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CHANGES IN RESTING-STATE FUNCTIONAL CONNECTIVITY FOLLOWING DELAY AND TRACE FEAR CONDITIONING ACQUISITION AND EXTINCTION

by

Douglas H. Schultz

A Thesis Submitted in

Partial Fulfillment of the

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ABSTRACT

CHANGES IN RESTING-STATE FUNCTIONAL CONNECTIVITY FOLLOWING DELAY AND TRACE FEAR CONDITIONING ACQUISITION AND EXTINCTION

by

Douglas H. Schultz

The University of Wisconsin-Milwaukee, 2014
Under the Supervision of Professor Fred J. Helmstetter

Consolidation is the process of stabilizing a recently acquired memory into a more permanent or durable form. Several studies with laboratory animals have uncovered valuable information about the process of consolidation, but less is known about the process of consolidation in healthy humans. The current study examined the consolidation of emotional memories in different brain circuits in healthy humans using resting-state fMRI. We used the acquisition and extinction of two variations of Pavlovian fear conditioning, delay and trace, which rely on slightly different circuits to examine changes in functional connectivity related to a general fear learning process and also to examine how these changes may differ in these circuits. We found that the acquisition of delay and trace fear conditioning involves similar circuitry including the amygdala, but that trace conditioning involves the addition of a few more brain regions to the general circuit including the hippocampus. Twenty-four hours following acquisition there was an increase in functional connectivity between the amygdala and several other brain areas including the hippocampus and medial prefrontal cortex for both the delay and trace groups suggesting that these changes reflect the consolidation of a general fear memory.

We also observed changes in connectivity that were specific to the trace group in brain regions thought to be specifically involved in trace conditioning including the medial prefrontal cortex and the retrosplenial cortex. By seven days after acquisition most of the changes in connectivity had returned to baseline. Extinction data revealed that the ventromedial prefrontal cortex was involved in forming this inhibitory memory and that connectivity between the amygdala and a region of ventromedial prefrontal cortex increased for the trace group following extinction. These results suggest that consolidation can be measured in healthy humans using resting-state fMRI and that these processes occur in the same circuits that are responsible during training.

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The development of non-invasive methods of functional imaging has led to a better understanding of the neural processes that occur in humans while they are forming or using a memory. However, these advancements led to studies that have primarily focused on the neural activity evoked by stimuli presented during either encoding or retrieval. While this stimulus-evoked activity is critical for the formation of memory, we know that a series of important events and patterns of activity in the nervous system continue for a period of time following the learning event to support the consolidation or storage of this memory. Memory consolidation can be addressed in animal models by disrupting or specifically measuring this activity at a variety of time points following learning. Until recently, questions regarding the process of consolidation in humans were difficult to answer. With the development of resting-state functional connectivity approaches to FMRI data we can now examine changes in the nervous system that may support the consolidation of memories in humans. This project will use Pavlovian fear conditioning, a well understood type of learning, to examine the changes in functional connectivity at different time points during the process of memory consolidation.

Pavlovian fear conditioning

Pavlovian fear conditioning is a type of learning in which a previously neutral conditional stimulus (CS) is paired with an aversive unconditional stimulus (UCS). After repeated pairings, the CS can evoke a learned conditional response (CR) that is usually similar to the unconditional response (UCR) evoked by the UCS alone (Pavlov, 1927). This type of learning can be assessed during the acquisition session, in which stimuli are paired, or in a specific test session that occurs following acquisition. Evidence of learning in human participants can be assessed by a variety of dependent measures

including: skin conductance (Prokasy & Ebel, 1967), heart rate (Obrist, Wood & Perez-Reyes, 1965), eye blinks (Cason, 1922) and fear potentiated startle (Brown, Kalish & Farber, 1951). Pavlovian fear conditioning has been used extensively in several model systems to investigate learning, memory and emotion.

Pavlovian fear conditioning can be done in several different ways. In *delay* conditioning the CS and the UCS can overlap temporally or be presented sequentially with no period of time between them. Delay conditioning has been examined extensively and the neural circuitry that supports delay conditioning is well understood. In *trace* conditioning the CS and UCS are both presented but there is a temporal gap between the offset of the CS and the onset of the UCS. This gap is referred to as the trace interval. Trace conditioning has received less attention in the literature and the neural circuitry that supports learning under these conditions is not as well understood. However, it is known that learning the association between the CS and the UCS when a stimulus free period is inserted between them recruits different neural circuitry.

Delay fear conditioning

The neural circuitry that supports fear learning in delay conditioning has been characterized by a variety of studies (for review see Fanselow and Poulos, 2005; Kim & Jung, 2006). In fear learning, an association is made between the sensory information related to the presentation of the CS and input that conveys information regarding the UCS or the affective or motivational consequence of the UCS. The amygdala is a critical region in fear conditioning because it is an area where this information converges. Specifically, the basolateral subdivision (BLA) of the amygdala is the region where CS

and UCS information converges. Plasticity in the BLA is critical for fear conditioning (Romanski, Clugnet, Bordi & LeDoux, 1993). The central nucleus (CeA) of the amygdala is also an important structure involved in fear learning. The CeA is the primary output structure of the amygdala (Veening, Swanson, & Sawchenko, 1984) and projections from the BLA to the CeA are important for the expression of fear behavior. The CeA then projects to several brain regions involved in the generation of fear behavior such as the periaqueductal gray (PAG) for freezing behavior, and the hypothalamus for autonomic responses (Gray & Magnuson, 1987). Although the BLA is considered the main region of signal convergence and is critical for fear learning, several other regions exhibit plastic changes that also support this type of learning. Wilensky and colleagues (2006) found that plasticity in the CeA was necessary for fear learning. Thus, there appear to be plastic changes in areas that are "downstream" of the CS-UCS association that are classically thought to play a larger role in fear expression, and these changes also support fear memory. Parsons and colleagues (2006) found that plasticity in the medial geniculate nucleus of the thalamus was necessary for fear learning when an auditory cue served as the CS. This is a region that is "upstream" of the CS-UCS association that is likely involved in the processing of the CS. The neural circuitry supporting fear conditioning is well defined. The BLA is the main area of signal convergence and is critical for fear learning. Additionally, plasticity in other regions that are both up and downstream of the CS-UCS association is also necessary. This suggests that fear memories are supported by plastic changes in the nervous system in several distributed regions of the brain (Helmstetter, Parsons, & Gafford, 2008).

A great deal of the information regarding the circuitry that supports fear conditioning was collected by manipulating the nervous system in laboratory animals. Technological and ethical limitations prevented researchers from being able to closely examine the neural correlates of fear learning in humans until the 1990s. The introduction of functional imaging has allowed researchers to examine the brain activity the supports fear learning in humans in a non-intrusive way.

Several neuroimaging studies have found amygdala activation during fear conditioning (e.g., Buchel, Morris, Dolan, & Friston, 1998; LaBar, Gatenby, Gore, LeDoux, & Phelps, 1998; Schultz, Balderston & Helmstetter, 2012). These data are consistent with the idea that the amygdala plays a critical role in fear learning. However, there is still a debate about the interpretation of this amygdala activity. One interpretation (Knight, Smith, Cheng, Stein, & Helmstetter, 2004b) is that the amygdala is critical in learning the CS-UCS association. This interpretation is supported by studies that show higher levels of amygdala activation early in training or after experimental contingencies have been changed when the association is being formed or modified. Another interpretation of the amygdala activity observed in neuroimaging studies is that it reflects the generation of a conditional response. Support for this interpretation comes from studies (Cheng, Knight, Smith, Stein, & Helmstetter, 2003; Cheng, Knight, Smith & Helmstetter, 2006) that used a method of analysis that focuses on modeling activity that is correlated with behavioral responses. Amygdala activity has also been interpreted as reflecting a response to novel stimuli, but the specific stimulus attributes that contribute to this response are still unclear (see Balderston, Schultz, & Helmstetter, 2011; Blackford, Buckholtz, Avery, & Zald, 2010). Although there is some debate around the

interpretation of the amygdala activity the basic finding is consistent with the findings in the non-human animal literature.

Neuroimaging studies in humans have found amygdala activation during a fear conditioning task, but they have also identified a number of other brain regions that seem to be involved in fear learning. The anterior cingulate cortex (ACC) is one region that has been identified in several neuroimaging studies of fear conditioning (Knight, Smith, Stein & Helmstetter, 1999; Phelps, Delgado, Nearing & LeDoux, 2004). One study (Dunsmoor, Bandettini & Knight, 2007) found that activity in the ACC corresponded to the rate of reinforcement during conditioning. The ACC showed more activity to a CS that was always paired with a UCS than to a different CS that was intermittently paired with a UCS. The authors suggest that this indicated that the ACC plays a role in coding the strength of the CS-UCS contingency. Other researchers have suggested that activity in the ACC during conditioning plays a slightly different role. The cortical thickness and CS evoked activity in the ACC both positively correlate with skin conductance responses during fear conditioning (Milad, Quirk, Pitman, Orr, Fischl, & Rauch, 2007). This suggests that ACC activity might be more closely related to fear expression. Another study (van Well, Visser, Scholte, & Kindt, 2012) found that individual differences in fear expression were positively correlated with activity in the amygdala and ACC. The relationship between fear expression and ACC activity existed despite the fact that participants showed a similar level of contingency knowledge as measured with UCS expectancy. This study supports the idea that ACC activity is more related to fear expression than it is to coding the strength of the CS-UCS association.

The insula has been identified in several neuroimaging studies of fear conditioning using a variety of different parameters (Buchel et al., 1998; Gottfried & Dolan, 2004; Phelps et al., 2004). It has been proposed that the insular cortex transmits a representation of fear to the amygdala (Phelps, O'Connor, Gatenby, Gore, Grillon, & Davis, 2001). However, the magnitude of the response in the insula seems to vary based on the rate of reinforcement. For example, Dunsmoor and colleagues (2007) found that insula activity was greater in response to a CS+ that was paired with the UCS 50% of the time compared to activity evoked by a CS+ that was paired with the UCS 100% of the time. This data is consistent with the idea that the insula is involved in introceptive processing (for review see Craig, 2009) because a predictable UCS is less aversive than an unpredictable UCS and would therefore evoke less insula activity.

Primary sensory areas of the cortex have also been implicated in fear conditioning. Sensory cortical involvement in fear conditioning can be characterized in different ways. Sensory regions can show larger responses to a stimulus that has been paired with an aversive UCS. Fear conditioning studies that used a visual stimulus as a CS have found increased activity in visual cortex when a CS is presented after learning (Cheng et al., 2003; Knight et al, 1999). Another study (Li, Howard, Parrish & Gottfried, 2008) found a similar effect in olfactory cortex when odors were used as a CS. These data suggest that associative learning can increase activity in the sensory regions that process CS information. Conditioning can also result in the CS activating a region of sensory cortex that is normally activated by the UCS. Another group identified a different type of sensory cortex activity involved in fear conditioning. They found that a CS that predicted a painful stimulus activated the same area of somatosensory cortex as

the painful stimulation alone (Yaguez, Coen, Gregory, Amaro, Altman, Brammer, Bullmore, Williams, & Aziz, 2005). This data demonstrates that the association between the CS and the UCS can result in the CS being able to activate a neural representation of the UCS that it predicts.

The amygdala has been identified as an integral brain structure that supports fear learning with a variety of different experimental approaches ranging from lesion studies in laboratory animal experiments to neuroimaging studies with human participants. The basolateral subregion of the amygdala is critical for fear learning, but recent experiments have suggested that fear learning also involves a variety of other brain structures including the CEA, thalamus, ACC, insula, and sensory cortex.

Trace fear conditioning

The neural circuitry that supports the acquisition of trace fear conditioning is similar to the circuitry for delay conditioning, but the inclusion of the trace interval necessitates the involvement of additional structures. Although the delay conditioning circuitry is well characterized, the circuitry supporting trace fear conditioning has received less attention in the literature but is also an active area of research.

The amygdala is a critical structure in delay conditioning and plasticity in the amygdala is necessary for this type of learning, but there is some conflicting evidence regarding whether or not the amygdala is required for trace fear conditioning. Raybuck and Lattal (2011) found that inactivation of the amygdala prior to training did not result in any deficit in trace conditioning, but it did result in an impairment in delay conditioned animals. However, disruption of protein synthesis in the amygdala during the period of

memory consolidation after training impairs both trace and delay conditioning (Kwapis, Jarome, Schiff, & Helmstetter, 2011). Additionally, another study found that even unilateral inactivation of the amygdala resulted in a deficit in trace conditioning (Gilmartin, Kwapis, & Helmstetter, 2012). There are some discrepancies in the literature, but there is evidence that neural activity and protein synthesis in the amygdala are necessary for both delay and trace fear conditioning. The amygdala may be equally important for both delay and trace fear conditioning, but there are other brain regions that are involved in trace but not delay conditioning.

Many studies have focused on the involvement of the hippocampus and prefrontal cortex in trace conditioning. The involvement of these brain regions in trace conditioning has primarily studied eyeblink as a CR. Eyeblink conditioning typically consists of an auditory CS being paired with either a puff of air to the eye or electrical stimulation of the area surrounding the eye. Although eyeblink conditioning is a paradigm that engages a different basic circuitry, it can provide information about what additional brain regions need to be involved when a trace interval is inserted between the CS and the UCS.

The hippocampus is necessary for the initial acquisition of trace eyeblink conditioning and during the consolidation period following training (Kim, Clark, & Thompson, 1995; Moyer, Deyo, & Disterhoft, 1990). However, the involvement of the hippocampus in trace conditioning is time dependent. Hippocampal lesions that occur 28 days after training do not impact trace conditioning performance (Takehara, Kawahara, & Kirino, 2003). This suggests that the hippocampus is involved during trace conditioning training and through a short consolidation window, but it does not seem to be the permanent site of the memory.

Evidence from trace conditioning studies in human participants is consistent in finding that the hippocampus is necessary for this type of learning. Eyeblink studies in human participants have found that amnesic patients with damage to the hippocampus acquire delay eyeblink conditioning, but fail to acquire when a trace conditioning paradigm is used (Clark & Squire, 1998). Neuroimaging studies in human participants have also found that the hippocampus is important in this paradigm. Hippocampal activity measured by FMRI has been observed during trace fear conditioning. Specifically, the hippocampus shows more activity in participants who are more temporally accurate in predicting the occurrence of the UCS (Knight et al., 2004a). This data suggests that the hippocampus may be involved in coding temporal information during trace conditioning. Further support for the involvement of the hippocampus in temporal coding during trace conditioning comes from electrophysiology experiments. Several studies have observed learning related changes in firing patterns in the hippocampus when compared to a pseudoconditioned control group (Gilmartin, & McEchron, 2005a) or a delay conditioned control group (Green & Arenos, 2007).

In summary, the hippocampus is necessary for trace fear conditioning and is required during acquisition and for a short consolidation period following training. However, the hippocampus does not appear to be necessary at more remote time points after training. This suggests that the hippocampus is not the site where trace conditioning memories are permanently stored. There is also support for the idea that the role of the hippocampus in trace conditioning is to code the temporal information regarding the timing of the UCS.

The medial prefrontal cortex (mPFC) has also received a great deal of attention in the trace conditioning literature. Disruption of the mPFC results in trace conditioning deficits while delay conditioning is intact (Kronforst-Collins, & Disterhoft, 1998; Runyan, Moore, & Dash, 2004; Weible, McEchron, & Disterhoft, 2000). This suggests that mPFC is specifically engaged when a trace interval is inserted between the CS and the UCS.

It has been proposed that the role of the mPFC in trace conditioning is to hold a representation of the CS in working memory during the stimulus free trace interval so that representation can be associated with the UCS. Electrophysiological recordings of mPFC neurons during trace conditioning provide support for this interpretation. Gilmartin and McEchron (2005b) found that neurons in the prelimbic region of the mPFC exhibited sustained increased activity specifically during the trace interval. Additionally, a FMRI study on trace conditioning found increased activity in frontal cortical regions during a trace interval compared to the period of time when the CS was present (Knight et al, 2004a). The mPFC maintaining a representation of the CS during the trace interval is also consistent with studies examining working memory. Working memory refers to the storage and processing information in the absence of environmental stimuli (Baddeley, 1992). Studies have found that the mPFC is engaged during working memory tasks (Carlson, Martinkauppi, Rama, Salli, Korvenoja, & Aronen, 1998; Ranganath, Cohen, Dam, & D'Esposito, 2004), and there is evidence that mPFC activity during the encoding of non-contiguous associations is positively related to subsequent recall performance (Hales, & Brewer, 2010).

The mPFC appears to be important for the acquisition, consolidation, and recall of trace conditioning memories even at long retention intervals. This is different from the role of the hippocampus. The hippocampus is necessary for the acquisition and initial consolidation of the trace conditioning memory, but hippocampal lesions at remote time points do not result in a memory deficit at test (Takehara et al., 2003). Medial prefrontal cortex lesions that occur 28 days after training result in memory deficits (Takehara et al., 2003). Furthermore, when neural activity in the mPFC is inhibited by a GABA agonist the recall of a remote trace memory is disrupted (Blum, Hebert, & Dash, 2006). The inhibition of neural activity in the mPFC at recent time points also disrupts the recall of trace memories.

In summary, the only difference between delay and trace conditioning is the insertion of a stimulus free period between the CS and the UCS. The amygdala is required for both of these types of learning. The insertion of this trace interval requires the addition of other brain areas into the neural circuitry that supports this type of associative memory. The hippocampus appears to be necessary for trace conditioning. Specifically, the hippocampus is involved during the training session and for a short period of consolidation following the training session. Hippocampal manipulations at remote retention intervals do not result in memory deficits so it does not seem to be involved in the long term storage of this type of memory. The hippocampus might be involved in coding the timing of the UCS. The mPFC is another brain region that is recruited during trace conditioning, but is not necessary for delay conditioning. The mPFC is necessary at remote time points and may be involved in maintaining a representation of the CS in working memory during the trace interval so that

representation can be associated with the UCS. The mPFC may also be involved in the long term storage of trace condition memories.

Extinction

Extinction occurs if a previously trained CS is repeatedly presented in the absence of the UCS. This results in a decrease in the magnitude or probability of expressing a CR (Pavlov, 1927). Initially, extinction learning was thought to weaken the association between the CS and the UCS and that this process of unlearning was ultimately responsible for the decrease in the CR (Rescorla, 1969). However, more recent theories of extinction learning emphasize that the original CS-UCS association is not erased, but that a new inhibitory CS-no UCS association is learned (for review see Bouton, 2004). Support for this interpretation comes from several sources including spontaneous recovery and renewal (for review see Myers & Davis, 2002). Most of the studies examining the neural circuitry that supports extinction learning have examined extinction after delay conditioning. The neural circuitry that supports extinction is not characterized as well as the circuit for fear acquisition, but there has been increased attention to understand fear extinction due to the possibility of applying this information in the treatment of anxiety disorders.

The mPFC has received a great deal of attention for its role in extinction. Lesions of the mPFC in rats do not result in deficits in fear acquisition or in the gradual decrease in CR expression during extinction training, but they do result in an extinction deficit when the animals are tested the next day (Quirk, Russo, Barron, & Lebron, 2000). This suggests that the mPFC is involved in the storage or retrieval of the extinction memory.

Neural recording data is also consistent with this idea. Cells in the infralimbic (IL) region of the mPFC show increases in firing during an extinction recall session (Milad & Quirk, 2002). Furthermore, the rate of firing from these cells was negatively correlated with measures of fear. The more these cells fired the less fear was observed during the extinction recall session. Temporary inactivation of the mPFC with a GABA agonist during extinction training also resulted in an extinction deficit when animals were tested the next day (Sierra-Mercado, Padilla-Coreano, & Quirk, 2010). Neuroimaging studies with human participants have also supported the mPFC playing a role in fear extinction. One study examined brain activity during acquisition, extinction and an extinction recall session (Phelps et al., 2004). During the extinction recall session activity in the vmPFC, a ventral region of the mPFC, was correlated with the degree of extinction recall as measured behaviorally with SCR. Another study found that the presentation of an extinguished CS+ evoked more activity in the vmPFC than the presentation of a non-extinguished CS+ (Milad, Wright, Orr, Pitman, Quirk, & Rauch, 2005). It has also been observed that cortical thickness in the vmPFC is positively correlated with the retention and recall of extinction memories (Milad, Quinn, Pitman, Orr, Fischl, & Rauch, 2005). There is a great deal of converging evidence from a variety of experimental models and approaches that the mPFC cortex is critical for fear extinction.

The exact mechanisms underlying the involvement of the mPFC in extinction are still under investigation. One current hypothesis is that activity in the mPFC during the recall of extinction results in inhibition of brain regions involved in the expression of fear behavior. Consistent with this idea, activation of the IL leads to a decrease in the responsiveness of output neurons located in the CEA (Quirk, Likhtik, Pelletier, & Pare,

2003). There are neurons in the mPFC that project to a set of inhibitory interneurons located in the amygdala which may serve to dampen activity in the fear expression circuitry during extinction (Likhtik, Pelletier, Paz, & Pare, 2005). If these inhibitory interneurons are eliminated following extinction training, the animal exhibits an extinction deficit (Likhtik, Popa, Apergis-Schoute, Fidacaro, & Pare, 2008). This suggests that the projections from the mPFC to these inhibitory interneurons may be the mechanism through which the mPFC can inhibit the expression of fear during the recall of extinction.

The hippocampus is another brain structure that is involved in extinction learning. The hippocampus plays a major role in contextual components of conditioning, and extinction learning is largely context specific. Therefore, the hippocampus might be involved in processing the current context and determining whether or not it is a context in which the CS is predictive of the UCS. However, research regarding the role of the hippocampus in extinction learning is inconsistent. Lesioning the hippocampus does not result in any deficit in the context dependent nature of extinction learning (Frohardt, Guarraci, & Bouton, 2000). Another study temporarily inactivated the hippocampus during extinction training and found that it abolished renewal (Corcoran & Maren, 2001). Renewal refers to the return of fear to an extinguished CS when the organism is returned to the original training context. Follow-up experiments by Corcoran and Maren (2004) found that some of the inconsistencies observed with the effect of hippocampal manipulations on extinction memories could be explained by methodological differences between experiments. Functional imaging studies on humans have also supported the idea that the hippocampus is involved in extinction learning. Knight and colleagues

(2004b) found a deactivation in the hippocampus during extinction training relative to a group that was exposed to CS-UCS pairings. Activation of the hippocampus has been observed during the recall of extinction, but this activation was only apparent when the conditional stimuli were presented in the context in which they were extinguished (Kalisch, Korenfeld, Stephan, Weiskopf, Seymour, & Dolan, 2006). Increases in hippocampal activity have also been detected during the recall of extinction for a CS that has undergone extinction relative to a CS that had been trained but not extinguished (Milad et al., 2007). The same study also found that hippocampal and mPFC activity was more positively correlated during the recall of extinction in participants that demonstrated a larger extinction effect behaviorally. Although there are some discrepancies in the literature, it appears that the hippocampus is involved in extinction. It is likely that the hippocampus is involved in determining whether or not an excitatory CS-UCS association or an inhibitory CS-no UCS association is used to guide behavior based on the context that the organism currently inhabits.

The amygdala is necessary for the acquisition of fear conditioning but it also plays a role in the process of extinction. Plasticity within the amygdala mediated by NMDA receptors during extinction training is necessary for extinction to occur (Falls, Miserendino, & Davis, 1992) and the disruption of AMPA receptors during extinction training also results in an extinction deficit (Kim, Lee, Park, Song, Hong, Geum, Shin, & Choi, 2007). Extinction relies upon plasticity in the amygdala during the extinction training session. Extinction learning is also dependent on plasticity in the amygdala during a window of consolidation following the extinction training session (Herry, Trifilieff, Micheau, Luthi, & Mons, 2006; Lu, Walker, & Davis, 2001). Human

neuroimaging studies have also found support for the amygdala playing a role in extinction learning. Differential amygdala activity during extinction learning has been observed between a previously trained CS+ relative to a CS- (LaBar et al, 1998), but this differential activity was evident on early extinction trials only. Another study compared CS evoked activity in a group of participants undergoing extinction training to a different group who were still presented with CS-UCS pairings (Knight et al., 2004b). The extinction group exhibited more amygdala activity than the group that was still receiving CS-UCS pairings. Phelps and colleagues (2004) conducted a similar experiment, but found that amygdala activity during extinction was decreased relative to amygdala activity during acquisition. This result is somewhat inconsistent with the data from Knight and colleagues (2004b), who observed amygdala activity during extinction, but not during acquisition. There were several methodological differences between the studies. Phelps and colleagues (2004) used a partial reinforcement schedule during acquisition which may have delayed the extinction process itself or some of the subtle attentional shifts which Knight and colleagues (2004b) interpreted as influencing amygdala activity. There are some discrepant findings in the literature, but there is a great deal of support for the involvement of the amygdala in extinction.

Systems consolidation

Delay and trace fear conditioning, as well as extinction, involve slightly different circuitry. However, the circuitry involved in learning and storing these memories can also change as time passes. The process of memory consolidation occurs at both a synaptic level and a systems level. Synaptic consolidation consists of creating and strengthening synaptic connections and is typically complete in the span of hours (for

review see Dudai, 2004). Systems consolidation is a slower process that involves reorganization of brain areas involved in storing the memory. This reorganization can involve a change in the brain areas that serve to support the memory over time (Frankland, & Bontempi, 2005).

The amygdala is an important brain structure for fear conditioning. Maren, Aharonov and Fanselow (1996) lesioned the BLA in rats at several time points surrounding delay fear conditioning and found that lesions made at any point from a week prior to conditioning to a month following conditioning disrupted memory. This suggests that the amygdala is still involved in the storage of a delay fear memory for up to a month after learning. Another study found that the amygdala was still necessary for the storage of a delay fear conditioning memory after 16 months (Gale, Anagnostaras, Godsil, Mitchell, Nozawa, Sage, Wiltgen, & Fanselow, 2004). These findings suggest that the amygdala is a site of permanent storage for a delay fear conditioning memory. At this time it is unknown whether or not the amygdala is a storage site for long-term trace fear conditioning memories.

The hippocampus is specifically important for trace conditioning. However, hippocampal lesions made 28 days after trace fear conditioning do not disrupt the memory (Takehara et al., 2003). A similar result has been observed on another hippocampal dependent process. The contextual component of delay fear conditioning is dependent on the hippocampus. Contextual fear is disrupted when the hippocampus is lesioned one day after training, but not when the lesion is made 7, 14, or 28 days following training (Kim & Fanselow, 1992). This same pattern has been observed in several different tasks in several different species although the amount of time before a

memory becomes independent of the hippocampus is variable (for review see McKenzie & Eichenbaum, 2011). These results suggest that tasks dependent on the hippocampus initially require the hippocampus for consolidation, but as time passes the memory may no longer rely on the hippocampus.

As consolidation occurs memories can be supported by brain structures that were not initially involved in storage. The systems consolidation theory suggests that as consolidation progresses a shift occurs from the initial structures involved to cortical structures. Consistent with this idea, immediate early gene (IEG) activity is increased in the anterior cingulate cortex 36 days following context conditioning and inactivation of the ACC 18 or 36 days but not at 1 or 3 days after conditioning resulted in memory deficits (Frankland, Bontempi, Talton, Kaczamarek, & Silva, 2004). Additionally, lesions of mPFC 28 days after trace conditioning disrupt memory, but lesions of the same region had little effect on memory when they were made 1 day after trace conditioning (Takehara et al., 2003). Another study used a method of cellular tagging to identify neurons that were involved in the formation of a contextual fear memory. Then the animals retrieved this memory either 2 or 14 days later. In the hippocampus and amygdala there was a greater overlap between the cells activated by learning and the cells activated by retrieval when the retrieval took place 2 days after training. In contrast, cells in the cortex showed a similar level of overlap in activity between training and retrieval regardless of whether or not retrieval took place 2 or 14 days after training (Tayler, Tanaka, Reijmers, & Wiltgen, 2013). Studies in humans have also found that the recall of recent memories activates the medial temporal lobe and the recall of more remote memories activates cortical areas (Smith, & Squire, 2009). Together these findings

suggest that cortical regions become more involved in the storage of memory at remote time points.

The neural circuitry supporting the acquisition and the storage of this memory can vary. Trace conditioning relies on a similar circuit as delay, but requires some additional resources. The circuitry that supports the acquisition and the storage of these memories is also dynamic over time. As the memory consolidates and moves into long term storage different brain regions support the memory. Systems consolidation predicts that some types of memories become less dependent on the initial circuitry that supported them and more dependent on the cortex.

Resting-state functional connectivity

Learning and memory are supported by plastic changes in the nervous system that modulate the efficacy of synaptic connections. The process of changing synaptic connections starts during a learning event and continues for a period of time as long term memory is formed and stored. The majority of neuroimaging studies examine brain responses evoked by stimuli during training or test and little attention has been focused on the neural activity occurring in the period of time following a task. If synaptic connections are being strengthened during this period of time following the task we should be able to observe and measure those changes. Functional connectivity analyses on neuroimaging data are another approach that can be applied to further our understanding of the changes in the nervous system that support learning and memory. Functional connectivity approaches can examine the degree of correlation in the neural signal in different brain regions. This approach can be applied to examining the

correlation between different brain regions while participants are performing a task. A functional connectivity approach can also be applied to neuroimaging data that is collected during a period of time when participants are not engaged in any particular task, referred to as resting-state.

Changes in functional connectivity have been observed during the performance of experimental tasks. One study used visual conditional stimuli and measured the correlation the BOLD signal in the amygdala and fusiform gyrus (Dunsmoor, Prince, Murty, Kragel, & LaBar, 2011). This study found that the correlation between the BOLD signal in these two regions increased throughout the progression of the conditioning session. Another study found that the functional connectivity of the visual cortex was increased when participants were presented with a stimulus that had previously been paired with a shock relative to a stimulus that had not been paired with shock (Damaraju, Huang, Barrett, & Pessoa, 2009). Functional connectivity also increases between the amygdala and the medial geniculate on CS+ trials relative to CS- trials when a visual CS and an auditory UCS are used (Iidaka, Saito, Komeda, Mano, Kanayama, Osumi, Ozaki, & Sadato, 2009). Connectivity between portions of the fear circuit also varies depending on whether or not participants expect to receive a shock (Linnman, Rougemont-Bucking, Beucke, Zeffiro, & Milad, 2011). This data provides support for the idea that functional connectivity is variable and that it can be modified during the performance of a task.

Functional connectivity can also be assessed when participants are not engaged in a specific experimental task. This paradigm is referred to as resting-state. Resting-state functional connectivity approaches focus on the spontaneous, low frequency (<0.1Hz) fluctuations that occur in the BOLD signal in the absence of direct stimulation (for

review see Fox & Raichle, 2007). Resting-state networks defined by selecting different seed regions are consistent with anatomical connectivity through white matter tracts (van den Huevel, Mandl, Kahn, & Pol, 2009). Resting-state networks have been identified in both rats and humans (Pawela, Biswal, Cho, Kao, Li, Jones, Schulte, Matloub, Hudetz, & Hyde, 2008). Additionally, resting-state connectivity maps are stable over time (Shehzad, Kelly, Reiss, Gee, Gotimer, Uddin, Lee, Margulies, Roy, Biswal, Petcova, Castellanos, & Milham, 2009).

A recent study examined how anxiety influences the resting-state connectivity profile of the amygdala (Kim, Gee, Loucks, Davis, & Whalen, 2011). In this study participants underwent a resting-state FMRI scan and were then given a questionnaire to assess both state and trait anxiety. Participants were divided into groups based on whether or not they scored high or low on the anxiety measure. A functional connectivity analysis was then conducted using the amygdala as a seed region. The low anxiety group showed a positive correlation between the vmPFC and the amygdala and a negative correlation between the dorsal medial PFC and the amygdala. The high anxiety group showed a negative correlation between the vmPFC and the amygdala and no correlation between the dorsal medial PFC and the amygdala. These results are consistent with the idea that the vmPFC is involved in the inhibition of fear and that more dorsal mPFC regions are involved in the expression of fear.

Another study compared the resting-state functional connectivity of the amygdala in a group of veterans with PTSD to that of a control group of veterans that saw combat, but did not develop PTSD. Participants in the control group showed a negative correlation between amygdala activity and activity in the dorsal mPFC. PTSD patients

lacked this negative correlation. PTSD patients instead showed a shift toward a more positive correlation between amygdala activity and activity in the hippocampus and the insula. These results are consistent with the results of Kim and colleagues (2011) in which high anxiety participants exhibited a similar breakdown of the negative correlation seen between the amygdala and the dorsal mPFC in controls. Additionally, the finding of increased connectivity between the amygdala and insula in PTSD patients is consistent with the involvement of the insula in fear conditioning (Sripada, King, Garfinkle, Wang, Sripada, Welsh, & Liberzon, 2012).

Although resting-state networks are relatively consistent across time, researchers have found changes in resting-state connectivity measures following behavioral tasks (Duff, Johnston, Xiong, Fox, Mareels, & Egan, 2008; Grigg & Grady, 2010; Schroeder, Weiss, Procissi, & Disterhoft, 2012; Stevens, Buckner, & Schacter, 2010). Studies have examined the effect of stress on the resting-state functional connectivity of the amygdala (Veer, Oei, Spinhoven, Buchem, Elzinga, & Rombouts, 2011). In this experiment one group of participants underwent a stress manipulation in which they were required to prepare for an impromptu five minute speech in addition to performing difficult math problems in front of a group of judges. During this period of time the control group was instructed to think about their favorite movie. Cortisol levels were increased in the stress group relative to the control group following the stress manipulation indicating that it was successful in evoking stress. Resting-state FMRI scans were done after the stress manipulation. The amygdala was used as a seed region for the functional connectivity analysis. The stress group showed increased functional connectivity between the amygdala and both ventral and dorsal regions of the PFC relative to the control group.

These results support the idea that the resting-state functional connectivity of the amygdala can be altered following a behavioral task.

Very little is currently known about the effects of learning on resting-state functional connectivity. One group measured the resting-state functional connectivity of the amygdala at several times across a two day procedure that involved both acquisition and extinction phases (Hermans, Kanen, Fernandez, & Phelps, 2011). They found that connectivity between the amygdala and a region of the mPFC increased across time. However, due to the nature of their experimental design it was impossible to determine whether or not those changes could be attributed to acquisition, extinction, or some other variable such as time. Another recent experiment examined the effect of trace conditioning on resting-state function connectivity in rabbits (Schroeder et al., 2012). Resting-state scans were collected on rabbits prior to any behavioral manipulation and served as a comparison for subsequent resting-state scans. Rabbits then underwent trace eyeblink conditioning for ten days. Thirty days later the rabbits were given a reminder trace conditioning session and resting-state FMRI data was collected again. The hippocampus was selected as a seed region due to its known involvement in trace conditioning. After conditioning, the hippocampus showed increased functional connectivity with the cerebellum, a critical brain region for eyeblink conditioning, and an area of the thalamus.

Recent work in our lab examined how the resting-state functional connectivity of the amygdala was altered following delay fear conditioning (Schultz, Balderston, & Helmstetter, 2012). We collected resting-state FMRI data immediately prior to and immediately following a fear conditioning session (Figure 1).

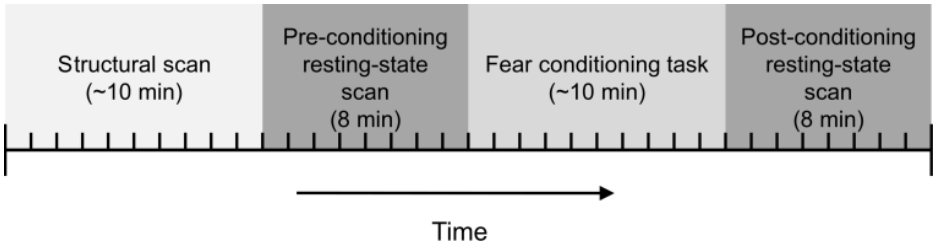

Figure 1. Diagram of the phases of the experiment.

Participants learned the contingencies based on UCS expectancy data (Figure 2A) and SCR data (Figure 2B). Previous research indicates that the amygdala is a critical brain region for fear learning so we created a region of interest (ROI) mask for each individual participant with a segmentation program in Freesurfer (Figure 3A). We found that the CS+ evoked a larger response in the amygdala than the CS- during the conditioning session and that this effect was bilateral (Figure 3B, 3C). Because the conditioning effect in the amygdala did not differ across hemispheres we used the mean signal from both the left and the right amygdala as the seed region for the resting-state functional connectivity analyses.

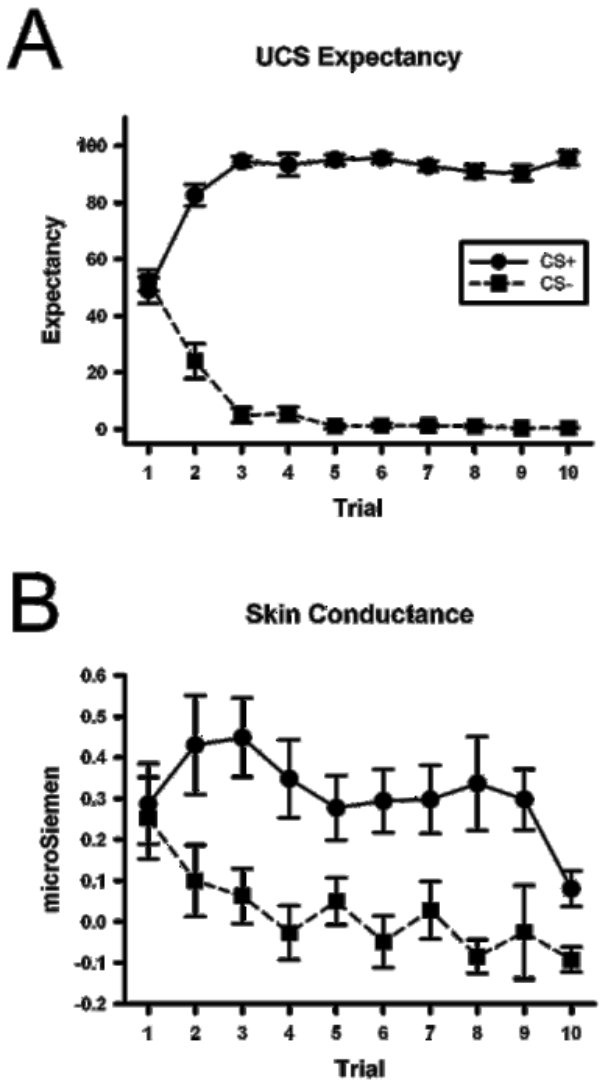

Figure 2. Participants learn differential delay fear conditioning on a measure of UCS expectancy and skin conductance. (A) Mean UCS expectancy ratings on CS+ and CS− trials during the conditioning task. (B) Mean SCR on CS+ and CS− trials during the conditioning task.

The resting-state functional connectivity of the amygdala was analyzed and compared for the resting-state scan before conditioning and the resting-state scan that followed conditioning. We found that the functional connectivity maps at each point in time were similar and consistent with a previous study of amygdala functional

connectivity at rest (Roy, Shehzad, Margulies, Kelly, Uddin, Gotimer, Biswal, Castellanos, & Milham, 2008). However, we found differences in the maps when we directly compared the map acquired prior to conditioning to the map acquired following the conditioning session.

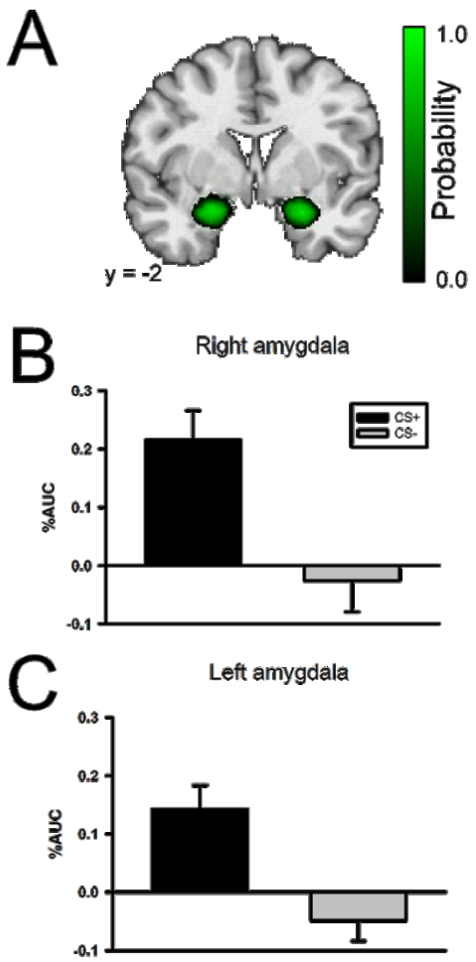

Figure 3. The left and right amygdala are both involved in delay fear conditioning. (A) An amygdala probability mask created by combining each participant's anatomical amygdala ROI. The color scale corresponds to the probability that the region is included in any of the participants' amygdala ROIs. Black indicates a low probability and bright green indicates high probability. (B) Bar graph depicting the AUC values for both the CS+ and CS− evoked BOLD response in the right amygdala during the conditioning task. (C) Bar graph depicting the AUC values for both the CS+ and CS− evoked BOLD response in the left amygdala during the conditioning task.

We found that a region in the superior frontal gyrus showed an increase in functional connectivity with the amygdala following fear conditioning (Figure 4). This was the only area that showed a significant difference in amygdala connectivity from the resting-state scan before conditioning to the resting-state scan following conditioning. Additionally, we found that changes in connectivity from pre to post-conditioning resting-state scans were related to behavioral performance during the conditioning session (Figure 5). Specifically, we found that functional connectivity changes between the amygdala and another region of the superior frontal gyrus were related to UCS expectancy performance. Individual participants that demonstrated better learning on the UCS expectancy measure showed greater increases in the connectivity between the amygdala and the superior frontal gyrus from pre to post-conditioning resting-state scans (Figure 5A-B). A similar association was found when we examined how behavioral performance as measured by SCR was related to changes in the functional connectivity of the amygdala from pre to post-conditioning sessions. SCR performance during the conditioning session was positively correlated with the change in functional connectivity between the amygdala and the ACC (Figure 5C-D).

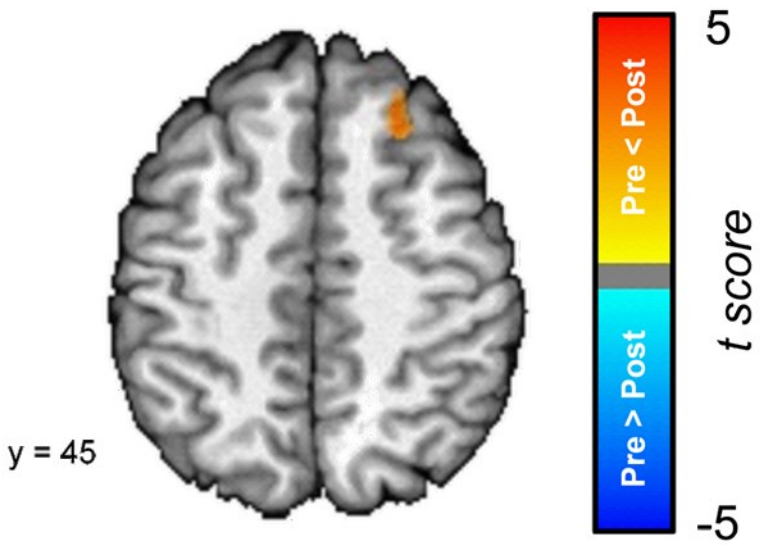

Figure 4. Functional connectivity between the amygdala and the dorsal medial prefrontal cortex increased following fear conditioning. The dorsal medial prefrontal cortex (superior frontal gyrus) [Talairach coordinates: 24, 30, 45] shows a significant increase in its connectivity with the amygdala following the conditioning task. The colors on the brain map correspond to the *t*-values on the color scale.

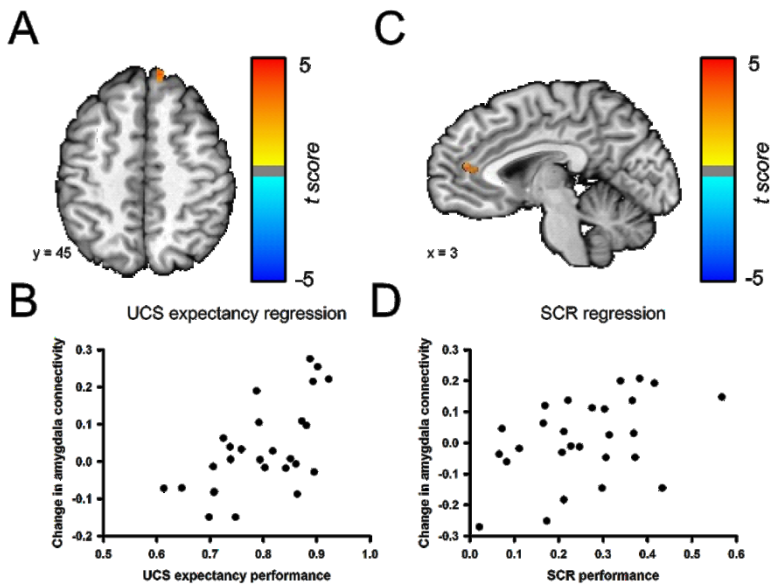

Figure 5. Changes in amygdala connectivity are related to behavioral performance during conditioning. (A) Connectivity changes between the amygdala and the superior frontal gyrus [Talairachcoordinates:11,48,9] are positively correlated with UCS expectancy performance during the conditioning task. (B) Scatterplot depicting UCS expectancy performance and change in connectivity between the amygdala and superior frontal gyrus. (C) Connectivity changes between the amygdala and the ACC [Talairach coordinates:3,13, −5] are positively correlated with SCR performance during the conditioning task. (D) Scatterplot depicting SCR performance and change in connectivity between the amygdala and the ACC.

Resting-state functional connectivity is a noninvasive method that can be used to understand the processes occurring in the human brain following a learning event that may serve in the storage of memory. Most human neuroimaging research to this point has focused on the brain activity evoked by stimuli during encoding or retrieval of memory. A resting-state functional connectivity approach to FMRI data will allow us to examine how functional connectivity is altered following learning, and the temporal dynamics of those changes. Previous data suggests that functional connectivity approaches to FMRI data have merit and that this approach can identify changes in connectivity induced by a behavioral task.

Aims

The goal of this experiment was to examine how resting-state functional connectivity is differentially modified by delay versus trace conditioning and to observe time-dependent changes in the fear network as a new long term memory is formed and stabilized. Delay and trace conditioning rely upon slightly different neural circuitry and we examined how resting-state functional connectivity changed between important components for each circuit. Previous neuroimaging work has typically focused on brain activity that is evoked by a stimulus during either encoding or retrieval of a memory. Resting-state procedures allowed us to examine changes in brain activity occurring following learning. We assessed resting-state functional connectivity immediately prior to and following a conditioning session, 24 hours following conditioning, 1 week following conditioning and immediately following extinction. This approach has furthered our understanding of how these changes support the consolidation of a memory.

Previous experiments have examined changes in functional connectivity following conditioning, but these experiments have primarily compared connectivity after conditioning to connectivity prior to conditioning. This design is a good first step, but several other factors could account for the observed changes in connectivity. Exposure to the CS or UCS could alter functional connectivity. The pre-post comparison is also confounded by time. It is possible the functional connectivity changes over time and that conditioning isn't actually altering connectivity. In order to address these methodological shortcomings we used an explicitly unpaired control group which was exposed to the stimuli, but did not learn an association between a CS and a UCS.

Another goal of this experiment was to compare the neural circuitry that supports the extinction of delay and trace conditioning. The acquisition of delay and trace conditioning rely on different brain regions and it is possible that extinction of these two different types of memory requires different brain regions as well. Furthermore, we examined how resting-state functional connectivity is altered by extinction learning by comparing connectivity following an extinction session.

Methods

Participants

Sixty-three right-handed, neurologically normal undergraduates from the University of Wisconsin-Milwaukee participated in the study. Twelve participants were excluded from analysis (nine due to excessive head movement during at least one of the functional scans, two due to equipment failure, and one who did not understand the task instructions). The final sample consisted of fifty-one (28 females) participants ranging from 18 to 32 years old (M = 21.65 years, SEM = 0.44). There were seventeen participants in each group. An additional six participants were excluded from the extinction analysis due to excessive movement during the extinction session or during the subsequent resting-state scan. The final sample for the extinction analysis included fifteen participants in each of the three groups. Participants received extra-credit in a psychology course, were paid $140, and received a picture of their brain. All participants provided informed consent. All procedures were approved by the Institutional Review Boards for human subject research at the University of Wisconsin-Milwaukee and the Medical College of Wisconsin.

Apparatus

Electrical stimulus

The UCS in the conditioning phase was a 500 ms duration electrical stimulation delivered via an AC (60 Hz) source (Contact Precision Instruments, Model SHK1, Boston, MA) through two surface cup electrodes (silver/silver chloride, 8 mm diameter, Biopac model EL258-RT, Goleta, CA). The electrodes were filled with electrolyte gel (Signa Gel, Parker Laboratories, Fairfield, NJ) and placed on the skin over the participant's right tibial nerve above the right medial malleous. Each participant individually determined the maximum UCS intensity used in the experiment prior to the start of the experiment in a work up procedure. The work up procedure consisted of no more than five presentations of the electrical stimulation. Each presentation was rated by the participant on a scale from 0 to 10 (0 = *no sensation*, 10 = *painful, but tolerable*). The intensity of the electrical stimulation was increased until the participant rated it as a 10. Participants were able to rate the stimulation higher than a 10 at which point the intensity was be decreased. The UCS intensity was set at the level that each participant rated as definitely painful, but tolerable.

UCS expectancy

Participants manipulated a joystick (Current Designs, Model HHSC-JOY-5, Philadelphia, PA) to report their expectancy of receiving the UCS throughout the conditioning portion of the study. The joystick controlled a cursor presented at the bottom of the visual display. Real-time feedback of the position of the cursor was continually presented. Participants were instructed to manipulate the joystick with their

right hand. Participants were given verbal instructions on how to use the joystick before the experiment began. They were instructed to place the cursor at 0 if they were certain that they would not experience the UCS, at 50 if they were not sure if they would experience the UCS, and at 100 if they were certain that they would experience the UCS. Participants were instructed to update the position of the cursor continuously throughout the conditioning portion of the experiment. Participants were not instructed about any of the potential relationships between the visual stimuli and the UCS.

Skin conductance

Skin conductance was recorded using a Contact Precision Instruments unit (Boston, MA) with a SC5 24-bit digital amplifier from Contact Precision Instruments at 200 Hz. Psychlab software (London, UK) was used for skin conductance data analysis. Skin conductance data was collected with electrodes (Biopac, Goleta, CA; Model EL258-RT) filled with electrolytic gel (Signagel, Parker Laboratories, Fairfield, NJ). The electrodes were attached to the sole of the left foot two centimeters apart.

Visual stimuli

The experiment was conducted using Presentation software (Albany, CA) on a PC computer. Visual stimuli and the UCS expectancy rating bar were presented to participants while they were in the scanner using a back projection system with prism glasses mounted on the head coil. The visual stimuli were a green trapezoid and an orange pentagon. Assignment of the visual stimuli to be the CS+ and the CS- was counterbalanced in the conditioning groups and the visual stimuli were presented to the unpaired control group in the same order as they appeared for the conditioning groups.

MRI

Whole brain imaging was conducted using a 3T short bore GE Signa Excite MRI system. Functional images were collected using a T2* weighted gradient-echo, echoplanar pulse sequence. We collected 4 mm sagittal slices (TR = 2 sec; TE = 25 ms; field of view = 24 cm; flip angle = 77°; voxel size = 3.75 × 3.75 × 4.0 mm) during the experiment. The resting-state runs consisted of two hundred forty whole brain scans. The acquisition and extinction runs consisted of two hundred ninety whole brain scans. High resolution spoiled gradient recalled (SPGR) images (1 mm slices) were collected in a sagittal orientation (TR = 8.2 sec; field of view = 24 cm; flip angle = 12°; voxel size = 0.9375 × 0.9375 × 1.0 mm) and served as an anatomical map for the functional images.

Procedure

Participants were given instructions on how to use the joystick to report their UCS expectancy rating and the UCS intensity work-up procedure was completed before they entered the scanner. Participants reported to the scanner on three different days over a seven day period of time. There were three different groups in the experiment. One group received delay fear conditioning. A differential conditioning paradigm was used. The visual stimulus that served as the CS+ was presented ten times and co-terminated with the shock UCS on all trials. The CS- was also presented ten times, but it was never be paired with the UCS. The stimuli were presented in one of two pseudorandom trial orders with the restriction that there could not be more than two consecutive trials of the same type. The visual stimuli assigned to be either the CS+ or the CS- were also counterbalanced. For the delay group the CS was 8 seconds in duration. The trace group

also received differential conditioning. The visual stimulus that served as the CS+ was presented ten times. The CS+ was 4 seconds in duration. A 3.5 second trace interval followed the CS+ before the presentation of the UCS. All CS+ presentations were followed by the UCS. Trial order and assignment of the CS+ and CS- was counterbalanced with the same method used in the delay group. The third group was an explicitly unpaired control group. The unpaired group was presented with the same visual stimuli and shocks as the other groups, but the shocks occurred during the intertrial interval and were explicitly unpaired with the visual stimuli. The visual stimuli were 8 seconds in duration and the same pseudorandom trial order and counterbalancing were used as in the other two groups.

On the first day, we collected a high resolution anatomy scan. The next scan was an eight minute resting-state scan. Participants were instructed to close their eyes, but remain alert during all of the resting-state scans. Following the resting-state scan participants were split into three groups for the acquisition functional scan. One group received delay fear conditioning acquisition. Another group received trace fear conditioning acquisition. A third group was presented with the same number of visual stimuli and shocks as both of the conditioning groups, but they were explicitly unpaired. This group served as a control for the exposure to the stimuli. After the acquisition scan participants received another resting-state scan. After the second resting-state scan, they completed a questionnaire designed to assess their knowledge of the associations during the acquisition session. Participants returned to the scanner 24 hours later. On the second day of the experiment we collected anatomical data and another resting-state scan. Participants then returned 6 days later for the final day of the experiment. The last day of

the experiment consisted of another anatomical scan followed by a resting-state scan. After the resting-state scan there was an extinction session during which we collected functional data. The extinction session consisted of presenting the same visual stimuli for the same number of trials as in the acquisition session, but the shock was not presented during the extinction session (see Figure 6). Following the extinction session there was another resting-state scan. Participants then completed a questionnaire regarding the experimental contingencies.

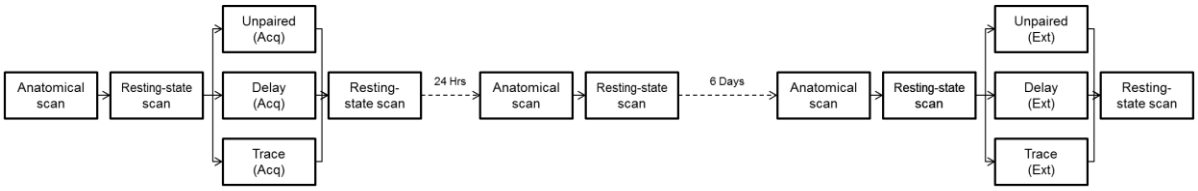

Figure 6. Design of the experiment.

Data analysis

UCS expectancy

UCS expectancy was analyzed by calculating the mean of the last second of the CS presentation for each trial type for the delay and unpaired group. Expectancy was calculated by taking the mean of the last second of the trace interval for each trial type in the trace group. Acquisition was assessed by examining the mean expectancy rating for each CS type across all ten trials. In order to examine both the retention of the original

memory and the process of extinction we split the extinction trials into the first three to test for retention and the last seven to examine extinction.

Skin conductance response

The SCR was analyzed by subtracting the mean value during a 2 second baseline from the peak value during the entire CS interval (Pineles, Orr, & Orr, 2009) for each trial. We also calculated the peak of the response during the trace interval period for the trace group. Acquisition was assessed by examining the mean SCR for each CS type across all ten trials. In order to examine both the retention of the original memory and the process of extinction we split the extinction trials into the first three to test for retention and the last seven to examine extinction.

General FMRI

Reconstruction and imaging processing were completed with AFNI (Cox, 1996). Raw data was motion corrected, passed through an edge detection algorithm and registered to the fifth volume of the functional run. The data was visually inspected for large head movements. Images containing large, discrete head movements were censored in the task scans. Censoring was not conducted on the resting-state data in the interest of not breaking the time series. Participants with excessive head movement were excluded from further analysis.

Acquisition and extinction FMRI

High-resolution structural scans were warped to Talairach space by manually placing anatomical markers (Balderston, Schultz, & Helmstetter, 2011). We used the

AFNI program 3dDeconvolve to model the mean impulse response function (IRF) evoked by the CS+ and the CS-. Head motion and motor processes associated with the gross movement of the UCS expectancy joystick were included as regressors of no interest. We then calculated the percent area under the curve (%AUC) of the mean IRF for each stimulus type. The resulting maps were blurred using a 4 mm full-width at half-maximum Gaussian kernel. These maps were used in the group analysis. Cluster thresholding (Forman, Cohen, Fitzgerald, Eddy, Mintun, & Noll, 1995) was used to correct for multiple comparisons across all the voxels in the whole brain volume with the use of Monte Carlo simulations in the AFNI program Alphasim. A CS type by group ANOVA was conducted on the %AUC data for the acquisition data. A CS type by group ANOVA was conducted on the %AUC data for the delay and trace group in the extinction session.

Resting-state FMRI

Variability in the BOLD time series accounted for by respiration and cardiac rhythm was removed from the raw data using previously published methods (Birn, Diamond, Smith, & Bandettini, 2006) in which cardiac and respiratory signals and their first harmonics serve as variables in a multiple regression analysis. Baseline, drift, and head motion effects will be removed from the time series using AFNI's 3dDeconvolve command. The mean signal from white matter and ventricle maps were included as regressors of no interest. A band pass filter was applied to the time series to attenuate frequencies above 0.1 Hz and below 0.01 Hz. The time series from several ROIs including the right and left amygdala, right and left hippocampus, right and left retrosplenial cortex, right and left insula, mPFC, and vmPFC was then extracted for each

participant. The amygdala, hippocampus, and insula ROIs were defined anatomically by Freesurfer segmentation (Destrieux, Fischl, Dale, & Halgren, 2010). The retrosplenial ROIs were defined anatomically by combining Brodmann areas 29 and 30. The mPFC ROI was selected based on the results from acquisition session. The vmPFC ROI was anatomically defined as a 6 mm radius sphere centered in the subgenual ACC [0, 21, 4]. The mean time series for these ROIs for each participant at each time point was calculated and then correlated with the time series from every other ROI at each time point for each participant. The individual r statistics were then normalized using a Fisher's z transformation. The normalized data was used to calculate all group level statistics. In order to test our hypotheses we ran two *t*-test contrasts on the connections between each ROI at each time point. The first contrast compared the connectivity for the unpaired group against the connectivity for both the delay and trace groups. This contrast was designed to examine general changes in connectivity related to fear learning regardless of whether or not it occurred in the delay or trace group. The second contrast compared the connectivity for the delay group to the connectivity for the trace group. This contrast was designed to examine differences in connectivity between the delay and trace groups. In order to correct for the multiple comparisons being tested across all of these connections at each time point we ran a permutation test. Each participant was randomly assigned to one of the three groups (Nichols & Holmes, 2002). We then ran all of the comparisons and tallied how many contrasts were significant at $p < .05$. We ran 500 iterations, examined the number of significant contrasts in the null distribution, and found that identifying more than 12 significant tests was unlikely due to chance, $p < .05$ (see Figure 7). We then used this as a threshold for all of the connectivity analysis.

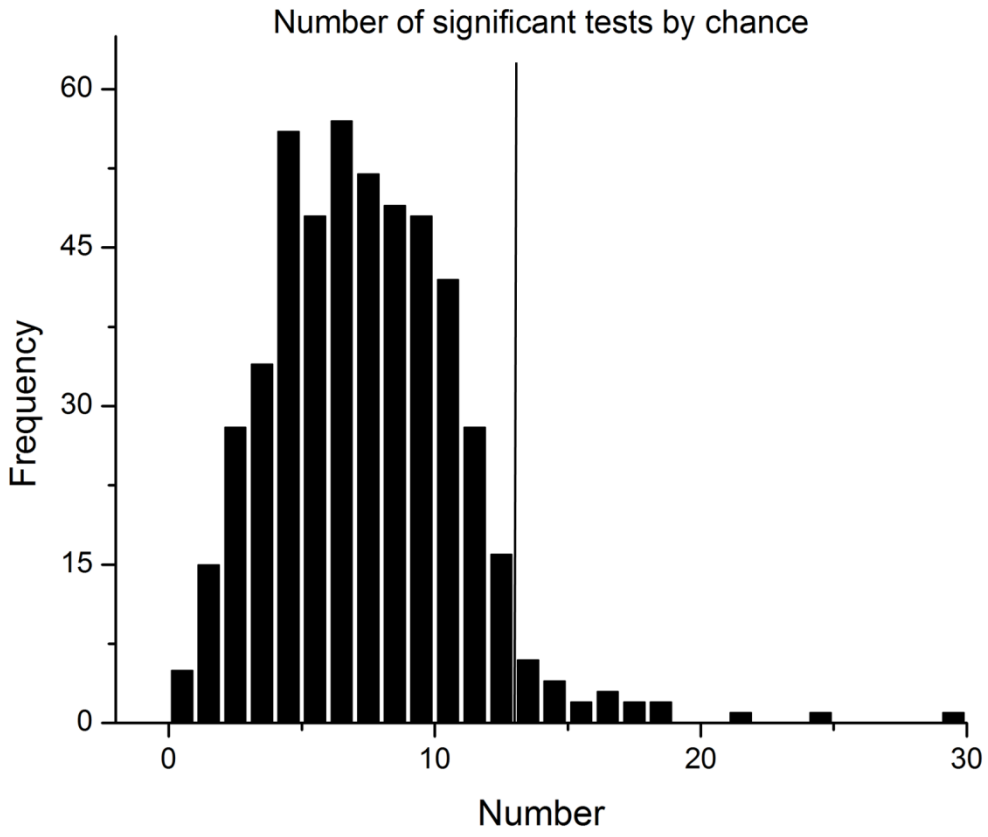

Figure 7. Histogram of permutation tests. Observing more than twelve significant contrasts is not likely due to chance.

Hypotheses

1. Fear conditioning (Acquisition)

We expected the delay and trace conditioning groups would both show learning related changes on both UCS expectancy and SCR measures. The unpaired control group would show a similar level of UCS expectancy and SCR to both visual stimuli. Brain activity in both of the conditioning groups should be consistent with previously published papers. The CS+ will evoke a larger response than the CS- in the amygdala in both the delay and trace conditioning groups. In the delay conditioning group we expected to see differential activity in the insula and visual cortex. We expected to see differential activity in the hippocampus and mPFC for the trace conditioning group. We planned to use the mPFC cluster identified as being specifically involved in trace conditioning as a functional region of interest in the resting-state connectivity comparisons.

2. Resting-state changes following acquisition

Based on previous work in our lab (Schultz et al., 2012), we expected to observe an increase in connectivity between the amygdala and dorsal PFC that was specific to learning following delay conditioning. Trace acquisition also involves the amygdala and this increase in connectivity may also exist in the trace conditioning group. We expected this immediate increase in connectivity to increase in strength over time. If this change in connectivity was supporting the consolidation of a fear memory then these functional connections would continue to increase in strength as the memory is consolidated. We expected to observe a

similar finding that was specific to the trace conditioning group in the hippocampus. We expected the trace conditioning group to show greater functional connectivity between the hippocampus, amygdala and mPFC as these regions are involved in the trace conditioning circuit. An alternative hypothesis was that we could observe a short-term increase in connectivity between the amygdala, hippocampus and dorsal PFC regions that was transient and is diminished by the 7 day time point. This type of finding could indicate that these changes in functional connectivity are important for the early stages of consolidation, but that the amygdala and hippocampus become less important hubs for the storage of this type of memory at longer time points. In this case, the interpretation would be consistent with a systems consolidation theory. A systems consolidation theory would also predict that the brain regions necessary for the initial learning of the association will show an increase in connectivity with other brain areas quickly following acquisition. As time passes the fear circuit could show stronger connectivity with a greater number of distributed cortical regions as the memory is transferred to long-term memory.

3. Fear conditioning (Extinction)

We expected the delay and trace groups would both show a decrease in CR magnitude and UCS expectancy ratings across the extinction session. For the delay group we expected to see amygdala activity during the extinction session based on previously published papers using a similar methodology (Knight, Smith, Cheng, Stein, & Helmstetter, 2004b; LaBar, Gatenby, Gore, LeDoux, & Phelps, 1998). The vmPFC has also been implicated in the recall of extinction.

We did not assess the recall of extinction in a separate session, but we could observe activity in the vmPFC during the extinction session. Finally, we expected activity in the retrosplenial cortex during the extinction session that was specific to the extinction of a trace conditioning memory. This structure has been implicated specifically in the extinction of trace conditioning memories in rats (Kwapis, Jarome, Gilmartin, Lee, & Helmstetter, 2012).

4. Resting-state changes following extinction

We expected to observe increases in functional connectivity between brain regions involved in extinction learning following the extinction session. Specifically, we expected to see increases in connectivity between the amygdala, hippocampus, and vmPFC for both the delay and trace conditioning groups. Additionally, we expected to see an increase in connectivity between the amygdala and retrosplenial cortex that was specific to the trace group.

Results

Acquisition

UCS expectancy

A CS type by group repeated measures ANOVA on the UCS expectancy data yielded a significant main effect for CS type, $F(1, 48) = 364.14$, $p < .001$. There was also a main effect for group, $F(2, 48) = 8.83$, $p < .001$. The CS type by group interaction was also significant, $F(2, 48) = 86.24$, $p < .001$. Follow up tests using the error term from the whole ANOVA revealed that there was a significant effect for CS type in the delay

group, $F(1, 16) = 305.83$, $p < .001$, and the trace group, $F(1, 16) = 231.03$, $p < .001$, but not for the unpaired group, $F(1, 16) = 0.14$, $p = .72$. These data indicate that participants in the delay and trace group give higher UCS expectancy ratings on CS+ trials relative to CS- trials and that the unpaired group does not display any differential expectancy for either of the visual stimuli (see Figure 8).

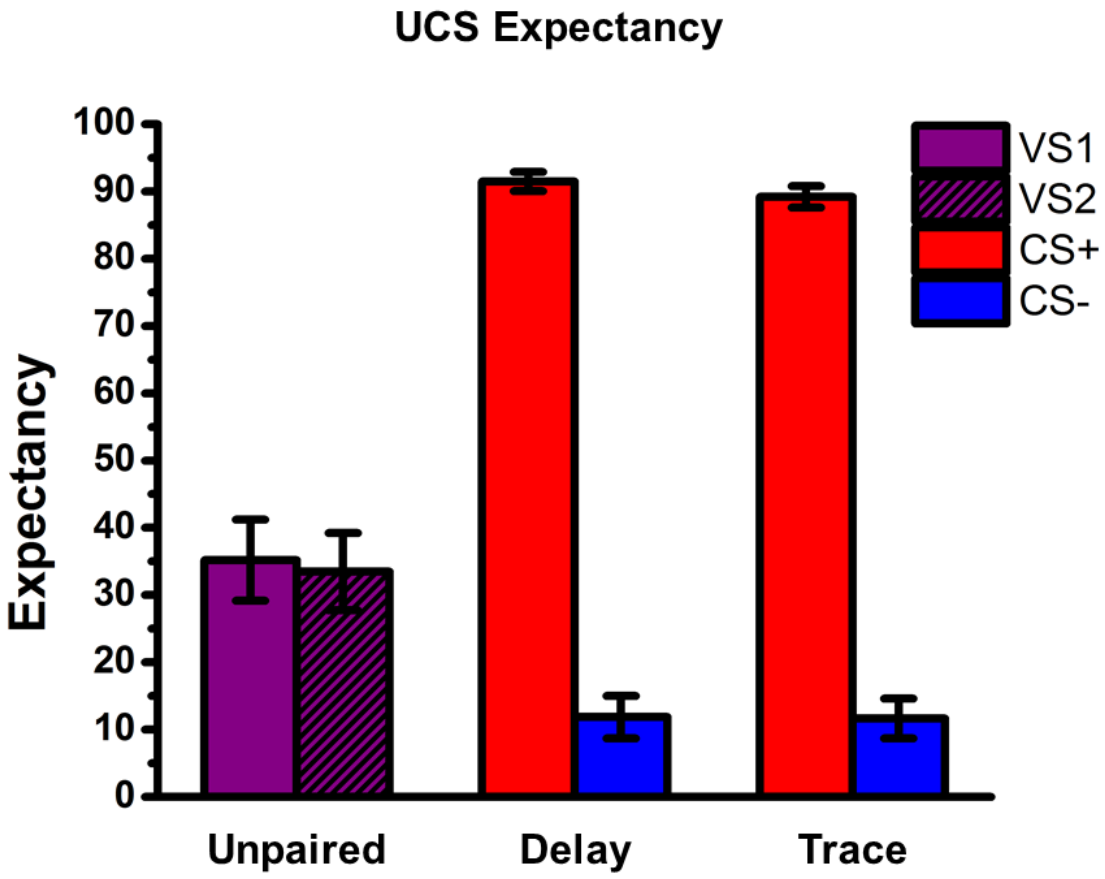

Figure 8. The delay and trace groups both show differential conditioning based on UCS expectancy data. Bars depict the mean UCS expectancy ratings during the acquisition session.

Skin conductance

A CS type by group repeated measures ANOVA on the SCR data yielded a significant main effect for CS type, $F(1, 48) = 36.92$, $p < .001$. There was also a main effect for group, $F(2, 48) = 21.79$, $p < .001$. The CS type by group interaction was also significant, $F(2, 48) = 8.79$, $p < .01$. Follow up tests using the error term from the whole ANOVA revealed that there was a significant effect for CS type in the delay group, $F(1, 16) = 15.54$, $p < .01$, and the trace group, $F(1, 16) = 38.14$, $p < .001$, but not for the unpaired group, $F(1, 16) = 0.13$, $p = .72$. These data indicate that participants in the delay and trace group develop differential SCR with larger amplitude response on CS+ trials relative to CS- trials and that the unpaired group does not display any differential SCR evoked by the visual stimuli (see Figure 9).

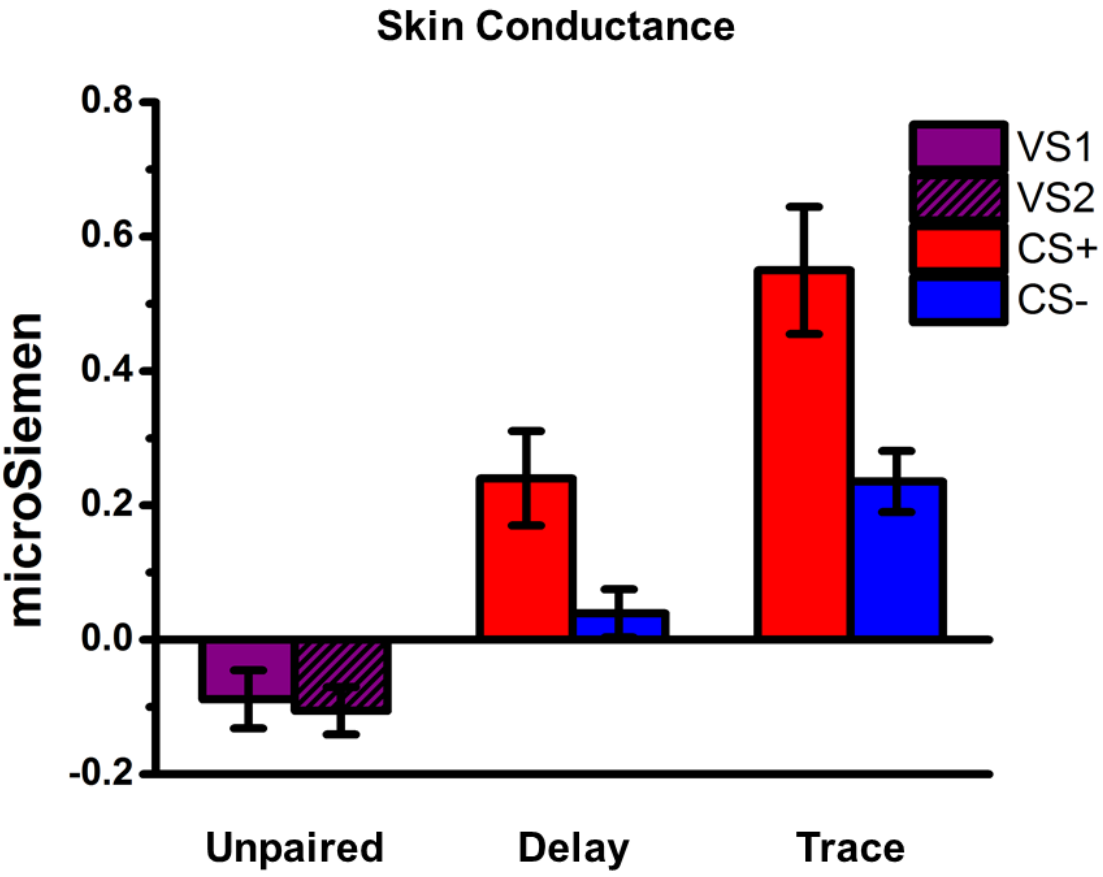

Figure 9. The delay and trace groups both show differential conditioning based on the skin conductance data. Bars depict the mean skin conductance response during the acquisition session.

Task FMRI

A whole-brain CS type by group ANOVA was conducted. Several brain areas showed a main effect for CS type (see Table 1). This effect was generally characterized by little differential activity evoked by either of the visual stimuli in the unpaired group and larger responses evoked by the CS+ relative to the CS- in both the delay and the trace groups (see Figure 10). A large cluster encompassing the thalamus and caudate

bilaterally showed a general effect of fear learning in both the delay and trace group consistent with previous research (Knight et al., 2004a). This contrast also revealed a large cluster including the cingulate gyrus and superior frontal gyrus. A previous study found that the ACC was involved in both delay and trace fear conditioning (Knight et al., 2004a) and the superior frontal gyrus has been identified in several delay fear conditioning studies (Kattoor, Gizewski, Kotsis, Benson, Gramsch, Theysohn, Maderwald, Forsting, Schedlowski & Eisenbruch, 2013; Schultz et al., 2012). This contrast also identified clusters in the insula, fusiform gyrus, cuneus and other regions. These regions have been identified in several delay fear conditioning experiments (Buchel et al., 1998; Dunsmoor et al., 2007; Gottfried & Dolan, 2004; Phelps et al., 2004).

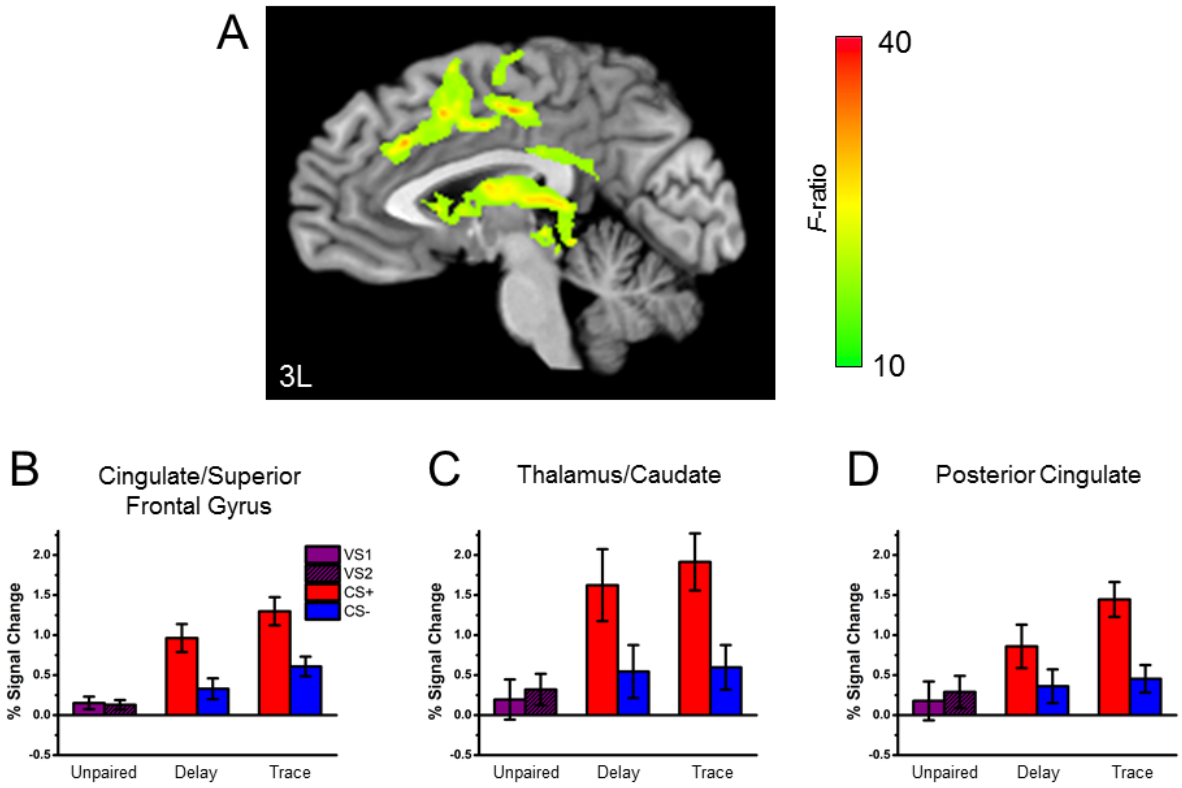

Figure 10. Several brain regions show a general fear learning effect characterized by increased BOLD activity on CS+ trials relative to CS- trials. (A) Brain image depicting regions characterized by a main effect for CS type. The color scale corresponds to the *F*-value. (B, C, D) Bar graph depicting the percent signal change values for the two visual stimuli in the unpaired group and the CS+ and CS- for the delay and trace groups for the corresponding cluster in the cingulate gyrus and superior frontal gyrus (B), thalamus and caudate (C), and posterior cingulate (D).

Main Effect for CS Type

			Talairach Coordinates		
Region	**Hemisphere**	**Volume (mm^3)**	**RL**	**AP**	**IS**
Thalamus / Caudate	Bilateral	6497	1.4	19.9	11
Cingulate Gyrus / Superior Frontal Gyrus	Bilateral	5748	0.1	-5.6	41.7
Inferior Frontal Gyrus / Insula	Right	2949	-51.6	-14.1	-0.7
Superior Temporal Gyrus / Insula	Left	1454	55.7	-6.2	1.9
Postcentral Gyrus	Left	935	3	37.6	76.2
Insula	Right	927	-52	27.3	20.9
Cingulate Gyrus / Posterior Cingulate Gyrus	Right	905	-1.4	34.1	25.7
Fusiform Gyrus / Declive	Right	514	-24.4	70.1	-14.1
Inferior Parietal Lobule	Left	442	56.9	41.3	23.5
Superior Temporal Gyrus / Insula	Right	415	-57.4	-1	5.6
Superior Frontal Gyrus	Right	339	-5.4	1.6	74.9
Cuneus	Right	298	-19.3	75.8	7.6
Medial Frontal Gyrus	Right	276	-1.9	10.6	57.3
Culmen	Right	273	-11.7	29.8	-7
Insula	Left	238	46.6	30.8	20.7

CS+ > CS- for all clusters

Table 1. The main effect of CS type during acquisition.

The whole brain ANOVA also identified regions showing a main effect for group (see Table 2). We observed two general patterns in this contrast. One pattern was characterized by larger CS evoked responses in both the delay and trace group relative to the unpaired group and one pattern was characterized by the largest CS evoked responses in the trace group followed by the delay group which was greater than the unpaired group. The main effect for group was largely driven by the CS by group interaction which is discussed in more detail below.

Main Effect for Group

			Talairach Coordinates		
Region	**Hemisphere**	**Volume (mm³)**	**RL**	**AP**	**IS**
Inferior Frontal Gyrus / Insula / BA 13 / BA 47	Right	3279	-48.4	-15.7	-0.4
Medial Frontal Gyrus / BA 6	Bilateral	1251	-0.7	-7.6	44.4
Insula / BA 13	Left	1068	43.8	-1.4	4.8
Culmen	Left	1010	10	32.6	-6
Superior Temporal Gyrus	Left	813	60	-5.7	3.9
Superior Frontal Gyrus	Right	693	-4.1	-10.2	70.7
Thalamus / Red Nucleus	Left	598	0.5	23.2	0.9
Insula / BA 13	Right	593	-54.6	30.6	21.1
Superior Temporal Gyrus / BA 22	Right	436	-58.3	55.3	14
Lingual Gyrus / Declive	Right	365	-7	74.9	10
Caudate Body	Right	310	-7.2	-4.3	6.7
Insula	Left	295	44.4	18.2	12.5
Lentiform Nucleus	Left	265	20.6	12.5	-6.2
Middle Frontal Gyrus	Right	259	-60.4	-11	42.4
Inferior Parietal Lobule	Left	236	49.7	41.3	22

Clusters characterized by U<D&T or U<D<T

Table 2. The main effect for group during acquisition.

The whole-brain ANOVA also identified several CS type by group interactions (see Table 3). This pattern was generally characterized by larger differential activation in the trace group relative to the delay and unpaired groups (see Figure 11). A cluster in the mPFC was characterized by this pattern which is consistent with previous research suggesting it plays a role specifically in trace conditioning (Gilmartin, Miyawaki, Helmstetter, & Diba, 2013; Kronforst-Collins, & Disterhoft, 1998; Runyan, Moore, & Dash, 2004; Weible, McEchron, & Disterhoft, 2000). The mediodorsal thalamas displayed a similar pattern of activity consistent with its role in trace conditioning and its known connections to mPFC (Powell & Churchwell, 2002). Other regions characterized by the same pattern include the insula, lingual gyrus, fusiform gyrus, and a cluster

including portions of the parahippocampal gyrus and posterior cingulate gyrus as well as several others (see Table 3).

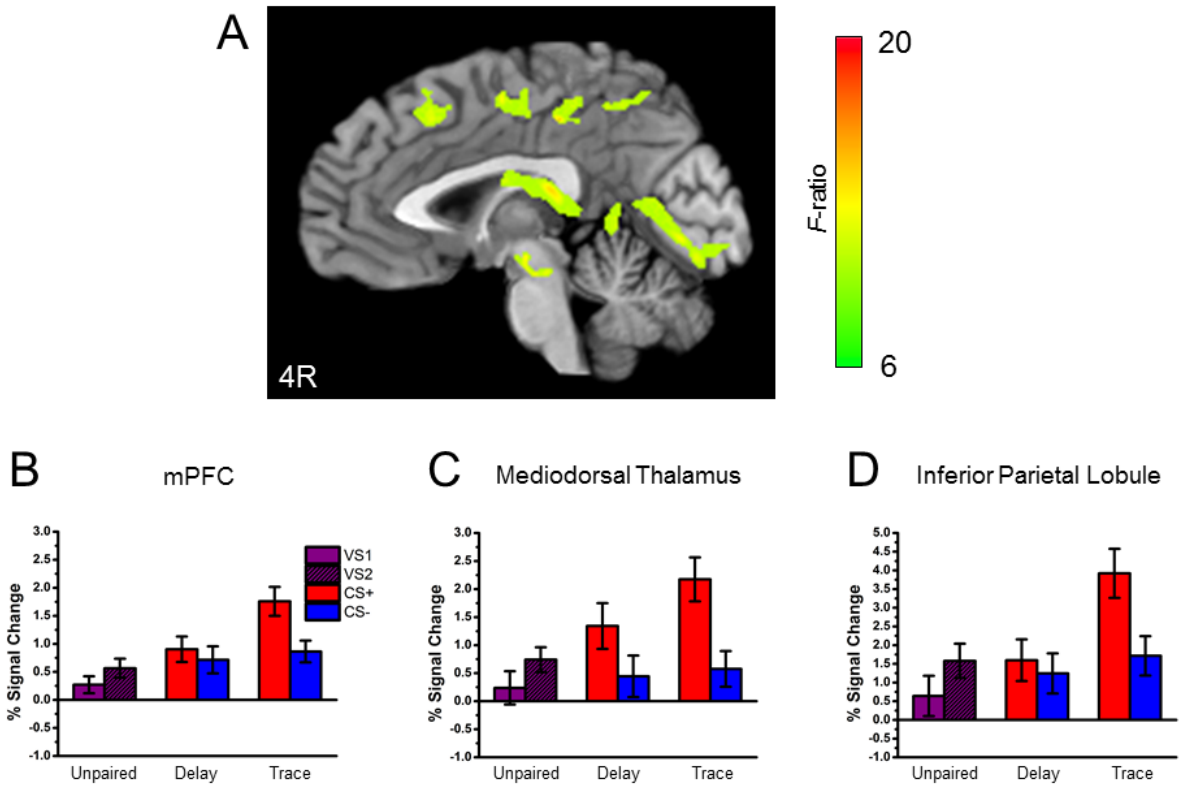

Figure 11. Some regions showed a trace specific effect characterized by greater differential BOLD activity between CS+ and CS- trials for the trace group relative to the delay and unpaired group. (A) Brain image depicting regions characterized by a CS type by group interaction. The color scale corresponds to the *F*-value. (B, C, D) Bar graph depicting the percent signal change values for the two visual stimuli in the unpaired group and the CS+ and CS- for the delay and trace groups for the corresponding cluster in the mPFC (B), mediodorsal thalamus (C), and inferior parietal lobule (D).

Group by CS Type Interaction

			Talairach Coordinates		
Region	Hemisphere	Volume (mm^3)	RL	AP	IS
Superior Temporal Gyrus / Insula	Right	1915	-53.4	-10.4	1
Superior Temporal Gyrus / Insula	Left	1894	52.3	-3.6	0.9
Postcentral Gyrus	Bilateral	1838	0.8	41.5	73.2
Thalamus	Bilateral	1553	-2.2	25.3	14.3
Lingual Gyrus	Bilateral	1246	-2.2	72.7	0.2
Fusiform Gyrus	Right	1022	-22.8	78.9	-14.2
Precuneus	Right	925	-5	62.3	65.4
Inferior Parietal Lobule	Left	827	64.6	42.6	23.6
Medial Frontal Gyrus	Bilateral	668	-2.6	-18	43.9
Medial Frontal Gyrus	Bilateral	616	-1.9	11.1	47.8
Paracentral Lobule	Bilateral	508	-0.2	30.6	45
Middle Temporal Gyrus	Left	493	57.4	75.4	16.8
Postcentral Gyrus	Left	343	58.1	19.9	14.8
Insula	Left	327	47.4	36.7	20
Parahippocampal Gyrus / Posterior Cingulate Gyrus	Left	252	7.2	37.4	6.8

Table 3. The CS type by group interactions for acquisition.

We predicted that the amygdala would be involved in both delay and trace conditioning, but we did not detect any significant clusters with the whole-brain ANOVA. In order to examine the role of the amygdala during acquisition we created an anatomical mask of the amygdala for each participant and examined the BOLD response with a CS type by group repeated measures ANOVA (see Figure 12). There was a significant main effect for CS type, $F(1, 48) = 6.3$, $p < .05$, a significant effect of group, $F(2, 48) = 8.35$, $p < .01$. The CS type by group interaction was not significant, $F(2, 48) = 1.98$, $p = .15$. Follow up tests revealed a significant effect of CS type for the delay, $F(1, 16) = 5.96$, $p < .05$, and trace group, $F(1, 16) = 6.19$, $p < .05$. However the unpaired group did not show a differential response to the visual stimuli, $F(1, 16) < 1$, $p = .89$.

These results suggest that the amygdala is involved in both delay and trace conditioning which is consistent with previous reports (Gilmartin et al, 2012; Kwapis et al., 2011).

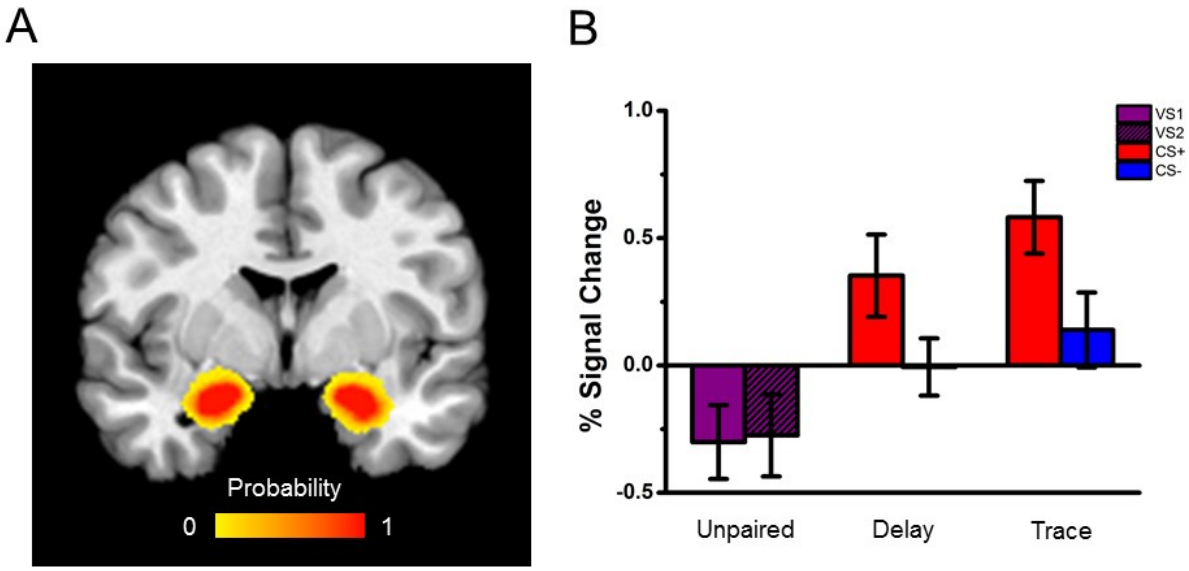

Figure 12. The amygdala is important for both delay and trace acquisition. (A) A probability mask created by combining each participant's anatomical amygdala ROI. The color scale corresponds to the probability that the region was included in any of the participants' amygdala ROI mask. Yellow colors indicate a low probability and red colors indicate high probability. (B) A bar graph representing the mean evoked response in the amygdala by the stimuli for each group during acquisition.

We expected that the hippocampus would be more important for trace than delay conditioning. However, we did not detect any significant clusters in the hippocampus proper with the whole-brain ANOVA. In order to examine the role of the hippocampus we used a similar anatomical mask approach for the hippocampus as previously discussed for the amygdala (see Figure 13). There was a significant main effect for CS type, $F(1, 48) = 5.52$, $p < .05$, a significant CS type by group interaction, $F(2, 48) = 5.42$, $p < .01$.

The main effect for group was not significant, $F(2, 48) = 3.10$, $p = .06$. Follow up tests revealed that the unpaired group, $F(1, 16) = 1.14$, $p = .30$, and the delay group, $F(1, 16) = 3.37$, $p = .09$, did not show differential responses, but the trace group showed a significantly larger response on CS+ trials compared to CS- trials, $F(1, 16) = 11.12$, $p < .01$. These results suggest that the hippocampus is important for trace conditioning as suggested by previous research (Clark & Squire, 1998; Kim, Clark, & Thompson, 1995; Knight et al., 2004a; Moyer, Deyo, & Disterhoft, 1990).

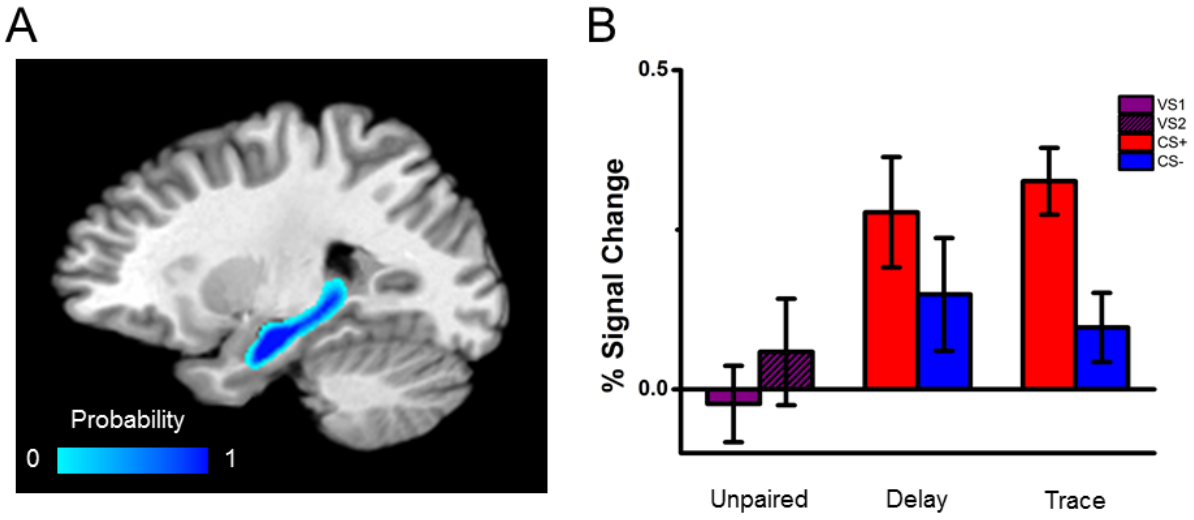

Figure 13. The hippocampus is especially important for trace acquisition. (A) A probability mask created by combining each participant's anatomical hippocampus ROI. The color scale corresponds to the probability that the region was included in any of the participants' hippocampus ROI mask. Light blue colors indicate a low probability and darker blue colors indicate high probability. (B) A bar graph representing the mean evoked response in the hippocampus by the stimuli for each group during acquisition.

Resting-state Functional Connectivity

At baseline we did not observe any significant differences in connectivity for the contrast designed to explore general fear learning effects (unpaired versus delay and trace). However, we did observe some differences in connectivity on the contrast between the delay and the trace group. The trace group showed increased connectivity between the right insula and vmPFC and also between the right amygdala and left retrosplenial cortex (see Figure 14). At baseline the groups were no different from one another because this scan occurred prior to the behavioral manipulation. We did not predict any differences on either contrast at this time point. Importantly, neither of the connections exhibit differences between either of the contrasts at any of the subsequent time points. Therefore, none of the differences observed at time points after conditioning could be due to differences between the groups prior to conditioning.

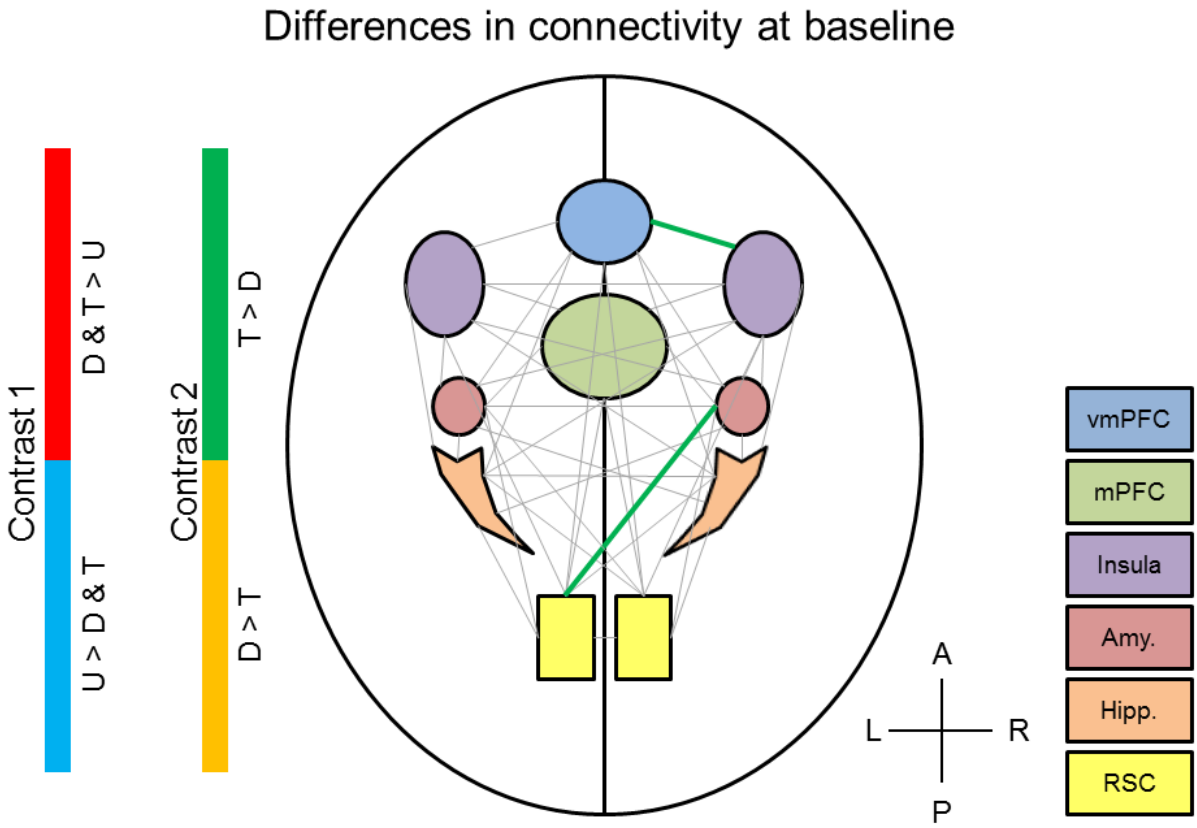

Figure 14. Network plot of differences in connectivity at baseline. The network plots depict regions of interest as shapes. The lines between the regions of interest represent all of the possible connections between the regions. We ran two contrasts. The first one compared connectivity for the unpaired group to that of both the delay and trace group. The second contrast compared connectivity between the delay and trace group. Gray lines indicate that there were no significant group differences in the correlation between the connected regions on either contrast. Any significant connectivity differences on the contrast comparing the delay and trace group to the unpaired group are depicted by a red line (increased connectivity between those nodes for the delay and trace groups relative to the unpaired group) or a blue line between nodes (increased connectivity between those nodes for the unpaired group relative to the delay and trace groups). Any significant connectivity differences on the contrast comparing the delay group to the trace group are depicted by a yellow line (increased connectivity between those nodes for the delay group relative to the trace group) or a green line between nodes (increased connectivity between those nodes for the trace group relative to the delay group).

Immediately following conditioning there were no significant differences on the general fear learning contrast, but we did observe significantly greater connectivity for the trace group relative to the delay group on the connection between the right amygdala and vmPFC and the connection between the mPFC and right retrosplenial cortex (see Figure 15). The increase in connectivity between the mPFC and the retrosplenial cortex for the trace group is consistent with previous studies suggesting that there regions are specifically engaged during learning and during the consolidation of trace memories (Kronforst-Collins, & Disterhoft, 1998; Runyan,et al., 2004; Weible et al., 2000; Gilmartin & McEchron, 2005b; Kwapis et al, unpublished data).

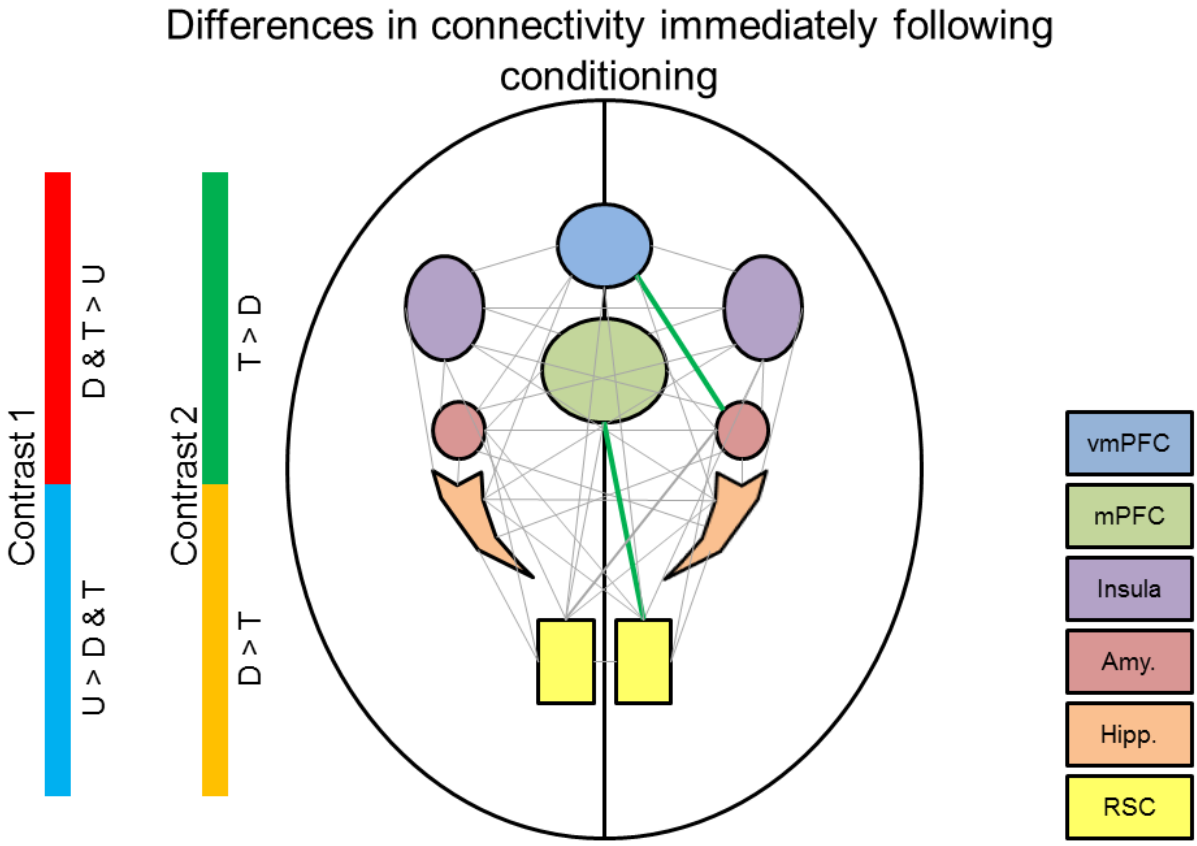

Figure 15. Immediately following acquisition the trace group shows increases in connectivity relative to the delay group between the right amygdala and vmPFC as well as between the mPFC and the right retrosplenial cortex. Differences in connectivity immediately following acquisition for both contrasts are depicted in a network plot.

Twenty-four hours after conditioning we observed increased connectivity for the delay and trace group relative to the unpaired group between the left amygdala and right hippocampus, left amygdala and right retrosplenial cortex, right amygdala and left hippocampus, right amygdala and mPFC. All of the differences in connectivity between the unpaired group relative to the delay and trace groups involved the amygdala which is consistent with it being involved in both delay and trace conditioning (Kwapis et al.,

2011; Gilmartin et al., 2012). Alternatively, we observed greater connectivity between the right insula and mPFC for the unpaired group relative to the delay and trace groups. We also identified increased connectivity for the trace group relative to the delay group between the mPFC and the left retrosplenial cortex which was consistent with the increase we observed between those structures in the trace group immediately following conditioning (see Figure 16).

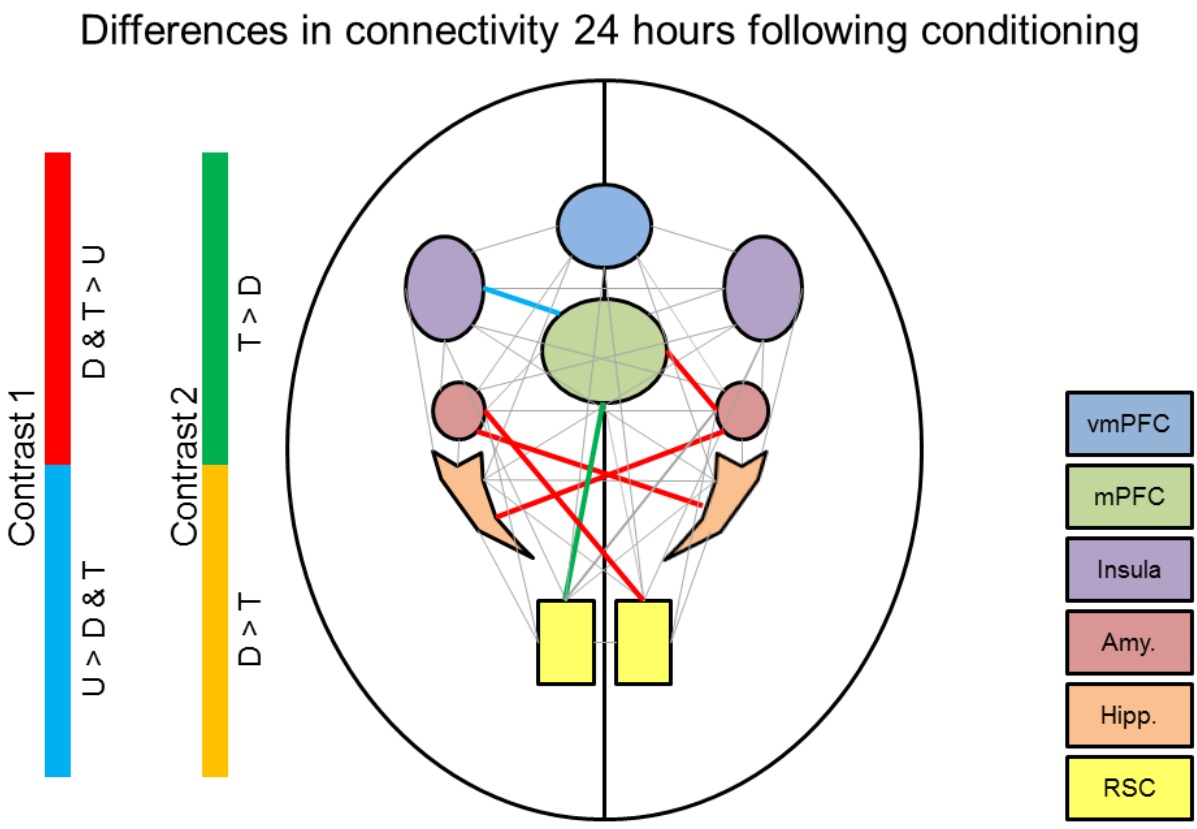

Figure 16. Twenty-four hours following acquisition the delay and trace groups both show increased amygdala connectivity relative to the unpaired group. The trace group shows increased connectivity between the mPFC and the retrosplenial cortex relative to the delay group. Differences in connectivity twenty-four hours following acquisition for both contrasts are depicted in a network plot.

Seven days following conditioning there was a significant increase in connectivity between the right hippocampus and right insula for the trace group relative to the delay group. There were no significant differences between the unpaired group and both the delay and trace groups (see Figure 17). The majority of differences in connectivity we observed at the 24 hour time point were no longer apparent 7 days following conditioning.

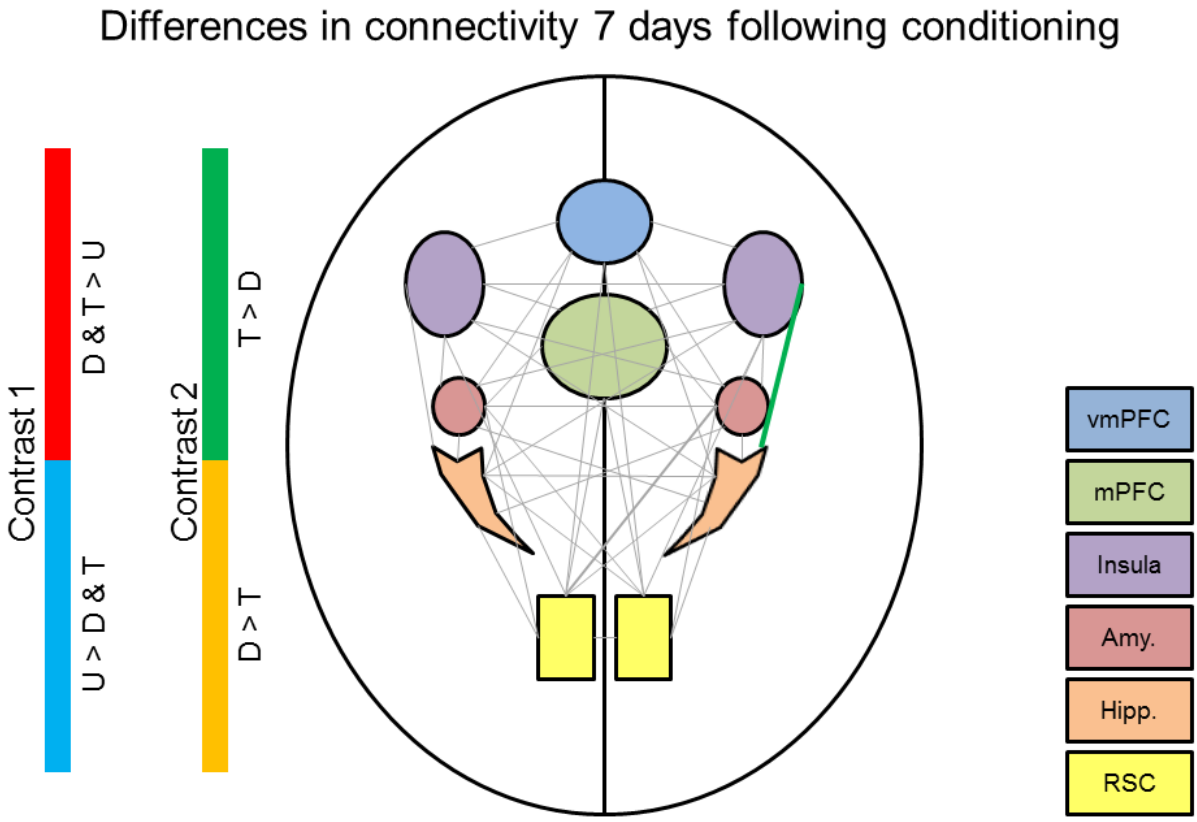

Figure 17. Seven days following acquisition most of the differences in connectivity we observed following learning had diminished. The trace group showed increased connectivity between the right hippocampus and the right insula. None of the other connections exhibited significant differences on either contrast. Differences in connectivity seven days following acquisition for both contrasts are depicted in a network plot.

Extinction

UCS Expectancy

Retention was measured by running a CS type by group ANOVA on the first three trials of the extinction session. A CS type by group repeated measures ANOVA on

the UCS expectancy data from the first three extinction trials yielded a significant main effect for CS type, $F(1, 42) = 30.98$, $p < .001$. There was not a significant main effect for group, $F(2, 42) = 0.33$, $p = .72$. The CS type by group interaction was significant, $F(2, 42) = 8.05$, $p < .01$. Follow up tests using the error term from the whole ANOVA revealed that there was a significant effect for CS type in the delay group, $F(1, 14) = 34.44$, $p < .001$, and the trace group, $F(1, 14) = 12.7$, $p < .01$, but not for the unpaired group, $F(1, 14) = 0.05$, $p = .83$. These data indicate that participants in the delay and trace group give higher UCS expectancy ratings on CS+ trials relative to CS- trials and that the unpaired group does not display any differential expectancy for either of the visual stimuli early in the extinction session. This data suggests the delay and trace groups retain the CS-UCS association as measured by UCS expectancy ratings (see Figure 18).

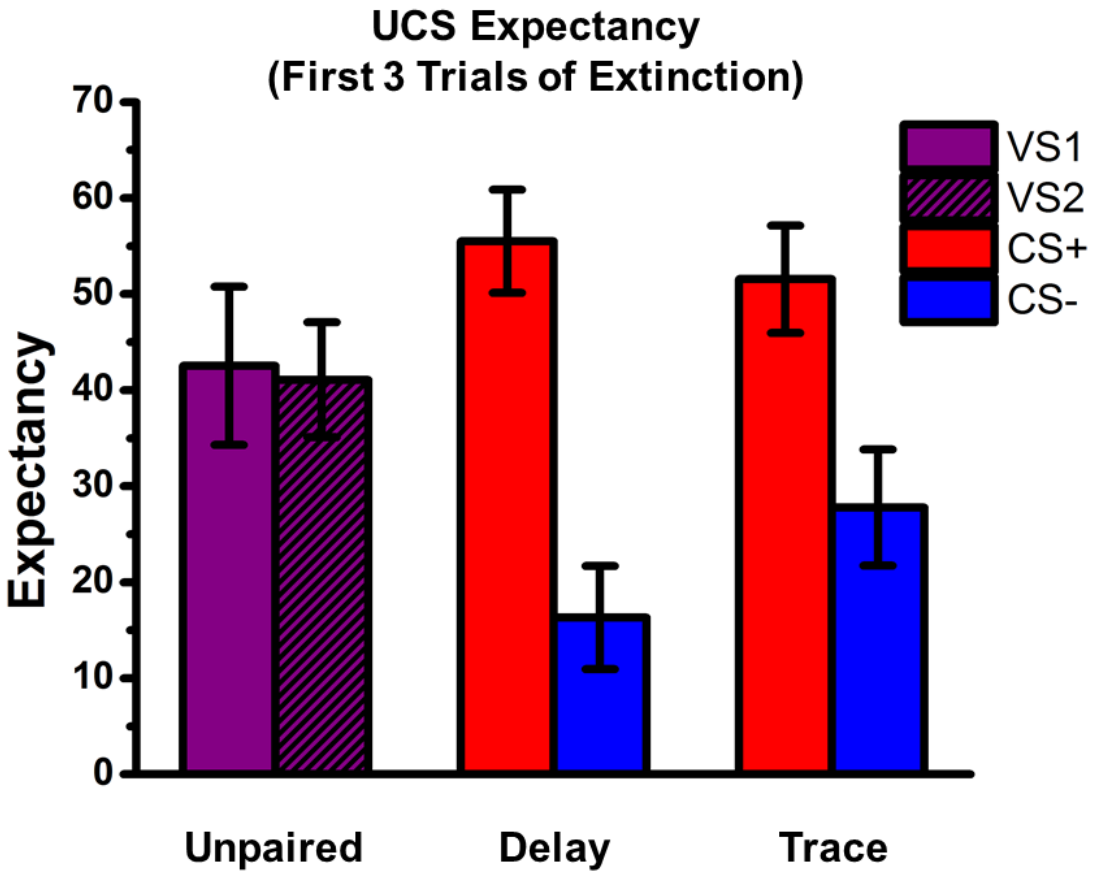

Figure 18. The delay and trace groups both show retained differential UCS expectancy ratings for the first three trials of the extinction session which occurred seven days after acquisition. Bars depict the mean UCS expectancy ratings during the first three trials of the extinction session.

Extinction was measured by running a CS type by group ANOVA on the last seven trials of the extinction session. A CS type by group repeated measures ANOVA on the UCS expectancy data from the last seven extinction trials revealed that there was not a significant main effect for CS type, $F(1, 42) = 0.28$, $p = .6$. There was a significant main effect for group, $F(2, 42) = 3.53$, $p < .05$. The CS type by group interaction was not significant, $F(2, 42) = 0.87$, $p = .43$. Follow up tests using the error term from the whole

ANOVA revealed that there was no significant effect for CS type in any of the groups, largest $F(1, 14) = 1.18$, $p = .3$. These data indicate that participants in the delay and trace group extinguished and do not demonstrate differential UCS expectancy during the last seven trials of the extinction session. (see Figure 19).

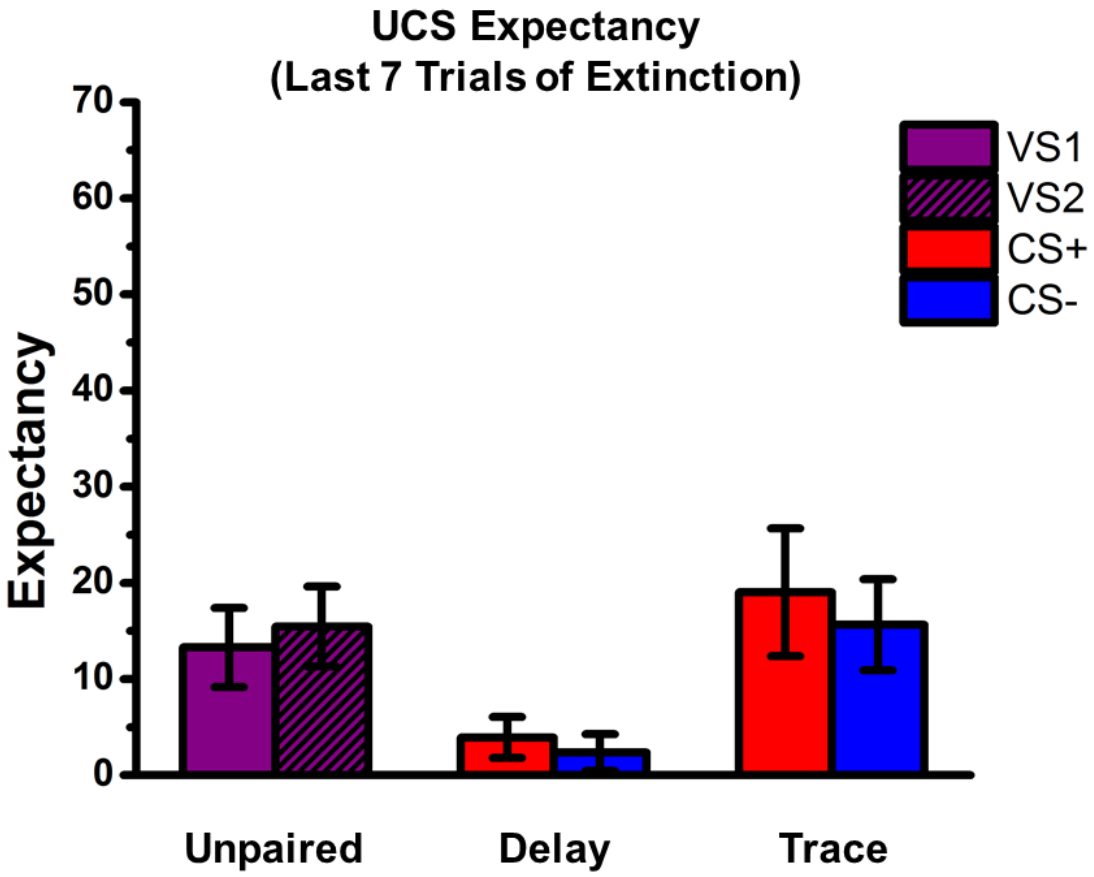

Figure 19. The delay and trace groups do not show differential UCS expectancy ratings during the last seven trials of the extinction session suggesting they learned that the CS+ no longer predicted the UCS. Bars depict the mean UCS expectancy ratings during the last seven trials of the extinction session.

Skin Conductance

Retention was measured by running a CS type by group ANOVA on the first three trials of the extinction session. A CS type by group repeated measures ANOVA on the skin conductance data from the first three extinction trials yielded a trend for the main effect CS type, $F(1, 42) = 3.95$, $p = .053$. There was a significant main effect for group, $F(2, 42) = 4.13$, $p < .05$. The CS type by group interaction was not significant, $F(2, 42) = 2.28$, $p < .12$. Follow up tests using the error term from the whole ANOVA revealed that there was not a significant effect for CS type in any of the groups, largest $F = 3.53$, $p = .08$. These data indicate that participants in the delay and trace group show slightly, but not significantly larger responses on CS+ trials relative to CS- trials early in the extinction session. This data suggests the delay and trace groups retain the CS-UCS association as measured by SCR (see Figure 20).

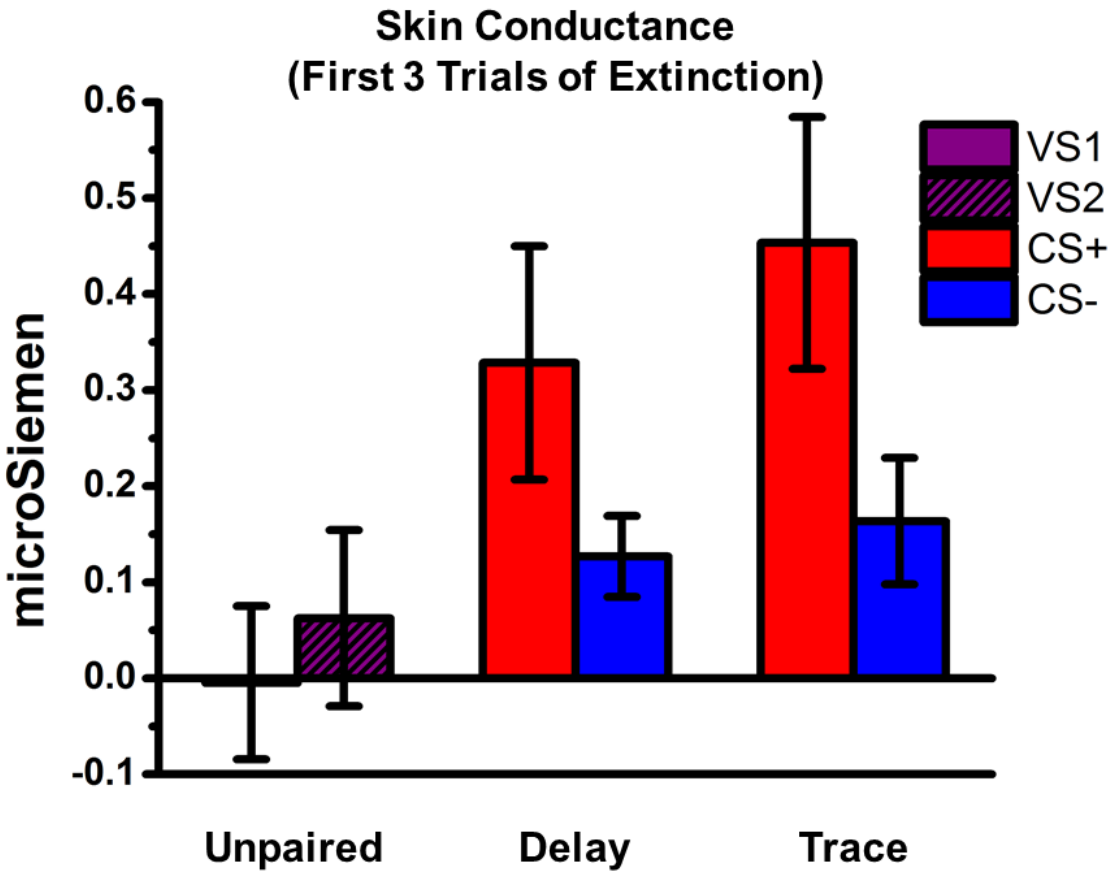

Figure 20. The delay and trace groups both show a larger skin conductance response on CS+ trials than CS- trials during the first three trials of the extinction session suggesting that the retained the acquisition memory. Bars depict the mean SCR during the first three trials of the extinction session.

Extinction was measured by running a CS type by group ANOVA on the last seven trials of the extinction session. A CS type by group repeated measures ANOVA on the SCR data from the last seven extinction trials revealed that neither main effect nor the interaction were significant, largest $F = 2.1$, $p = .16$. These data indicate that participants in the delay and trace group extinguished and do not demonstrate differential SCR during the last seven trials of the extinction session. (see Figure 21).

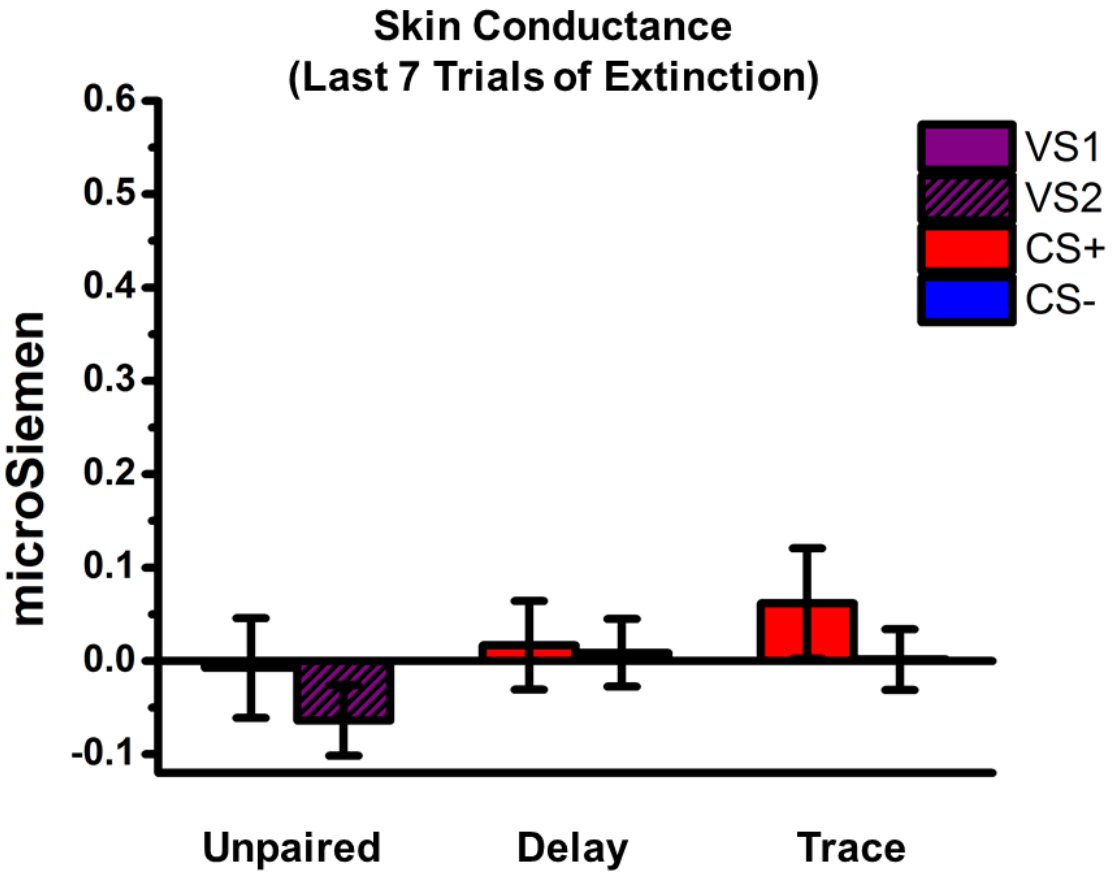

Figure 21. The delay and trace groups do not show differential SCR during the last seven trials of the extinction session suggesting they learned extinction. Bars depict the mean SCR during the last seven trials of the extinction session.

Task FMRI

Our primary hypotheses involved the extinction of the CS-UCS association formed during acquisition. The unpaired group did not receive CS-UCS pairings during acquisition. The UCS expectancy and SCR data from the acquisition session and the extinction session reveal that the unpaired group did not learn a CS-UCS association. We therefore excluded the unpaired group from the task FMRI analysis during extinction.

We conducted a whole-brain CS type by group ANOVA on the extinction data for the delay and the trace group.

Several brain areas showed a significant main effect for CS type (see Table 4). All of these regions were characterized by a larger response on CS+ trials relative to CS- trials for both the delay and trace groups (see Figure 22). We observed differential activity in the insula during extinction which is consistent with previous studies (Gottfried & Dolan, 2004; Phelps et al., 2004). We also saw differential activity in the caudate and the inferior frontal gyrus which have been previously reported during FMRI studies examining the BOLD response (Phelps et al., 2004) and cerebral blood flow (Molchan, Sunderland, McIntosh, Herscovitch, & Schreurs, 1994).

Main Effect for CS Type

			Talairach Coordinates		
Region	Hemisphere	Volume (mm^3)	RL	AP	IS
Insula	Left	966	37.3	-15.2	-4.4
Lingual Gyrus/	Right	588	-15.4	79.9	-6.9
Posterior Cingulate Gyrus	Right	581	-0.8	24.6	25.1
Inferior Frontal Gyrus	Right	417	-49	-15.8	-3.6
Inferior Frontal Gyrus	Right	386	-35.3	-18	-5.4
Cuneus	Right	342	-11.5	74.4	15.1
Thalamus	Right	296	-3.5	25.3	13.4
Caudate	Left	257	0.6	-0.3	10.6
Thalamus	Left	211	1.4	10.1	17.2
Superior Frontal Gyrus	Right	202	-0.9	-8.4	68
Clusters characterized by CS+>CS-					

Table 4. Main effect for CS type during extinction.

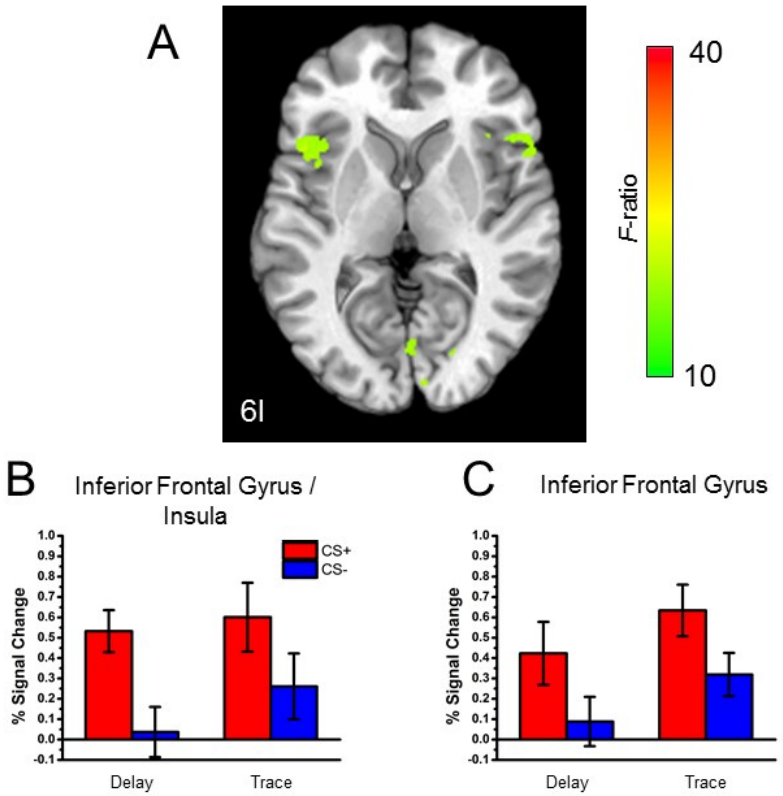

Figure 22. Some regions showed a general fear extinction effect that was consistent for both the delay and trace group. CS type main effect during extinction for the delay and trace groups. (A) Brain image depicting regions characterized by a main effect for CS type. The color scale corresponds to the *F*-value. (B, C) Bar graph depicting the percent signal change values for the CS+ and CS- for the delay and trace groups for the corresponding cluster in the inferior frontal gyrus and insula (B), and inferior frontal gyrus (C).

There was also a significant main effect for group in several brain regions (see Table 5). All of the significant effects on this contrast were characterized by a larger BOLD response in the trace group relative to the delay group. We observed a main effect for group in the inferior parietal lobule which previous studies have suggested is involved

during the acquisition of trace fear conditioning (Haritha, Wood, Ver Hoef, & Knight, 2013).

Main Effect for Group

			Talairach Coordinates		
Region	Hemisphere	Volume (mm^3)	RL	AP	IS
Inferior Parietal Lobule	Left	1189	48.4	42.7	56.7
Inferior Frontal Gyrus	Right	470	-55.2	-12.6	31.2
Precentral Gyrus	Right	440	-44.4	18.3	60.1
Postcentral Gyrus	Right	337	-0.5	47.2	71
Superior Frontal Gyrus	Left	226	23.1	1.7	67

All cluster characterized by Trace>Delay

Table 5. Main effect for group during extinction.

One cluster in the vmPFC showed a significant CS type by group interaction (see Figure 23). In this region the delay group showed a larger CS+ response relative to the CS-. The trace group did not exhibit a differential response in this area. Activation of the vmPFC during extinction is consistent with several studies suggesting that the vmPFC is involved in extinction and the retention of an extinction memory (Phelps et al., 2004; Milad et al., 2005). However, we expected that the vmPFC would be involved in extinction for both the delay and the trace group.

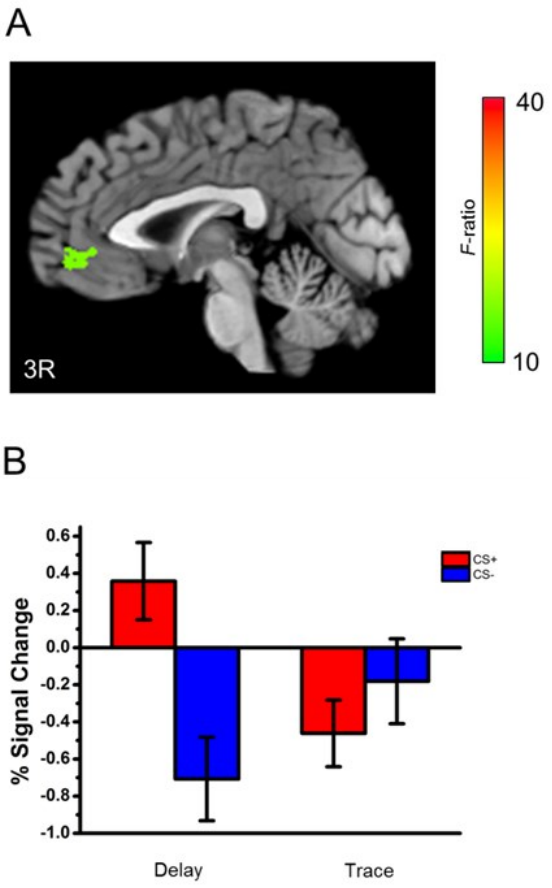

Figure 23. The vmPFC is involved in extinction for the delay group. CS type by group interaction during extinction for the delay and trace groups. (A) Brain image depicting regions characterized by the CS type by group interaction. The color scale corresponds to the *F*-value. (B) Bar graph depicting the percent signal change values for the CS+ and CS- for the delay and trace groups for the corresponding cluster in the vmPFC.

Resting-state Functional Connectivity

Immediately following extinction there were no significant differences on the general fear learning contrast, but we did observe significantly greater connectivity for the trace group relative to the delay group on the connection between the right amygdala

and the vmPFC. The trace group also had significantly greater connectivity between the left amygdala and the vmPFC (see Figure 24). The increase in connectivity between the vmPFC and the amygdala for the trace group is consistent with previous studies suggesting that vmPFC is acting to inhibit amygdala activity after extinction learning (Quirk et al., 2003), however if this finding reflects a general effect of extinction learning we would have expected to see the same pattern in the delay group.

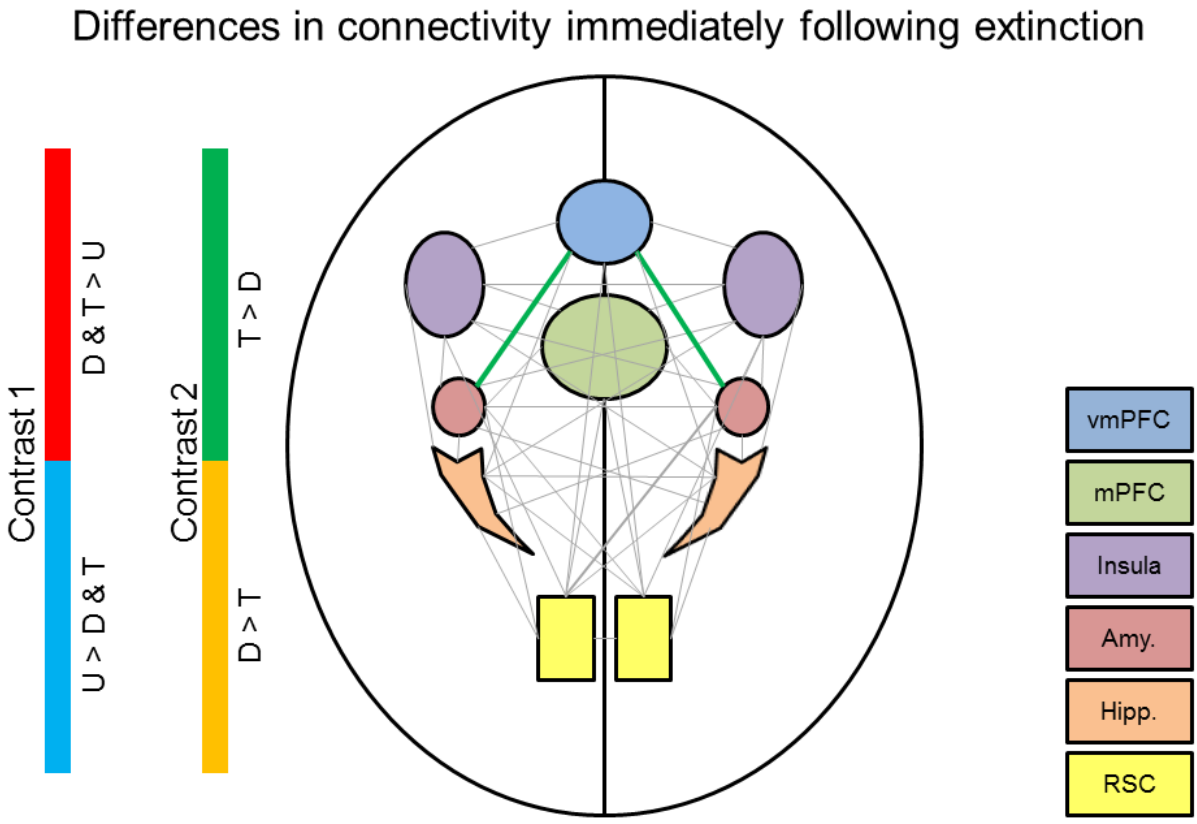

Figure 24. Immediately following extinction the trace group shows increases in connectivity relative to the delay group between the amygdala and vmPFC. Differences in connectivity immediately following extinction for both contrasts are depicted in a network plot.

Correlating functional connectivity and memory

We correlated resting-state functional connectivity with memory performance in order to further explore this relationship. We created a metric reflecting memory which was defined as the difference in UCS expectancy ratings on the first CS+ and CS- trial during the extinction session. Larger numbers reflect a better memory for the relationships between the different CSs and the UCS. These values were normalized within the delay and trace groups which led to a score for each participant that reflected their memory for the contingencies relative to the other participants in the same group. The unpaired group did not learn a predictive relationship between the CS+ and the UCS so they were excluded from this analysis. We observed increases in connectivity for both the delay and trace group involving the amygdala at the twenty-four hour time point, so we focused the analysis at this time point.

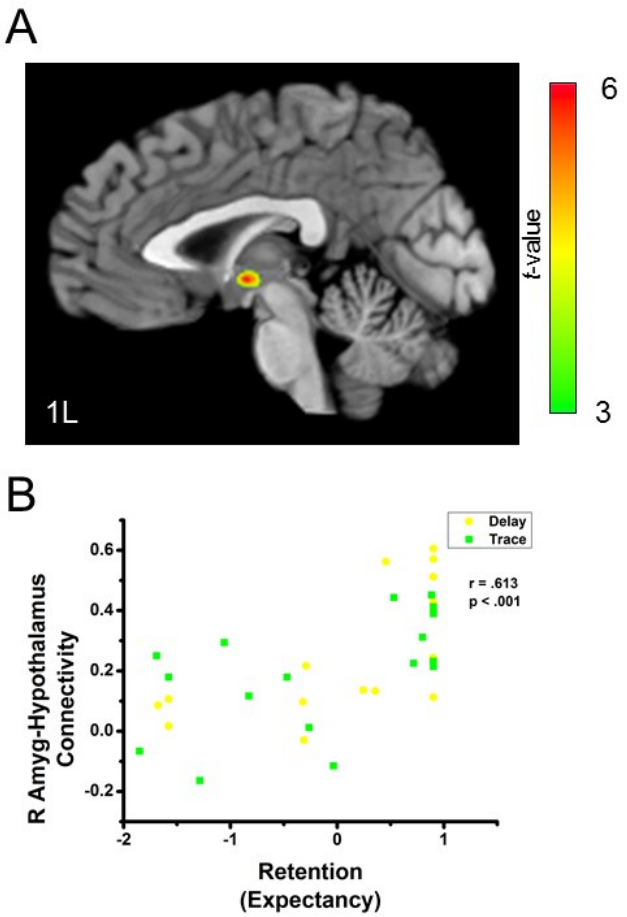

Figure 24. Connectivity between the right amygdala and the hypothalamus twenty-four hours after conditioning is correlated with memory retention measured seven days following conditioning for both the delay and trace groups. (A) Brain image depicting regions characterized by this correlation. The color scale corresponds to the *t*-value. (B) Scatterplot of the connectivity between the right amygdala and hypothalamus at twenty-four hours and memory retention scores for the delay and trace groups.

We found a positive correlation between memory for the CS-UCS contingencies seven days following acquisition and connectivity between the right amygdala the hypothalamus twenty-four hours after conditioning for the delay and trace groups (see Figure 24). The central nucleus of the amygdala projects to the hypothalamus which is involved in the generation of autonomic fear responses (Gray & Magnuson, 1987). We

also found a positive correlation between memory for the CS-UCS contingency seven days following acquisition and connectivity between the left amygdala and the ACC for the delay and trace groups (see Figure 25). Several studies have suggested that fear memories rely more on the ACC as the amount of time increases from acquisition (Frankland et al., 2004; Wheeler, Teixeira, Wang, Xiong, Kovacevic, Lerch, McIntosh, Parkinson, & Frankland, 2013). We ran the same correlation analysis for the amygdala on each of the other time points to see if this relationship was specific to the twenty-four hour time point or if it was a general effect across time. We did not find a significant correlation between memory scores and connectivity between the amygdala and the hypothalamus or the ACC at any of the other time points, largest $r = .149$, $p = .39$.

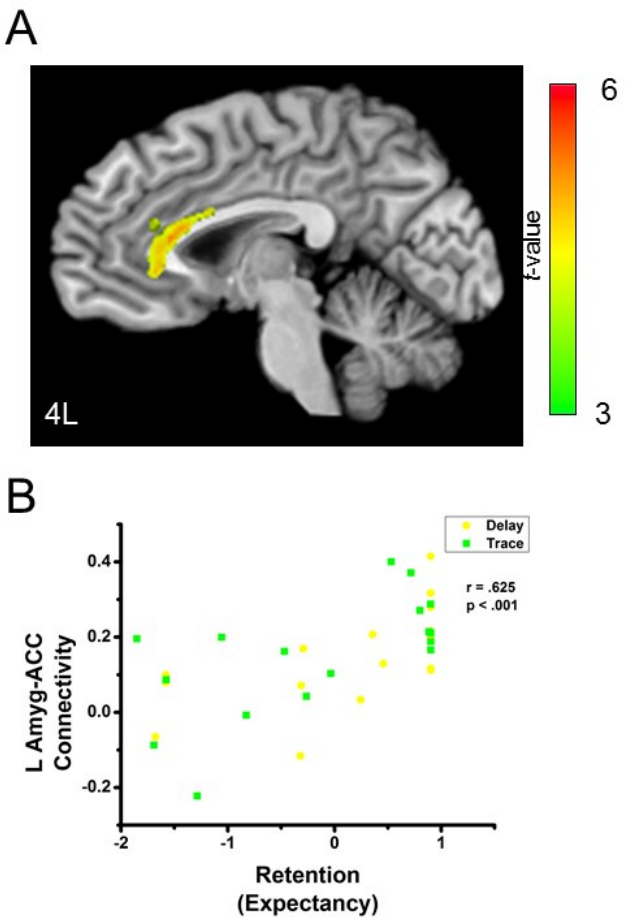

Figure 25. Connectivity between the left amygdala and the anterior cingulate cortex twenty-four hours after conditioning is correlated with memory retention measured seven days following conditioning for both the delay and trace groups. (A) Brain image depicting regions characterized by this correlation. The color scale corresponds to the *t*-value. (B) Scatterplot of the connectivity between the left amygdala and anterior cingulate cortex at twenty-four hours and memory retention scores for the delay and trace groups.

In order to explore correlations between memory and functional connectivity that might be specific to either the delay or trace group we looked at connectivity with the mPFC at the twenty-four hour time point. The trace group showed increased connectivity between the mPFC and the retrosplenial cortex at this time point in the between group analysis. We found a significant positive correlation between memory scores and

connectivity between the mPFC and the inferior parietal lobule in the trace group, but not in the delay group (see Figure 26). Previous studies have suggested that the inferior parietal lobule is specifically engaged in trace conditioning (Haritha, Wood, Ver Hoef, & Knight, 2013). We ran the same correlation analysis for the mPFC on each of the other time points to see if this relationship was specific to the twenty-four hour time point or if it was a general effect across time. We did not find a significant correlation between memory scores and connectivity between the mPFC and the inferior parietal lobule in the trace group prior to conditioning or immediately following conditioning, largest $r = .43$, $p = .083$. However, we did see a significant positive correlation for the trace group at the seven day time point, $r = .53$, $p < .05$.

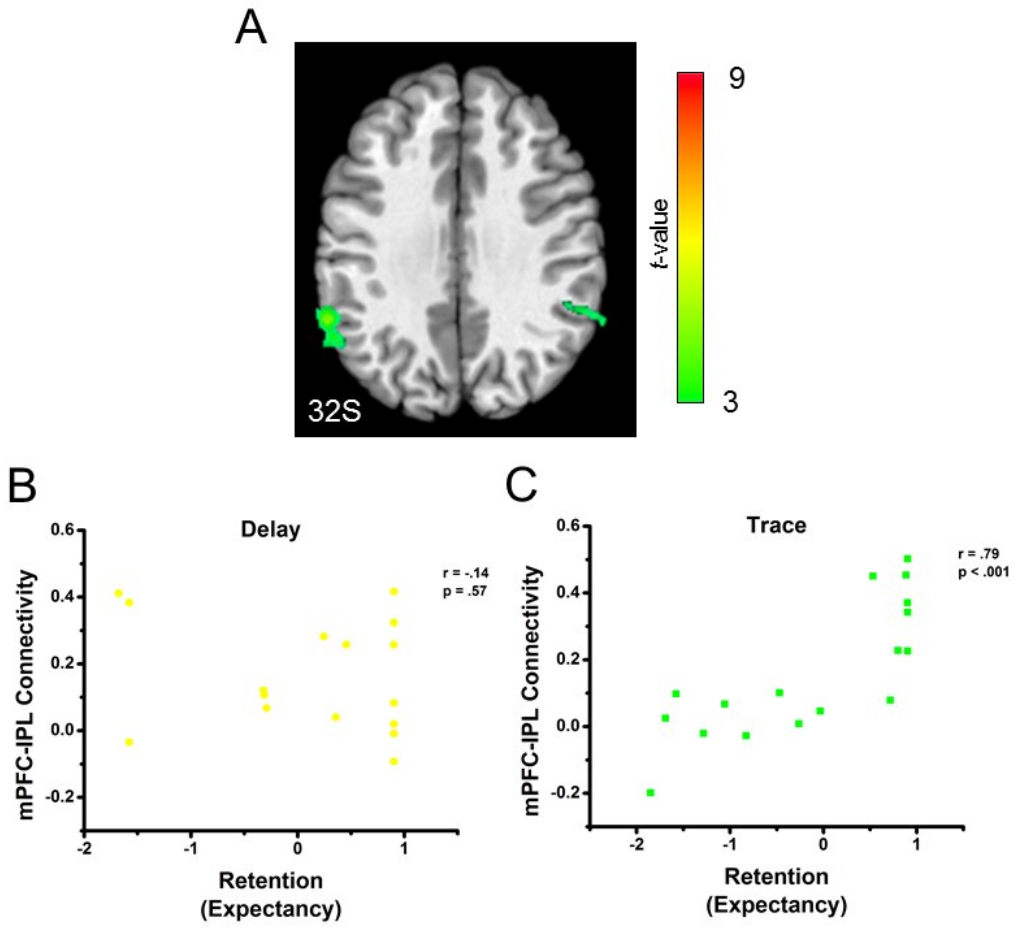

Figure 26. Connectivity between the medial prefrontal cortex and the inferior parietal lobule twenty-four hours after conditioning is correlated with memory retention measured seven days following conditioning for trace group, but not the delay group. (A) Brain image depicting regions characterized by this correlation. The color scale corresponds to the *t*-value. (B) Scatterplot of the connectivity between the medial prefrontal cortex and the inferior parietal lobule at twenty-four hours and memory retention scores for the delay group. (C) Scatterplot of the connectivity between the medial prefrontal cortex and the inferior parietal lobule at twenty-four hours and memory retention scores for the trace group.

Discussion

This study examined the acquisition and extinction of delay and trace fear conditioning in humans. Additionally, we examined time-dependent changes in resting-state functional connectivity following learning in the different networks that support delay and trace conditioning. We found that the delay and trace groups showed evidence of learning as measured by UCS expectancy and skin conductance relative to an explicitly unpaired control group. The delay and trace groups showed similar levels of behavioral performance during acquisition and we identified a variety of brain regions that were involved in fear acquisition for both groups. We also identified several brain regions that were involved in the acquisition of trace conditioning that were not active for the delay group. Resting-state functional connectivity was assessed immediately following conditioning, twenty-four hours following conditioning, and seven days following conditioning. We observed several increases in functional connectivity at the twenty-four hour time point that were common to both the delay and trace groups and largely involved the amygdala. We also found several increases in functional connectivity that were specific to the trace group and involved regions required for the acquisition of trace conditioning. We also found that extinction resulted in a decrease in differential responses to the CS+ and the CS- on both UCS expectancy and skin conductance measures for both delay and trace groups. Brain activity during extinction paralleled the behavioral results in several areas that have been previously reported in extinction studies. During extinction we found differential activity in an anterior portion of vmPFC for the delay group, but not for the trace group. However, the trace group

showed increased connectivity between the subgenual ACC portion of the vmPFC and the amygdala.

Behavioral evidence of delay and trace fear acquisition

Numerous studies have examined the acquisition of delay fear conditioning in humans while measuring UCS expectancy and SCR (Cheng, Richards, & Helmstetter, 2007; Dunsmoor et al., 2007; Knight et al., 2009; Schultz et al., 2012). Fewer studies have examined these variables during both delay and trace fear conditioning (Knight et al., 2004; Knight, Nguyen, & Bandettini, 2006). In the present study we replicated previous studies and found evidence for differential conditioning on UCS expectancy and SCR measures in both delay and trace fear conditioning. The magnitude of these effects was also consistent between the delay and trace group. This indicates that any of the subsequent differences that we observed in functional connectivity cannot be attributed to differences in how well the groups acquired differential fear responses.

Stimulus evoked BOLD activity during acquisition

A whole-brain ANOVA of the acquisition data revealed a general fear learning effect in which the delay and trace group both showed differential activity by the CS+ and the CS-. We observed a large region including the bilateral thalamus and caudate consistent with previous delay fear conditioning studies (Dunsmoor et al., 2011; Schiller, Levy, Niv, LeDoux, & Phelps, 2008), trace conditioning studies (Buchel, Dolan, Armony, & Friston, 1999), and studies examining both delay and trace conditioning (Knight et al., 2004b). We also observed a general fear learning effect in the insula which is consistent with several previous fear conditioning studies examining both delay

(Knight, Nguyen, & Bandettini, 2005; Morris & Dolan, 2004; Phelps et al., 2001; Schultz et al., 2012) and trace conditioning (Buchel et al., 1999; Nitschke, Sarinopoulos, Mackiewicz, Schaefer, & Davidson, 2006). We also found a general fear learning effect in a large cluster containing portions of the cingulate gyrus and the superior frontal gyrus which have both been observed in previous fear conditioning studies (Buchel et al., 1998; Knight et al., 1999; LaBar et al., 1998; Milad et al., 2007).

We did not observe a general fear learning effect in the amygdala using a whole-brain analysis approach. However, when we defined the amygdala anatomically and extracted the BOLD signal from this mask we did see a general fear learning effect in the amygdala which is consistent with previous FMRI data with human participants using delay (Cheng et al. 2003; LaBar et al., 1998; Schultz et al., 2012) and trace procedures (Buchel et al., 1999; Carter, O'Doherty, Seymour, Koch, & Dolan, 2006; Cheng et al., 2006). The present data is also consistent with laboratory animal studies suggesting that protein synthesis (Kwapis et al., 2011) and activity (Gilmartin et al., 2012) in the amygdala are required for both delay and trace fear conditioning. Our data support the idea that the amygdala is a critical region for the acquisition of both delay and trace fear conditioning.

The whole-brain ANOVA on the acquisition data also revealed several regions that exhibited greater differential activity in the trace group compared to the delay and unpaired group. This pattern of data suggests that these regions are specifically involved in the acquisition of trace fear conditioning. We observed activity specific to the trace group in the mPFC which is consistent with several studies (Gilmartin, Miyawaki, Helmstetter, & Diba, 2013; Kronforst-Collins, & Disterhoft, 1998; Runyan, Moore, &

Dash, 2004; Weible, McEchron, & Disterhoft, 2000). It has been hypothesized that sustained activity during the trace interval in this region may serve to hold a representation of the CS through the empty trace interval so that it can be associated with the UCS (Gilmartin & McEchron, 2005b). We observed a similar pattern in the mediodorsal thalamus which is consistent with data from a lesion experiment in rabbits where the mediodorsal thalamus damage resulted in the retarded acquisition of a trace CR (Powell & Churchwell, 2002). Other studies have shown that mediodorsal thalamic lesions result in deficits in reversal learning (Buchanan, 1991) and when long interstimulus intervals are used (Buchanan, Penney, Tebbutt, & Powell, 1997) suggesting that the mediodorsal thalamus is critical under more complex or difficult conditioning conditions. We also observed trace specific activity in a region of the posterior cingulate cortex that includes portions of the retrosplenial cortex. This finding is consistent with a study in which rabbits with a retrosplenial cortex lesion were unable to acquire a trace eyeblink response (Solomon, Vander Schaff, Thompson, & Weisz, 1986). The trace specific activity we observed during acquisition was largely consistent with previous trace conditioning studies.

We did not observe a trace specific effect in the hippocampus using a whole-brain analysis approach. However, when we defined the hippocampus anatomically and extracted the BOLD signal we observed a larger differential response for the trace group relative to the delay and unpaired group. The delay group did show a non-significantly larger response evoked by the CS+ relative to the CS-. This is consistent with data suggesting that patients with hippocampal lesions cannot acquire trace eyeblink conditioning (Clark & Squire, 1998). Our data is also consistent with the hypothesis that

the hippocampus is important for timing the CR in humans (Knight et al., 2004a) and laboratory animals (Gilmartin, & McEchron, 2005a; Green & Arenos, 2007). Alternatively, the hippocampus is not required for delay eyeblink conditioning (Berger & Orr, 1983; Schmaltz & Theios, 1972), but disruptions of septo-hippocampal communication can retard delay eyeblink conditioning (Berry & Thompson, 1979) suggesting that hippocampal involvement in delay conditioning serves a modulatory role. More evidence for the modulatory role of hippocampal activity in delay conditioning comes from a fear conditioning paradigm in which the explicit contingency awareness was manipulated on a trial by trial basis. Conditional skin conductance responses were observed on both aware and unaware trials, but differential hippocampal activity was only observed on aware trials (Knight, Waters, & Bandettini, 2009). The trend for larger CS+ evoked responses relative to CS- evoked responses in the hippocampus for the delay group in the current study may not reflect the necessity of this region, but the influence of modulatory activity associated with the explicit processes of fear learning.

The results from the acquisition session of the current experiment were consistent with previous studies. We observed a general fear learning effect characterized by differential activity evoked by the CS+ and the CS- for both the delay and trace groups in several regions including the amygdala, insula, thalamus, caudate, and cingulate gyrus. These regions have been previously shown to be involved in the general fear learning circuit. We also observed trace specific activity in several regions including the hippocampus, mPFC, mediodorsal thalamus, and posterior cingulate gyrus. These regions have been implicated in trace conditioning in a variety of different species and different preparations.

Resting-state functional connectivity prior to and following acquisition

All of the groups were treated the same prior to acquisition and we did not expect to observe any differences at this time point. Prior to acquisition we did not observe any differences in connectivity related to general fear learning. This contrast examined the difference between connectivity for the unpaired group and compared it to the connectivity for both the delay and trace groups. However, we did observe a few differences in a contrast comparing the delay and trace groups. The trace group showed greater connectivity between the right insula and vmPFC relative to the delay group. The trace group also showed greater connectivity between the right amygdala and left retrosplenial cortex. Importantly, we did not observe any difference in connectivity in these same connections following acquisition on either contrast indicating that the differences observed at later time points were not due to differences present at baseline.

Immediately following acquisition we observed increased connectivity between the mPFC and the right retrosplenial cortex for the trace group relative to the delay group. Previous studies have suggested that these two regions are both important during the acquisition of trace conditioning and during the consolidation period following trace learning (Kronforst-Collins, & Disterhoft, 1998; Runyan,et al., 2004; Weible et al., 2000; Gilmartin & McEchron, 2005b; Kwapis et al, unpublished data). We also observed increased connectivity between the right amygdala and the vmPFC in the trace group relative to the delay group immediately following acquisition. The vmPFC has been identified as an important structure for the inhibition of fear responses during extinction learning (Quirk, Likhtik, Pelletier, & Pare, 2003; Sierra-Mercado, Padilla-Coreano, & Quirk, 2010). However, recent data has also suggested that the vmPFC may be involved

in the maintenance of an autonomic response when there is a gap of time between the signal for a reward and the delivery of the reward (Rudebeck, Putnam, Daniels, Yang, Mitz, Rhodes, & Murray, 2014). This function would be consistent with increased communication between the amygdala and the vmPFC if the vmPFC is playing a role in sustaining an autonomic response that is typically generated in the amygdala.

We expected to observe increases in connectivity for the delay and trace group relative to the unpaired group in a few regions immediately following acquisition. Previous work in our lab found an increase in connectivity between the amygdala and the superior frontal gyrus or the dorsomedial PFC immediately following acquisition (Schultz et al., 2012). Another study used a similar design and found decreases in connectivity between the amygdala and the dorsal ACC immediately following conditioning, although the region of dorsal ACC detected was much more posterior (Feng, Feng, Chen, & Lei, 2013). We did not observe an increase in connectivity between the amygdala and the mPFC for the delay and trace group relative to the unpaired group immediately following acquisition. These results conflict with the findings from Schultz and colleagues (2012). However, the current study included both delay and trace conditioning while the previous study only used delay conditioning. The previous study also included several more participants. Furthermore, the current study defined the mPFC as a region that was specifically engaged in trace conditioning. This region did not perfectly overlap with the region originally described by Schultz and colleagues (2012). It is possible that the increase in connectivity between those two regions immediately following conditioning is more subtle and more power would be needed to detect it. It is also possible that the amygdala connectivity changes following

acquisition in the previous study and the region in the current study defined functionally as being specifically involved in trace conditioning are not modified in the same way. The results from the current study are also somewhat inconsistent with the data from Feng and colleagues (2013). There are several differences in methodology across the two studies. Feng and colleagues (2013) used a much different conditioning preparation that consisted of shorter duration CS presentations. They also used partial reinforcement and the UCS was a fearful picture rather than an electric shock. It is difficult to equate the type of learning in these studies considering all of these factors and the fact that Feng and colleagues (2013) did not collect any autonomic or UCS expectancy measures that can be compared.

Twenty-four hours following acquisition we observed general fear learning related increases in connectivity between several regions including: the left amygdala and right hippocampus, left amygdala and right retrosplenial cortex, right amygdala and left hippocampus, right amygdala and mPFC. All of these connections showed increased connectivity for both the delay and trace groups relative to the unpaired group. All of the general fear learning increases in connectivity involved the amygdala which is consistent with previous data suggesting that the amygdala is important for both delay and trace fear conditioning (Kwapis et al., 2011; Gilmartin et al., 2012). Increases in connectivity for the delay and trace group relative to the unpaired group between the amygdala and the hippocampus may reflect the consolidation of the explicit or declarative components of fear conditioning that are thought to rely on the hippocampus (Knight et al., 2009; Squire & Zola, 1996). We also observed increased connectivity between the right amygdala and the mPFC for the delay and trace group relative to the control group twenty-four hours

following acquisition. This is similar to the effect reported by Schultz and colleagues (2012) for increased connectivity following delay fear conditioning, although the current study observes this effect twenty-four hours after acquisition rather than immediately after acquisition.

Twenty-four hours following acquisition we observed increased connectivity for the trace group relative to the delay group between the mPFC and the retrosplenial cortex. This difference in connectivity was apparent immediately following conditioning, as well as twenty-four hours following acquisition. These regions have previously been implicated in trace learning and it appears that increases in connectivity between these regions may reflect processes involved in the consolidation of this type of memory.

Seven days following acquisition we did not observe any differences in connectivity reflecting a general fear learning process. The extensive increases in connectivity involving the amygdala twenty-four hours following acquisition were no longer present seven days after the acquisition session. We did observe increased connectivity for the trace group relative to the delay group between the right hippocampus and the right insula. This was the only difference in connectivity between the groups that we detected seven days following acquisition. The increase in connectivity for the trace group between the hippocampus, which is important for trace conditioning, and the insula, which is involved in interoceptive processing (Craig, 2009), might reflect the integration of the trace training parameters and the pain associated with the presentation of the UCS.

The time course of changes in functional connectivity

Few studies have examined the time course of changes in functional connectivity induced by a behavioral manipulation. A recent study examined changes in resting-state functional connectivity immediately following a motor learning task, approximately thirty minutes later, and also six hours after learning (Sami, Robertson, & Miall, 2014). This study found that resting-state connectivity was dynamic across these multiple time points. Initially, connectivity changes were localized to motor networks, but as the consolidation process unfolded connectivity revealed more distributed patterns across the brain. Similar to the results of the current study, Sami and colleagues (2014) observed limited changes in connectivity in some networks immediately following learning that showed larger changes six hours after learning.

Another recent study found changes in resting-state functional connectivity immediately following a task, but more robust changes twenty-four hours following the task (Harmelech, Preminger, Wertman, & Malach, 2013). Harmelch and colleagues (2013) used a real-time neurofeedback task where participants were asked to activate a region of dorsal ACC using a general cognitive strategy. Participants were given feedback via auditory tones that indicated the level of activity in the dorsal ACC. Resting-state functional connectivity maps were calculated immediately following the neurofeedback task and twenty-four hours later using the dorsal ACC as a seed region. Increases in functional connectivity were observed immediately following the task, but these increases were much more robust at the twenty-four hour time point.

Both of these studies demonstrate that differences in resting-state functional connectivity can be observed for several hours following a behavioral manipulation. These findings are consistent with the current study which shows the most robust differences in functional connectivity occur twenty-four hours following conditioning. It is interesting that the largest changes in resting-state connectivity are observed after a period of time when consolidation is occurring. The twenty-four hour time point is especially interesting because it likely includes a night of sleep, which numerous studies have suggested is an important factor in the consolidation of memory (for review see Rasch & Born, 2013; Stickgold & Walker 2007).

The current study observed increases in functional connectivity between the amygdala and several other brain regions for the delay and trace groups twenty-four hours following acquisition. These increases in connectivity may reflect the ongoing consolidation of fear memories. These changes in connectivity could reflect several different processes involved in consolidation. Some researchers have suggested that the primary role of the amygdala during the formation of emotional memories is to modulate activity in other brain areas where the memory is ultimately stored (McGaugh, 2004; Paz & Pare, 2013). However, several studies have indicated that plasticity in the amygdala is critical during the consolidation window and that the amygdala is an important site for the long-term storage of fear memories (Romanski et al., 1993; Gale et al., 2004). Another possible explanation is that the amygdala is part of a general fear learning circuit and that plastic changes in the amygdala as well as other distributed regions in the circuit all contribute to the storage of fear memories (Helmstetter et al., 2008). The current study measured changes in functional connectivity following fear conditioning. This type

of measurement is focused on the correlations in the BOLD signal between regions. This type of analysis cannot determine the directionality of these connections or if activity in one region is the cause for changes in activity in another region. Advances in FMRI analysis techniques as well as technological advances that can increase the rate at which BOLD data is sampled may allow researchers to determine directionality and the causal relationship between signal in different brain areas in the near future (Deshpande & Hu, 2012).

Behavioral evidence of retention and extinction of delay and trace fear memories

The delay and trace groups both acquired differential fear responses on the UCS expectancy and SCR measure. Participants underwent extinction training seven days following acquisition. In order to assess the integrity of the original memory we examined the UCS expectancy and SCR data from the first three trials of this extinction session. We found that the delay and trace group both retained the acquisition memory as evidenced by differential UCS expectancy ratings. The SCR data showed a similar trend toward significant differential responses, although the effect was not as robust. Extinction learning was assessed by comparing responses to the CS+ and the CS- during the last seven trials of the extinction session. As we expected, the delay and trace group both demonstrated extinction learning by no longer exhibiting differential responses on either the UCS expectancy or SCR measure. These effects are consistent with previous human fear extinction studies (Knight et al., 2004b; Milad et al., 2007; Sokol & Lovibond 2012).

Stimulus evoked BOLD activity during extinction

A whole-brain ANOVA of the extinction data revealed a general extinction effect consistent for the delay and trace group. We observed a general extinction effect in the insula, caudate, and inferior frontal gyrus which is consistent with several previous delay fear conditioning studies (Gottfried & Dolan, 2004; Phelps et al., 2004; Molchan et al., 1994). We also observed an extinction effect in the vmPFC that was only evident in the delay group. Activity in the vmPFC during extinction is consistent with several previous studies suggesting that the vmPFC engaged during extinction learning and during the recall of an extinction memory (Phelps et al., 2004; Milad et al., 2005). We expected that the vmPFC would be involved in the extinction process for both delay and trace conditioning, however we only observed this effect in the delay group. This could indicate that trace extinction relies on slightly different circuitry or that it is supported by more distributed activity that is difficult to detect with FMRI.

Resting-state functional connectivity following extinction

Immediately following extinction the trace group showed increased connectivity relative to the delay group between the right amygdala and the vmPFC. The trace group also had significantly greater connectivity between the left amygdala and the vmPFC. We did not observe any significant differences on the general fear extinction contrast. The increase in connectivity between the vmPFC and the amygdala for the trace group is consistent with previous studies suggesting that vmPFC is involved in extinction learning (Quirk et al., 2003). If this effect was a general extinction learning process we would have expected to see it in the delay group as well. However, we did see activity related to

extinction in the delay group in a more anterior portion of the vmPFC. It is possible that the discrepancy between the stimulus evoked activity during extinction and the increases in connectivity following extinction for the delay and trace groups is due to differences in the loci of the vmPFC involvement in extinction. In fact, vmPFC activation observed during extinction training or the recall of extinction has been reported in different areas within the vmPFC across different studies (Milad et al., 2007; Phelps et al., 2004). The acquisition of trace conditioning requires more complex and distributed brain areas, and it is possible that the extinction of a trace memory relies on more distributed regions or even distributed cells within a region making it more difficult to detect the effect with FMRI methods.

Changes in functional connectivity following learning are related to memory

We found a relationship between resting-state functional connectivity twenty-four hours following acquisition and memory based on UCS expectancy seven days after acquisition. The delay and trace group both showed a positive correlation between memory scores at the seven day time point and connectivity between the amygdala and hypothalamus as well as the amygdala and ACC, both regions involved in the generation of fear responses (Gray & Magnuson, 1987; Milad et al, 2007; van Well et al., 2012). This relationship may reflect the strengthening of connections between the amygdala where the CS and UCS information converges and regions involved in the generation of fear responses. This increased connectivity may support an increase in the efficiency of the expression of fear responses. We also observed a relationship between connectivity between the mPFC and the inferior parietal lobule twenty-four hours following acquisition and memory scores collected seven days after learning. This effect was only

evident in the trace group. Although the relationship between memory scores and connectivity for the trace group was strongest twenty-four hours following acquisition, it was also apparent seven days after conditioning immediately prior to the memory assessment. The mPFC and inferior parietal lobule have both been identified as being important for trace learning (Gilmartin et al., 2013; Kronforst-Collins, & Disterhoft, 1998; Haritha et al., 2013; Runyan et al., 2004; Weible et al., 2000). In the current study we observed a relationship between the connectivity of these two regions that have been specifically implicated in trace conditioning and subsequent memory scores that was apparent in the trace group, but not the delay group. This suggests that connectivity within the trace conditioning circuit is associated with memory for the acquisition of trace conditioning.

Conclusions

In this study we examined resting-state functional connectivity following the acquisition and extinction of delay and trace fear conditioning. During the acquisition session we found activity reflecting a general fear learning effect in several brain regions including the amygdala. We also observed activity that was specific to trace conditioning in several other brain areas including the mPFC and hippocampus. Resting-state functional connectivity was increased between several regions and the amygdala for both the delay and the trace group twenty-four hours following acquisition. We found increased functional connectivity for the trace group relative to the delay group in brain regions supporting trace conditioning. The most robust differences in functional connectivity were apparent twenty-four hours following acquisition and most of those increases had diminished by seven days after conditioning. During the extinction session

we observed activity for both the delay and the trace group in regions previously reported in the extinction of delay conditioning. There was an extinction effect in an anterior region of the vmPFC for the delay group. Functional connectivity following the extinction session was increased between the amygdala and the subgenual ACC for the trace group, but not the delay group. We also found correlations between functional connectivity twenty-four hours following conditioning and a measure of memory collected seven days after conditioning. Some of these relationships were evident for both the delay and trace group and one relationship was specific for the trace group. We believe that changes in resting-state functional connectivity following conditioning can be observed for several hours and that these changes reflect the process of memory consolidation.

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Wilensky, A., Schafe, G., Kristensen, M., & LeDoux, J. (2006). Rethinking the fear circuit: the central nucleus of the amygdala is required for the acquisition, consolidation and expression of Pavlovian fear conditioning. *J Neurosci, 26*, 12387-96.

Yaguez, L., Coen, S., Gregory, L., Amaro, E., Altman, C., Brammer, M., Bullmore, E., Williams, S., & Aziz, Q. (2005). Brain response to visceral aversive conditioning: a functional magnetic resonance imaging study. *Gastroenterology, 128*, 1819-29.

CIRRICULUM VITAE

Douglas H. Schultz

Place of birth: Montevideo, MN

Dissertation title: Changes in resting-state functional connectivity following delay and trace fear conditioning acquisition and extinction

Education History

Southwest Minnesota State University
Major: Psychology
Degree: B.A.

University of Wisconsin-Milwaukee
Major Professor: Fred Helmstetter, Ph. D.
Major: Neuroscience
Minors: Behavior Analysis, Neurobiology
Degree: MS, Ph, D

Awards and Honors

APA Dissertation Research Award	2013
UWM Psychology Department Summer Fellowship	2013
UWM Psychology Graduate Student Research Award	2012
UWM Clinical and Translational Science Institute Research Assistantship	2010
UWM Graduate Student Research Symposium Presentation Award (1st Place)	2010
John and Lynn Schiek Award in Behavior Analysis	2008
UWM Graduate Student Research Symposium Presentation Award (2nd Place)	2008
UWM Graduate Student Travel Award	2007
UWM Chancellor's Graduate Student Award	2005, 2006
Southwest Minnesota State University – Graduated *Magna Cum Laude*	2004

Publications

Schultz, D., & Helmstetter, F. (2013). Dissociation between implicit and explicit responses in postconditioning UCS revaluation after fear conditioning in humans. *Behavioral Neuroscience*, *127*, 357-68.

Balderston, N., **Schultz, D**., Baillet, S., & Helmstetter, F. (2013). How to detect amygdala activity with megnetoencephalography using source imaging. *Journal of Visualized Experiments* (76), e50212, doi:10.3791/50212.

Larson, C., Baskin-Sommers, A., Stout, D.M., Balderston, N., **Schultz, D**., Curtin, J., Kiehl, K., & Newman, J. (2013). The interplay of attention and emotion: Top-down

attention modulates amygdala activation in psychopathy. *Cognitive, Affective, & Behavioral Neuroscience*. doi: 10.3758/s13415-013-0172-8.

Balderston, N., **Schultz, D**., & Helmstetter, F. (2013). The effect of threat on novelty evoked amygdala responses. *PLoS One*, 8(5): e63220. doi:10.1371/journal.pone.0063220.

Schultz, D., Balderston, N., & Helmstetter, F. (2012). Resting-state connectivity of the amygdala is altered following Pavlovian fear conditioning. *Frontiers in Human Neuroscience,* **6**:242. doi: 10.3389/fnhum.2012.00242.

Balderston, N., **Schultz, D**., & Helmstetter, F. (2011). The human amygdala plays a stimulus specific role in the detection of novelty. *Neuroimage, 55*, 1889-1898.

Schultz, D., & Helmstetter, F. (2010). Classical conditioning of autonomic fear responses is independent of contingency awareness. *Journal of Experimental Psychology: Animal Behavior Processes*, *36*, 495-500.

Schultz, D., Balderston, N., Cheng, D., Geiger, J., & Helmstetter, F. (in preparation). Conditional stimulus BOLD activation overlaps with shock evoked activity in somatosensory cortex.

Schultz, D., Balderston, N., & Helmstetter, F. (in preparation). A direct comparison of auditory and visual fear conditioning in humans using FMRI.

Abstracts

Schultz, D., Balderston, N., Newman, J., Larson, C., & Helmstetter, F. (2012). Brain activity during fear conditioning in incarcerated psychopaths. Poster presented at Society for Neuroscience meeting in New Orleans.

Schultz, D., Balderston, N., Hannula, D., & Helmstetter, F. (2012). Using eye movement and pupil dilation measures to examine the role of awareness in fear conditioning. Poster presented at Pavlovian Society meeting in Jersey City, NJ.

Schultz, D., Balderston, N., & Helmstetter, F. (2011). The amygdala shows increased functional connectivity with the dorsal mPFC following fear conditioning. Poster presented at Society for Neuroscience meeting in Washington DC.

Schultz, D., Balderston, N., & Helmstetter, F. (2011). The amygdala shows changes in functional connectivity following fear conditioning. Poster presented at the Pavlovian Society meeting in Milwaukee.

Balderston, N., **Schultz, D**., & Helmstetter, F. (2011). Neuromagnetic amygdala responses during trace fear conditioning without awareness. Poster presented at Society for Neuroscience meeting in Washington DC.

Schultz, D., Balderston, N., & Helmstetter, F. (2010). Auditory and visual CS fear conditioning both result in modality specific changes in sensory cortex that correlate with activity in the amygdala and striatum. Poster presented at Society for Neuroscience meeting in San Diego.

Balderston, N., **Schultz, D**., & Helmstetter, F. (2010). The effect of threat on novelty evoked amygdala responses. Poster presented at Society for Neuroscience meeting in San Diego.

Schultz, D., Balderston, N., & Helmstetter, F. (2009). A direct comparison of differential auditory and visual fear conditioning in humans using FMRI. Poster presented at Society for Neuroscience meeting in Chicago.

Balderston, N., **Schultz, D**., & Helmstetter, F. (2009). Novel faces but not scenes drive BOLD responses in the amygdala. Poster presented at Society for Neuroscience meeting in Chicago.

Schultz, D., Balderston, N., & Helmstetter, F. (2009). Somatosensory cortex activation by a visual stimulus that signals shock. Poster presented at Organization for Human Brain Mapping meeting in San Francisco.

Balderston, N., **Schultz, D**., & Helmstetter, F. (2009). BOLD response to novelty in the human amygdala. Poster presented at Organization for Human Brain Mapping meeting in San Francisco.

Schultz, D., Balderston, N., Schramm, C., & Helmstetter, F. (2008). Conditional stimulus evoked BOLD activation overlaps with shock evoked activity in somatosensory cortex. Poster presented at Society for Neuroscience meeting in Washington DC.

Balderston, N., **Schultz, D**., Schramm, C., & Helmstetter, F. (2008). In a direct comparison of novelty and emotional valence, novelty evokes larger magnitude BOLD responses in the amygdala. Project presented at the Society for Neuroscience meeting in Washington DC.

Schramm, C., **Schultz, D**., Balderston, N., & Helmstetter, F. (2008). Impact of repeated versus novel exposure to conditional stimuli on human brain activation during Pavlovian fear conditioning. Poster presented at Society for Neuroscience meeting in Washington DC.

Schultz, D., Geiger, J., Balderston, N., & Helmstetter, F. (2007). Classical conditioning of autonomic fear responses is independent of contingency awareness. Poster presented at the Pavlovian Society meeting in Austin, TX.

Schultz, D., Geiger, J., Balderston, N., & Helmstetter, F. (2007). Classical conditioning of autonomic fear responses is independent of contingency awareness. Program No. 526.18. *2007 Abstract Viewer/Itinerary Planner.* Washington DC: Society for Neuroscience.

Balderston, N., Geiger, J., **Schultz, D**., & Helmstetter, F. (2007). Masked presentations of simple visual stimuli and learning without awareness in human fear conditioning. Program No. 526.19. *2007 Abstract Viewer/Itinerary Planner*. Washington DC: Society for Neuroscience.

Balderston, N., Geiger, J., **Schultz, D**., & Helmstetter, F. (2007). Learning without awareness in human fear conditioning. Poster presented at Pavlovian Society meeting in Austin, TX.

Geiger, J., **Schultz, D**., Balderston, N., & Helmstetter, F. (2007). Functional neuroimaging of context dependent fear reinstatement in humans. Program No. 526.17. *2007 Abstract Viewer/Itinerary Planner*. Washington DC: Society for Neuroscience.

Schultz, D., Geiger, J., Balderston, N., & Helmstetter, F. (2007). Contextual modulation of human brain activity during reinstatement of conditioned fear. Poster presented at the 13th annual Organization for Human Brain Mapping meeting in Chicago, IL. Available on CD-ROM in *NeuroImage, Vol. 36, Sup. 1*. Poster No. 131 TH-AM.

Schultz, D., Richards, J., & Helmstetter, F. (2006). UCS revaluation in human fear conditioning. Program No. 366.4. *2006 Abstract Viewer/Itinerary Planner*. Washington DC: Society for Neuroscience.

Colloquia and Symposia

Examining the consolidation of delay and trace fear memories in humans. Neuroscience and Physiology Brown Bag. January 2014.

Resting-state connectivity of the amygdala is altered following fear conditioning. Neuroscience and Physiology Brown Bag. December 2011.

The role of awareness in human fear conditioning. Graduate Research Symposium. April 2011.

Cortical contributions to human fear conditioning. Medical College of Wisconsin Lunch and Learn Symposium. June 2010.

The effect of retention interval on postconditioning UCS inflation in human fear conditioning. Master's Thesis. May 2010.

Learning related alterations in sensory processing in human fear conditioning. Graduate Research Symposium. April 2010.

The role of expectancy in human fear learning. Neuroscience and Physiology Brown Bag. November 2008.

Classical conditioning of autonomic fear responses is independent of contingency awareness. Graduate Research Symposium. April 2008.

A prospective FMRI study of postconditioning UCS devaluation in human fear conditioning. Medical College of Wisconsin FMRI Brown Bag. June 2007.

Postconditioning UCS devaluation in human fear conditioning. Neuroscience and Physiology Brown Bag. May 2007.

Invited Talks

Rutgers University (2014): The nature of human fear memories and how these memories are stored. (Invited by Dr. Michael Cole).

Laureate Institute for Brain Research (2014). The storage and nature of human fear memories. (Invited by Dr. Justin Feinstein).

Membership in Professional Associations

American Psychological Association	2013-present
Organization for Human Brain Mapping	2006-present
Pavlovian Society	2006-present
Society for Neuroscience	2006-present
Psi Chi – National Honor Society in Psychology	2003-present

Professional Positions

1. Teaching Assistant, University of Wisconsin-Milwaukee

Fall 2005, Introduction to Psychology	(Christopher Flessner)
Spring 2006, Introduction to Psychology	(Kristin Flora)
Fall 2006, Introduction to Psychology	(Dr. Katie Mosack)
Spring 2007, Introduction to Psychology (online)	(Dr. Diane Reddy)
Fall 2007, Introduction to Psychology (online)	(Dr. Diane Reddy)
Fall 2009, Physiological Psychology	(Dr. James Moyer)
Spring 2010, Psychological Statistics	(Dr. Anthony Greene)

2. Research Assistant, University of Wisconsin-Milwaukee

Spring 2008, Graduate Research Assistant	(Dr. Fred Helmstetter)
Fall 2010, Graduate Research Assistant	(Dr. Fred Helmstetter)
Spring 2010, Graduate Research Assistant	(Dr. Fred Helmstetter)
Fall 2011, Graduate Research Assistant	(Dr. Fred Helmstetter)
Spring 2011, Graduate Research Assistant	(Dr. Fred Helmstetter)

Fall 2012, Graduate Research Assistant	(Dr. Fred Helmstetter)
Spring 2013, Graduate Research Assistant	(Dr. Fred Helmstetter)
Fall 2013, Graduate Research Assistant	(Dr. Fred Helmstetter)
Spring 2014, Graduate Research Assistant	(Dr. Fred Helmstetter)

3. Project Assistant, University of Wisconsin-Milwaukee

Fall 2008, Graduate Project Assistant	(Dr. Fred Helmstetter)
Spring 2009, Graduate Project Assistant	(Dr. Fred Helmstetter)