbation of nicotinic acetylcholinergic signaling in the PrL facilitates acquisition of TFC (Raybuck & Gould, 2010). Additionally, extra-cellular signal regulated kinase (ERK) phosphorylation, a marker of neuronal plasticity (Selcher *et al.*, 1999), increases in the PrL following acquisition of trace conditioning (Runyan & Dash, 2004; Runyan *et al.*, 2004). Further, recording studies demonstrate that during the trace interval, cells in the PrL differentially fire across acquisition of trace conditioning (Gilmartin & McEchron, 2005b), with firing increasing as the trace-US relation is acquired, and optogenetic inactivation of mPFC during the trace interval blocks acquisition of trace conditioning (Gilmartin *et al.*, 2013b). Indeed, even one month after conditioning, lesions or inactivation of the mPFC (centered around the PrL) still produce deficits in responding to a trace conditioned cue (Beeman *et al.*, 2013; Quinn *et al.*, 2008), suggesting that the PrL may be a site of long-term storage for trace fear learning.

Because the PrL is heavily interconnected with both the hippocampus and amygdala (Hoover & Vertes, 2007; Vertes, 2004), it is possible that the PrL plays a critical role in trace fear conditioning by maintaining a representation of the CS in working memory during

the trace interval. Indeed, in humans the dorsolateral prefrontal cortex, an analogous region in terms of function and connectivity (Vertes, 2004), is active during working memory dependent tasks such as n-back, as well as during human trace fear conditioning (Barbey *et al.*, 2013; Jansma *et al.*, 2000), though the exact role of this region in trace fear conditioning is not yet clear. Additionally, more complex accounts of the role of the PrL involving contingency degradation are also worth consideration.

Anterior Cingulate Cortex

The anterior cingulate cortex (ACC) is involved in both attention and working memory dependent tasks in both humans and rodents (Dalley *et al.*, 2004; Hartley & Speer, 2000; Peterson *et al.*, 1999). In humans, the ACC is activated during trace conditioning trials (Büchel *et al.*, 1999; Knight *et al.*, 2004). In rodents, the key involvement of the ACC in acquisition of trace fear conditioning has been revealed in studies showing that transcription factors are expressed in the ACC following trace conditioning and lesions to the ACC prior to training produce deficits in acquisition of trace learning (Han *et al.*, 2003; Weitemier & Ryabinin, 2004). Additionally, recording studies suggest that pyramidal neurons in the ACC are involved in acquisition of trace fear learning (Steenland *et al.*, 2012) and trace fear conditioning drives immediate changes in neural excitability in this region (Descalzi *et al.*, 2012). Indeed, facilitation of intra-cellular signaling via over expression of Ca²⁺/calmodulin dependent protein kinase IV enhances trace conditioning and long-term potentiation (a molecular model of learning) in the ACC (Wu *et al.*, 2008). However, electrolytic lesions to the ACC failed to disrupt acquisition of trace eyeblink conditioning (McLaughlin *et al.*, 2002), which generally involves many more trials than fear conditioning despite the much shorter trace interval (Burman & Gewirtz, 2004; Moyer *et al.*, 1990). Thus, the ACC may be involved in early attentional processing of the CS, having a critical role in CS-US association formation during conditioning with few trials in trace fear conditioning (Han *et al.*, 2003), but not being required for learning the timed conditioned response in trace eyeblink conditioning (McLaughlin *et al.*, 2002). Though speculative, such a selective role for the ACC in trace conditioning may make trace fear conditioning a useful assay for dissecting attentional and timing circuitry.

Entorhinal and Perirhinal Cortices

Studies suggest that both entorhinal and perirhinal cortices are involved in trace conditioning. Lesions or inactivation of these brain regions disrupts trace fear conditioning (Esclassan *et al.*, 2009a; Kholodar-Smith *et al.*, 2008), and muscarinic acetylcholinergic signaling in these regions is also implicated in trace conditioning (Esclassan *et al.*, 2009a; Kholodar-Smith *et al.*, 2008), but the relative role of each region is not yet clear. In the perirhinal cortex, NMDA lesions produce deficits in trace-cued learning (Kholodar-Smith *et al.*, 2008), though lesions also disrupt contextual fear conditioning, suggesting generalized deficits in fear learning or expression (for review, see Kent & Brown, 2012). In the entorhinal cortex, lesions produce selective deficits in trace conditioning without affecting context or delay conditioning (Esclassan *et al.*, 2009a). It should be noted that in either brain region, antagonism of muscarinic acetylcholine receptors selectively disrupts trace fear conditioning (Bang & Brown, 2009; Esclassan *et al.*, 2009a). Collectively, these findings suggest critical roles for both these regions in trace fear conditioning.

The connectivity of the entorhinal cortex may make it particularly well situated to process complex CS-US relations during fear conditioning. The entorhinal cortex is a primary input to the hippocampus and may act as a bridge between the hippocampus and amygdala to facilitate complex cue learning during Pavlovian fear conditioning tasks (Morales *et al.*, 2007; Vertes, 2006). In fact, entorhinal cortex is critically involved in latent inhibition of delay-fear conditioning (Lewis & Gould, 2007; Lewis & Gould, 2007). Additionally, the

entorhinal cortex hosts persistent firing neurons that maintain firing for fixed periods following stimulation, a mechanism that may support timing models as well as working memory (Hasselmo & Brandon, 2008). Indeed, cholinergic modulation of Layer 3 output from the entorhinal cortex to the hippocampus is critical to trace conditioning (Suh *et al.*, 2011), suggesting that the entorhinal cortex is involved in CS processing during conditioning. Thus, both perirhinal and entorhinal cortices appear to be critically involved in trace fear conditioning, and persistent firing neurons within these regions likely support learning of CS-US temporal relations.

Amygdala

Although the role of the amygdala in delay and contextual fear conditioning has been well characterized (for review, see Fanselow & Poulos, 2005; Pape & Pare, 2010), its role in trace conditioning is less well understood. In trace conditioning, a number of additional brain regions critically contribute to formation of a CS-US association, thus it is not clear how these regions (particularly the DH and mPFC) interact with the amygdala to generate fear learning. Indeed, such a distributed network is involved that it may be possible for multiple components to host CS-US associations and drive expression of those associations (Raybuck & Lattal, 2011). Thus, insertion of an empty trace interval between the CS and US may fundamentally change how these two stimuli are associated at a neurobiological level. Indeed, studies show mixed evidence for amygdala involvement in trace fear learning.

Similar to delay and contextual fear conditioning, lesions to the amygdala prior to conditioning produce deficits in trace fear conditioning (Selden *et al.*, 1991). Although it may be the case that these types of amygdalar lesions cause general deficits in fear output, recent studies show that inactivation of the amygdala with muscimol or disruption of protein synthesis with anisomycin can disrupt trace fear learning (Gilmartin *et al.*, 2012; Guimarães *et al.*, 2011; Kwapis *et al.*, 2011), and that cholinergic signaling in the amygdala can selectively modulate trace fear learning (Baysinger *et al.*, 2012). The variety of these manipulations suggests that the amygdala is critically involved in trace fear conditioning. However, in contrast to these findings in rats, in mice we have shown that inactivation of the amygdala with muscimol fails to affect trace conditioning, though in the same mice it generates robust deficits in contextual fear learning, and in delay conditioned animals the expected deficits appear (Raybuck & Lattal, 2011). These findings demonstrate that the specific role of the amygdala in trace fear conditioning remains to be determined.

Collectively these reports suggest that there may be some procedural variables that these effects depend upon. For instance, variations in CS length, length of trace interval, or number of training trials may shift animals from amygdala involvement in trace conditioning to amygdala-independent trace fear learning. While it is not yet clear what conditions are required for trace conditioning to generate amygdala-independent fear learning, these findings have been replicated recently in rats (Thompson, et al. 2012). Further work on this issue will be necessary to determine how trace conditioning can generate amygdala-independent fear learning. This issue may shape how we conceive human emotional learning, because like trace conditioning, human learning often involves significant trace intervals as well as cortical processing. The possibility that trace conditioning may occur independent of amygdala activity may have clear clinical implications as models of circuitry generated in these animal models are generalized to human populations with generalized anxiety disorders, such as post-traumatic stress disorder (PTSD).

A larger question that emerges from these independent examinations of brain regions that support trace fear conditioning is how this network of regions interacts to support learning of a trace fear association. Several studies have manipulated the function of multiple regions

in trace conditioning, including inactivation of the mPFC, DH, and amygdala (Guimarães *et al.*, 2011), nicotinic signaling in the mPFC, DH, and VH (Raybuck & Gould, 2010), and independent inactivation of acetylcholine release (Flesher *et al.*, 2011). Few studies, however, have examined the effects of simultaneous manipulations of multiple brain regions, which is necessary to determine how these structures interact to support trace conditioning, or how this network is affected by inactivation of a single component. A recent study used a disconnection approach to examine the interaction between the mPFC and hippocampus (Gilmartin *et al.*, 2012). Interestingly, Gilmartin *et al.* (2012) found that unilateral inactivation of the VH or amygdala was sufficient to impair trace fear conditioning, whereas bilateral inactivation of the prelimbic mPFC was required to cause an impairment. Though an interactive and compensatory network of brain regions may best explain variations in roles for hippocampus and amygdala in trace fear conditioning, much additional work will be necessary to determine how these various regions interact to support trace fear learning.

Bridging the gap between theoretical and neurobiological approaches

The complicated circuitry that underlies trace fear conditioning provides some insight into how and where different theoretical mechanisms may occur. For example, the idea that a stimulus trace must be maintained in working memory until the US occurs is consistent with literature on hippocampal and prefrontal cortical involvement in working memory and attention. If the function of these structures is impaired, the ability to maintain that stimulus trace in working memory is lost, resulting in little to no conditioning. Because in delay fear conditioning the CS and US co-terminate, the working memory demands are fewer, resulting in strong conditioning, even when the hippocampal or prefrontal cortical functions are impaired.

The involvement of the medial prefrontal cortex in trace fear conditioning also speaks to the potential inhibitory, safety signal component of trace conditioning. Although the relation between inhibitory learning in trace fear conditioning and inhibitory learning that may occur during extinction is not clear, there is a documented role for the medial prefrontal cortex in both forms of learning (Peters *et al.*, 2009; Santini *et al.*, 2008; Stafford *et al.*, 2013). This may suggest that this structure plays a similar role in both procedures with excitatory and inhibitory processes involved in extinction and trace conditioning being mediated by different regions of the mPFC.

Finally, many of the structures described above that are involved in trace fear conditioning have been implicated in studies of timing (e.g., Díaz-Mataix *et al.*, 2013). Indeed a recent study suggests that a distinction can be made between the role of the hippocampus in associative learning and timing during trace conditioning. In an appetitive task, Tam and Bonardi (2012) found that hippocampal inactivation disrupted the spread of behavior during the trace interval. The interesting implication of this finding is that it is possible for hippocampal manipulations to disrupt timing without necessarily altering associative learning. Thus, when the various behavioral properties of different neurobiological systems are considered, it is very possible that a given manipulation will alter behavior through different possible mechanisms depending on the targeted region.

Applications and Conclusion

The simple insertion of a temporal gap between a CS and a US alters the theoretical and neurobiological systems that underlie learning. The study of trace fear conditioning has revealed novel insights about the ways in which animals encode temporal information, as well as the ways in which these signals are processed in the brain. A clear conclusion from

behavioral work is that differences in performance as a function of the time of CS and US delivery may reflect any of several mechanisms. Multiple learning processes – association formation, timing, inhibition – may contribute to the behavioral differences between trace and delay fear conditioning. At a basic science level, the continued refinement of behavioral approaches to test theories of these different processes will continue to reveal novel insights into the mechanisms that underlie timing and learning in general.

At a translational level, the study of trace conditioning in rodents may reveal insights about higher cognitive function in humans. Studies of the neural and cognitive substrates of trace fear conditioning suggest that both humans and rodents rely on similar mechanisms to form a trace conditioned CS-US association. Additionally, there is some face validity to trace fear conditioning, in that predictive cues are most useful if they do not coincide with an event but rather if they allow for some anticipatory response. In humans and animals, predictive cues in real-world situations are likely to be temporally dissociated from positive or aversive outcomes. Thus, disruption of the circuitry supporting trace conditioning could underlie a number of anxiety disorders in humans, such as, post-traumatic stress disorder, generalized anxiety disorders, and specific phobias. Indeed, trace conditioning has been used to examine disruption of learning by exposure to drugs of abuse (Davis & Gould, 2007; Gould *et al.*, 2004; Hunt *et al.*, 2009; Raybuck & Gould, 2009), ontology of the development of learning (Barnet & Hunt, 2005; Hunt & Richardson, 2007; Hunt *et al.*, 2006; Wagner & Hunt, 2006), effects of drugs of abuse on development (Wagner & Hunt, 2006; Yttri *et al.*, 2004), and as a screen for cognitive disruption in mouse disease models for schizophrenia (Brzózka & Rossner, 2013; Mao *et al.*, 2009), fragile X syndrome (Hayashi *et al.*, 2007; Zhao *et al.*, 2005), and Alzheimer's disease (Kaczorowski *et al.*, 2011; Ohno *et al.*, 2006). Though speculative, the above points imply that more work should focus on examining the role of cognitive and neural mechanisms involved in the formation of temporally discontinuous associations in both natural and pathological conditions.

A particularly powerful aspect of trace fear conditioning is that it provides a rapidly acquired model of working memory and attention-dependent associative learning that may well reflect human learning mechanisms. Trace conditioning provides an excellent avenue through which these mechanisms can be mapped onto cortex dependent higher-order human learning. As a fast, translatable model of learning, trace conditioning may be well utilized as a translational bridge between simpler learning models and human learning.

Acknowledgments

Preparation of this article was supported by grants DA007262 and DA031537 to JDR and by grants MH077111, DA025922, and DA018165 to KML.

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