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Metabolic Mapping of Rat Brain Activity Associated with Conditioned Fear Extinction and Renewal, and Improvement of Extinction Memory by the Metabolic Enhancer Methylene Blue

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Metabolic Mapping of Rat Brain Activity Associated with Conditioned Fear Extinction and Renewal, and Improvement of Extinction Memory by the Metabolic Enhancer Methylene Blue

by

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Dedication

with love to my father Ratko Krsmanović

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Metabolic Mapping of Rat Brain Activity Associated with Conditioned Fear Extinction and Renewal, and Improvement of Extinction Memory by the Metabolic Enhancer Methylene Blue

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Changes in brain metabolism associated with the consolidation, extinction and recall of fear memories were investigated in rats using two complementary brain metabolic mapping approaches. First, fluorodeoxyglucose (FDG) metabolic mapping technique was used to track the stimulus-evoked changes in brain glucose uptake that mostly occur during the first ten minutes following the FDG administration. Second, cytochrome oxidase (CO) histochemistry, which is well-suited for tracking long-term changes in brain metabolic capacity, was utilized. By combining these two techniques, brain structures involved in fear extinction memory consolidation and retention were compared to brain regions that displayed altered metabolic activity during conditioned fear memory recall. Additionally, since memory consolidation requires expenditure of energy, enhancement of brain oxidative phosphorylation through CO activity increase was tested as a possible way for improving extinction memory retention in rats. Low doses of the metabolic enhancer methylene blue (MB) were used to

enhance CO activity in the post-extinction training period, to test the hypothesis that neurons with high metabolic demand which are engaged in consolidation and retention of the extinction memory would benefit most from the presence of a metabolic-enhancing drug. The results suggest that during conditioned fear renewal, the auditory conditioned stimulus activates the neural representation of the footshock unconditioned stimulus, thus supporting Pavlov's stimulus-substitution model of classical conditioning. Quantitative CO histochemistry revealed that Pavlovian fear acquisition training increased metabolic capacity in several brain regions, including medial prefrontal cortex (mPFC) and septum, while extinction training reduced CO activity to levels comparable to the pseudorandom group. A functional neural network model of extinction explored how the direct influences on regions such as mPFC and amygdala might change between fear extinction recall and fear renewal. Finally, the third experiment illustrated that MB might be a useful adjunct to exposure therapy, since it improved consolidation and retention of fear extinction in our animal model of specific phobias.

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Chapter 1: Introduction

Fear conditioning and extinction in rodents have been used as models for studying etiology of certain anxiety disorders, as well as for modeling behavioral therapy procedures based on extinction. Fear extinction takes place when a conditioned stimulus (CS) that acquired associative strength through repeated temporal pairing with an aversive unconditioned stimulus (US) is presented repeatedly without reinforcement. Behaviorally, extinction responding resembles reversal of acquisition, although phenomena like spontaneous recovery, renewal and reinstatement of conditioned responding (CR) clearly argue against extinction as complete reversal of the original conditioning. Spontaneous recovery is a term that describes a loss of extinction performance due to a passage of time. Renewal refers to a loss of extinction performance when a subject is tested outside of the extinction context. Reinstatement is a recovery of excitatory responding to an extinguished stimulus due to exposure to the US or another strong stimulus. In humans, similar return of fear phenomena can be observed following a successful completion of extinction-based therapy. Since all these phenomena constitute at least a partial return to pre-extinction behavioral responding, extinction learning seems to be a form of inhibitory learning through which CR becomes repressed. Patients with anxiety disorders, such as phobias, will occasionally experience return of fear when faced with an extinguished CS in environments which differ from the extinction setting. This return of fear can be modeled in rodents using fear renewal training paradigm. Fear renewal can be evoked by testing extinction memory in a context different from extinction context. A common measure of fear conditioning and renewal in rodents is the

amount of freezing which animals display to CS during the probe trials. Freezing is a species-specific defensive response, and it is thought to represent a modal action pattern normally displayed at a certain time point in a predatory imminence continuum (Fanselow, 1989; Domjan, 1998). In this study, freezing was defined as a complete lack of movement (except for rapid, shallow breathing), and was conceptualized to represent an ecologically relevant display of associative learning, since rats naturally freeze when they detect a sign (CS) that a predator (US) is very near.

Metabolic mapping of neural substrates that play a role in fear conditioning, extinction and renewal in rodents would improve our understanding of system-level brain changes that may take place in humans with anxiety disorders such as specific phobias. Better understanding of neurobiological substrates of fear extinction and renewal might also lead to improved therapeutic approaches for treatment of phobias and other anxiety disorders. There is a need to decrease the cost and aversive nature of exposure therapy, and increase its effectiveness with adjunct therapeutics, in order to increase its availability. Recent evidence showed that the cognitive enhancer d-cycloserine (DCS) could improve extinction learning in both rats and humans (Davis, 2002; Ressler et al., 2004). Therefore, we investigated whether administration of a metabolic enhancer methylene blue (MB) could enhance retention of an extinguished conditioned response in rats. Unlike DCS, which acts on a specific neurotransmitter system, MB is proposed to improve metabolic capacity of neurons during the critical time of memory consolidation. Together, information obtained from the metabolic mapping studies of fear extinction and renewal, and findings from the MB administration experiments will contribute to better

understanding of the mechanisms governing the neural processing of an extinguished CS, and lead to a possible development of a novel adjunct therapeutic compound for exposure therapy.

Extinction of conditioned fear

Extinction of conditioned behavior was first described by Ivan Pavlov, who noticed that when conditioned stimuli that were originally paired with an US no longer reliably predicted that US, the conditioned responding (CR) to those CSs decreased (Pavlov, 1927). In addition to describing behaviors accompanying the extinction of CR, Pavlov also hypothesized that the decrease in conditioned responding resulted from inhibitory influence of higher (cortical) brain areas on brain regions responsible for the generation of CR. Since then, many conditioning experiments and theories followed, some in agreement with Pavlov's idea that extinction learning is new, inhibitory learning that is superimposed onto the existing memory of conditioning episodes, and others challenging it. For example, extinction of CR has been viewed as stemming from a decrease in CS associative value resulting from an unexpectedly absent US (Rescorla & Wagner, 1972), reversal of the acquisition process (Richards, Farley, & Alkon, 1984) or even forgetting (Christofi, Nowicky, Bolsover, & Bindman, 1993). At present, it seems certain that extinction is not a form of forgetting, since repeated presentations of the non-reinforced CS are required for extinction, as opposed to forgetting, which results from a mere passage of time. However, it is possible that different forms of extinction (depending on the learning paradigm used), might involve a combination of different mechanisms, so that generalizations across them might be unwarranted. For example, it is possible that extinction of primarily cerebellar learning (such as eye-blink conditioning) is a better example of unlearning (Mauk & Ohyama, 2004) whereas extinction of fear conditioning is more akin to new learning.

Extinction also resembles another well-studied learning paradigm, habituation. Indeed, many of the phenomena that are closely associated with habituation, such as stimulus specificity, exponential decay, dishabituation (or disinhibition), spontatenous recovery and a direct relationship to stimulus frequency are also found in extinction (Domjan, 1998; Cain, Blouin, & Barad, 2003; Kamprath & Wotjak, 2004).

However, an extinguished stimulus has an ambiguous meaning through competing memories of both reinforced learning (conditioning) and non-reinforced training (extinction); in habituation, the stimulus does not acquire a predictive associative value that is by definition necessary for extinction. It is possible that habituation-like processes might contribute to a decrease in behavioral responding observed during the extinction of conditioned fear, since habituation process constitutes a universal feature of any stimulus-elicited behavior (Domjan, 1998). A recent study by Kamprath and Wotjak (2004) supports a possibility that auditory fear conditioning extinction leaves associative conditioning properties intact, but instead of inhibiting the CR specifically, it relies on a more general process of habituation. The most accurate conceptualization of conditioned fear extinction will probably need to take into account both associative and non-associative aspects of extinction training. For example, it has been proposed that qualitative (a particular CS predicts the US), and evaluative (US was aversive) aspects of conditioning might be differentially sensitive to extinction (Vansteenwegen, Francken, Vervliet, De Clercq, & Eelen, 2006). In the present study, the control pseudorandom group had the same number of tone stimulus presentations, and presumably accounted for any habituation to the tone that might have occurred during the course of extinction

training. Despite the uncertainty regarding the exact mechanism, fear extinction remains a good model to study the ways in which anxiety disorder therapies based on extinction might be augmented to improve their efficacy in clinical settings.

Extinction-based therapies for treatment of anxiety disorders

In the middle of the last century, learning principles established in the laboratory setting were first applied to clinical problems such as phobias. One of the first attempts of modern behavior therapy was Wolpe's desensitization technique (Wolpe, 1958). Since then, the field of behavioral therapy has undergone many changes, including the rise of cognitive behavior therapy, which emphasized learning research with human subjects. However, the need to understand maladaptive behavior as an example of choice behavior being influenced by competing contingencies (O'Donohue, 1998) revived interest of applying current learning research to clinical problems. Indeed, better understanding of general principles of behavior derived from animal and human research will doubtlessly lead to improved behavioral modification procedures.

Exposure therapy in clinical settings resembles extinction learning. During extinction, CS is presented repeatedly without the US, resulting in diminished CR. Exposure therapy forces the patient to experience the CS in a controlled setting, thus presumably allowing a new CS-noUS association to form. Unfortunately, exposure therapy can be aversive and difficult to complete, as illustrated by 18% dropout rates in patients undergoing exposure therapy for social phobia (Federoff & Taylor, 2001). Therefore, techniques and agents that

could reduce the span of exposure therapy might be beneficial in decreasing its attrition.

Exposure-based therapies of anxiety disorders are not the only effective way to reduce symptoms associated with anxiety (cognitive therapy, acceptance and commitment therapy, meditation and self-examination therapy, among others, have been reported successful in treatment of anxiety), but they are widely used and can be readily studied using fear extinction in animals. Furthermore, for certain anxiety disorders, such as stimulus-specific phobias and post-traumatic stress disorder (PTSD), exposure therapy alone seems to be more effective than any other single treatment option (Nemeroff et al., 2006). When exposure therapy for an anxiety disorder is combined with medication, it can greatly improve outcome for patients with partial response to medication alone, including fewer occurrences of relapse (Bystritsky, 2004). Traditionally, anxiolytic medication or heterocyclic serotonin enhancers, such as imipramine, have been used as adjunct therapeutics in treatment of anxiety disorders (Craske, 1999). However, use of these compounds is not entirely justified by research findings. While imipramine administration during exposure therapy has been found to produce more improvement in patients than exposure therapy alone (Telch, Agras, Taylor, Roth, & Gallen, 1985; Barlow, Gorman, Shear, & Woods, 2000), patients treated with imipramine also show more relapse, and due to side effects associated with its anticholinergic properties, also tend to drop out of treatment more frequently than patients treated with exposure therapy alone (Barlow et al., 2000). More recently, selective serotonin reuptake inhibitors (SSRIs) have been used in treatment of anxiety disorders, also with mixed results (Ferguson, LaVia, & Crossan, 1999; Ballenger, 2004). Therefore,

development of novel adjunct therapeutics is warranted and might prove beneficial in combination with exposure therapy. For example, use of virtual reality exposure combined with d-cycloserine, a partial N-methyl-d-aspartate (NMDA) receptor agonist, has been shown to result in better outcome for acrophobics than exposure therapy alone (Ressler et al., 2004).

Finally, it is of interest to note that since anxiety disorders represent a broad category of various conditions, it is important to choose the therapeutic approach wisely. Often, just the right timing and combination of interventions tailored to each patient's specific disorder and individual history can make a difference in success rate. Also, care should be taken when medications are combined with behavioral therapy, since in certain instances use of medications can impede the benefits of behavioral therapeutic approach (Barlow et al., 2000). Lastly, there are many variants of exposure therapy, and as recent research shows (Powers, Smits, & Telch, 2004) even seemingly minor modifications in the exposure protocol can considerably influence the final outcome.

Neural basis of fear extinction

Because conditioned fear extinction presumably models both inhibitory learning and behavioral exposure therapy, it has been extensively studied in animals and humans. However, the neural basis of extinction is not well understood, especially in humans, where there are only a few studies using non-invasive imaging techniques of system-level neural processing that takes place during fear extinction (Gottfried & Dolan, 2004; Phelps, Delgado, Nearing, & Ledoux, 2004; Knight, Smith, Cheng, Stein, & Helmstetter, 2004; Straube,

Glauer, Dilger, Mentzel, & Miltner, 2006). This handful of human studies is mostly in agreement with animal literature, which implicates the medial prefrontal cortex-amygdala interaction in the extinction of conditioned fear (Quirk, Russo, Barron, & Lebron, 2000; Herry & Mons, 2004; Milad, Vidal-Gonzalez, & Quirk, 2004; Pare, Quirk, & Ledoux, 2004; Garcia, Chang, & Maren, 2006; Morgan, Schulkin, & Ledoux, 2003) One of the first studies that examined the role of the medial prefrontal cortex (mPFC) in fear extinction was conducted by Morgan et al. (1993), who reported that rats with mPFC lesions had an increased resistance to extinction. Since damage to the prefrontal cortex had long been known to produce emotional disturbances and an increase in certain perseverative responses (Nauta, 1971; Goldman-Rakic, 1987; Sotres-Bayon, Bush, & Ledoux, 2004), Morgan et al. speculated that the resistance to extinction following mPFC lesions corresponded to perseverative tendencies in the emotional realm. Furthermore, the authors proposed that mPFC-amygdala connections normally allowed an animal to adjust its emotional behavior when environmental circumstances changed, and that a loss of prefrontal influence on the amygdala might bring about the reduced capacity of people with anxiety disorders to regulate their emotions. Subsequent research has examined the role of mPFC in extinction in more detail. For example, when brain lesions were limited to only dorsal or ventral mPFC (vmPFC), it was found that vmPFC lesions selectively impaired extinction over days without increasing the expression of fear within trials (Morgan & Ledoux, 1995). Quirk et al. (2000) followed this work with further lesions of yet a smaller subdivision of vmPFC, and found that lesions of the infralimbic cortex (IL) in rats also interfered with between-days extinction. In other words, IL is not necessary for extinction learning, but it does play a role in either

consolidation or retrieval of fear extinction. Furthermore, IL stimulation has been found to simulate extinction learning through experiments that paired the tone CS with brief IL stimulation, which resulted in reduced freezing in rats (Milad et al., 2004; Milad & Quirk, 2002). Data from our laboratory also support the hypothesis that mPFC plays a role in the retrieval of fear extinction memory. Barrett et al. (2003) demonstrated that mice with higher prefrontal metabolic activity more successfully inhibited the CR when presented with an extinguished auditory CS.

When evaluating animal studies that employ rodent subjects, it is important to consider the debate about the development of the prefrontal cortex in non-primate species. Nevertheless, it does seem that certain regions of mPFC, such as the orbital frontal cortex, anterior cingulate, IL and prelimbic cortex are present in rodents and other non-primate animals (Uylings, Groenewegen, & Kolb, 2003). However, certain regions might not have direct homology between species, as illustrated by differences in their connectivity with other cortical and subcortical regions. For instance, the dorsal part of the anterior cingulate in rats seems to be an intermediate between macaque's precentral medial area and prelimbic cortex, thus sharing some properties with the macaque premotor cortex, while prelimbic and infralimbic area in the rat may have characteristics in common with the cingulate and orbital areas of macaques (Conde, Maire-Lepoivre, Audinat, & Crepel, 1995).

It has been proposed that basolateral amygdala (or perhaps just the lateral amygdaloid nucleus) plays a role in the acquisition and extinction of conditioned fear (Fanselow & Ledoux, 1999; Akirav, Raizel, & Maroun, 2006; Berlau & McGaugh, 2006), in part because this region receives converging sensory input from auditory, somatosensory and visual areas (Ghashghaei &

Barbas, 2002). Basolateral nucleus of the amygdala (BLA) projects to the intercalated amygdaloid cells (Pare et al., 2004), which synapse onto the central amygdaloid nucleus, an area that organizes the conditioned fear response via its influence on ventromedial and lateral hypothalamus (VMH, LH) and ventrolateral periaqueductal grey (VLPAG), among others. This fear response could be inhibited by the top-to-bottom control of the amygdala firing by the medial prefrontal cortex, more specifically the infralimbic cortex (Quirk et al., 2000). According to Pare et al. (2004), IL sends projections to the GABA-ergic intercalated cells of the amygdala, thus inhibiting the activation of the medial central nucleus by the BLA when the CS is encountered in the extinction context. This model of CR inhibition in fear extinction might be too simplistic, since it does not account for how the contextual influence modifies extinction responding.

One possible way for contextual cues to exert their influence during extinction memory retrieval is through the interaction of the hippocampal formation with the basal nucleus of the amygdala, as proposed by Sotres-Bayon et al. (2004). However, since bilateral lesions of the basal amygdaloid nucleus have no effect on fear extinction (Anglada-Figueroa & Quirk, 2005), another possibility is that hippocampal formation directly influences the vmPFC, thus modifying vmPFC control of the amygdala.

Anatomical, as well as electrophysiological studies of these regions show that there are strong functional connections between the hippocampus and mPFC (Jay & Witter, 1991; Tierney, Degenetais, Thierry, Glowinski, & Gioanni, 2004; Ishikawa & Nakamura, 2003; Conde et al., 1995). These anatomical and electrophysiological findings, together with the lesion studies and behavioral findings (Corcoran, Desmond, Frey, & Maren, 2005; Quirk et al., 2000; Sotres-

Bayon, Cain, & Ledoux, 2006), suggest that hippocampal inputs to mPFC cells may subserve contextual constraints on the retrieval of cued fear extinction. In addition, other brain areas, such as auditory regions TE1 and MGD (Ledoux, Sakaguchi, & Reis, 1984; Teich et al., 1989) have also been shown to play a role in fear extinction. While the current opinion favors the role of certain brain areas, such as MGD, as simple relay stations to, for example, amygdala, it is not unlikely that these regions also contribute to the processing and storage of the associative properties of the conditioned stimuli. A consistent finding from our laboratory using metabolic mapping techniques has been that the auditory system activity results not only from the tone CS processing, but also from the associative effects of excitatory conditioning (Gonzalez-Lima & Scheich, 1984a; Gonzalez-Lima, Finkenstadt, & Ewert, 1989; Gonzalez-Lima, 1992; Jones & Gonzalez-Lima, 2001a; Jones & Gonzalez-Lima, 2001b; Barrett, Shumake, Jones, & Gonzalez-Lima, 2003). When one considers the complexity of the conditioned response in a fear conditioning paradigm, it is easy to imagine that even if mPFC alone can inhibit the behavioral freezing response to an extinguished CS, other brain regions are surely required to accomplish extinction of other aspects of learned CR, such as increased arousal, changes in blood pressure, heart rate etc.

Animal and human studies point to involvement of additional brain regions, such as bed nucleus of stria terminalis, cuneate nucleus and nucleus accumbens in extinction (Langa, Davish, & Ohmanc, 2000; Seillier, Seillier, Majchrzak, Marchand, & Di Scala, 2005; Phelps et al., 2004). Although some of these findings might stem from baseline anxiety state rather than CS-specific activation, it is likely that as more extinction studies are compiled using both

animal and human research, the current model of neural circuitry of fear extinction will be expanded to include more than just a couple of critical brain regions.

One of the aims of this study was to examine whether recall of fear extinction alters metabolic activity in some of the aforementioned areas, such as mPFC. Another aim was to examine which brain regions play a role in consolidation and storage of extinction memory, which was made possible through use of cytochrome oxidase (CO) histochemistry. While most other metabolic mapping techniques, such as functional magnetic resonance imaging (fMRI), positron emition tomography (PET) and fluorodeoxyglucose (FDG) autoradiography allow quantification of stimulus-evoked brain activity, CO histochemistry measures changes resulting from long-term alterations in metabolic demand. Therefore, this technique can be used to assess training-related brain activity changes, as opposed to stimulus-evoked short-term brain activation. Cumulative changes in CO activity might correspond to regional activation observed during extinction memory recall, since it is likely that areas which are involved in extinction memory storage might also play a role in the recall of that memory. Therefore, the hypothesis that mPFC might show metabolic changes related to both extinction storage and recall will be tested.

Based on review of literature, mPFC is a good candidate for a brain region that might participate in both extinction memory storage and extinction memory recall. As noted already, there are areas of mPFC, such as IL, that play a role in retrieval or consolidation of fear extinction (Quirk et al., 2000). In addition, Bontempi et al. (1999) have shown that time-dependent reorganization of the neural circuitry shifts memory-storage toward the neocortical areas. In particular,

frontal, temporal and anterior cingulate cortex show region-specific changes in metabolic activity as a function of the timing of the memory retrieval. The altered metabolic activity in the medial prefrontal cortex observed in the present study may reflect the participation of this brain area in organized access to and use of previously stored information. Without use of a technique such as CO histochemistry, it is difficult to differentiate neural activation stemming from memory retrieval from activation associated with memory storage, because one invariably needs to conduct a memory-specific retrieval test to assess memory consolidation. A dual function of mPFC is proposed in extinction memory in this study: one is mPFC activation that is related to the immediate task completion (memory recall), and the other is a process that forms a memory for that task (consolidation).

It is also possible that there are certain brain regions that might show extinction-related changes during memory recall which are not necessarily involved in the long-term storage of extinction memory. One possible candidate for this type of effect is the amygdala. Although activation of the amygdala has been found during both fear conditioning recall and fear extinction recall (Maren, 2001; Maren & Quirk, 2004; Marsicano et al., 2002; Phelps & Ledoux, 2005), this activation is not long-lasting (Schafe, Doyere, & Ledoux, 2005), suggesting that amygdaloid neurons play a role in the initial processing, but not long-term storage of extinction memory. In the Breiter et al. (1996) fMRI study, rapid habituation of the amygdala response to fearful stimuli was observed, which may provide an important clue to the time-limited function of the amygdala in the stream of affective information processing.

Other regional effects are likely to be associated with the extinction training, and might not necessarily be assessable with the FDG mapping technique. For instance, it is probable that some aspects of the affective dimension of fear extinction training might be encoded by septal region. It has been proposed that when a CS is paired with a US, separate and independent associations are formed with the sensory and affective components of the US (Wagner & Brandon, 1989). Extinction training could exert different effects on the CS association with the sensory and affective properties of the US. If there was such a dissociation in conditioned fear extinction in rats, then one might speculate that brain regions that show long-term training and memory related metabolic capacity changes might be the regions associated with affective aspects of learning, such as septum (Sheehan, Chambers, & Russell, 2004). Lateral septum, for example, has been implicated in behavioral freezing response to an aversive stimulus. Mice showing fear-induced freezing express high levels of c-Fos specifically in the ventral lateral septum (Mongeau, Miller, Chiang, & Anderson, 2003), and both the elicitation and the inhibition of fear responses are associated with learning-related changes in LS neurons (Jones et al., 2001a; Sheehan et al., 2004). Extinction of conditioned fear could lead to long-lasting changes in metabolic capacity in the lateral septum, or another area related to representation of affective properties of an extinguished CS. The aforementioned predictions, as well as the general hypothesis that both recall and consolidation of fear extinction involve multiple brain regions which form a functional neural network necessary for successful fear extinction, will be tested.

Renewal of conditioned fear

One of the by-products of extinction learning that will be described in more detail is renewal of CR. Renewal of CR has been demonstrated in both animals and humans (Bouton & King, 1983; Rodriguez, Craske, Mineka, & Hladek, 1999; Mineka, Mystkowski, Hladek, & Rodriguez, 1999), and it is thought to resemble some aspects of return of fear following exposure therapy. Renewal takes place when an extinguished stimulus is encountered outside the extinction context. Commonly, renewal in animals has been studied in a so-called ABA paradigm, in which subjects receive fear acquisition training in one context (context A), extinction training in another context (context B) and are then returned to the acquisition context A and tested. Another possibility is to conduct acquisition training in context A, extinction in context B and to test the animals in a third, neutral context C (the ABC renewal paradigm).

Context refers to any environmental (physical), temporal or internal (interoceptive) cues that accompany extinction learning. Since an extinguished CS has more than one possible meaning, these contextual cues come to play an important role in determining which behavioral response is selected during extinction memory recall. Bouton (1983) has developed a memory-based approach to many conditioning phenomena including renewal, in which conditioning and extinction experiences are proposed to be represented as distinct “CS–US” and “CS–noUS” memories. Therefore, subsequent responding to that CS is determined by which one of these memories is activated, and contextual cues act as an occasion setter that can activate the memories. An

important distinction between the two types of memories is that activation of the "CS–US" memory is independent of the context in which conditioning occurred, whereas retrieval of the "CS–noUS" memory is coupled to the extinction context. This idea explains CR renewal as due to the extinguished CS being tested outside the context that retrieves the "CS–noUS" memory.

Another theory that has been developed to account for contextually controlled inhibition of CR locates the inhibition between the context and the US. According to this view, the extinction-associated context increases the threshold at which the US representation can be activated (Rescorla, 1979). Once the animal is tested outside of the extinction context, this threshold is lowered and the US representation can be easily constructed. Traditionally, loss of extinction performance due to a context shift has been viewed as a memory retrieval failure stemming from unmatched extinction and test contexts (Morgan & Riccio, 1994). However, other conceptualizations of context-dependent renewal ascribe inhibitory influence of context on CS-US association, and this inhibitory input is likely to be lost with a context switch (Bouton & Nelson, 1998).

If contextual inhibitory input is lost during renewal, then a tone CS should activate the excitatory US footshock representation in somatosensory systems. On the other hand, if loss of extinction performance results from a memory retrieval failure, then the CS might not activate US representation in these systems, but rather an association between the neural effects of CS and the CR might become activated. One possible method for differentiating between the proposed mechanisms which might account for the renewal effect is assessing the system-level changes in the neural substrates that accompany acquisition, extinction and renewal of fear conditioned response. By considering, for

example, whether CS and US neural representations are activated during CR renewal, one can investigate the proposed activation of the US representation in the absence of the US.

Neural substrates of renewal

To date, there are no published human imaging studies of fear renewal, and only a handful of studies in rodents that implicate dorsal hippocampus (DHC) as a brain region associated with renewal effects. Specifically, Corcoran et al. (2005) have found that inactivation of DHC impairs both ABA and ABC fear renewal in rats. However, other findings are in disagreement with these results (Frohardt, Guarraci, & Bouton, 2000; Wilson, Brooks, & Bouton, 1995). Other than hippocampal formation, no other lesion or inactivation studies have been conducted to investigate involvement of areas such as mPFC, amygdala or sensory areas in conditioned fear renewal. Since Bouton proposed that contextual cues gate the recall of extinction memory (Bouton et al., 1983; Bouton et al., 1998), it was hypothesized that the hippocampal formation might show altered metabolic activity associated with fear renewal. It was also speculated that during fear renewal presentation of the extinguished CS might activate the neural representation of the US, and that this “CS-US” stimulus substitution (Pavlov, 1927) might be gated by the hippocampal formation. This hypothesis was tested in specific aim 1 one of this study using FDG metabolic mapping technique, which provides an index of stimulus-evoked neural activity.

Fluorodeoxyglucose (FDG)

Similarly to CO histochemistry, FDG imaging technique is based on a principle that electrical activity of brain cells is coupled to oxidative cellular metabolism (Sokoloff, 1992). As neurons metabolize their primary fuel source glucose to produce ATP, cellular regions with highest energy expenditure, such as regions of the neuronal membrane with the highest sodium load, utilize a high percentage of the available glucose. FDG is a glucose analogue that is taken up by the metabolically active cells, but unlike glucose, it is not broken down by the cellular enzymatic processes, but rather accumulates within the cell as FDG-6-phosphate (Gonzalez-Lima, 1992). As FDG is injected in tracer amount, its presence, or the presence of its metabolites, does not interfere with normal functioning of the cell.

FDG is thought to be taken up by all active regions of the membrane, and therefore FDG could accumulate inside both neuropil and cell bodies (Gonzalez-Lima, 1992). Since FDG metabolically potentially maps electrical activity of the entire cell membrane, its use is well-suited for obtaining a functional map of the regions involved in a processing of a distinct stimulus. Because glucose is ubiquitously used throughout the entire brain, FDG has an advantage over other functional metabolic mapping techniques, such as immediate early gene expression mapping techniques, since immediate early genes are not equally distributed and expressed throughout the entire brain (Herdegen & Leah, 1998). However, FDG mapping technique does not provide cellular resolution like the CO technique, but since the first aim of this study was to map stimulus-evoked

brain activity changes at the regional and system levels, FDG mapping was chosen as the most appropriate method for that specific aim.

Cytochrome oxidase

Cytochrome oxidase (CO) is a mitochondrial enzyme that catalyzes the terminal step in cellular oxidation. Cellular oxidation is of critical importance for brain cells, since glucose is the primary fuel in the brain. Glucose is oxidized to carbon dioxide and water in a process called oxidative phosphorylation, in which CO catalyzes the transfer of electrons from its substrate cytochrome c to molecular oxygen to form water, thus generating proton gradient necessary for adenosine-tri-phosphate (ATP) production (Hevner, Duff, & Wong-Riley, 1992). In neurons, energy demand associated with generation of electrical neurotransmission and consequent resetting of the trans-membrane electrochemical potential by the sodium-potasium pump is estimated to consume about 50% of the energy budget of the rodent brain (Attwell & Laughlin, 2001). Overall, as much as 74% of the energy expenditure in the brain is from metabolic demands of integrative and signaling activity of neurons (Attwell et al., 2001).

As neurons metabolize glucose to produce ATP, higher energy demand is met by an increase in CO activity (Wong-Riley, 1989). This coupling between neuronal activity and energy metabolism allows the utilization of cytochrome oxidase activity as a reliable indicator of neuron's oxidative capacity (Wong-Riley, 1989). An increase in CO activity is mainly controlled transcriptionally by the regulation of the protein amount. Because of this, changes in CO activity may take a relatively long time (days) to manifest. However, it is possible that some of the changes in CO activity might become apparent as early as within few hours of altered metabolic demand (Bennet, Roelfsema, Pathipati, Quaedackers, &

Gunn, 2006). Regardless, it is well-established that under physiological conditions CO activity is regulated by its relative amount, as opposed to its molecular activity (turnover number) (Wong-Riley et al., 1997). Experimental manipulations that change neuronal activity over a period of days result in corresponding changes in CO activity (Wong-Riley et al., 1997; Gonzalez-Lima, 1992; Tremere & Pinaud, 2005). CO histochemistry provides a useful way to map the neural circuits in which learning-related changes take place.

Thus, use of CO histochemistry to visualize a long-lasting change in brain metabolic capacity can be very advantageous in studies aimed at quantifying memory retention-related changes in brain metabolism. In particular, quantitative CO histochemistry, a technique developed by Dr. Gonzalez-Lima (1998), is a sensitive method with cellular resolution that allows assessment of long-term alterations in enzymatic activity which develop over the entire period of an experiment. When combined with other metabolic mapping techniques, such as FDG, quantitative CO histochemistry can help us better understand how learning and memory functions may be organized in an intact brain.

Methylene blue

Methylene blue (MB) is a dye that has a metal ion center which can accept and donate electrons. Hence, MB is a redox agent that can exist in one of two forms: an oxidized state, in which it is blue in color, and a reduced, colorless state, also known as leucomethylene blue. As early as 1886 Erlich discovered that when MB is injected into live rats, it had an affinity for neurons. Since then, MB has been used as a supravital stain because of its propensity to enter living, metabolically active cells. Some other uses of MB include methemoglobinemia treatment, treatment of urinary tract infections and treatment of ifosfamide-induced encelopathy (Raj, Bertolone, & Jaffe, 2004; Nickel JC, 2005; Gosselin, Hodge, & Smith, 1976)

Upon MB administration, MB crosses the blood-brain barrier (Peter, Hongwan, Küpfer, & Lauterburg, 2000) and enters the mitochondria (Visarius, Stucki, & Lauterburg, 1997). When it crosses the charged lipid membranes, MB is in its reduced state. Once inside the mitochondria, it is likely that there is a balance between the oxidized and the reduced MB, which in turn can either accept or donate electrons to the mitochondrial respiratory chain complexes found in the inner mitochondrial membrane. Enhanced electron flow through the mitochondrial enzymatic chain complexes can lead to increased CO activity and enhanced ATP production. Hence, by acting as an electron shuttle in the inner mitochondrial membrane, MB can aid neurons in meeting their metabolic energy demands.

However, only low doses (1-10 mg/kg) of MB have been proven effective for enhancing memory (Martinez, Jensen, Vasquez, McGuiness, & McGaugh, 1978; Riha, Bruchey, Echevarria, & Gonzalez-Lima, 2005). High doses (50-100 mg/kg) can actually have a detrimental effect on learning and memory (Martinez et al., 1978), and lead to the formation of methemoglobin and even death (Riha et al., 2005; Burrows, 1984). Although MB is considered to be a safe drug by the FDA, the proper dosing is of critical importance.

Because administration of a low-dose MB has been found to improve memory retention in a one-step inhibitory avoidance task (Martinez et al., 1978), as well as spatial memory in both untreated rats and rats treated with the CO inhibitor sodium azide (Callaway, Riha, Wrubel, McCollum, & Gonzalez-Lima, 2002; Callaway, Riha, Bruchey, Munshi, & Gonzalez-Lima, 2004), it was hypothesized that the administering of low-dose MB following conditioned fear extinction might improve retention of extinction memory. Furthermore, because MB administration in vitro and in vivo enhances brain oxygen consumption (Riha et al. 2005) and improves rat brain CO activity (Callaway et al., 2004), it was speculated that MB administration following fear extinction might enhance brain CO activity in regions with increased metabolic demand, thereby improving extinction memory retention.

Methylene blue as an adjunct to exposure therapy

Exposure therapy for anxiety disorders is a rather effective behavioral therapeutic method, however, the level of compliance can present a problem. It is not uncommon for patients to start the exposure therapy, but then drop out

before completion (Hembree et al., 2006). The possibility of return of anxiety following the therapy is problematic as well, so finding a way to decrease the amount of time needed to complete exposure therapy regimen, as well as increase the retention of that learning would be beneficial. Attempts at complementing exposure therapy with adjunct therapeutic agents have been made for many years now. Recently, Davis et al. (2002) and Ressler et al. (2004) hypothesized that since fear extinction involved new learning, and NMDA receptors are known to play a role in learning (Cooke & Bliss, 2005), improved function of NMDA receptors during extinction learning might result in better fear extinction. This was proven to be the case in both animals undergoing extinction of a fear-potentiated startle (Davis et al., 2002) and humans undergoing virtual reality exposure to a feared CS (Ressler et al., 2004).

The approach taken by our laboratory was that since learning and memory consolidation required expenditure of energy (ATP), a metabolic enhancer, such as MB, might prove beneficial as an adjunct to exposure therapy. In specific aim 3 of this study, low-dose MB was first administered following fear extinction training, in order to test its effects on extinction memory consolidation and storage. Peter et al. (2000) have found that MB accumulates in the brain following intraduodenal administration, and that although its half-life in whole blood is approximately 5.6 hours, the concentration of MB in the brain one hour following the MB injection is ten times of that found in blood. In the sodium azide study (Callaway et al., 2002), 1 mg/kg MB dose had an effect only after repeated injections, and in other pilot studies conducted in our laboratory using this dose, repeated MB administration was shown to be more effective than a single MB injection. This may be because it takes a certain concentration of MB in brain

mitochondria for its energy production enhancing-effects to take place. By slowly accumulating in brain tissue over a period of days, MB might reach a desirable concentration in brain cells without having any adverse peripheral effects (such as methemoglobinemia) that could result from a single, larger dose of this compound. The most effective concentration of MB required to achieve the most benefit without any non-specific side effects was investigated by Riha et al. (2005), and it was found to be below 10 mg/kg in rats. However, Riha et al. used only a single dose of MB prior to testing, and other unpublished work completed in our laboratory suggested that repeated low-dose of MB may be more effective than a single higher dose for its optimal memory-enhancing effects.

In the present study, a low-dose (4 mg/kg) MB was administered either during, or following the extinction training protocol, over a period of 4-5 days. Since previous evidence suggested that repeated MB injections may be necessary for the MB-memory enhancing effects, the extinction training during which MB was administered along with extinction sessions lasted four days. This allowed administration of 4 repeated MB injections during fear extinction learning and memory consolidation. When MB was administered over five days following the extinction training protocol, it was hypothesized that it would improve retention of extinction memory. In both cases (MB administered for 5 days following 2 days of extinction or MB administered during the 4 days of extinction training), MB was found to enhance retention of extinction memory, suggesting that MB is a possible candidate for adjunct drug that could improve exposure therapy.

Chapter 2: Brain activity associated with context-dependent renewal of conditioned fear

This is the first mapping study of the brain activity associated with the renewal of an extinguished freezing conditioned response (CR). Rats were given radiolabeled fluorodeoxyglucose (FDG) to map brain effects of an extinguished tone during context-dependent renewal of the CR. A tone conditioned stimulus (CS) was paired with a footshock unconditioned stimulus (US) in a first context, followed by CS extinction in a second context and CR renewal in a third context. Control rats were treated identically, except that footshocks were presented pseudorandomly. Extinction effects included decreased FDG uptake in anterior cingulate, and increased FDG uptake in ventral medial hypothalamic and ventral basal thalamic nuclei, as well as increased uptake in central and basolateral amygdaloid nuclei. Conditioned rats with CR renewal had increased tone-evoked activity in auditory system (auditory cortex, medial geniculate, inferior colliculus, lateral lemniscus nuclei), as well as increased activity in somatosensory nuclei (external cuneate, solitary tract, spinal trigeminal and vestibular nuclei). In addition, perirhinal cortex, anterior lateral hypothalamus and ventrolateral periaqueductal grey also showed renewal effects. Renewal effects in the auditory regions suggest that the CS retained its excitatory associative effects after extinction, since these effects were not present in the pseudorandom group. Brain-behavior correlation analysis indicated that the metabolic activity of the external cuneate nucleus (US somatosensory relay) and ventromedial hypothalamus strongly predicted the CR in the renewal group. Context-

dependent fear renewal was associated with the neural activation in the perirhinal cortex, reactivation of the excitatory CS representation in the auditory system and the neural reactivation of the US representation in somatosensory pathways in the absence of the US. These findings support Pavlov's stimulus-substitution theory as a neural mechanism contributing to the renewal effect.

Introduction

Fear conditioning is considered one of the pathogenic mechanisms in anxiety disorders (Craske, 1999). Behavioral exposure therapy methods aimed at decreasing anxiety and fear are largely based on principles of extinction, which has recently been characterized not only from a behavioral but from a neural perspective as well (Barrett et al., 2003; Myers & Davis, 2002; Quirk et al., 2000). Since extinction does not erase the original fear association (Barrett et al., 2003; Bouton et al., 1998), there is a risk of return of fear in a person suffering from an anxiety disorder, although completing successful extinction-based therapy (Rodriguez et al., 1999; Mystkowski, Craske, & Echiverri, 2002). Rodriguez et al. (1999) and Mineka et al. (1999) found context-specific return of fear in people with stimulus-specific phobia, and Milad et al. (2005) demonstrated context dependency of extinction recall in humans. Therefore, from a therapeutic standpoint, it would be useful to have a better understanding of the neural substrates that underlie renewal of conditioned fear responses.

Pavlovian fear conditioning in rodents can be readily accomplished through repeated pairing of a neutral CS, such as a tone, with an US, such as a footshock. With CS-US pairings, a conditioned response (CR) to the CS develops. During the aversive tone-footshock conditioning, one of the CRs displayed by rodents is freezing, defined by a lack of movement. Conversely, when a well-trained CS is repeatedly presented in the absence of the US with which it was originally paired (extinction), the CR is no longer displayed. CR renewal refers to the recovery of the CR to an extinguished CS produced by a

shift away from the contextual cues that were present during extinction (Bouton et al., 1983; Bouton & Moody, 2004).

In the present study, the neural substrates of renewal were mapped in intact animals by assessment of brain metabolic activity as revealed by the regional uptake of a radiolabeled glucose analog, fluorodeoxyglucose (FDG). Tone-evoked brain differences in control and conditioned groups of rats exposed to the same CS and US were used to identify which brain regions and systems played a part in CR renewal. The obtained data were further evaluated in light of previous FDG studies of Pavlovian conditioning (Gonzalez-Lima & Scheich, 1984b), extinction (Barrett et al., 2003), blocking (Jones et al., 2001b) and differential inhibition (Jones et al., 2001a).

It was hypothesized that just like extinction is not forgetting or unlearning of the original CS-US association (Barrett et al., 2003), renewal is not forgetting of the extinction training, as suggested by the savings in the rate of re-extinction (Quirk, 2002). Therefore, it was anticipated that the increased metabolic activity in the prefrontal cortex associated with the behavioral inhibition of an extinguished CR (Barrett et al., 2003; Quirk et al., 2000) would be absent in the renewal group, and that other regions, especially those implicated in CS and US processing, would show elevated activity as compared to the control group. Moreover, we tested the prediction that contextual cues influenced the activation of the CS and the US neural representation, with the final behavioral output governed through a stimulus substitution mechanism.

Materials and Methods

Subjects

Subjects were 36 one-month old male Long-Evans rats purchased from Harlan, Inc. (Indianapolis, IN). Rats were divided into three experimental groups (extinction, pseudorandom and renewal groups; n = 12 per group). All subjects were housed under standard laboratory conditions (12 hour light/dark cycle), 3 per cage, with food and water available ad libitum. Upon their arrival to the colony, rats were handled daily for a week to habituate them to the experimenters. At the time of FDG administration subjects weighed 172 g on average (range 157-187 g). The protocol used was approved by the University of Texas Institutional Animal Care and Use Committee and complied with all applicable federal and NIH guidelines.

Apparatus

The acquisition phase of the experiment took place in MED Associates (St. Albans, VT) sound-attenuated operant chambers (context A) illuminated by a bright red light. The chambers had a speaker mounted at the top, through which Wavetek Sweep/Modulation generators (Wavetek, San Diego, CA) produced 65 dB frequency-modulated tones of 1-2 kHz, and 15 seconds in duration. The chambers were also equipped with a grid floor through which an electric shock could be delivered as programmed into a MED-PC protocol supplied by MED Associates. Before each session the chambers were cleaned with diluted detergent which also served as an olfactory cue for this context.

The extinction phase of the experiment took place either in context B or context C. Context B was a metal cage with a distinct metal floor, a diluted iodine odor cue and dim red light illumination. Context C consisted of a clear plastic cage with a speaker mounted on top, dim white light and diluted Bioclean (Nitritex Ltd, Ontario, Canada) odor cue.

Behavioral training

Initial pilot studies determined the parameters best suited to produce a strong renewal effect in the ABC paradigm (Bouton and Moody, 2004). The present experiment was optimized to produce a robust FDG renewal effect, whereas previous FDG studies were optimized to produce strong extinction effects (Barrett et al., 2003).

Table 1. Experimental design

		Group		
Day	**Procedure**	Renewal (different extinction and testing contexts)	Extinction (same extinction and testing contexts)	Pseudorandom (same contexts without acquisition or extinction)
1-2	Habituation 1 hr/day	exposure to context A	exposure to context A	exposure to context A
3-4	Acquisition 15 min/day	CS -> US x 4/day context A	CS -> US x 4/day context A	CS,US x 4/day context A
5-	Probe 10 min	3 CS presentations in context C -> CER	3 CS presentations in context C -> CER	3 CS presentations in context C -> no CER
5-6	Extinction 1 hr/day	CS in context B 18/day	CS in context C 18/day	CS in context C 18/day
7-	Probe 10 min	3 CS presentations in context C -> CER	3 CS presentations in context C -> no CER	3 CS presentations in context C -> no CER
7-	FDG 1 hr	CS in context C	CS in context C	CS in context C

Acquisition phase

Animals were habituated to the conditioning chambers for two days, one hour each day. After habituation, the extinction and the renewal groups received two days of four tone-shock pairings. US consisted of a footshock of 0.5 mA, 0.75 sec in duration and it co-terminated with a tone CS. Each CS was divided into five three second bins and subjects' behavior was scored for each of the five bins. Pre-CS behavior was recorded for the 15 sec preceding each CS presentation. The CR that was measured was freezing behavior, defined as lack of any movement except for rapid, shallow breathing, with all 4 feet on the floor. In addition to measuring freezing behavior, experimenters also documented number of fecal boli and urinations during each session as an additional measure of autonomic system arousal, which is correlated but not synonymous with fear. Subjects in the pseudorandom group were presented with the same number of tones and shocks, but these stimuli were paired only once out of eight total stimulus presentations. Pseudorandomly trained subjects were used to control for any possible non-associative effects of stimulus presentation. The average inter-trial interval (ITI) was three minutes. Presentation of stimuli was controlled by computer programs created using MED-PC behavioral programming language (MED Associates). Twenty four hours following the acquisition training, one ten-minute probe trial consisting of three CS presentations was conducted out of the acquisition context to test CR independently of any possible contextual influence. Freezing behavior was measured in three-second bins, over the entire period of CS presentation (15 seconds), as well as 15 seconds prior to the onset of the CS (pre-CS freezing). The pre-CS freezing measure was used to account for any

non-specific freezing due to generalization from context A to C or sensitization effects of the US. Based on the probe trial scores, animals were matched into the predetermined experimental groups.

Extinction phase

Extinction training was conducted in context C (extinction and pseudorandom groups) or context B (renewal group) over a period of two days, and it consisted of one hour-long sessions of 18 CS presentations with an average 3 minute ITI. This particular protocol was based on pilot studies, and it was chosen as an optimal training procedure for reliable ABC renewal effect. Contexts B and C were counterbalanced between different cohorts of animals.

FDG test

Twenty four hours following the extinction training, another ten-minute probe trial was conducted as described earlier, followed by FDG administration two hours later. All subjects received an intraperitoneal injection of 18 μCi/100 gm body weight of 14C(U)-FDG (specific activity, 300-360 mCi/mmol; American Radiolabeled Chemicals) in 0.1 ml of sterile saline. The renewal group subjects were tested out of their extinction context, and the final FDG session context was kept identical for all groups to ensure similar conditions during the critical FDG uptake period. During this period, subjects were exposed to an hour-long 5 seconds on, 1 second off CS tone. This repeated frequency of tone presentations has been determined from previous FDG studies to produce optimal CS-evoked buildup of FDG for mapping the regional effects of the tone in the brain (Jones et

al., 2001b). Because most of the FDG uptake takes place during the first ten minutes following the intraperitoneal administration of the compound (Gonzalez-Lima, 1992), the cumulative amount of freezing to the CS was measured as the CR for the first ten minutes of the FDG session. Following the FDG test, rats were decapitated and their brains were quickly removed and frozen in -40°C isopentane, then stored in plastic bags in a -40°C freezer.

FDG autoradiography

The standard FDG autoradiographic procedure (Gonzalez-Lima, 1992) was performed on 40 μm thick frozen brain sections. Briefly, frozen sections were picked up on slides and immediately dried on a 60°C hot plate. Then, slides and standards of known 14C concentration were apposed to radiographic film for a period of 14 days. Films were developed, dried and stored in protective covers. After the films were developed, subjects were dropped out of the final analysis if they showed poor FDG uptake, which was most likely caused by injection errors. The final number of subjects was n = 9 for extinction and pseudorandom groups, and n=10 for the renewal group.

Image analysis

FDG uptake was quantified by film densitometry as described previously by Jones and Gonzalez-Lima (2001b). Briefly, developed films were placed on a light box and optical density of images was captured by the video camera. Digitized images were corrected for film and light box artifacts through subtraction of the background (film background and optical distortions from

camera and light source) by image analysis software JAVA (Jandel Scientific Corp.). 14C standards from Amersham were used to calculate a calibration curve for each film and convert optical density values to 14C incorporation per gram of brain tissue. The film developing parameters were optimized to obtain maximum linearity between isotope concentration and optical density. Each region of interest (ROI) was measured from three adjacent sections, and the mean optical density value was used for calculating the FDG uptake. Mean activity readings were expressed as nanocuries per gram of tissue. Additionally, for each slide containing the ROIs, the overall FDG uptake was determined for the entire set of sections (white and gray matter) by means of the object threshold function in the JAVA software. The average of all readings served as an index of a whole brain isotope incorporation.

Statistical analysis

Behavioral data were analyzed based on the post-acquisition, extinction and post-extinction freezing scores, as well as FDG session freezing scores. Repeated measures ANOVA with tests for simple effects was used for differences within (pre and post extinction) and between groups. ANOVA was used to analyze the freezing scores although these scores fall into a category of discrete variables (score can be 0, 1, etc. to 5), since they approximate a continuous freezing time (0 to 15 sec) variable.

FDG uptake data were analyzed for the presence of outliers, and one subject was excluded from the renewal group due to its consistently extreme values (more than three standard deviations away from the mean) in all of the ROIs examined. After it was determined that there were no between group

differences in the whole brain FDG incorporation, regional activity was normalized using whole brain uptake values for each subject to account for individual variability in FDG uptake. Since two batches of FDG had to be used in this study, analysis of covariance (ANCOVA, 95% confidence interval) with FDG batch as a covariate was done to compensate for any batch-related variations in labeling intensity across individuals. Because brain regional analysis included 92 regions of interest, multiple group comparisons were limited to avoid the large number of possible multiple comparisons among groups that would have led to inflated Type I errors.

In addition, correlations between regional 14C incorporation and the behavioral index of CR renewal, as well as interregional within-group correlations were examined to determine the pattern of relationships among the various brain regions and systems. More specifically, partial Pearson product-moment correlations were obtained for each region that showed a significant between-group difference (as determined by ANCOVA) in FDG metabolism. Only the correlations that remained significantly different ($p < 0.05$) from zero throughout all permutations of the jackknife procedure were included in order to avoid inflated Type I errors. A jackknife procedure sequentially drops each subject and calculates correlations repeatedly to avoid outliers that might inflate Type I errors caused by the large number of interregional correlations computed relative to the sample sizes. Interregional correlations were transformed to z-scores in order to compare between-group correlations using the formula $z = (z_{ij(group1)} - z_{ij(group2)}) / \sqrt{1/(ngroup1-3)+1/(ngroup2-3)})$. The functional index of ROI activity was also correlated with the behavioral measures of fear, with positive

correlations indicating a linear relationship between increased FDG uptake and fearfulness.

Results

Behavioral results

Following the acquisition training in context A, the renewal and extinction groups showed very high levels of freezing to the tone CS in the post-acquisition probe session in context C, while the pseudorandom group showed a small level of freezing that was similar to the pre-CS freezing counts. The pre-CS freezing measure was used to account for any non-specific freezing due to generalization from context A to C or sensitization effects of the US. The difference in the amount of freezing (Fig. 1a) between the animals that experienced paired CS-US presentations vs. the pseudorandom group was significant, with the pseudorandom rats freezing 85% less to the CS than either the extinction ($F_{(1,17)} = 12.3$; $p < 0.01$) or the renewal group rats ($F_{(1,17)} = 13.6$; $p < 0.01$).

Figure 1. Mean freezing counts

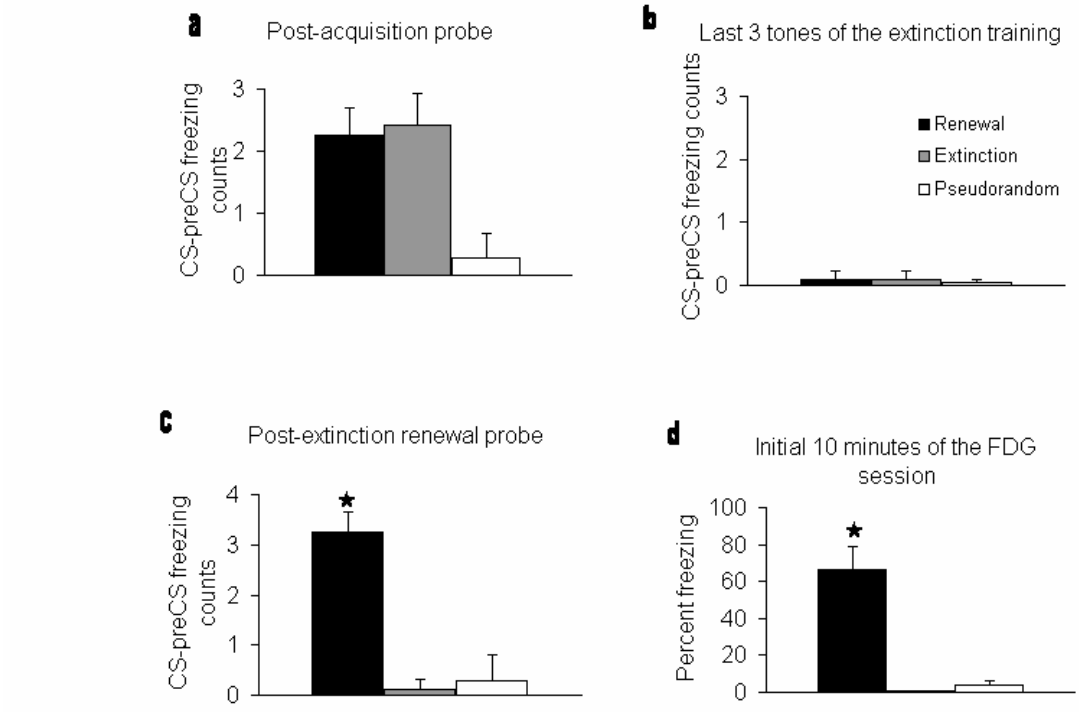


Figure 1. PreCS freezing was measured (a-c) during the 15 seconds preceding the CS presentations. a) Post-acquisition probe CS-preCS freezing scores (average of three CS presentations ± SE). The CR was scored out of the acquisition context during tone alone presentations. The pseudorandom group showed significantly less freezing to the CS than the extinction and the renewal group. b) Difference in freezing scores 15 seconds before and during the final three tone presentations of extinction training. All animals displayed almost complete suppression of freezing, indicating a successful completion of extinction training. c) Average probe trial freezing during the three tone presentations in either the extinction context or the novel context (renewal). d) Freezing behavior during the first ten minutes of the FDG session. Duration of the CR was scored in seconds and converted to total percentage of time spent freezing. * $p < 0.01$

Freezing scores recorded during the last three extinction trials with the CS indicate that all three groups exhibited very low freezing to the CS (Fig. 1b). ANOVA revealed no statistical differences between groups ($F_{(2,26)} = 0.25$; $p > 0.05$). While pseudorandom group subjects did not differ in their post-acquisition and post-extinction level of freezing ($p > 0.05$), there was a large significant difference ($F_{(1,17)} = 70.2$; $p < 0.01$) between the post-acquisition probe and the last three extinction scores for both extinction and renewal groups. Therefore, both groups that received paired tone-shock presentation successfully extinguished their CR over the two days of extinction training.

Twenty four hours following the extinction training, a post-extinction probe session was conducted to test for contextually-influenced renewal of CR (Fig. 1c). During this probe session, both pseudorandom and extinction group rats (extinguished and tested in the same context) showed very low CR to the three tones presented during the probe trial. In contrast, renewal group rats (tested out of the extinction context) froze significantly more than extinction group rats during the CS presentations ($F_{(1,18)} = 20.87$; $p < 0.01$).

Extinction and renewal were further assessed by comparing freezing during the three tones of the post-acquisition probe with freezing during the post-extinction renewal probe trial conducted 24 hours following the end of extinction training. Data were analyzed with a 3 × 2 (Group × Probe) repeated measures ANOVA, with the average of the CS-preCS scores from all three trials from the post-acquisition and post-extinction probes serving as repeated measures. When freezing displayed by the subjects was compared for all three groups, there was a significant difference between the post-acquisition probe ($F_{(2,26)} = 7.9$; $p < 0.05$)

and the renewal probe ($F_{(2,26)}$ = 22; $p < 0.05$). While pseudorandom group subjects had low freezing scores in both post-acquisition and post-extinction probe sessions, extinction group rats decreased their CR as a result of extinction training, so that during the post-extinction probe session they did not differ from the pseudorandom group ($p > 0.05$), although they were significantly different after the acquisition ($p < 0.05$). Conversely, the extinction group was significantly ($p < 0.05$) different from the renewal group only during the post-extinction renewal probe. Pairwise comparisons confirmed that renewal group subjects maintained their difference ($p < 0.05$) from the pseudorandomly trained rats during both post-acquisition and renewal probe trials. A highly significant two-way (Group × Probe) interaction was obtained for freezing ($F_{(1,17)}$ = 90; $p < 0.05$), which indicates that extinguished rats showed a substantial reduction in freezing after extinction, and renewal group rats showed as robust level of freezing in the novel context as they did immediately following the acquisition.

Freezing was also scored during the first 10 minutes after the FDG session because nearly all the FDG uptake occurs during this period (Jones et al., 2001b). FDG session freezing scores (Fig. 1d) indicate that, on average, rats in the renewal group froze more during the first 10 minutes of the session than the pseudorandom ($F_{(1,17)}$ = 22.34; $p < 0.05$), and the extinction group rats ($F_{(1,18)}$ = 28.93; $p < 0.05$). This suggests a strong and relatively long-lasting effect of context shift on behavioral performance in renewal group subjects. Behavioral observations confirmed that extinction learning is new learning that does not erase the original memory of acquisition, and that extinction memory recall depends on the testing context.

Brain activity results

Overall, out of 92 brain regions examined, 21 showed significantly different mean regional FDG uptake between the groups (Table 2) as determined by ANCOVA at 95% confidence interval.

Table 2. Regional significant FDG differences evoked by the tone during context-dependent renewal of conditioned fear.

Renewal effects by ROI	Renewal	Extinction	Pseudorandom
Auditory cortex (TE2)	668 ± 28	657 ± 26	607 ± 28
Medial geniculate nucleus, dorsal (MGD)	851 ± 18	774 ± 17	790 ± 18
Medial geniculate nucleus, medial (MGM)	815 ± 23	765 ± 21	774 ± 22
Inferior colliculus nucleus, external (ICE)	768 ± 33	674 ± 30	616 ± 32
Inferior colliculus nucleus, central (ICC)	1060 ± 46	932 ± 43	790 ± 45
Inferior colliculus nucleus, dorsal (ICD)	663 ± 31	619 ± 29	578 ± 31
Lateral lemniscus nucleus, dorsal (LLD)	661 ± 26	614 ± 24	586 ± 26
Lateral lemniscus nucleus intermediate, (LLI)	784 ± 36	740 ± 34	679 ± 36
Lateral lemniscus nucleus ventral, (LLV)	745 ± 26	730 ± 24	685 ± 26
External cuneate nucleus (ECU)	790 ± 37	627 ± 34	596 ± 36
Solitary tract nucleus (SOL)	635 ± 33	510 ± 31	508 ± 33
Spinal trigeminal nucleus (ST)	627 ± 26	548 ± 24	550 ± 25
Vestibular nucleus (VE)	972 ± 27	967 ± 25	915 ± 26
Perirhinal cortex (PER)	597 ± 17	582 ± 16	556 ± 16
Anterior lateral hypothalamus (ALH)	662 ± 42	545 ± 39	550 ± 41
Ventrolateral periaqueductal grey (VLPAG)	424 ± 21	397 ± 19	367 ± 20

Extinction effects by ROI	Renewal	Extinction	Pseudorandom
Anterior cingulate (CG2)	841 ± 24	749 ± 23	846 ± 24
Ventral medial hypothalamic nucleus (VMH)	441 ± 29	502 ± 27	419 ± 28
Ventral basal thalamic nucleus, lateral (VBL)	750 ± 40	899 ± 40	727 ± 40
Central medial thalamic nucleus (CM)	697 ± 44	779 ± 41	680 ± 43
Caudate putamen, caudal (CPC)	621 ± 21	631 ± 19	580 ± 20
Ventral cochlear posterior (VCP)	813 ± 35	886 ± 33	772 ± 34
Primary visual cortex (V1)	900 ± 40	808 ± 37	915 ± 39
Central amygdaloid nucleus (CeA)	449 ± 32	525 ± 30	429 ± 32
Basolateral amygdaloid nucleus (BLA)	655 ± 56	827 ± 52	691 ± 55

Regional FDG uptake ± standard errors is calculated as (ROI/whole brain mean global activity) x pseudorandom group whole brain mean activity and expressed as nanocuries per gram tissue wet weight.

Renewal effects

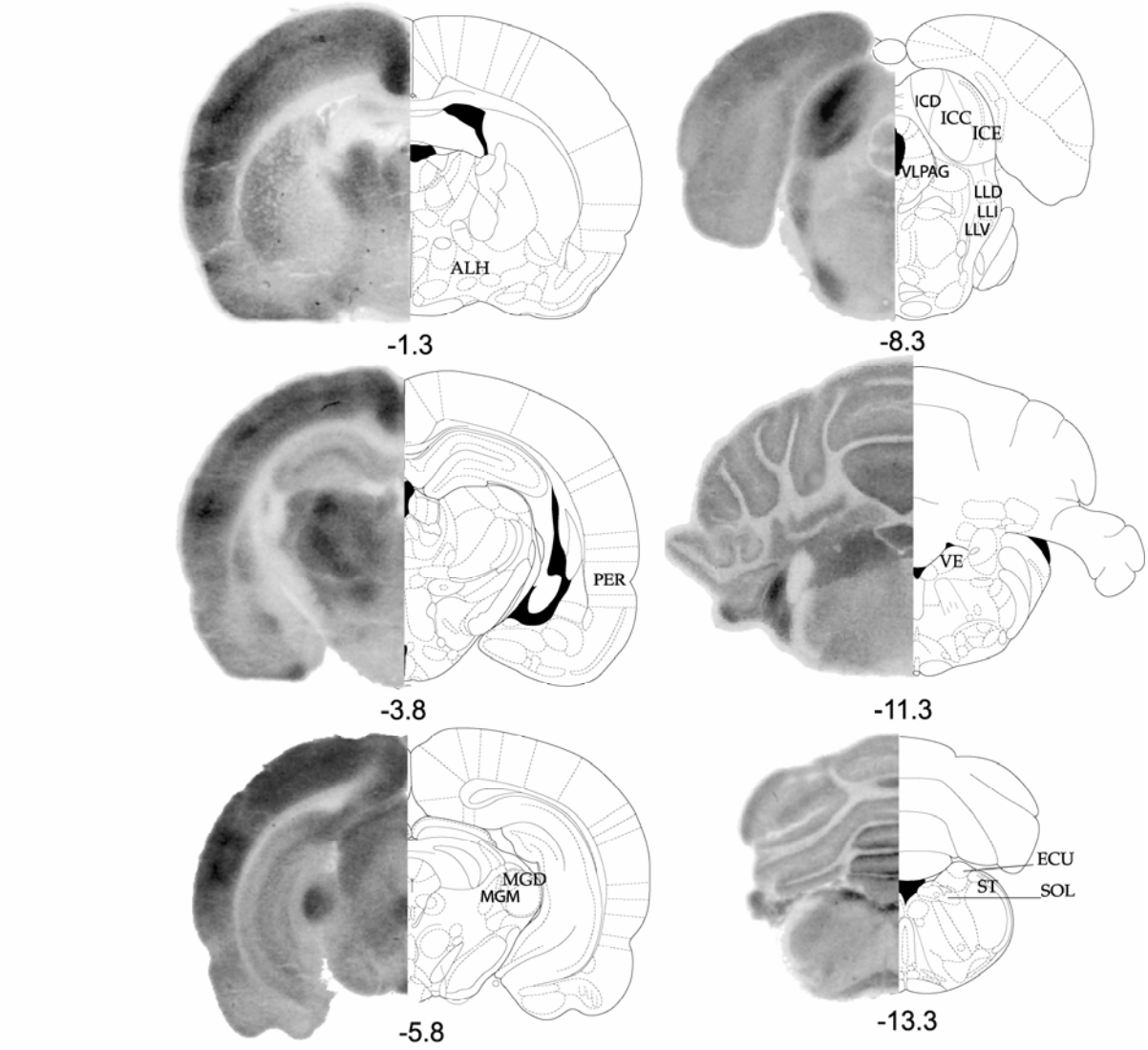
In auditory regions, the CS evokes metabolic activity not only as a physical stimulus (tone), but also through its learned signal value (Gonzalez-Lima et al., 1984a; Gonzalez-Lima et al., 1989; Gonzalez-Lima & Agudo, 1990). The largest FDG uptake increases (20%-25%) were seen in the external and central inferior collicular nuclei. However, lower (lateral lemniscus) and upper (medial geniculate thalamic nucleus and auditory cortex) levels of auditory system also showed significantly increased (8-15%) FDG uptake in the renewal group. Taken together, these excitatory effects in the auditory system, and especially in the inferior colliculus, may reflect not only auditory sensory processing (which was the same tone in the three groups), but tone-evoked associative effects specific to the renewal group. Because the effects seen in the renewal group were not present in the pseudorandom group, the data suggest that the extinguished CS retained its associative effects in a different context.

Out of the four somatosensory regions that showed significant renewal effects, the external cuneate had the highest percent increase in the FDG uptake in the renewal group (32%), while spinal trigeminal and solitary tract nuclei showed increases of 14% and 24%, respectively, compared to the pseudorandom group. Vestibular nucleus FDG uptake in the renewal group was 6% higher than in the pseudorandom group.

In addition to auditory and somatosensory system activation, three more areas showed renewal effects. These areas were perirhinal cortex, anterior lateral hypothalamus and ventrolateral periaqueductal grey. The size of the

renewal effects in these brain regions measured between 7% and 20 %, as compared to the pseudorandom group.

Figure 2. Coronal diagrams of locations of regions with renewal effects on FDG uptake by bregma level



Extinction effects

Regional extinction effects mostly consisted of increased FDG uptake in the extinction subjects, as compared to the other groups. The only region to show a decrease in FDG uptake was anterior cingulate cortex (CG2), in which a decreased (11%) FDG uptake was found in the extinction group as compared to pseudorandom and renewal groups. Conversely, rats in the extinction group showed increased (16-20%) FDG uptake in the lateral ventral basal thalamic nucleus as compared to the other two groups. Extinction group metabolic activity was also increased (17%) in ventral medial hypothalamic nucleus and central amygdaloid nucleus as compared to the pseudorandom group (Table 2). Finally, basolateral amygdala (BLA) had a 20% increase in the FDG uptake as compared to the FDG uptake in this region in renewal group subjects, suggesting that BLA plays a role in the recall of extinction memory. This finding, combined with emerging literature about differential properties of various amygdaloid nuclear subdivisions and their role in fear conditioning and extinction prompted us to examine FDG uptake in the amygdala in more detail (Table 3). However, the additional data did not yield any significant effects among the examined groups.

Table 3. Means and standard errors of regional FDG uptake in renewal and control subjects, with 99 % confidence intervals.

Structure	Group	Mean			Lower 99%	Upper 99%
Dorsal frontal cortex (DFC)	Renewal	796	±	32	706	887
	Extinction	825	±	30	740	909
	Pseudorandom	814	±	32	725	903
Medial frontal cortex (MFC)	Renewal	794	±	28	714	874
	Extinction	804	±	27	729	878
	Pseudorandom	834	±	28	755	912
Prelimbic cortex (PRL)	Renewal	795	±	32	703	886
	Extinction	797	±	30	713	882
	Pseudorandom	768	±	32	678	857
Medial orbital cortex (MO)	Renewal	870	±	30	784	955
	Extinction	820	±	28	741	900
	Pseudorandom	852	±	30	768	936
Lateral orbital cortex (LO)	Renewal	1005	±	17	956	1053
	Extinction	994	±	16	949	1039
	Pseudorandom	1014	±	17	966	1062
Agranular insular cortex (AI)	Renewal	782	±	46	654	910
	Extinction	778	±	42	659	897
	Pseudorandom	796	±	45	670	922
Lateral frontal cortex (LFC)	Renewal	783	±	39	673	893
	Extinction	816	±	37	713	918
	Pseudorandom	830	±	39	722	939
Infralimbic anterior cortex (ILA)	Renewal	769	±	45	643	895

	Extinction	690	±	42	572	807
	Pseudorandom	704	±	44	580	828
Infralimbic posterior cortex (ILP)	Renewal	755	±	53	607	903
	Extinction	721	±	49	583	859
	Pseudorandom	761	±	52	615	906
Anterior cingulate cortex (CG2)	Renewal	841	±	24	772	909
	Extinction	749	±	23	685	813
	Pseudorandom	846	±	24	779	914
Granular insular cortex (GI)	Renewal	728	±	48	592	863
	Extinction	749	±	45	622	875
	Pseudorandom	730	±	48	597	863
Nucleus Accumbens core (ACBC)	Renewal	590	±	59	424	756
	Extinction	615	±	55	460	770
	Pseudorandom	596	±	58	432	760
Nucleus Accumbens shell (ACBS)	Renewal	516	±	49	379	652
	Extinction	539	±	45	412	666
	Pseudorandom	536	±	48	401	671
Medial septal nucleus (MS)	Renewal	585	±	51	442	729
	Extinction	630	±	48	496	763
	Pseudorandom	621	±	50	480	762
Vertical limb diagonal band nucleus (VDB)	Renewal	579	±	54	428	730
	Extinction	615	±	50	474	756
	Pseudorandom	601	±	53	452	750
Lateral septal nucleus (LS)	Renewal	532	±	36	431	632
	Extinction	543	±	33	449	636
	Pseudorandom	523	±	35	425	622

Caudate putamen Anterior (CPUA)	Renewal	802	±	34	707	896
	Extinction	772	±	31	684	860
	Pseudorandom	820	±	33	727	912
Primary somatosensory area (S1)	Renewal	748	±	24	681	814
	Extinction	773	±	22	711	835
	Pseudorandom	765	±	23	700	830
Medial preoptic area (MPA)	Renewal	540	±	51	396	683
	Extinction	544	±	48	410	677
	Pseudorandom	524	±	50	383	665
Lateral preoptic area (LPO)	Renewal	631	±	50	490	772
	Extinction	628	±	47	496	759
	Pseudorandom	608	±	49	469	747
Horizontal limb diagonal band nucleus(HDB)	Renewal	744	±	48	608	879
	Extinction	714	±	45	588	841
	Pseudorandom	667	±	48	534	801
Caudate putamen posterior (CPUP)	Renewal	645	±	47	513	776
	Extinction	719	±	44	596	842
	Pseudorandom	661	±	46	531	790
Anterior hypothalamus (AHY)	Renewal	415	±	22	352	478
	Extinction	404	±	21	346	463
	Pseudorandom	393	±	22	331	455
Anterior lateral hypothalamus (ALH)	Renewal	662	±	42	544	779
	Extinction	545	±	39	436	655
	Pseudorandom	550	±	41	434	665
Periventricular hypothalamus (PVH)	Renewal	395	±	10	369	422
	Extinction	407	±	9	382	432

	Pseudorandom	395	±	9	369	422
Posterior cingulate cortex (CGP)	Renewal	916	±	19	863	968
	Extinction	900	±	17	851	949
	Pseudorandom	923	±	18	871	975
Mediodorsal thalamic nucleus (MD)	Renewal	836	±	42	718	954
	Extinction	901	±	39	791	1011
	Pseudorandom	819	±	41	703	935
Central medial thalamic nucleus (CM)	Renewal	697	±	44	574	819
	Extinction	779	±	41	665	894
	Pseudorandom	680	±	43	559	800
Ventral basolateral thalamic nucleus (VBL)	Renewal	750	±	43	631	870
	Extinction	897	±	40	785	1008
	Pseudorandom	730	±	42	613	848
Ventromedial hypothalamic nucleus (VMH)	Renewal	441	±	29	361	522
	Extinction	502	±	27	426	577
	Pseudorandom	419	±	28	340	499
Medial amygdaloid nucleus (MEA)	Renewal	544	±	43	423	665
	Extinction	567	±	40	454	680
	Pseudorandom	489	±	43	370	609
Central amygdaloid nucleus (CEA)	Renewal	449	±	32	358	540
	Extinction	525	±	30	440	610
	Pseudorandom	429	±	32	339	518
Basolateral amygdaloid nucleus (BLA)	Renewal	655	±	56	499	812
	Extinction	827	±	52	682	973
	Pseudorandom	691	±	55	537	845
Lateral amygdaloid nucleus (LA)	Renewal	477	±	18	425	529

	Extinction	486	±	17	438	534
	Pseudorandom	489	±	18	437	540
Intercalated cells of amygdala (ITC)	Renewal	444	±	17	396	493
	Extinction	451	±	16	406	496
	Pseudorandom	455	±	17	408	503
Central medial amygdaloid nucleus (CEM)	Renewal	354	±	14	313	394
	Extinction	363	±	13	325	400
	Pseudorandom	372	±	14	332	412
Cortical amygdaloid nucleus (COA)	Renewal	433	±	29	351	514
	Extinction	472	±	27	396	548
	Pseudorandom	453	±	29	373	534
Anterior pirifirom cortex (APIR)	Renewal	467	±	28	389	545
	Extinction	468	±	26	395	541
	Pseudorandom	509	±	27	432	586
Piriform cortex (PIR)	Renewal	632	±	26	558	706
	Extinction	609	±	25	540	678
	Pseudorandom	623	±	26	550	695
Posterior parietal cortex (PPA)	Renewal	763	±	24	695	831
	Extinction	742	±	23	678	805
	Pseudorandom	748	±	24	681	815
Field CA1 of hippocampus (CA1)	Renewal	475	±	10	447	502
	Extinction	472	±	9	447	498
	Pseudorandom	465	±	10	437	492
Field CA2 of hippocampus (CA2)	Renewal	525	±	25	454	595
	Extinction	532	±	23	466	598
	Pseudorandom	534	±	25	464	604

Field CA3 of hippocampus (CA3)	Renewal	522	±	18	472	573
	Extinction	523	±	17	476	570
	Pseudorandom	513	±	18	463	563
Dentate gyrus (DG)	Renewal	483	±	13	447	520
	Extinction	488	±	12	454	521
	Pseudorandom	462	±	13	426	497
Habenula (HB)	Renewal	883	±	36	783	983
	Extinction	841	±	33	747	934
	Pseudorandom	844	±	35	746	943
Perirhinal cortex (PER)	Renewal	597	±	17	550	644
	Extinction	582	±	16	538	626
	Pseudorandom	556	±	16	510	603
Caudal caudate putamen (CPC)	Renewal	621	±	21	563	680
	Extinction	631	±	19	576	685
	Pseudorandom	580	±	20	523	638
Caudate putamen grey matter (CPUGRY)	Renewal	657	±	21	598	715
	Extinction	664	±	19	610	718
	Pseudorandom	676	±	20	618	733
Entorhinal cortex (ENT)	Renewal	491	±	17	442	539
	Extinction	501	±	16	455	546
	Pseudorandom	476	±	17	428	524
Medial mammilary nucleus (MM)	Renewal	650	±	69	457	843
	Extinction	696	±	64	516	876
	Pseudorandom	709	±	68	520	899
Primary auditoty cortex (TE1)	Renewal	799	±	17	751	848
	Extinction	811	±	16	766	856

	Pseudorandom	798	±	17	750	845
Secondary auditory cortex (TE3)	Renewal	638	±	26	564	712
	Extinction	669	±	25	600	738
	Pseudorandom	639	±	26	567	712
Primary visual cortex (V1)	Renewal	900	±	40	788	1012
	Extinction	808	±	37	703	912
	Pseudorandom	915	±	39	805	1026
Associative visual cortex, medial (V2M)	Renewal	845	±	36	742	947
	Extinction	792	±	34	696	887
	Pseudorandom	839	±	36	738	940
Associative visual cortex, lateral (V2L)	Renewal	809	±	37	704	913
	Extinction	769	±	35	672	867
	Pseudorandom	803	±	37	701	906
Superificial grey layer superior colliculus (SCSUP)	Renewal	755	±	34	660	850
	Extinction	745	±	32	656	833
	Pseudorandom	786	±	33	692	880
Intermediate grey layer superior colliculus (SCINT)	Renewal	710	±	23	646	775
	Extinction	673	±	21	613	734
	Pseudorandom	703	±	23	639	767
Deep grey layer superior colliculus (SCDEEP)	Renewal	722	±	32	631	813
	Extinction	663	±	30	579	748
	Pseudorandom	675	±	32	586	764
Midbrain reticular formation (MRF)	Renewal	617	±	21	559	675
	Extinction	578	±	19	524	632

	Pseudorandom	609	±	20	552	666
Hippocampal field CA1, caudal (CCA1)	Renewal	541	±	14	501	581
	Extinction	518	±	13	481	555
	Pseudorandom	521	±	14	482	560
Hippocampal field CA2, caudal (CCA2)	Renewal	525	±	25	454	595
	Extinction	532	±	23	466	598
	Pseudorandom	534	±	25	464	604
Hippocampal field CA3, caudal (CCA3)	Renewal	522	±	18	472	573
	Extinction	523	±	17	476	570
	Pseudorandom	513	±	18	463	563
Subiculum (SUB)	Renewal	483	±	13	447	520
	Extinction	488	±	12	454	521
	Pseudorandom	462	±	13	426	497
Medial geniculate nucleus, dorsal (MGD)	Renewal	851	±	18	801	901
	Extinction	774	±	17	727	820
	Pseudorandom	790	±	18	741	839
Medial geniculate nucleus, medial (MGM)	Renewal	815	±	23	751	878
	Extinction	765	±	21	706	824
	Pseudorandom	774	±	22	712	837
Medial geniculate nucleus, ventral (MGV)	Renewal	760	±	29	679	841
	Extinction	715	±	27	639	790
	Pseudorandom	722	±	28	643	802
Ventral tegmental area (VTA)	Renewal	476	±	16	432	521
	Extinction	453	±	15	411	495
	Pseudorandom	475	±	16	431	519
Retrosplenial cortex (RS)	Renewal	821	±	27	745	896

	Extinction	785	±	25	714	855
	Pseudorandom	811	±	27	736	885
Auditory associative cortex (TE2)	Renewal	668	±	28	588	747
	Extinction	657	±	26	583	731
	Pseudorandom	607	±	28	529	685
Inferior colliculus, dorsal (ICD)	Renewal	663	±	31	575	751
	Extinction	619	±	29	537	700
	Pseudorandom	578	±	31	491	664
Inferior colliculus, central (ICC)	Renewal	1060	±	46	932	1189
	Extinction	932	±	43	813	1052
	Pseudorandom	790	±	45	663	916
Inferior colliculus, external (ICE)	Renewal	768	±	33	677	860
	Extinction	674	±	30	589	759
	Pseudorandom	616	±	32	526	705
Lateral lemniscus nucleus, dorsal (LLD)	Renewal	661	±	26	587	734
	Extinction	614	±	24	545	683
	Pseudorandom	586	±	26	514	658
Lateral lemniscus nucleus, intermediate (LLI)	Renewal	784	±	36	682	886
	Extinction	740	±	34	645	836
	Pseudorandom	679	±	36	578	779
Lateral lemniscus nucleus, ventral (LLV)	Renewal	745	±	26	671	818
	Extinction	730	±	24	661	798
	Pseudorandom	685	±	26	613	757
Periaqueductal grey, dorsal (PAGD)	Renewal	413	±	22	350	475
	Extinction	385	±	21	327	444
	Pseudorandom	369	±	22	307	431

Periaqueductal grey, ventrolateral (VLPAG)	Renewal	424	±	21	366	482
	Extinction	397	±	19	342	451
	Pseudorandom	367	±	20	309	424
Trapezoid body (TB)	Renewal	653	±	58	491	814
	Extinction	675	±	54	525	826
	Pseudorandom	707	±	57	548	866
Superior olivary nucleus, medial (MSO)	Renewal	746	±	66	560	932
	Extinction	774	±	62	601	947
	Pseudorandom	787	±	65	604	970
Superior olivary nucleus, lateral (LSO)	Renewal	753	±	66	568	938
	Extinction	799	±	61	626	971
	Pseudorandom	801	±	65	619	983
Ventral cochlear nucleus, anterior (VCA)	Renewal	576	±	37	471	681
	Extinction	633	±	35	535	730
	Pseudorandom	579	±	37	476	682
Cerebellar hemisphere, medial (CHM)	Renewal	470	±	23	405	535
	Extinction	487	±	22	427	548
	Pseudorandom	468	±	23	404	532
Cerebellar hemisphere, lateral (CHL)	Renewal	532	±	24	463	600
	Extinction	515	±	23	451	578
	Pseudorandom	521	±	24	454	589
Deep cerebellar nucleus (DPCB)	Renewal	679	±	16	635	723
	Extinction	699	±	15	658	740
	Pseudorandom	675	±	15	631	718
Vestibular nucleus (VE)	Renewal	972	±	27	896	1047
	Extinction	967	±	25	897	1038

	Pseudorandom	915	±	26	840	989
Dorsal cochlear nucleus (DC)	Renewal	1067	±	49	930	1203
	Extinction	1053	±	45	926	1179
	Pseudorandom	990	±	48	856	1124
Ventral cochlear nucleus, posterior (VCP)	Renewal	813	±	35	715	911
	Extinction	886	±	33	794	977
	Pseudorandom	772	±	34	675	869
Cerebellar vermis (CBV)	Renewal	642	±	28	565	720
	Extinction	604	±	26	532	677
	Pseudorandom	607	±	27	531	684
External cuneate nucleus (ECU)	Renewal	790	±	37	687	894
	Extinction	627	±	34	531	724
	Pseudorandom	596	±	36	494	698
Solitary tract nucleus (SOL)	Renewal	635	±	33	541	729
	Extinction	510	±	31	423	597
	Pseudorandom	508	±	33	416	600
Parvocellular reticular nucleus (PCR)	Renewal	557	±	18	507	608
	Extinction	542	±	17	496	589
	Pseudorandom	537	±	18	488	587
Spinal trigeminal nucleus (ST)	Renewal	627	±	26	555	700
	Extinction	548	±	24	481	616
	Pseudorandom	550	±	25	479	621

Regional FDG uptake ± standard errors is calculated as (ROI/whole brain mean global activity) x pseudorandom group whole brain mean activity and expressed as nanocuries per gram tissue wet weight.

Interregional within-group FDG uptake correlations

In the renewal group, FDG uptake in auditory and amygdaloid nuclei showed high positive intercorrelations, while somatosensory nuclei showed high negative correlations with auditory and amygdaloid nuclei (Table 4). Additionally, amygdaloid nuclei were highly intercorrelated in the renewal group, and VLPAG also had high positive intercorrelations with auditory areas. In general, extinction group showed an opposite correlative pattern as compared to the renewal group, while the pseudorandom group showed a mixed pattern of correlative activity as compared to the other groups.

Table 4. Inter-regional within-group correlations of FDG activity

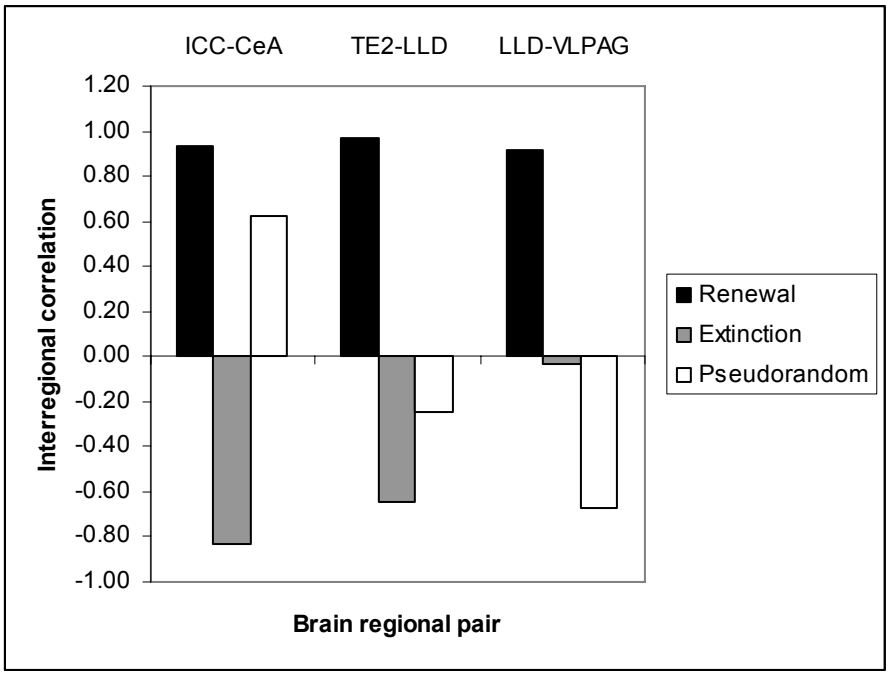
		Group	
Intercorrelated regions	Renewal	Extinction	Pseudorandom
Auditory system intercorrelations			
TE2-LLD	0.97	-0.64	-0.24
ICD-ICE	0.87	0.17	0.5
ICE-LLD	0.86	0.29	-0.45
ICD-LLD	0.85	-0.12	0.08
LLD-LLI	0.96	0.6	0.4
LLD-LLV	0.91	0.54	-0.31
LLI-LLV	0.95	0.95	0.18
Auditory system correlations with extraauditory regions			
TE2-VLPAG	0.95	0.07	0.11
ICD-VLPAG	0.88	0.46	0.31
ICE- VLPAG	0.86	-0.54	0.18
LLD- VLPAG	0.92	-0.03	-0.67
MGD-ALH	0.79	0.57	-0.2
ICC-CeA	0.93	-0.83	0.62
ICC-BLA	0.9	-0.24	0.23
LLI-ECU	-0.79	0.51	-0.33

Amygdala correlations with other regions			
CeA-ST	-0.92	-0.09	0.36
BLA-ST	-0.8	0.02	0.29
CeA-VBL	-0.72	0.5	0.58
CeA-VMH	0.81	0.71	0.99
BLA-VMH	0.91	-0.17	0.58
CeA-BLA	0.85	0	0.55

The left column lists regional pairs with significant within-group correlations that survived jackknife procedure.

Analysis of inter-regional FDG uptake correlations transformed to z-scores yielded three regional pairs that showed a statistically reliable difference between the renewal and other groups (Figure 3).

Figure 3. Inter-regional correlations that were found to be significantly ($p < 0.05$) different between the groups, as determined by between-group z-score



Brain-behavior correlations

Of the brain regions that showed significant mean activity changes or inter-regional correlations in FDG uptake, two regions were significantly ($p < 0.05$) correlated with the freezing behavior during the FDG session. For the renewal group, these regions were the external cuneate nucleus (ECU) and the ventral medial hypothalamic nucleus (VMH). The high negative correlation ($r = -0.77$) between the freezing behavioral measure and ECu activity suggests that renewal subjects with higher FDG uptake in the external cuneate exhibit less freezing to the CS during the FDG session. On the other hand, a positive correlation ($r = 0.77$) between freezing during the first ten minutes of the FDG session and FDG uptake in the VMH implies that this region plays a role in the expression of renewal (Table 5).

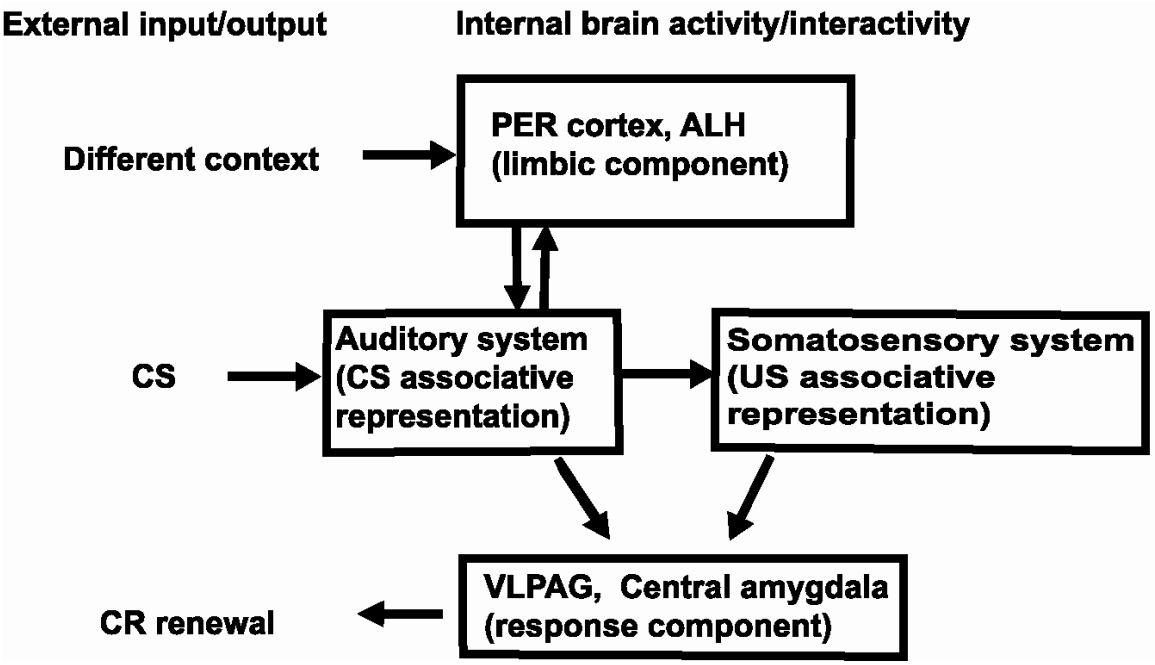
Table 5. Regional FDG uptake- behavior correlations in renewal group

ROI	r	p
Auditory CS-related regions		
Auditory cortex (TE2)	0.55	0.16
Medial geniculate nucleus, dorsal (MGD)	-0.09	0.83
Medial geniculate nucleus, medial (MGM)	-0.42	0.30
Inferior colliculus nucleus, external (ICE)	0.35	0.39
Inferior colliculus nucleus, central (ICC)	0.57	0.14
Inferior colliculus nucleus, dorsal (ICD)	0.21	0.63
Lateral lemniscus nucleus, dorsal (LLD)	0.60	0.11
Lateral lemniscus nucleus, intermediate(LLI)	0.55	0.16
Lateral lemniscus nucleus, ventral (LLV)	0.53	0.18
Somatosensory US-related regions		
External cuneate nucleus (ECU)	-0.77	0.03
Solitary tract nucleus (SOL)	-0.39	0.34
Spinal trigeminal nucleus (ST)	-0.42	0.31
Vestibular nucleus (VE)	-0.65	0.09
Context and CR-related regions		
Perirhinal cortex (PER)	-0.04	0.93
Anterolateral hypothalamus (ALH)	0.32	0.44
Ventrolateral periaqueductal grey (vlPAG)	0.47	0.24
Ventromedial hypothalamus (VMH)	0.77	0.03
Central amygdala (CeA)	0.63	0.09

Discussion

Regional metabolic increases between renewal and comparison groups suggest that context-dependent fear renewal in the ABC paradigm is mediated by the neural reactivation of the CS and US associative representations involving auditory system excitatory responses to the tone CS, and reactivation of the US representation in somatosensory pathways in the absence of the US (Figure 4).

Figure 4. Stimulus-substitution model of ABC renewal



Contextual input in the ABC fear renewal

Renewal effects in perirhinal cortex (PER) suggest that contextual processing is of relevance in the ABC renewal of conditioned fear. Although there are no lesion studies of PER in renewal, PER lesions disrupt auditory, visual and contextual fear conditioning, as well as fear-potentiated startle (Rosen et al., 1992; Sacchetti, Lorenzini, Baldi, Tassoni, & Bucherelli, 1999; Burwell, Bucci, Sanborn, & Jutras, 2004). Nerad and Bilkey (2005) have found that PER electroencephalogram oscillation in the rat cortex is modulated by environmental familiarity. Lesions of rostral PER following fear conditioning interfere with CRs elicited by auditory stimuli when these stimuli are presented in contexts that differ from the initial conditioning context (Corodimas & Ledoux, 1995). Therefore, PER may participate in novel/familiar context discrimination during ABC renewal. In addition, PER has reciprocal connections to the hippocampus (Naber, Witter, & Lopes da Silva, 1999; Burwell et al., 2004). Hippocampal lesions impair renewal in some studies (Corcoran & Maren, 2004; Ji & Maren, 2005), but not others (Wilson et al., 1995; Frohardt et al., 2000). Perhaps hippocampal lesions disrupt PER functioning related to memory, especially novelty/familiarity discriminations (Bussey, Muir, & Aggleton, 1999; Liu & Bilkey, 2001; Murray & Richmond, 2001).

Although anterior lateral hypothalamus (ALH) has been associated with regulation of sympathetic output, there is some suggestion that ALH might also be involved in the integration of cognitive and autonomic responses related to environmental dangers (Krout, Mettenleiter, Karpitskiy, Van Nguyen, & Loewy,

2005). Footshock can activate ALH, while auditory CSs activate both ALH and PER (Campeau et al., 1997; Li, Ericsson, & Sawchenko, 1996). Cholinergic stimulation of ALH potentiates duration of immobility (de Oliveira, Del Bel, & Guimaraes, 2000), and ALH c-fos expression in rats is upregulated following exposure to cat odor (Staples, Hunt, Cornish, & McGregor, 2005). Exposing rats to the environment in which they had previously received footshock significantly increases the number of Fos immunoreactive cells in LH (Beck & Fibiger, 1995). This suggests that LH neurons might play a role in detection of environmental dangers, and modify behavioral responses to those dangers through their connections with limbic cortex and sympathetic outflow.

Auditory system activity during conditioned response (CR) renewal

FDG uptake increased in auditory nuclei in the renewal group as compared to the pseudorandom group, suggesting that the extinguished tone still retained its behavioral excitatory associative value after extinction. ICC, the region with the 25% percentage increase in FDG uptake in the current study has previously shown increased metabolic activity following conditioning of a tone with an aversive stimulus (Barrett et al., 2003; Gonzalez-Lima et al., 1990; Gonzalez-Lima, 1992; Jones et al., 2001b). ICE, which also showed a tone-evoked FDG increase in the renewal group, contains representations of both auditory and somatosensory stimuli (Aitkin, Kenyon, & Philpott, 1981). ICE activation suggests that its overlapping neural representations of the auditory CS

and the somatosensory US may be important for the Hebbian-type interaction of the CS and US effects on the brain.

Lesions of IC, MG or its projections to amygdala lead to decreased freezing after auditory conditioning (Ledoux et al., 1984). The behavioral excitatory component of the CS was transmitted from IC to higher auditory centers, as supported by elevated MGD and auditory cortex activity in the renewal group. IC and MGD activation during conditioned fear renewal may represent CS-US associative effects (McIntosh & Gonzalez-Lima, 1998; Jones et al., 2001b). It appears that during renewal of an extinguished CR signaled by a tone, auditory neurons are critically important in representing the associative properties of the CS leading to CR renewal.

Somatosensory system activation during CR renewal

Several somatosensory nuclei (external cuneate, solitary tract, spinal trigeminal and vestibular) had increased tone-evoked activity in the renewal group as compared to extinction and pseudorandom groups, suggesting that during renewal of conditioned responding there is a CS-evoked reactivation of the US representation in the absence of the US. Solitary tract nucleus (SOL) has been implicated in conditioning effects by lesion studies (Grigson, Shimura, & Norgren, 1997), and electrophysiological recording studies (Giza, Ackroff, McCaughey, Sclafani, & Scott, 1997). Elevated activity in both spinal trigeminal and solitary tract nuclei has been found with FDG metabolic mapping of the Kamin blocking effect (Jones et al., 2001b), and McCaughey et al. (1997) found a CS-evoked bursting pattern in SOL after both acquisition and extinction of a

conditioned taste aversion. Activation of somatosensory areas during renewal of CR suggests a reactivation of US representation in rats that had been exposed to CS-US pairing.

Elevated FDG uptake in the ECu in the renewal group complements the Barrett et al. (2003) discovery that recall of fear conditioning acquisition elevates metabolic activity of ECu. Furthermore, while extinction training in this study led to functional coupling between ECu and a network of lower auditory regions, no such interaction was found following acquisition (Barrett et al., 2003). In the present study, although ECu was significantly correlated with the behavioral index of renewal, interregional within-group correlations revealed that ECu was not functionally coupled to the other regions that showed increased metabolic activity. Thus, while both acquisition and renewal of conditioned fear responses lead to elevated CS-evoked ECu metabolism and its functional disconnect from other regions, in renewal subjects the increased freezing response was significantly correlated with decreased ECu activity. This inverse relationship between ECu activation and freezing index was also found in Jones and Gonzalez-Lima (2001b) FDG study of blocking. In each of these studies, ECu activity was evoked by tone-alone presentations, indicating that differences resulted from animals' prior experiences with that tone, not to the presence of a CR.

Brain regions contributing to CR behavioral output

Ventrolateral periaqueductal grey (VLPAG) and anterior lateral hypothalamus (ALH) both showed increased metabolic activity in the renewal

group. Opioid receptor activation in the VLPAG has recently been associated with the extinction of conditioned fear (McNally, Pigg, & Weidemann, 2004), and increased Fos expression in VLPAG is linked to contextual conditioned fear (Carrive, Leung, Harris, & Paxinos, 1997). Electrical stimulation of VLPAG elicits freezing behavior in rats (Vianna, Graeff, Brandao, & Landeira-Fernandez, 2001) and VLPAG neurons respond following the stimulation of central amygdala (Costa Gomez & Behbehani, 1995), since VLPAG is one of the projection targets of the central nucleus of amygdala (Price & Amaral, 1981; Post & Mai, 1978). Another projection of the central amygdala is to the lateral hypothalamus (Price et al., 1981). This region seems to be involved more in the autonomic conditioned response, and even though in this study we did not measure any autonomic CRs (such as arterial pressure response), it is likely that the increased metabolic activity in the ALH in the renewal group is related to this response.

Extinction effects

The decreased anterior cingulate cortex (CG2) FDG uptake found in the extinction group as compared to pseudorandom and renewal group indicate that this area has diminished metabolic activity during extinction memory recall. Considering that both direct electrical stimulation and focal metabotropic glutamate receptor agonist administration in CG2 induces Pavlovian fear memory (Tang et al., 2005), it is conceivable that reduced metabolic activity in this region might be associated with reduced fear memory recall. Because CG2 sends projections to the periaqueductal grey (An, Bandler, Ongur, & Price, 1998) and basolateral amygdala (Gabbott, Warner, Jays, Salway, & Busby, 2005), it is

possible that CG2 activity may affect both the endogenous analgesia system and amygdaloid nuclei which are thought to be important for the formation of fear extinction memory (Sotres-Bayon et al., 2004; Falls, Miserendino, & Davis, 1992; Barad, 2005). Decreased FDG uptake in anterior cingulate cortex in extinction group subjects complements the findings of Straube et al. (2006) in which attenuated anterior cingulate cortex activation was found following cognitive-behavioral therapy for specific phobia.

Although the 20% increase in FDG uptake in extinction subjects compared to renewal subjects found in the basolateral amygdaloid nucleus (BLA) seemed somewhat contradictory to several findings implicating amygdala in acquisition, and possibly storage of conditioned fear (Goosens & Maren, 2001; Phelps et al., 2005; Kim & Jung, 2006), our findings are in agreement with many other studies that link BLA to fear extinction. In fact, BLA was the first brain structure to be linked to extinction by Falls et al. (1992), in a study which showed that memory of extinction training could be blocked by infusion of an NMDA receptor antagonist in the BLA during extinction of fear-potentiated startle. In addition, infusion of NMDA receptor agonist facilitates fear extinction after intra-amygdala infusion (Davis, 2002).

Considering that FDG metabolic mapping method does not distinguish between increased excitatory or inhibitory neural signaling, the observed increase in FDG uptake in the BLA only reflects increased glucose demand. Therefore, tone-evoked increased FDG uptake in BLA might result from either increased glutamatergic neurotransmission or, alternatively, increased release of GABA. Indeed, it has been proposed that extinction learning involves mPFC activation of inhibitory interneurons within the BLA (Grace & Rosenkranz, 2002;

Bauer & Ledoux, 2004). The observed increase in metabolic activity in BLA in the extinction group as compared to renewal group in this study reflects importance of this region in fear conditioning and extinction.

Likewise, increased metabolism in central amygdala (CeA) in the extinction group as compared to pseudorandom group also suggests that this subdivision of the amygdala might play a role in fear extinction, likely through its modulation by prefrontal cortex. Although between-group changes in FDG uptake were not detected in regions such as infralimbic cortex (IL) in this study, possibly due to the experimental design favoring strong renewal of the freezing response, anterior cingulate cortex cortex (CG2) did show a decreased metabolic activity in the extinction group. As outlined before, CG2 projects to BLA, which in turn can modulate CeA output via intercalated cells (Pare et al., 2004), with the medial part of the CeA constituting the main output pathway for amygdala projections that influence behavioral and autonomic responses (Sotres-Bayon et al., 2004). Since Tang et al. (2005) showed that stimulation of CG2 produces fear-like freezing response in rodents, it is conceivable that decreased CG2 neuronal activity in extinction group might be functionally related to metabolic changes in amygdaloid nuclei considered to mediate the CR response.

The ventral medial hypothalamic nucleus (VMH) was another brain region that showed significantly increased FDG uptake in extinction group as compared to pseudorandom group. Nerve fibers containing excitatory amino acids project from the medial hypothalamus to NMDA receptors in PAG, and it is thought that this pathway plays a role in the mediation of defensive behaviors (Schubert, Shaikh, & Siegel, 1996). Blockade of GABAA receptors in ventromedial hypothalamus results in increased fear behavior (Zagrodska, Romaniuk,

Wieczorek, & Boguszewski, 2000), suggesting that increased metabolism in VMH might be related to increased GABA transmission in this area, rather than increased excitatory activation. This hypothesis needs to be tested in order to better understand the role of VMH activity in extinction and renewal of fear responses.

Extinction effects were also observed in the lateral ventral basal thalamic nucleus (VBL), commonly known as the ventral posterolateral thalamic nucleus. It is a major relay station for the extroceptive and proprioceptive impulses to the cortex. In addition to receiving fibers from the spinothalamic tract (Ledoux, Farb, & Ruggiero, 1990) and having reciprocal connections with the primary sensory cortex (Landry & Deschenes, 1981), VBL also receives projections from the dorsal nucleus of the lateral lemniscus (Yasui et al., 1983) and the external an pericentral nuclei of the IC (Linke & Schwegler, 2000). Therefore, this region is in position to integrate somatosensory inputs from the spinal cord with auditory information and to transmit that information to the medial central amygdala and accessory basal amygdaloid nucleus (Linke et al., 2000; Turner & Herkenham, 1991). Increased metabolism in the extinction group at this level suggests that signals for inhibition of freezing behavior likely originate from higher brain centers, but it also implies that there is a component of thalamic involvement in relaying of the salient extinguished conditioned stimuli. In summary, extinction effects included increased FDG uptake in ventral medial hypothalamic, ventral basal thalamic, and central and basolateral amygdaloid nuclei, as well as decreased FDG uptake in the anterior cingulate cortex.

Hippocampal role in the ABC fear renewal paradigm

Based on animal brain lesion and inactivation work (Corcoran et al., 2004; Ji et al., 2005; Maren, Aharonov, & Fanselow, 1997a) it was hypothesized that dorsal hippocampus (DHC) might be a region contributing to context-dependent renewal in the ABC paradigm. However, other studies found that fornix or hippocampal lesions have no effect on ABA renewal (Frohardt et al., 2000; Wilson et al., 1995), and no metabolic brain differences were found between renewal, extinction and pseudorandom groups in the DHC in the current study. One possible explanation is that in the ABC renewal paradigm the testing context is not ambiguous (no excitatory training or extinction had taken place there), and there is some evidence that the hippocampus is not necessary in unambiguous contexts (Barad, 2005). Rather, in a neutral context such as the testing context encountered during the FDG session in this study, behavioral responses may be modulated through the perirhinal cortex, brain region that did show an increase in the FDG uptake following CR renewal. Perirhinal cortex has reciprocal connections with entorhinal and parahippocampal cortices (Suzuki & Amaral, 1994), and neurophysiological studies have shown that these cortical regions contribute in an important way to normal memory function. Dorsal hippocampus, perirhinal cortex and subiculum are reciprocally connected (Swanson & Cowan, 1977), which makes it likely that a lesion of any one of these areas would affect the normal functioning of the whole system. While the dorsal hippocampus might contribute to a renewal effect, our metabolic mapping technique identified perirhinal cortex as a limbic area that was more active during renewal of CR.

Functional brain connectivity indicated by inter-regional correlations

Analysis of inter-regional within-group correlations of FDG activity in the renewal group indicated that auditory and amygdaloid nuclei, as well as auditory areas and VLPAG were highly positively intercorrelated. On the other hand, somatosensory regions showed high negative correlations with auditory and amygdaloid nuclei in the renewal group. Generally, the extinction group showed an opposite correlative pattern as compared to renewal. Although there were many pairs of brain regions in the renewal group that showed significant correlative activity, only three brain regional pairs were found to differ between the groups. These inter-correlated pairs were: ICC-CeA, TE2-LLD and LLD-VLPAG. In each case, animals displaying fear renewal showed high positive intercorrelation between these regional pairs, while the extinction group showed negative correlations between these brain regions. The functional connectivity patterns of these regions offer an insight into how conditioned fear renewal in the ABC paradigm might be gated. Auditory regions seem to strongly influence central amygdala and VLPAG, two brain areas thought to be essential for the compilation of the freezing behavioral response. It is conceivable that the activation of the neural context representation influences the auditory system, resulting in the functional network of inter-correlated brain regions that direct the freezing behavioral response (Fig 4).

Summary

Overall, the data presented support that CR renewal is not due to one brain region switching the CR on and off. Rather, activation of the neural representation of a novel context influences the processing of the extinguished CS in such a way that a US representation is constructed in the absence of the footshock. Activation of the CS and US neural representation, in turn, results in activation of the CR output areas, thus leading to CR renewal. Neural differences found between renewal and control group support the notion that "sensory learning represents a distributed property of neuronal maps which may be demonstrated in any system which meets the appropriate requirements of time and space convergence of CS and US inputs" (Gonzalez-Lima, 1992). As demonstrated by renewal, memories can build upon each other, but these building blocks themselves seem to retain their strong foundation in the physical substance of functional neuronal networks. Contextual influence might modify responding to the CS based on how much arousal it provides. Therefore, it seems beneficial to conduct extinction training in varying environments (for example conduct exposure treatments in nonclinical settings), thus changing the arousal level that accompanies the learning. In this way, the "test" context would be more likely to resemble the extinction context, which has been shown to decrease renewal of CR in fear-conditioned rats (Thomas, Larsen, & Ayres, 2003; Gunther, Denniston, & Miller, 1998). This approach might lead to better retention of CR inhibition for anxiety patients who show return of fear following behavioral exposure therapy.

Chapter 3: Cytochrome oxidase metabolic mapping of conditioned fear acquisition and extinction in rats

Introduction

The goal of this study was to evaluate training-evoked cumulative changes in brain metabolic capacity associated with Pavlovian fear conditioning and extinction in rats. Neuronal metabolic capacity is largely determined by the ability to use ATP for the resetting of membrane potentials involved in synaptic communication (Wong-Riley, 1989). ATP production itself is limited by enzymatic complexes which create a proton gradient that is used by ATP synthase. The rate-limiting mitochondrial respiratory chain enzyme for the oxidative metabolism of neurons that is coupled to ATP production is the mitochondrial enzyme cytochrome oxidase (CO) (Capaldi, 1990). Brain areas with a higher level of maintained neuronal activity, such as cortical ocular dominance columns in visual cortex, are rich in CO (Wong-Riley, 1989). Thus, quantitative CO histochemistry can be used to determine alterations in neuronal metabolic capacity resulting from the entire training period. This technique provides a more stable representation of the metabolic state than the fluorodeoxyglucose (FDG) technique, which measures short-term stimulus-evoked neuronal energy demand (Gonzalez-Lima & Cada, 1998).

CO metabolic brain mapping provides an alternative approach for investigating brain neural circuits mediating fear conditioning and extinction, and it is a well-suited method for quantifying more stable neuronal metabolic capacity changes that reflect prolonged training. Considering that fear extinction is only

possible following fear acquisition, then CO histochemistry will delineate brain circuits that are engaged following the cumulative processing of both excitatory (acquisition) and inhibitory (extinction) learning and memory.

In this experiment, quantitative histochemistry of CO was used to examine cumulative differences in rat brain regional metabolic capacity related to conditioned fear acquisition and extinction training. Utilization of the CO methodology allowed us to examine neural circuits implicated in across-days learning of Pavlovian conditioning and extinction, as opposed to memory retrieval. As the training-evoked energy demand to process and consolidate fear-related memories changes, so does the production of the CO enzyme (Wong-Riley, 1989; Gonzalez-Lima et al., 1998). Earlier findings from our laboratory have demonstrated that auditory fear conditioning in rats results in long-term metabolic capacity changes in the auditory system, confirming that mapping the CO metabolic activity using quantitative histochemistry can be used to examine long-lasting effects of behavioral training on the brain (Poremba, Jones, & Gonzalez-Lima, 1998). Another recent study illustrated that several days of the excitatory tone-footshock conditioning in rats changed CO activity in septohippocampal areas (Conejo et al., 2005).

The first hypothesis of this study was that sensory areas associated with the CS processing would show long-term training effects in both acquisition-only and acquisition followed by extinction groups, as compared to the pseudorandom group. Specifically, we expected to see learning-related changes in neuronal cytochrome oxidase metabolic capacity in the auditory system, since we used a tone as the CS, and because an earlier study that used auditory fear conditioning with suppression of drinking as the conditioned response in rats found alterations

in CO activity in auditory system structures following this type of associative learning (Poremba et al., 1998). Second, it was hypothesized that the acquisition + extinction group animals might show metabolic capacity changes in the medial prefrontal cortex, specifically the infralimbic cortex, since there is accumulating evidence that this brain area is important in retention (Gonzalez-Lima & Bruchey, 2004) and consolidation (Milad et al., 2002) of fear extinction. Third, it was anticipated that brain regions involved in the processing of the affective aspects of fear conditioning might undergo changes in metabolic capacity related to conditioning and extinction. One possible candidate for such a region is the septal area, since changes in CO activity resulting from Pavlovian fear conditioning have been found in the septal region (Conejo et al., 2005). It was also speculated that since the pseudorandomly trained control group essentially undergoes contextual fear conditioning, subjects in this group might show learning-related metabolic capacity changes in regions known to be involved in contextual cue processing, mainly the hippocampal formation (Corcoran et al., 2005; Maren & Fanselow, 1997b).

Materials and Methods

Behavioral training and brain processing

Alternating sections of rat brains from pseudorandom and extinction groups used in the experiment described in Chapter 2 were also used in this experiment. Acquisition-only group animals (n=10) were trained as described in Chapter 2, except that they did not receive CS-alone presentations during the

extinction phase of the training. All other parameters (contextual cues, probe trials) were kept identical.

Table 6. Experimental training protocol

		Group		
Day	**Procedure**	Acquisition only (excitatory tone)	Acquisition + Extinction (excitatory then inhibitory tone)	Pseudorandom (excitatory context)
1-2	Habituation	Exposure to context A	Exposure to context A	Exposure to context A
3-4	Excitatory training	CS -> US context A	CS -> US context A	CS,US context A
5-	Probe	3 CS presentations in context B -> CER	3 CS presentations in context B -> CER	3 CS presentations in context B -> no CER
5-6	Inhibitory training	Context B	CS in context B	CS in context B
7-	Probe	3 CS presentations in context B -> CER	3 CS presentations in context B -> no CER	3 CS presentations in context B -> no CER
7-	FDG/saline	Context B	CS in context B	CS in context B

Acquisition-only animals were injected with saline (as opposed to FDG) and placed in the FDG testing context for 60 minutes prior to decapitation. Next, their brains were quickly extracted, frozen in isopentane and stored at -40°C. Following collection of all brains, 40μm sections were cut in a Reichter-Jung cryostat at -20°C, picked up on clean glass slides and stored in sealed slide boxes at -40°C until further processing.

Quantitative cytochrome oxidase histochemistry

Twelve additional rats were used for the preparation of CO standards. These rats were allowed food and water ad libitum and were housed under the standard laboratory rearing conditions (12h light/dark cycle). Following decapitation, brains were removed and stored at 4°C in sodium phosphate buffer (pH 7.6) until all brains were collected. The unfixed brains were then homogenized in a large glass homogenizer kept at 4°C. Aliquots of brain homogenate were placed in chilled plastic tubes and briefly centrifuged at low speed (2000 rpm) in a cold (4°C) centrifuge to remove air bubbles. These aliquots were frozen in the same manner as the experimental brains and stored at -40°C. Immediately prior to the CO histochemistry procedure, the brain homogenate was sectioned at 5 different thicknesses (10, 20, 40, 60 and 80 μM) and two sets were included with each batch of incubation medium. This is a necessary procedure performed to generate a single regression equation between CO activity and optical density of the sections for the comparison of all tissue in the experiment. The CO enzymatic activity of the brain homogenates was determined using spectrophotometry.

Spectrophotometry of cytochrome oxidase activity

Materials

For the spectrophotometric assay, the following reagents (all materials were purchased from Sigma, St. Louis, MO) were prepared: 1) Isolation buffer (pH 7.0): 21.7 g sucrose, 0.076 g EDTA, 0.2644 g Trizma HCl, 0.039 g Trizma base, distilled water to 0.2 liters; 2) Dialysis buffer (pH 7.0): 1.405 g potassium phosphate monobasic, 2.082 g sodium phosphate dibasic, distilled water to 0.5 liters; 3) 10% sodium deoxycholate in distilled water; 4) 1% solution of cytochrome c from the horse heart in the dialysis buffer; and 5) 500 μM MB (Faulding Pharmaceuticals, Paramus, NJ) in distilled water.

Procedure

1) Ascorbic acid (0.15 g) was added to the 1% solution of cytochrome c to reduce it. The solution was inverted to mix and stored on ice in the dark.

2) A sephadex PD-10 column was equilibrated with 25 ml of the dialysis buffer, and then reduced cytochrome c solution was run through it. The eluate was collected and stored on ice.

3) After excess ascorbic acid was removed, the absorbance at 550 nM wavelength of the 10% cytochrome c solution was determined in triplicate. Next, several grains of sodium hydrosulfate were added to the cuvette, and another reading was taken. The first reading should be no less than 95% of the second reading.

4) Utilizing the first readings from the previous step, the concentration of the cytochrome c in the solution was determined, and then the number of ml needed to make 0.07% cytochrome c solution was calculated according to the formula:

(0.07*(# ml desired))/(((absorbance/extinction coefficient)/100)*12.384)= x µl cytochrome c

The extinction coefficient was determined for our spectrophotometer, Shimadzu model UV-1201V, and it was found to be 8.69.

5) An aliquot of brain tissue homogenate was then homogenized in cold isolation buffer (1 g tissue per 4 ml buffer).

6) 50 µl of this solution was mixed with 3.75 ml of isolation buffer and 200 µl of the 10% deoxycholate stock, vortexed briefly and incubated for 5 minutes at room temperature. Afterwards, the solution was vortexed again and placed on ice. This working solution was used within 30 minutes.

7) 990 µl of the diluted cytochrome c solution was placed in the cuvette and warmed up to 37˚ C. Next, 10 µl of the tissue solution was added, mixed in and placed in the spectrophotometer.

8) Change in the absorbance at 550 nm (optical absorption peak for reduced cytochrome c) was recorded over a two minute time period.

9) Steps 7 and 8 were repeated, and the absorbance data were collected by measuring at least five separate samples of the brain homogenate solution.

10) The units of reduced cytochrome c were calculated by dividing the change in the absorbance during the first minute by the extinction coefficient. To determine cytochrome oxidase activity in the brain homogenate, this number is then divided by 0.000025 to give a final µmol cytochrome c/min/g tissue value.

The cytochrome oxidase staining procedure

Frozen brain slides were carefully picked to assure best possible section quality. Because of the large number of slides processed, it was necessary to run CO histochemistry in several batches. Each batch contained the slides from the same Bregma level and two CO standard slides. This way, the between-batch differences could be accounted for, and analysis of the between-region differences was done on the slides that were processed at the same time. Frozen slides were first immersed for 5 minutes in 10% phosphate-buffered sucrose (0.1 M, pH 7.6) containing 0.5% glutaraldehyde to aid adherence of the sections to the slides. Following this, three five-minute washes in 10% phosphate-buffered sucrose were done to remove red blood cells and to gradually warm tissue to room temperature. Slides were then preincubated in Tris buffer (0.05 M, pH 7.6) containing 275 mg/l cobalt chloride, 10% sucrose and 0.5% dimethylsulfoxide. This 10 min preincubation step involving metal intensification through cobalt binding has been shown to enhance CO staining (Poremba et al., 1998). Slides were then rinsed in phosphate buffer and incubated in the 37 $^{\circ}$C incubator for one hour in an oxygen-saturated phosphate buffer containing diaminobenzidine tetrachloride and dimethylsulfoxide. Following this incubation step, tissue was fixed by a thirty minute immersion in room-temperature 4% formalin in 10% sucrose phosphate buffer. Next, tissue was dehydrated through a series of five-minute incubations in ethanol dilutions, ending with two submersions in 100% EtOH. Finally, slides were cleared by immersions in xylene and then coverslipped using Permount (Fisher Scientific, Pittsburgh, PA).

Image analysis

Using a stereotaxic atlas of the rat brain (Paxinos & Watson, 1997), as well as a cytochrome oxidase atlas of the rat brain (Gonzalez-Lima, 1998), representative sections for each brain region were chosen for densitometric analysis. The optical density (OD) in the CO stained sections was analyzed using an image-processing system consisting of a high-gain video camera, a Targa-M8 image capture board, a 486 computer, Sony color monitor, DC-powered illuminator, and JAVA software (Jandel Scientific, San Rafael, CA). The system was calibrated using an OD step tablet (Kodak, Rochester, NY) and the OD of the brain sections was used as a chromatic indicator of CO activity. Possible optical artifacts from the camera were corrected through background subtraction. A calibration curve was created with the standards using a single regression equation (Jones et al., 2001b) in order to account for between-batch staining differences. These standards were linear in the 10 to 80 μM range of thickness used. Multiple readings were taken from left and right sides of each section to avoid artifacts. For each brain region analyzed, 12 readings per region per brain were taken (four readings per section from three adjacent sections per brain). For each region measured, the size of the densitometer window was set to approximate one quarter of the size of the whole region. Adjacent measurements were taken and averaged to sample the entire region. The averaged measures resulted in one mean value per region for each subject. The size of the window was held identical across subjects, as was the number of readings for each ROI. Thus the area measured was constant across all animals and was limited to the anatomical region of interest.

Statistical analysis

In addition to the individual regions of interest, a measure of global brain activity was taken by averaging the CO activity units from all ROIs examined for any given subject. Although this average only reflects CO activity in the grey matter, it has been demonstrated that the whole brain CO activity estimate obtained in this manner is very closely correlated to the global brain average calculated from the entire set of sections in the series of a given subject (grey and white matter, r=0.98, Bruchey unpublished data). This global measure of activity was used as a reference for group differences in overall metabolic activity. Further data analysis proceeded only when it was determined that there were no between-group differences in these global measures (one-way ANOVA, $p > 0.05$).

Analysis of variance (ANOVA) was used to examine between-group differences in freezing behavior and regional brain CO activity. Since there were many regions of interest that were examined, Tukey's honest significant difference (HSD) post-hoc test was used to test the statistical significance of the results at $p \leq 0.05$. Using this approach reduced the risk of inflated Type I error rates associated with multiple tests while not discarding true differences as a result of being overly conservative. An alternative approach could have been to treat functional groups of brain areas as repeated measures on CO brain activity, and test for group main effect with an omnibus test. This approach has been justified previously (McIntosh & Gonzalez-Lima, 1995), and it is a compromise between treating regions of the brain as independent and treating all values as part of one measure. When this approach was applied to regions hypothesized to

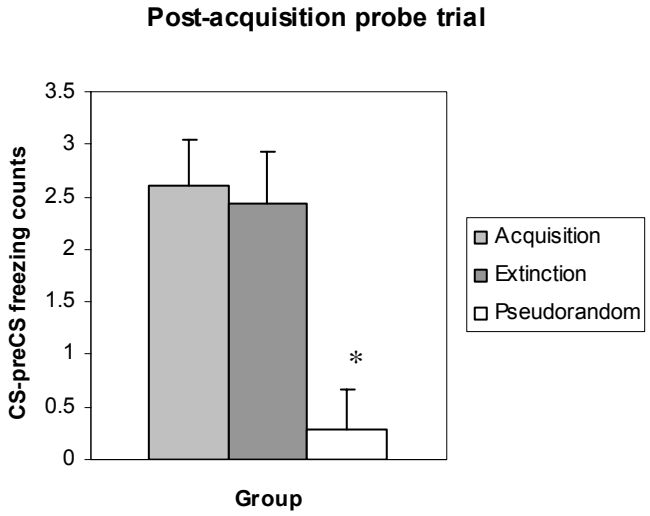
undergo training-related changes in CO activity, such as auditory system regions, the results did not differ from the results obtained using ANOVA.

Results

Behavioral results

In the post-acquisition probe trial, acquisition-only and acquisition + extinction groups showed similarly high levels of the conditioned freezing responses (Fig 5), indicating that rats in both groups successfully learned the predictive value of the tone CS. The post-acquisition probe trial was conducted out of the acquisition context, in order to assess the tone-evoked CR, without the presence of possibly confounding contextual cues. Although the CS-preCS freezing counts during this probe trial were not maximal for the two conditioned groups, freezing to the CS alone was near its upper limit value of 5. The pre-CS freezing often persisted during the entire 3 minute ITI following the first CS presentation, thus making the pre-CS count somewhat high and decreasing the CS-preCS measure. The pseudorandom group, on the other hand, displayed significantly less freezing to the CS than the acquisition-only ($F_{(1,18)} = 13.9$; $p < 0.01$) and the acquisition + extinction group ($F_{(1,17)} = 12.3$; $p < 0.01$), suggesting that in the pseudorandom group, the tone CS did not acquire associative properties as it did in the other two groups. For succinctness, henceforth I will refer to “acquisition-only” group as the acquisition group and “acquisition + extinction” group as the extinction group; however, it is important to keep in mind that cytochrome oxidase histochemistry reveals learning-related changes in brain regional metabolic capacity resulting from the entire training period.

Figure 5. Mean ± SE CS-preCS freezing counts for the post- acquisition probe trial. *$p < 0.05$



Since acquisition group rats did not undergo extinction training, their "post-extinction" probe trial freezing scores did not differ ($F(1,8) = 0.23$, $p > 0.05$) from the post-acquisition scores presented in Fig 5. Acquisition and pseudorandom group post-extinction probe trial freezing was already described in Chapter 2, page 53.

Regional brain metabolic capacity changes

The hypothesized CO activity changes in the auditory system in either acquisition or extinction group were not found in this study. However, the hypothesized neuronal metabolic capacity changes in the mPFC were observed; specifically, the anterior infralimbic (ILA) and the posterior infralimbic (ILP) areas showed between-group differences following fear acquisition and extinction training. Infralimbic cortex has been implicated in fear extinction memory consolidation or retrieval (Milad et al., 2002), and therefore we considered this area most likely to display long-lasting enhancement of neuronal metabolic capacity following the extinction training. Instead, it was found that both ILA and ILP had decreased CO activity as compared to the acquisition group ($F_{(2,26)} = 4.43$, $p = 0.02$; $F_{(2,26)} = 3.6$, $p = 0.04$), and that in the acquisition group, ILP metabolic capacity was also significantly higher ($F_{(2,26)} = 4.71$, $p = 0.02$) than in the pseudorandom group. Another mPFC region that showed between-group difference in this study was the anterior cingulate cortex. In the acquisition group, there was a 22-25% increase in CO enzymatic activity in this area as compared to the extinction and pseudorandom groups. The third brain area hypothesized to undergo training-induced metabolic capacity changes was the septal area. Acquisition of fear conditioning increased CO activity in the lateral septum by

24% as compared to the pseudorandom group. CO capacity in medial septum as well as in the ventral diagonal band was also elevated (20-22% compared to the pseudorandom), suggesting that the some of the aspects of emotional learning that accompanies fear conditioning might be preserved in these areas (Gray & McNaughton, 1983; Delamater, 2004). Interestingly, extinction group CO activity measured in LS and VDB was 17% lower than the acquisition group CO activity. It seems that extinction training conducted with the parameters utilized in this study was linked to CO enzymatic activity decreases (as compared to acquisition group) in prefrontal cortex and septal areas, thus making CO activity in these brain areas similar to CO activity measured in the pseudorandom group.

Table 7. Regional activity of cytochrome oxidase in the brains of rats that underwent tone-footshock fear conditioning, tone extinction and pseudorandom training. Means and standard errors of CO activity units expressed in μmol/min/g tissue wet weight were obtained by averaging measurements from the three consecutive brain sections per neuronal area for each subject.

GROUP	**Acquisition**			**Extinction**			**Pseudorandom**		
ROI	Mean		SE	Mean		SE	Mean		SE
MFC	290	±	12	306	±	11	310	±	10
DFC	313	±	10	311	±	8	327	±	9
LFC	314	±	14	315	±	10	338	±	10
AI	312	±	15	313	±	16	336	±	13
LO	301	±	12	323	±	9	334	±	9
MO	292	±	11	310	±	10	317	±	11
PRL	278	±	9	293	±	12	301	±	11
ILA	283	±	9	235	±	14	256	±	12
ILP	265	±	8	220	±	15	212	±	14
CG2	284	±	15	232	±	9	226	±	6
CPUA	261	±	16	223	±	10	208	±	4
GI	239	±	15	210	±	6	205	±	6
LS	241	±	11	203	±	5	194	±	8
MS	224	±	13	200	±	5	186	±	5
VDB	246	±	14	202	±	7	201	±	7
ACBS	252	±	20	217	±	11	199	±	5
ACBC	261	±	19	221	±	10	204	±	4
S1	153	±	23	195	±	24	220	±	23

CPUP	140	±	21	173	±	20	203	±	23
MPA	136	±	21	179	±	23	200	±	20
LPO	136	±	18	183	±	23	196	±	20
HDB	141	±	20	187	±	25	210	±	22
AHY	266	±	10	263	±	15	298	±	22
LH	259	±	9	271	±	17	323	±	32
PVH	231	±	13	249	±	17	261	±	21
CGPB	212	±	7	264	±	30	259	±	23
MD	193	±	7	256	±	27	235	±	20
CM	185	±	7	235	±	25	221	±	18
VBL	199	±	5	235	±	23	211	±	17
VHM	188	±	6	226	±	22	217	±	16
CEA	168	±	7	210	±	21	187	±	14
MEA	181	±	5	245	±	26	218	±	21
BLA	189	±	6	248	±	27	221	±	22
PPA	210	±	10	210	±	4	283	±	23
CA1	166	±	7	168	±	6	199	±	18
CA3	208	±	11	204	±	5	243	±	19
DG	220	±	12	222	±	6	278	±	24
HB	219	±	13	218	±	8	250	±	17
PER	192	±	14	185	±	4	235	±	20
CPC	225	±	17	222	±	6	288	±	27
MM	169	±	19	169	±	7	184	±	6
ENT	148	±	17	142	±	10	166	±	11
VTA-ant	139	±	14	134	±	12	154	±	10
DLG	157	±	10	157	±	9	176	±	10

RS	204	±	8	204	±	13	227	±	12
SUB	174	±	9	179	±	11	198	±	12
CCA1	157	±	8	157	±	7	171	±	10
CCA2	203	±	9	199	±	12	207	±	12
CCA3	184	±	9	202	±	12	211	±	13
MGD	184	±	9	207	±	13	210	±	11
MGM	190	±	8	208	±	14	205	±	11
MGV	198	±	10	200	±	15	209	±	10
TE1	216	±	9	211	±	8	230	±	12
TE3	200	±	9	202	±	8	219	±	14
VTA-post	118	±	9	130	±	13	125	±	13
CGD	163	±	3	183	±	11	171	±	9
CGLV	160	±	3	171	±	10	166	±	9
ICD	158	±	4	179	±	8	159	±	5
ICC	176	±	3	179	±	8	182	±	10
ICE	151	±	4	153	±	7	152	±	10
LLD	165	±	6	156	±	6	154	±	5
LLI	170	±	9	147	±	8	147	±	8
LLV	172	±	11	129	±	14	135	±	17
TE2	167	±	4	176	±	6	174	±	7
VCA	193	±	7	181	±	8	178	±	5
LSO	189	±	3	168	±	5	182	±	7
MSO	180	±	5	165	±	3	172	±	6
TB	157	±	6	148	±	5	154	±	7
CHL	173	±	6	163	±	4	168	±	7
CHM	184	±	6	170	±	4	171	±	6

VE	212	±	13	207	±	16	186	±	3
DPCB	185	±	4	179	±	6	176	±	5
DC	221	±	18	202	±	12	191	±	9
VCP	184	±	5	179	±	9	175	±	5
CBV	189	±	10	202	±	10	200	±	11
ECU	198	±	26	236	±	21	240	±	33
ST	168	±	9	184	±	7	200	±	11
PCR	155	±	9	171	±	8	167	±	12
SOL	177	±	14	221	±	13	188	±	7

All abbreviations are listed in table 3.

In addition to mPFC and septal regions that showed increased CO activity in the acquisition group as compared to the extinction group, CO activity increases were also present in lateral superior olivary nucleus (LSO), which showed a 20% increase in CO activity in the acquisition group as compared to extinction group.

Increased CO capacity in the acquisition group as compared to pseudorandom group was found in the nucleus accumbens, in which cells in both the core (ACBC) and the shell (ACBS) compartments showed elevated CO activity (28% and 27%, respectively) three days following the excitatory fear conditioning.

The only brain region which showed increased neuronal metabolic capacity following extinction training, as compared to the acquisition group, was the solitary tract nucleus (SOL). In this region, a 25% increase in CO activity was measured in the extinction group as compared to the acquisition only group.

The final hypothesis, that the pseudorandom group might be particularly attentive to contextual cues (since in this training paradigm the absence of a tone CS makes it difficult to accurately predict the shock US), and thus may show increasing energy demand in the hippocampal formation, was confirmed. Contextual conditioning effects (pseudorandom group CO activity greater than both acquisition and extinction group) were found in posterior parietal cortex (PPA) and dentate gyrus (DG), which showed a 25% and 20-21% increase in CO activity, respectively. Furthermore, the pseudorandom group also showed an 18% increase in perirhinal cortex (PER) and a 22% increase in caudal caudate-putamen CO activity as compared to acquisition group.

Table 8. Means and standard errors of CO activity for ROIs that showed significant ($p < 0.05$) between-group differences in CO activity.

	Acquisition		**Extinction**		**Pseudorandom**	
Acquisition greater than both extinction and pseudorandom	Mean	SE	Mean	SE	Mean	SE
ILP	265 ±	8	220 ±	15	212 ±	14
CG2	284 ±	15	232 ±	9	226 ±	6
LS	241 ±	11	203 ±	5	194 ±	8
VDB	246 ±	14	202 ±	7	201 ±	7
Acquisition greater than extinction						
ILA	283 ±	9	235 ±	14	256 ±	12
LSO	189 ±	3	168 ±	5	182 ±	7
Acquisition greater than pseudorandom						
CPUA	261 ±	16	223 ±	10	208 ±	4
MS	224 ±	13	200 ±	5	186 ±	5
ACBS	252 ±	20	217 ±	11	199 ±	5
ACBC	261 ±	19	221 ±	10	204 ±	4
Extinction greater than acquisition						
SOL	177 ±	14	221 ±	13	188 ±	7

Pseudorandom greater than both acquisition and extinction			
PPA	210 ± 10	210 ± 4	283 ± 23
DG	220 ± 12	222 ± 6	278 ± 24
Pseudorandom greater than extinction			
PER	192 ± 14	185 ± 4	235 ± 20
CPC	225 ± 17	222 ± 6	288 ± 27

Discussion

The main findings of this study include: a) the observed changes in cytochrome oxidase enzymatic activity in the prefrontal cortex and septal area following Pavlovian fear conditioning and extinction, and b) changes in CO activity in the hippocampal formation following pseudorandom presentations of CSs and USs in an excitatory context. While rats which underwent acquisition training showed elevated CO activity in anterior cingulate and infralimbic cortex, as well as in the septal area, extinction animals showed a relative decrease in CO activity in these areas. Furthermore, pseudorandom group animals seemed to have undergone contextual conditioning during the training period, as suggested by the elevated CO activity in perirhinal cortex and dentate gyrus in this group. These sustained neuronal activity-associated changes in energetic demand, and the pertinent literature relating the abovementioned brain regions and their proposed role in fear conditioning and extinction, are discussed below.

Medial prefrontal cortex in Pavlovian fear conditioning and extinction

As noted in Chapter 1, in recent years there has been increased interest in the neural substrates of Pavlovian conditioning and extinction. One of the brain regions that plays a prominent role in discussion of systems-level neural processes that accompany fear conditioning and extinction is medial prefrontal cortex, and especially the infralimbic region of the mPFC. However, literature regarding involvement of medial prefrontal cortex in rodent fear conditioning and extinction is equivocal. There are some reports that mPFC lesions do not disrupt

conditioned fear acquisition and extinction (Gewirtz, Falls, & Davis, 1997; Garcia et al., 2006; Morgan & Ledoux, 1999), and others that show that mPFC plays an important, if not essential role in these processes (Quirk et al., 2000; Santini, Ge, Ren, Pena, & Quirk, 2004). Recent evidence demonstrates that infusion of the GABA agonist muscimol into the rat infralimbic cortex enhances conditioned fear extinction (Akirav et al., 2006). It has also been proposed that mPFC may influence extinction performance through processes occurring during acquisition rather than only during extinction (Morgan et al., 2003). Some of the ambivalence probably stems from different methodological approaches used to measure mPFC changes (Milad et al., 2002; Barrett et al., 2003; Herry et al., 2004), and some of it can likely be due to differences in the training protocols utilized by various researchers (Santini et al., 2004; Barrett et al., 2003; Milad et al., 2002). For example, time interval between fear acquisition learning and extinction training may play an important role in fear conditioning and extinction memory consolidation, and in the differential recruitment of brain regions such as mPFC (Akirav et al., 2006). Therefore, the functional involvement of mPFC in the acquisition and extinction of conditioned fear responses is not yet fully understood.

The observed CO changes in the infralimbic cortex (IL) in the present study support involvement of IL in fear-inducing situations. Following the acquisition of Pavlovian fear conditioning, ILP showed a 25% increase in CO activity in the acquisition group. In contrast, when fear acquisition training was followed by CS extinction training, CO activity in this region decreased to where it more closely resembled CO activity found in the pseudorandomly trained group. A similar trend was seen for the ILA, suggesting that the increased IL CO activity

is associated with the acquisition of fear conditioning, and extinction training has a tendency to attenuate this increase. These results are similar to fMRI findings in which spider phobics show a therapy-induced attenuation of prefrontal cortical activity (Paquette et al., 2003). Increased mPFC activity has also been proposed to correlate with the maintenance of anxiety during a repeated confrontation with phobia-related stimuli (Straube et al., 2006), suggesting that attenuation of metabolic activity in this brain area might be important for successful fear extinction.

Research findings that support the role of IL in fear extinction in rodents assign an inhibitory role to this region. It is thought that through an increase in the IL output, the central nucleus of amygdala is inhibited, resulting in reduction of the CR (Pare et al., 2004; Sotres-Bayon et al., 2006). Milad et al. (2004) have shown that stimulating IL can lead to quicker extinction of the CR. Our data support the role of IL as a region that can modulate CR inhibition. Prior to training, mPFC might exert an overall inhibitory role on lower brain regions, as suggested by many studies demonstrating loss of behavioral inhibition following mPFC injury (Lacroix, Spinelli, White, & Feldon, 2000; Miller & Cohen, 2001; Schneider & Koch, 2005). After Pavlovian fear conditioning, which renders a behavioral response such as conditioned freezing appropriate, there may be a decrease in mPFC inhibition of regions such as the amygdala. Following the extinction training, the IL region of mPFC might regain inhibitory control over behavioral responding so that the more appropriate reduction of CR is expressed. Our data showed that 24 hours following the extinction of Pavlovian fear conditioning, there was a decrease in energy demand in IL cortex, possibly indicating reduced firing rates of inhibitory interneurons in this brain region.

Decrease in GABA release could result in differential modulation of IL output neurons which synapse on neurons in response-output regions (such as the amygdala). More specifically, increased firing rates of the IL output cells might result in more activation of intercalated amygdaloid cells, which would lead to net inhibition of CeA output, thereby decreasing the freezing CR.

Alternatively, CO activity changes measured in IL may have directly resulted from changes in neuronal firing rates of IL output neurons, not in the indirect changes in their activity stemming from decreased inhibitory input. At the systems level of analysis used in this study, differentiation between the proposed mechanisms of CO activity change was not attempted. Future studies could address this question, because, unlike FDG metabolic mapping technique, CO histochemistry can provide a cellular-level resolution of metabolic brain changes. Since CO activity changes recorded in IL in this study could be due to one or both of the mechanisms described above, the important information obtained is less the direction of the CO activity changes, and more the evidence that Pavlovian fear conditioning and extinction alter oxidative energy metabolism in the IL.

The biphasic nature of CO changes recorded in this study also suggests that CO enzymatic activity may be adapting to variations in brain metabolic demand a bit more quickly than previously hypothesized. Under certain circumstances, CO activity might change after only a few hours of altered demand for energy, as opposed to days or weeks (Bennet et al., 2006). Future studies are needed to better characterize time course of CO activity changes associated with increased or decreased energy demand in an intact brain. Nonetheless, CO enzymatic activity changes are likely to persist in the brain until

there is a shift in metabolic demand associated with the new learning experiences, which may also change functional networking between different brain regions.

Anterior cingulate cortex and conditioned fear extinction

While fear conditioning increases regional cerebral blood flow (Fischer, Andersson, Furmark, & Fredrikson, 2006), c-fos expression (Beck et al., 1995) and stimulus-related functional activation in the anterior cingulate (Knight, Smith, Stein, & Helmstetter, 1999), role of this brain region in conditioned fear extinction is more unclear. Some report an increase in differential blood oxygenation level-dependent (BOLD) response during conditioned fear extinction in this area (Phelps et al., 2004), while others report decreased anterior cingulate activity following fear extinction (Bremner et al., 2005; Straube et al., 2006). In our study, CO activity in the anterior cingulate cortex was found to be elevated 3 days following tone-footshock conditioning, while no differences in CO activity were found between pseudorandomly trained animals and animals that underwent extinction training.

Since anterior cingulate cortex (CG2) has several proposed functions and anatomical subdivisions, it is possible that differential findings regarding its role(s) in conditioned fear extinction could be reconciled through a careful dissociation of all aspects of fear conditioning and training, as well as consideration of the exact neuroanatomical correlates of metabolic changes measured. The “affect” component of CG2 modulates autonomic activity and internal emotional responses, contributing to internal context representation, while the “cognition” division is engaged in response selection associated with

skeletomotor activity and responses to noxious stimuli (Devinsky, Morrell, & Vogt, 1995). Because feature extraction from the environment and sensory-motor integration have been associated with anterior cingulate cortex (Vogt & Miller, 1983), then the CO activity increase in this area following fear conditioning might reflect CG2 involvement in the assemblage of the conditioned freezing response. In the extinction group, anterior cingulate CO activity was not as elevated as it was in the acquisition group. Acquisition-only group also had elevated CO activity in anterior caudate-putamen, as compared to the pseudorandom group, further suggesting that acquisition group animals sustained elevated preparation for action, motor control and performance monitoring.

However, CG2 most likely functions as more than only a motor-response integrating region. It has been suggested that mPFC areas such as CG2 might be a part of a core anxiety system relevant for sustained evaluation of potential danger and the subjective experience of fear (Rauch, Savage, Alpert, Fischman, & Jenike, 1997). Furthermore, CG2 has been implicated in the elicitation and control of sympathetic autonomic arousal (Critchley, 2003), which is one of the main physiological features present during the processing of phobogenic stimuli. Perhaps the most convincing argument for role of CG2 in fear extinction comes from a recent fMRI study which demonstrated that subjects with a specific phobia showed greater responses in anterior cingulate than control subjects when presented with videos of feared stimuli (Straube et al., 2006). Cognitive-behavioral therapy strongly reduced phobic symptoms and hyperactivity of anterior cingulate, suggesting that increased activation in CG2 is associated with specific phobias, while an attenuation of this brain response correlates with successful therapeutic intervention (Straube et al., 2006). CG2's role in

multimodal processing and sensorimotor integration could facilitate a modality-specific involvement in the expression of fear, as in the case of specific phobias, or our tone CS-evoked freezing. CG2 metabolic activity measures obtained with both FDG and CO methodology in this study seem to be in agreement with this proposition.

Septal areas and fear conditioning

We hypothesized that Pavlovian fear conditioning might lead to CO activity changes in septal areas since the US (footshock) used in this study is neurogenic (or emotional or psychological), and in view of evidence supporting a critical involvement of the limbic structures in affixing an emotional valence to environmental events. However, the direction of CO activity changes in the septum was difficult to predict. A large number of studies suggest that the excitation of LS neurons can inhibit fear, whereas fear can be disinihibited following the inhibition of LS neurons (Desmedt, Garcia, & Jaffard, 1999; Sheehan et al., 2004; Sparks & Ledoux, 1995; Brady & Nauta, 1953; Vouimba, Garcia, & Jaffard, 1998). On the other hand, there is also evidence which shows that enhanced activity within the septum promotes stimulus-induced fearful behavioral responses (Thomas & Evans, 1983; Sheehan et al., 2004; Thomas, 1988) while damage to the septum decreases the expression of fear (Solomon, Solomon, van der Schaaf, & Perry, 1983; Asaka, Seager, Griffin, & Berry, 2000). For example, Mongeau et al. (2003) found that mice which displayed freezing behavior had increased neural activity in the lateral septum, as measured by the c-fos method. The lateral septum, through its connectivity with amygdala and hippocampal formation, plays a role in establishing and modifying the CS-US

association (Desmedt et al., 1999). Medial septum also plays a role in fear conditioning and extinction. Classical conditioning is impaired following lesions of the medial septum (Rokers, Mercado, Allen MT, Myers, & Gluck, 2002), and Siegel (1976) described a more rapid extinction of an appetitive CR in animals with lesions of medial septum. A theoretical model of septohippocampal dynamics predicts that levels of medial septal activity should be low during extinction (Rokers et al., 2002). Our data lend support to findings that medial and lateral septal areas play a role in Pavlovian fear acquisition and extinction, possibly by modulating mPFC and VDB influences on the CR. Previous data from our laboratory showed decreased FDG uptake in medial and lateral septum of rats showing conditioned response inhibition (Jones et al., 2001a) and a recent study by Conejo et al., (2005) found negative neurobehavioral correlations between CO activity in the medial septum and conditioned fear. The septal region is an attractive candidate for one of the brain areas in which pharmacological intervention may have some bearing on anxiety disorders. When GABA-ergic benzodiazepines are injected into LS, fear in animals is reduced, possibly through disinhibition of septal output (Sheehan et al., 2004). Electrical stimulation of LS and benzodiazepine administration into LS both inhibit activation of amygdala neurons (Yadin, Thomas, Grishkat, & Strickland, 1993; McGregor, Hargreaves, Apfelbach, & Hunt, 2004), suggesting that increasing GABA-ergic output of LS neurons may contribute to a decrease in anxiety.

LS can also contribute to regulation of nucleus accumbens (NAcc) function, whose neurons are proposed to form an interface between limbic and motor areas (Mogenson & Yang, 1991). NAcc can influence dopamine release in the caudate putamen and thereby speed the rate at which an action will be

carried out (Mogenson et al., 1991). This brain region is also thought to play a role in motor response planning via its projections to prefrontal cortex (Groenewegen, Wright, Beijer, & Voorn, 1999). Since levels of GABA released into the NAcc from lateral septum regulate its activity (Sheehan et al., 2004), and given our results showing an increase in CO activity in both lateral septum and NAcc in the acquisition group, CO activity in NAcc might be attributable to CR-related changes.

Hippocampal formation in fear conditioning

While the acquisition and extinction groups received tone-footshock pairings during the conditioning phase of their training, pseudorandom group animals could not utilize the auditory stimulus as a reliable signal for the imminent US. Therefore, the training paradigm used with this control group closely resembles contextual conditioning, which is thought to engage the hippocampal formation (Maren et al., 1997b; Maren et al., 1997a; Fanselow, 2000; Rudy, Huff, & Matus-Amat, 2004). Indeed, following the pseudorandom training, we found large (20-25%) increases in CO activity in some of the hippocampal regions in the pseudorandom group. The pattern of CO activity found between the conditioned and pseudorandomly trained groups in the hippocampal formation and the septum fit the model of increased septal activity when the CS-US paired procedure is used, as opposed to increased hippocampal and unaffected septal activity when contextual conditioning is used (Desmedt et al., 1999). Unlike the mPFC, in which changes in CO activity might reflect differential inhibitory interneuron neurotransmission, excitatory output neuron firing, or both, hippocampus is composed of mostly excitatory neurons.

An electron microscopy study by Megias et al. (2001) characterized the total synaptic input to CA1 pyramidal cells and estimated only 1700 inhibitory inputs compared with 30,000 excitatory inputs. The inhibitory synapses account for only 5% of the total synaptic input to these cells, suggesting that there is a great excess of excitatory input which is balanced by what seems a trivial inhibitory drive. Although this seeming imbalance in the excitatory and inhibitory inputs is probably attenuated by the positions of these synapses (inhibitory ones likely closer to the cell body and able to exert stronger influence on action potential generation), the most likely explanation for the observed CO activity changes in the hippocampus is the increased excitatory neurotransmission in this region.

The finding that the posterior parietal cortex and caudal caudate-putamen both had higher CO activity in the pseudorandom group as compared to the other two groups also suggests that the pseudorandomly trained rats were engaged in the processing of contextual stimuli. Posterior parietal cortex is considered a large associative cortical region, where afferents from different sensory modalities are integrated to provide the basis for perceptual processes such as space perception (Cavada & Goldman-Rakic, 2004; Andersen, 1997), and caudal caudate-putamen receives overlapping representations of sensorimotor, visual and auditory information (McGeorge & Faull, 1989). Both of these areas likely participate in the neural processing of environmental dangers. CO activity changes in dentate gyrus and the perirhinal cortex found in the pseudorandom group, as compared to acquisition and extinction groups, support the view that these brain areas play a role in contextual fear conditioning (Corodimas et al., 1995; Riedel, Casabona, Platt, Macphail, & Nicoletti, 2000; Ferreira, Moreira, Ikeda, Bueno, & Oliveira, 2003).

Additional brain regional effects

CO activity increases were found in the acquisition group as compared to the pseudorandom group in the nucleus accumbens and the anterior caudate-putamen. Both of these regions have been linked to the consolidation of memories of the emotionally arousing experiences through their connections with the amygdala (McGaugh, 2004). Nucleus accumbens, in particular, might be "…a critical locus of converging BLA and hippocampal modulatory influences on memory consolidation" (McGaugh, 2004). Nucleus accumbens core region neurons have been found to participate in fear memory for both contextual and discrete stimuli associated with the US (Thomas, Hall, & Everitt, 2002). Therefore, this brain region may contribute to consolidation and storage of excitatory fear conditioning memory.

Another region of interest for Pavlovian fear conditioning and extinction is nucleus of the solitary tract (SOL). SOL is a brain stem nucleus that sends noradrenergic projections to the amygdala, thus modulating amygdaloid output to other brain regions involved in memory consolidation (McGaugh, 2004). In the present study SOL showed increased metabolic capacity in the extinction group, as compared to the acquisition group, suggesting that SOL may be an important contributor to fear extinction memory consolidation.

In conclusion, our results support earlier findings suggesting that quantitative CO histochemistry can be successfully used as an alternative metabolic mapping technique to evaluate regional effects of learning in the brain (Poremba et al., 1998; Conejo, Gonzalez-Pardo, Vallejo, & Arias, 2004). We found that fear conditioning and extinction training involved infralimbic and

anterior cingulate cortex, as well as septum, nucleus accumbens and solitary tract nucleus. We also found evidence of hippocampal involvement in contextual fear conditioning using this technique. The use of CO histochemistry provides a way to assess metabolic changes in the brain which result from the entire training period, thus making these results complementary to the stimulus-evoked regional metabolic changes measured with the FDG metabolic mapping technique.

Chapter 4: Extinction memory improvement by the metabolic enhancer methylene blue

Introduction

MB is a non-neuroleptic dye previously used safely in humans as a neuroprotective metabolic agent for treatment of dementia, depression and drug-induced encephalopathy (Naylor, Martin, Hopwood, & Watson, 1986; Wainwright & Crossley, 2002). MB serves as a redox compound that at low doses (1-5 mg/kg) improves mitochondrial respiration (Visarius et al., 1997) and prevents free radical damage (Salaris, Babbs, & Voorhees III, 1991). Low-dose MB acts on the electron transport chain, and increases cellular oxygen consumption by a well-known mechanism of action that involves accepting electrons from molecular oxygen (Lindahl & Öberg, 1961). When MB acts as an alternative electron acceptor in mitochondria, it also inhibits the production of superoxide by competing with molecular oxygen (Salaris et al., 1991).

Low-dose MB increases brain cytochrome oxidase activity after intraperitoneal administration in rats (Callaway et al., 2004). Cytochrome oxidase is the terminal enzyme in the electron transport, and it catalyzes the utilization of molecular oxygen to form water and ATP in the process known as oxidative phosphorylation (Wong-Riley, 1989). The brain is the organ most dependent on oxidative phosphorylation for the production of metabolic energy (Sokoloff, 1992). Therefore, MB could be used to increase brain cytochrome oxidase activity and thereby improve oxidative energy metabolism in the brain.

Low doses of MB significantly enhance memory retention in both aversive and appetitive tasks. For example, post-training MB administration improves

memory retention tested 24 h after inhibitory avoidance training (Martinez et al., 1978), as well as memory retention of tone-footshock conditioning tested 22 days later (Wrubel, unpublished data). Corresponding low doses of MB also increase spatial memory retention in a holeboard food search task (Callaway et al., 2002, 2004). Intrigued by these findings, we have investigated MB as a possible metabolic enhancer that could improve retention of memory for extinction of a conditioned response. Specifically, in the first two experiments, we tested whether 4 mg/kg of MB injected for five days post-extinction could enhance the retention of extinction of a conditioned freezing response in rats, without affecting general fearfulness and anxiety.

Extinction is a behavioral phenomenon characterized by the reduction of the conditioned response that occurs as a consequence of non-reinforcement. Pavlov (1927) hypothesized that the neural mechanism of extinction involved the formation of cortical circuits that inhibited a conditioned response by counteracting the previously acquired excitatory associations between the conditioned stimulus (CS) and the unconditioned stimulus (US). We hypothesized that if post-extinction MB enhances extinction memory by increasing brain cytochrome oxidase activity, the brain regions involved in consolidation of extinction memory would be more activated in rats showing better retention of extinction. Therefore, in this study we investigated whether low-dose MB administered after extinction would enhance extinction memory through a mechanism involving increased cytochrome oxidase activity in the rat brain.

The second hypothesis tested with the third experiment was that if a low-dose of MB can enhance the retention of extinction learning, then rats

administered MB during the extinction would have less spontaneous recovery of CR when tested several days after the extinction training. We chose to test for the spontaneous recovery of CR either 10 or 22 days following the completion of extinction training based on Quirk's (2002) study that showed that conditioned freezing to the tone gradually recovers with time to reach 100% by day 10, and on unpublished data from our laboratory that show that MB's memory-enhancing effects can be long-lasting (22 days).

Materials and methods

Subjects

Subjects were 63 Long-Evans male rats (n = 16 in experiment 1, n = 23 in experiment 2, n = 24 in experiment 3; Harlan, Houston, TX) weighing an average of 135g at the start of the experiment. Each rat was handled daily for 3 minutes for 7 days prior to the start of the experiment. Rats were given food and water ad libitum. They were housed 3-4 rats per cage and kept on a 12-h light/dark cycle. All procedures were conducted in agreement with the American Association for the Accreditation of Laboratory Animal Care and were approved by the Institutional Animal Care and Use Committee.

Experiment 1

Behavioral training

We used the same apparatus described previously in chapter two. Behavioral training lasted 12 days (Table 9).

Table 9. Experimental Design

Day	Procedure	Context	Treatment
1 - 2	Habituation	A	Context exposure
3 - 4	Acquisition	A	Tone-shock pairing
5 - 6	Extinction	B	Tone alone presentations
7 - 12	Probe with tone	B	Post-extinction MB or saline

On days 1 and 2 animals were carried one at a time in a dark container to an operant chamber (MED Associates, St. Albans, VT) illuminated with a red light bulb and enclosed in a sound-attenuated box. They were allowed to explore the chamber for one hour, after which they were placed back in their home cage. On days 3 and 4 subjects underwent acquisition training, in which four CS tones (15-second, 65 dB, 1-2 kHz frequency modulated tones, generated by two Wavetek Sweep/Modulation generators) coterminated with a 0.5 mA, 0.75 second footshock (US). The acquisition sessions lasted 15 minutes each, with an average inter-trial interval of three minutes. Animals were placed back in their home cages after the completion of each session. On day 5, one 10-minute post-acquisition probe trial was conducted with three 15-second CS tones in a different context which consisted of a clear plastic cage (10" x 7.5" x 6") with a speaker mounted on top.

Freezing was defined as a rat having all four feet on the floor, with shallow, rapid breathing and minimal head and vibrissae movement for at least three seconds. Freezing was scored for 15 seconds prior to the CS onset as well as during CS presentations in 3-second bins. The normalized freezing score was computed as average CS freezing counts minus pre-CS freezing counts. Two hours after the post-acquisition probe session rats underwent extinction training in the probe context. On the first day of extinction training, rats were presented every three minutes for an hour with a non-reinforced CS tone (18 tones per session). On the second day of extinction training, animals received another 18 non-reinforced CS presentations; then 2 hours later they were exposed to a series of 5-second on, 1-second off CS presentations for another hour to

simulate the testing session used in our previous studies with fluorodeoxyglucose (Barrett et al., 2003).

Subjects were returned to their home cages following the extinction training, and 30 minutes later eight rats were injected intraperitoneally with saline, and another eight rats were injected with 4 mg/kg methylene blue (MB, Faulding Pharmaceuticals) dissolved in saline. This dose was selected because it is the same one given chronically to humans without side-effects (Naylor et al., 1986; Peter et al., 2000). Twenty four hours after the injection, animals were tested for freezing responses during the pre-CS period of 15 seconds and during the CS presentation using the same protocol as for the post-acquisition freezing probe session. After the post-extinction probe session, animals were transferred back into their home cage, left for 30 minutes, and then injected with saline or MB. The injections were administered 3 more times, 24 hours apart, for a total of 5 drug administrations and 5 post-extinction probe sessions. After the last probe session, rats were decapitated and their brains quickly extracted.

Brain biochemistry

One brain hemisphere was rapidly frozen in isopentane and stored at -40°C. The other hemisphere was homogenized on ice using glass tissue homogenizer and frozen in isopentane. Our spectrophotometric procedure described in chapter 3 was used to determine absolute units of cytochrome oxidase activity in brain homogenates.

Experiment 2

Behavioral training

A control experiment with separate groups of rats was conducted to rule out that the MB effects on post-extinction freezing found in experiment 1 could be due to nonspecific increases in motor activity or decreased fearfulness. One group of rats (n=11) was injected with saline, a second group of rats (n=6) was injected once with MB (4 mg/kg, i.p.) and a third group of rats (n=6) was given repeated MB injections as in experiment 1 (one injection daily for five days).

All the parameters were the same as in experiment 1 except that the three groups of rats were evaluated in an automated open field activity monitoring system from MED Associates (St. Albans, Vermont, USA). The open field chamber (17" x 17" x 12") had clear plastic and a white Plexiglas floor. Four arrays of 16 parallel infrared motion detector beams spaced 1" apart and located 1" and 7" above the chamber floor were used to record behavioral measures. The measures recorded were: ambulatory distance, ambulatory time, ambulatory counts, stereotypic time, stereotypic counts (defined as movement without displacement), resting time, vertical counts (defined as number of upper beam brakes), vertical time and number of center zone entries (center defined as 38% of the area). The chambers were cleaned between animals with a mild detergent solution. After a five minute habituation period in the room, rats were placed individually in the chamber facing one corner and their motor behavior was automatically recorded for ten minutes.

Fear-related behavior was evaluated during the first five minutes of exposure to the open field, by testing for thigmotaxic behavior (time in periphery)

before injections, or 24 h after one injection of MB or saline, or 24 h after the last of five MB injections.

Experiment 3

The goal of this experiment was to investigate whether methylene blue (MB) can be used to postpone the occurrence of spontaneous recovery of CR in rats without having any non-specific motor or anxiolytic effects.

Behavioral training

We used the same apparatus described for experiment 1. Behavioral training lasted 30 days. On days 1 and 2 the animals were carried one at a time in a dark container to an operant chamber (MED Associates, St. Albans, VT) illuminated with a red light bulb and enclosed in a sound-attenuated box. They were allowed to explore the chamber for one hour, after which they were placed back in their home cage. On days 3 and 4 the subjects underwent acquisition training, in which four CS tones (15-second, 65 dB, 1-2 kHz frequency modulated tones, generated by two Wavetek Sweep/Modulation generators) coterminated with 0.5 mA, 0.75 second footshocks (US). The acquisition sessions lasted 15 minutes each, with an average inter-trial interval of three minutes. Animals were placed back in their home cages after the completion of each session. On day 5, one 10-minute post-acquisition probe trial was conducted with three 15-second CS tones in a different context which consisted of a clear plastic cage (10” x 7.5” x 6”) with a speaker mounted on top, a novel betadine odor cue and dim white light illumination.

The conditioned response (CR) measured was freezing behavior, which was scored for 15 seconds prior to the CS onset as well as during CS presentations in 3-second bins. The normalized freezing score for probe trials was computed as average of CS freezing counts minus pre-CS freezing counts. Extinction sessions freezing scores were represented as freezing to the tone CS, because animals showed tendency to continue freezing throughout the inter-trial interval, especially during the first few CS presentations in the initial extinction session.

Two hours after the post-acquisition probe session, the rats underwent extinction training in the probe context. There were four days of extinction training, and on each day, the rats were presented every three minutes with a non-reinforced CS tone (9 tones per session, 30 minutes total). Following the first extinction training session, they were placed back in their home cages for 30 minutes, then injected intraperitoneally with either saline or 4 mg/kg methylene blue (MB, Faulding Pharmaceuticals, Paramus, NJ) dissolved in saline. Twenty four hours after the injection, animals underwent another extinction session, followed by a subsequent MB or saline injection. This was repeated two more times for a total of 4 extinction sessions and four injections. Thus, the cumulative amount of time dedicated to extinction sessions was kept the same as in experiment 1 (120 minutes), as was the total number of non-reinforced CS presentations (36). Freezing was scored during all four extinction training sessions. Twenty four hours following the last day of extinction training and the last drug administration, animals were tested in a post-extinction probe session (3 CS tones in 10 minutes) and then evaluated in a light-dark exploration testing chamber.

The light-dark exploration test has previously been used to assess the anxiety level in rodents (Gaalen et al., 2002), so we employed it to measure possible non-specific motor or anxiety-related effects of repeated MB administration. The automated open field activity monitoring system from MED Associates (St. Albans, Vermont, USA) consisted of an open field chamber (17" x 17" x 12") with clear plastic walls and a white Plexiglas floor divided in half by a dark insert. After a five minute habituation period in the room, rats were placed individually in the dark compartment of the chamber and their motor behavior was automatically recorded for ten minutes. Animals were placed in the center of a dark part of the open field, but could move freely between the two compartments through a small opening located in the center of the dark insert. Four arrays of 16 parallel infrared motion detector beams spaced 1" apart and located 1" and 7" above the chamber floor were used to record behavioral measures. Latency for animals to enter the light chamber was also scored by a blind observer independently of the computer-generated measure of time to enter the light zone. The measures recorded for each compartment were: ambulatory distance, ambulatory time, ambulatory counts, stereotypic time, stereotypic counts (defined as movement without displacement), resting time, vertical counts (defined as number of upper beam brakes), vertical time, latency to transition between two zones and a number of zone entries. The chambers were cleaned with a mild detergent solution between animals.

Following this testing session, all rats were left undisturbed in their home cages for nine days. On the tenth day, the animals were tested in the light-dark box test again, and two hours later the first post-extinction probe session was conducted identical to the post-acquisition probe session. Rats were then placed

back in their home cages for another 12 days, after which a second probe session was conducted identical to the first one. The timing of the post-extinction probe sessions conducted was based on the Quirk (2002) study showing that after successful extinction training it takes an average of 10 days for most rats to show spontaneous recovery of CR, and on the work done in our laboratory showing that MB effect on memory retention can be observed 22 days following its administration (unpublished data). Therefore, we chose to test at these two time points in order to examine retention of extinction memory as demonstrated by a delay of spontaneous recovery in animals treated with the repeated doses of MB.

Statistical analysis

Repeated measures ANOVA followed by Tukey's post-hoc tests was used to examine the effects of MB administration on behavioral performance during the extinction and probe trials. Group differences in spectrophotometric cytochrome oxidase values and average probe trial measures of freezing were compared using 2-tailed Student's t-tests between 2 independent groups, with significant group differences tested at two-tailed $p < 0.05$. The t and p values are reported in the results section.

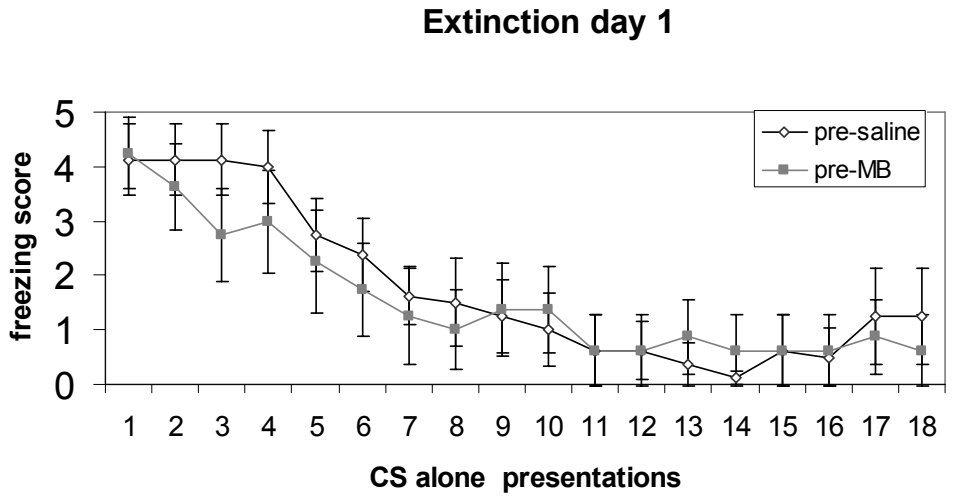
Results

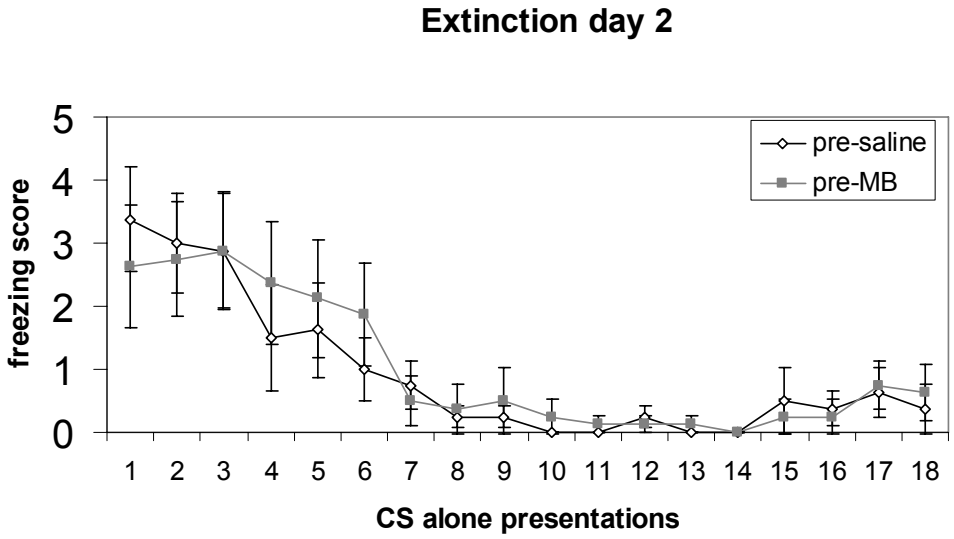
Experiment 1

Acquisition and extinction behavior

All subjects showed similar acquisition and extinction prior to the beginning of MB or saline administrations. On a 0 to 5 scale, with a 0 behavioral score indicating no freezing, and a score of 5 representing maximum possible freezing counts, the mean pre-saline group CS freezing score was 4.7 ± 0.22, and the mean pre-MB group CS freezing score was 4.29 ± 0.42. During the two days of extinction, all rats showed similar extinction curves (Fig. 6).

Figure 6. Mean ± SE freezing counts during the two days of extinction, before MB and saline injections.

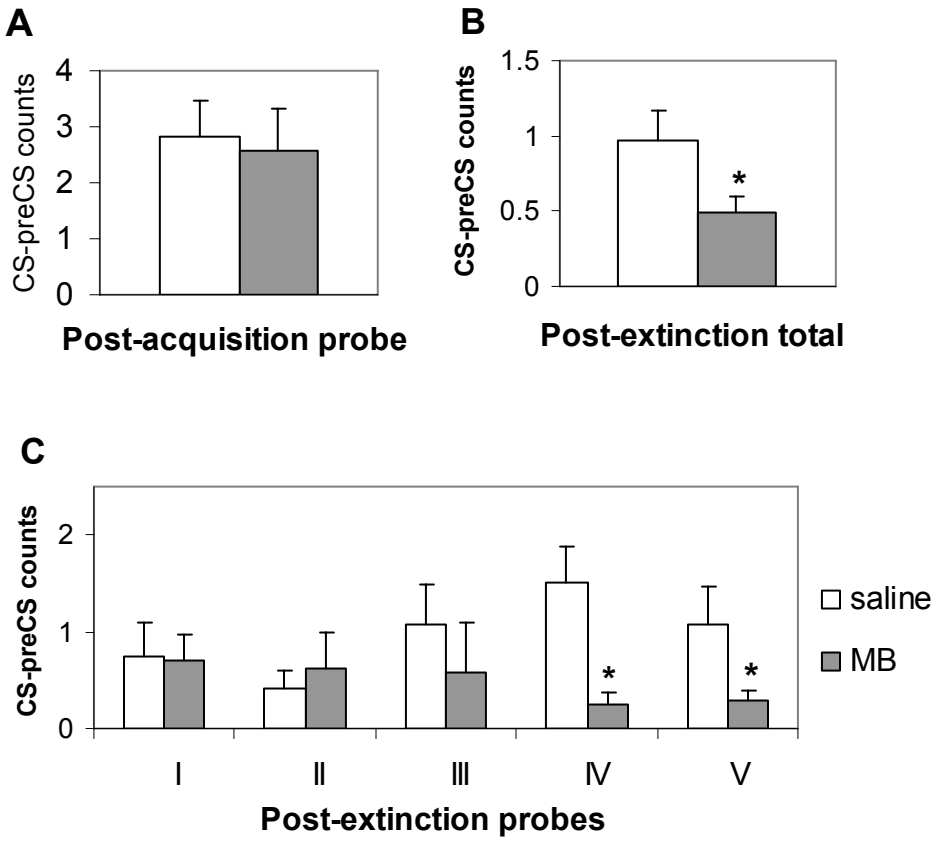




Effects of MB on the retention of extinction

The effects of MB and saline were evaluated during pre-CS (15 seconds before tone) and CS periods (15 second tone) in five post-extinction probes (probes I-V, with 3 tones each). Each probe is reported as the mean of the three Cs-preCS freezing counts. There were no significant group differences in pre-CS freezing (saline: 0.31 ± 0.13, MB: 0.45 ± 0.23, $t = -0.546$, $p = 0.593$). Normalized freezing scores were computed as CS – preCS freezing counts for each trial for each subject, and mean group results are shown in Fig. 7. Figure 7A shows that after two days of acquisition training with 4 tones (CS) coterminating with footshocks (US), all animals showed similar CS - preCS freezing scores in the post-acquisition probe trial.

Figure 7. Behavioral effects of methylene blue on the extinction of conditioned freezing. (A) Mean ± SE freezing scores (CS-preCS freezing counts) after two days of acquisition training before the onset of MB/saline treatments; (B) mean ± SE total post-extinction freezing scores in the MB-treated and saline-treated groups; (C) mean ± SE freezing scores in the MB-treated and saline-treated groups in post-extinction probes I - V. * $p < 0.05$.



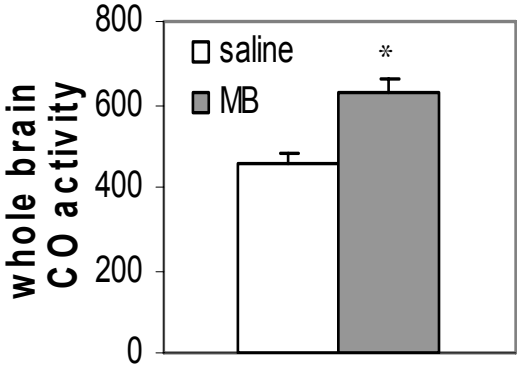
The averaged post-extinction freezing CS – preCS scores were significantly lower in animals receiving MB as compared to the control group (Fig. 7B), suggesting that MB enhanced retention of the extinction memory. Overall, there was a group effect of MB administration ($t = 2.328$, $p = 0.048$), with saline-administered animals freezing about twice as much as MB-treated animals.

Figure 7C shows that the MB-treated animals had a longer-lasting extinction effect as seen in probes IV and V. On average, saline-treated rats showed freezing scores that were five times greater than those of MB-treated rats in the last two probes. But in post-extinction probe I there were no significant differences ($p > 0.05$) in the freezing scores between the two groups. Thus, repeated daily MB injections improved retention of extinction memory in MB subjects as compared to the saline-injected subjects.

Effect of MB on absolute brain cytochrome oxidase activity

The brain homogenates of MB-treated animals showed an overall increase in absolute units of cytochrome oxidase activity that was 38% greater than the activity measured in brains from the saline-treated rats ($t = -4.116$, $p = 0.001$). Figure 8 shows the spectrophotometric analysis of the rate of cytochrome c oxidation in the brain homogenates of MB-treated and control rats. Animals receiving MB after extinction showed both greater absolute brain metabolic activity and greater retention of extinction memory.

Figure 8. Overall absolute brain cytochrome oxidase (CO) activity units (μM/min/g) in the brains of MB-treated and saline-treated groups. Asterisk indicates significant group difference at $p < 0.001$.



Experiment 2

Effects of MB on general motor activity and fearfulness

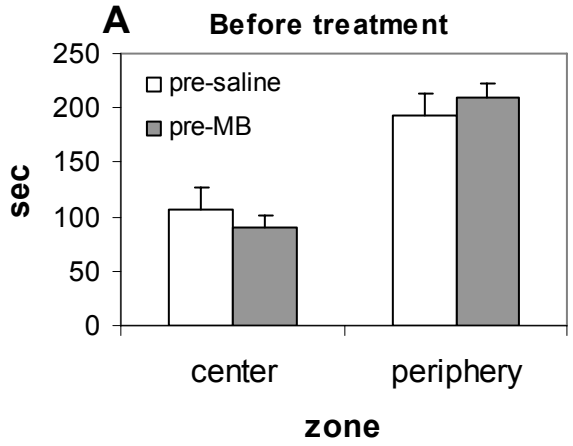
In experiment 1, the subjects treated with saline or MB showed no group differences in pre-CS freezing behavior and average number of fecal boli during the post-extinction probe sessions (saline: 1.85 ± 1.19; MB: 1.75 ± 1.21; $t = -0.198$, $p = 0.847$). To further evaluate whether there were nonspecific motor and fear-related effects of MB, a second experiment was conducted with separate groups of rats treated with saline or with one or five MB injections. Experiment 2 showed that there were no differences in motor activity measures in the open field after saline or repeated MB injections ($p > 0.05$, Table 10).

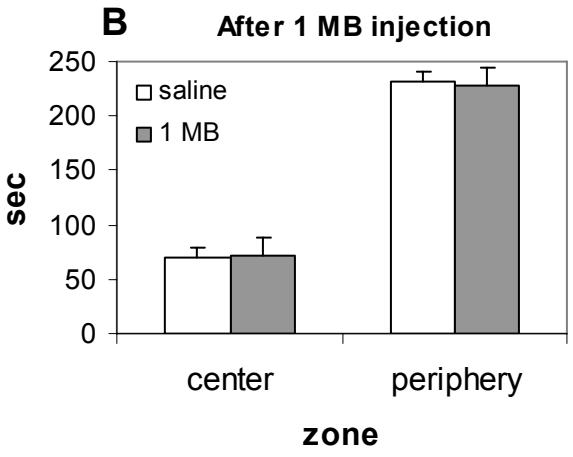
Table 10. General activity measures (mean ± SE) in saline and MB-treated groups

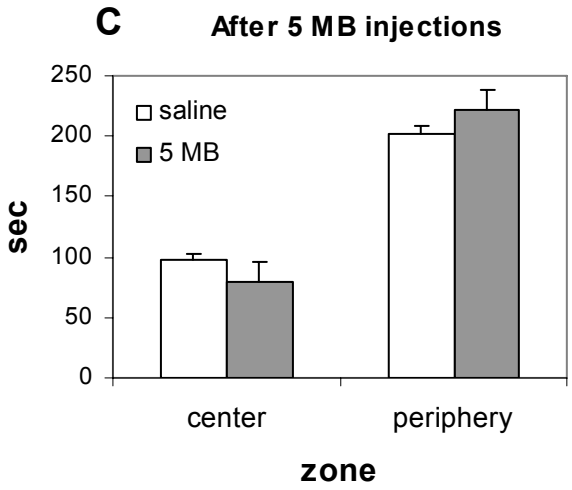
Measures	Saline	1 MB	5 MB
Ambulatory Counts	1014 ± 192	715 ± 164	796 ± 155
Vertical Counts	83 ± 7	79 ± 8	76 ± 9
Center Zone Entries	421 ± 59	379 ± 52	358 ± 64

Fear-related behavior evaluated with center-avoidance/thigmotaxic behavior also served to rule out that MB effects were simply due to a general decreased fearfulness as opposed to enhanced extinction memory. Subjects spent a similar amount of time in the center and periphery of the open field in each group: before treatment (Fig. 9A), after 1 MB injection (Fig. 9B) or after 5 daily MB injections (Fig. 9C), indicating that there was no evidence of general decreased fearfulness with repeated MB injections.

Figure 9. Behavioral effects of methylene blue on fear-related behavior. Mean ± SE time spent in center and periphery (A) prior to any treatment; (B) 24 h following one MB injection; (C) 24 h after five daily MB injections.






C
After 5 MB injections
250
200
150
100
50
0
sec
saline
5 MB
center
periphery
zone

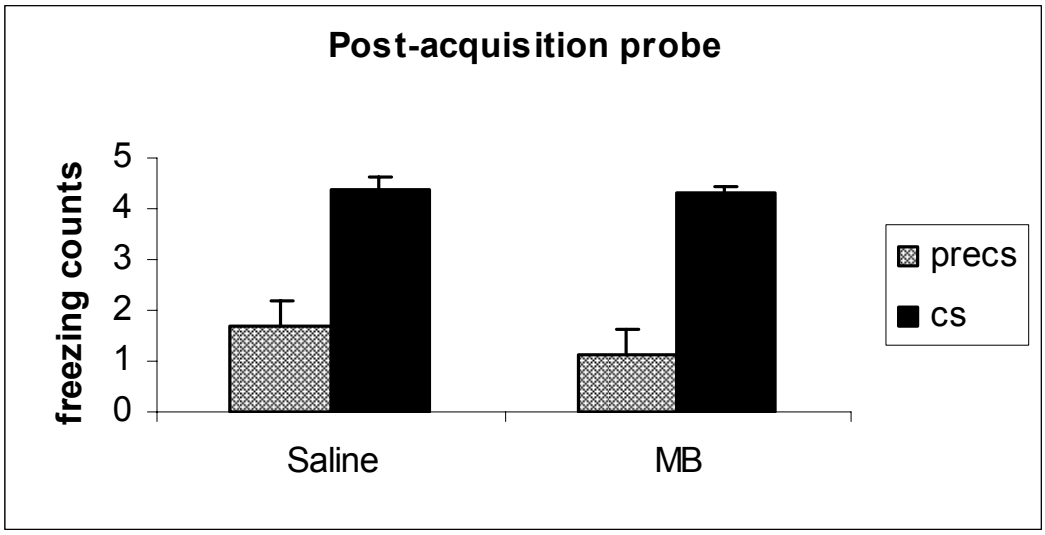
Experiment 2, together with the similar pre-CS freezing in MB and saline groups in experiment 1, served to rule out that repeated MB injections may simply increase levels of motor activity. Therefore, MB administration modified post-extinction CS-evoked conditioned freezing, as opposed to general motor activity.

Experiment 3

Acquisition and Extinction Behavior

Following the excitatory tone-footshock conditioning in spontaneous recovery study, animals were matched into two groups based on their post-acquisition probe freezing scores (Figure 10).

Figure 10. Post-acquisition freezing to the tone CS.



Consequently, there were no significant differences (preCS $F_{(1,22)}$ = 0.74, p = 0.4; CS $F_{(1,22)}$ = 0.2, p = 0.66) in the amount of CR displayed by rats in either group prior to the start of extinction. Likewise, repeated measures ANOVA revealed no differences between groups on the first day of extinction training ($F_{(1,22)}$ = 0.57, p = 0.46, Fig. 11a), suggesting that all animals responded similarly to both the acquisition training and subsequent extinction. Thirty minutes following the first extinction session, rats were injected with either saline or 4 mg/kg MB. Twenty four hours later they underwent a second extinction session, in which nine additional non-reinforced tone conditioned stimuli were presented (Fig 11b). During the second extinction session, the extinction learning curve was similar for both saline-injected and MB-injected rats. However, MB administration seemed to enhance retention of extinction, so that overall freezing to the CS was lower in the MB group ($F_{(1,22)}$ = 5.92, p < 0.05). A similar trend in conditioned responding to the tone CS was observed during the third extinction session ($F_{(1,22)}$ = 4.6, p < 0.05, Fig 11c). However, by the fourth day of extinction training, the difference in freezing behavior between the groups disappeared ($F_{(1,22)}$ = 0.1, p > 0.05, Figure 11d), suggesting that with a sufficient number of CS-alone presentations, saline-treated animals were able to extinguish their CR as well as the MB-treated animals.

Figure 11. Mean + SE freezing counts to the tone CS during the four extinction sessions.

Fig 11a.

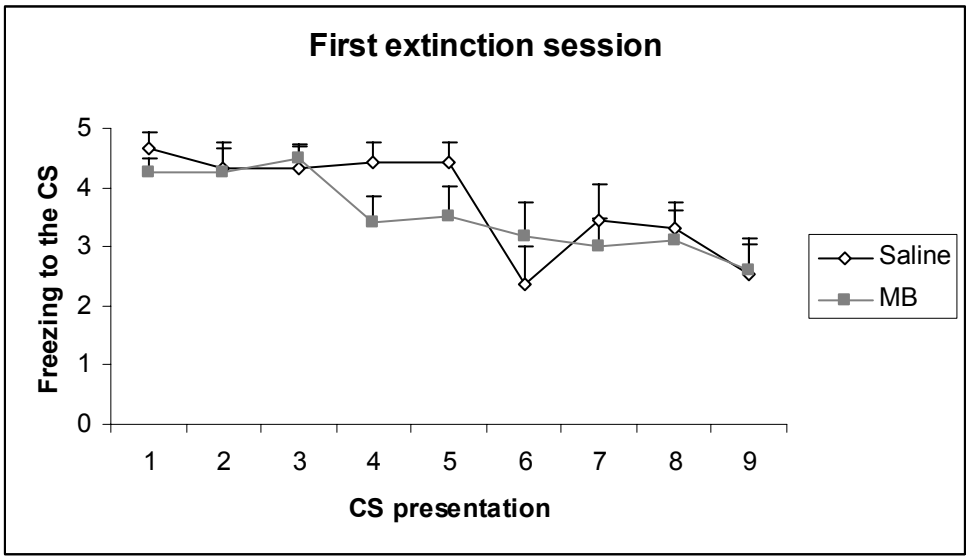


Fig 11b.

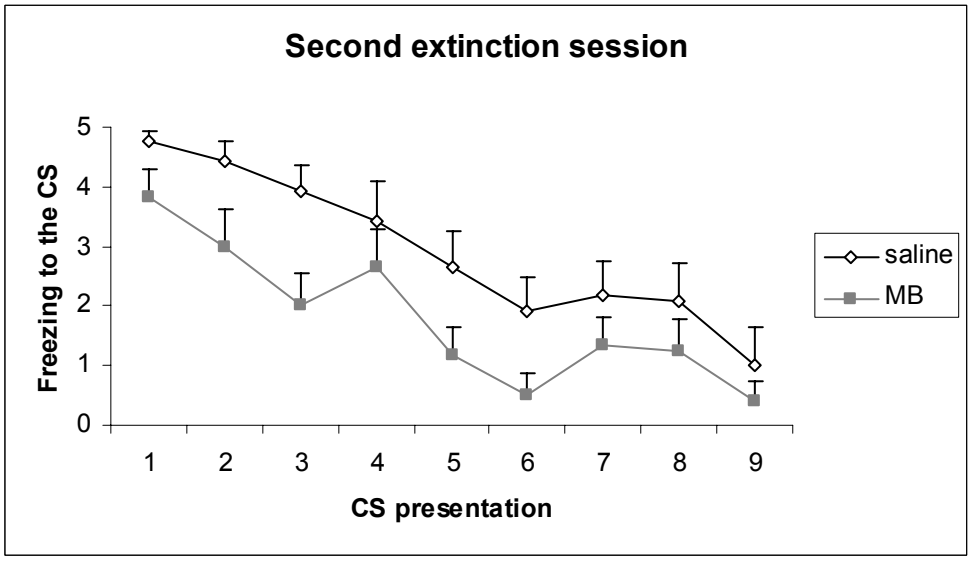


Fig 11c.

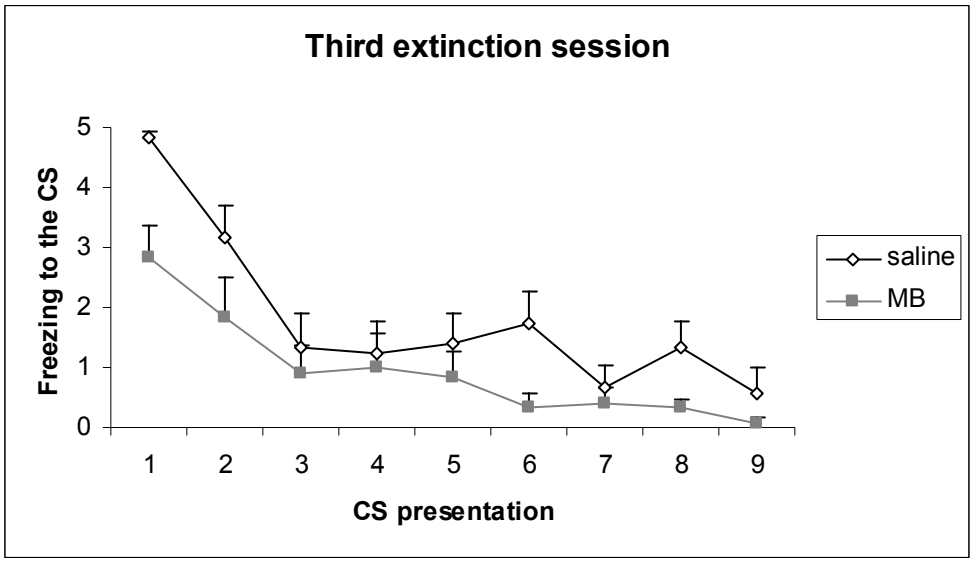
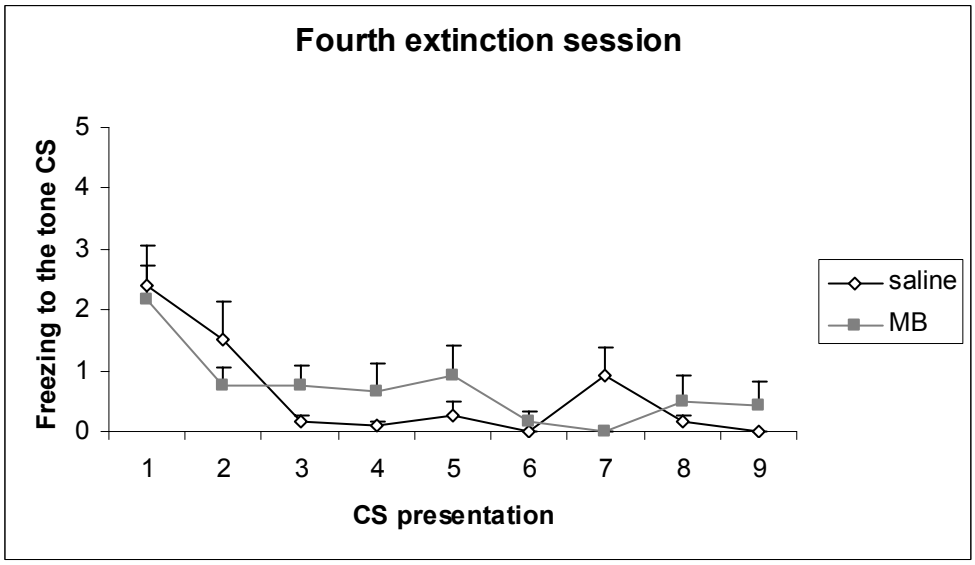
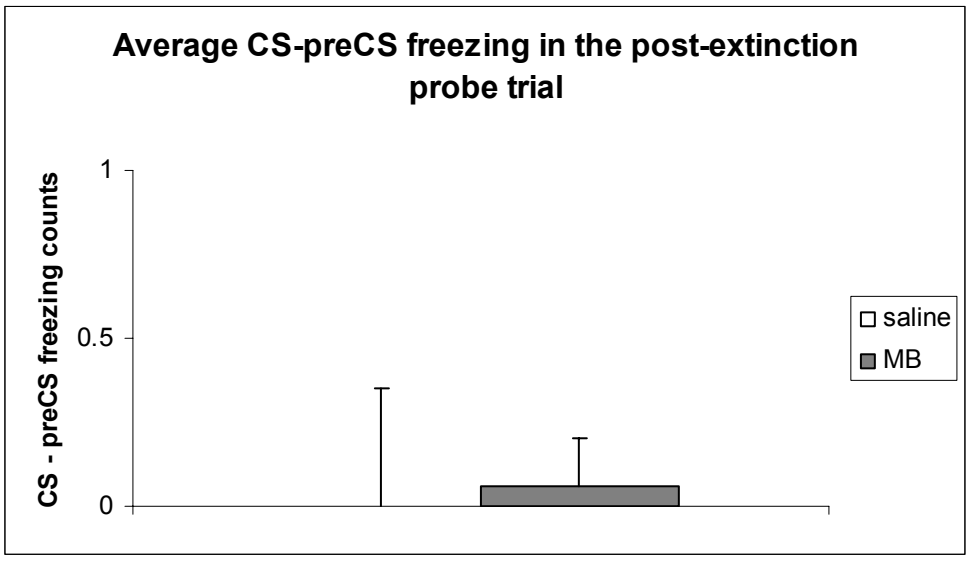


Fig 11d



Repeated measures ANOVA showed a significant main effect of group ($F_{(1,22)} = 4.65$, $p = 0.04$) for the extinction sessions during which MB was on board. Twenty-four hours after the last extinction session and the final MB administration, rats were tested in a probe trial conducted in the extinction context. No significant ($F_{(1,22)} = 0.74$, $p = 0.4$) differences in freezing to the CS were found between the saline and the MB-treated rats in this probe trial (Fig 12).

Figure 12. Mean + SE of CS-preCS freezing counts in the post-extinction probe trial.



Light-dark test

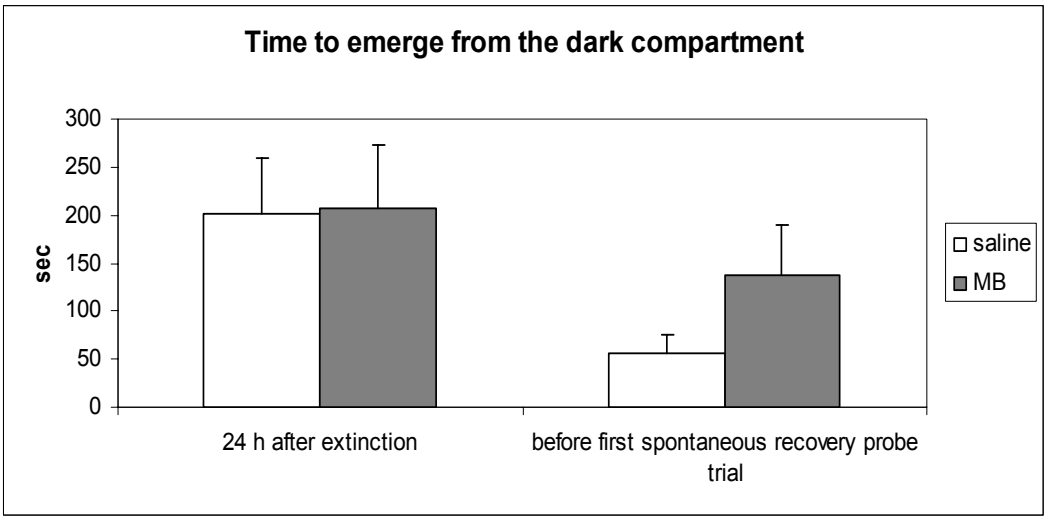
After the post-extinction probe trial, animals were also tested in an open field equipped with a light-dark insert, in order to assess locomotor activity and anxiety levels. This test was repeated 10 days later (prior to the first probe trial for the spontaneous recovery of CR). On both occasions, the open field light-dark exploration test revealed no significant ($p > 0.05$) differences in any of the measured variables, including the time to enter the light compartment, total amount of time spent in the light compartment and number of light-dark zone entries (Table 11).

Table 11. Light-dark exploration test measures recorded either 24 hours following the last MB injection or 10 days later.

	Day 1											
	Light						Dark					
Measure	Saline		SE	MB		SE	Saline		SE	MB		SE
Ambulatory distance (cm)	1022	±	191	1122	±	231	1255	±	191	1202	±	124
Ambulatory time (sec)	19	±	4	20	±	5	23	±	4	22	±	3
Ambulatory counts	405	±	93	494	±	56	523	±	92	473	±	75
Stereotypical time (sec)	81	±	14	94	±	13	91	±	14	71	±	14
Stereotypical counts	1353	±	225	1596	±	203	1610	±	264	1269	±	246
Resting time (sec)	174	±	37	220	±	38	203	±	36	162	±	36
Vertical counts	23	±	5	32	±	4	36	±	6	32	±	6
Vertical time (sec)	23	±	4	26	±	6	42	±	8	36	±	7
Light-dark zone entries	24	±	2	21	±	2	23	±	2	21	±	2

	Day 10											
	Light						Dark					
Measure	Saline		SE	MB		SE	Saline		SE	MB		SE
Ambulatory distance (cm)	1478	±	126	1466	±	383	1122	±	134	1217	±	674
Ambulatory time (sec)	22	±	13	25	±	9	20	±	9	22	±	13
Ambulatory counts	635	±	77	627	±	231	504	±	73	573	±	351
Stereotypical time (sec)	108	±	25	102	±	22	65	±	21	65	±	32
Stereotypical counts	1936	±	131	1831	±	359	1202	±	128	1205	±	606
Resting time (sec)	230	±	25	241	±	22	132	±	68	132	±	68
Vertical counts	36	±	3	32	±	11	35	±	4	33	±	17
Vertical time (sec)	39	±	6	39	±	16	53	±	7	52	±	34
Light-dark zone entries	24	±	2	24	±	9	24	±	2	25	±	10

Figure 13. Average time ± SE to emerge from the dark compartment in light-dark exploration test

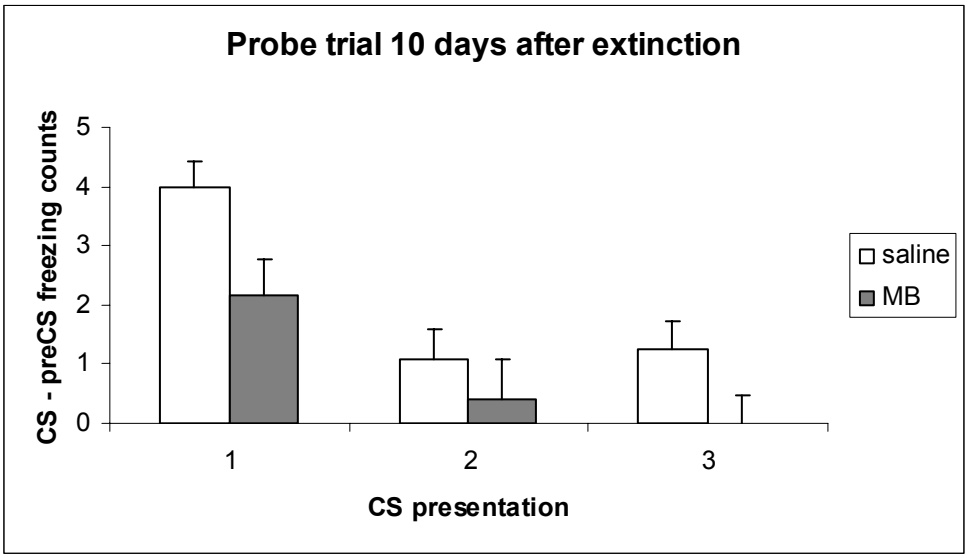


Therefore, it does not appear that the single or repeated MB dose used in this study had any non-specific motor or anxiolytic effects after repeated administration, as measured by this method.

Spontaneous recovery of CR

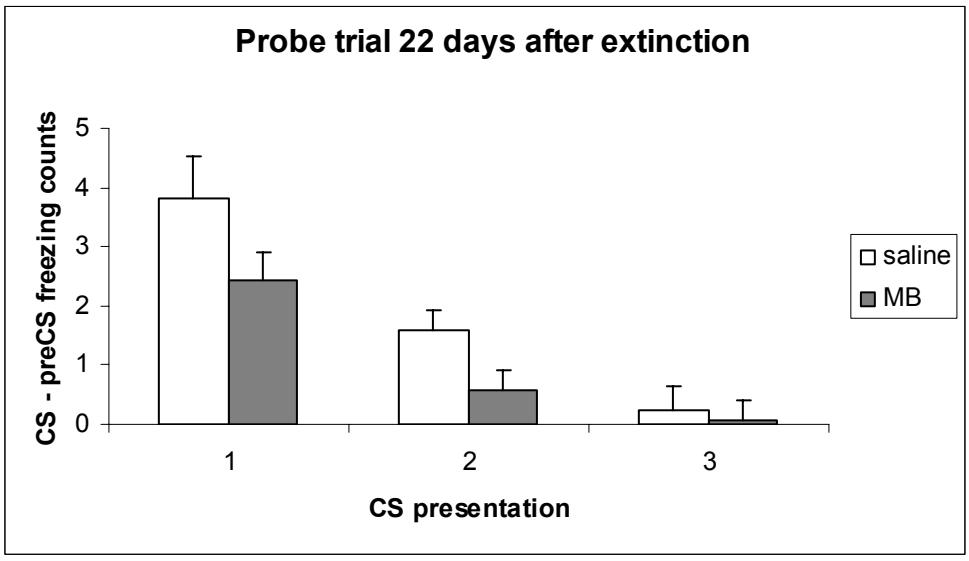
Spontaneous recovery of conditioned freezing was assessed 10 days after the final extinction session (Fig. 14), following the same procedure used for the post-extinction probe. During this probe trial, MB-treated rats showed significantly less spontaneous recovery of their freezing response than the saline-treated rats ($F_{(1,22)} = 4.65$, $p = 0.04$).

Figure 14. Mean + SE CS-preCS freezing counts for the first probe test of spontaneous recovery of CR.



Twenty-two days after the last extinction session was conducted (twelve days following the first spontaneous recovery probe trial), the animals were tested again in another probe trial (Fig 15), and the MB-treated animals showed less spontaneous recovery of the CR as compared to the saline group subjects ($F_{(1,22)} = 5.82$, $p = 0.02$).

Figure 15. Mean + SE CS-preCS freezing counts for the second probe test of spontaneous recovery of CR.



There were no differences in the amount of fecal boli or urinations on any of the training or testing days ($p > 0.05$, Student's t-test). We also monitored the weight of all subjects throughout the duration of experiment 3, and found no differences in MB-treated subjects as compared to saline-treated animals.

Discussion

Experiment 1 showed that MB administered repeatedly in the post-extinction period enhanced the retention of extinction of the CS-evoked conditioned freezing response. Rats receiving MB exhibited enhanced extinction memory that was associated with an absolute increase in brain energy metabolism, as revealed by cytochrome oxidase activity. Experiment 2 showed that repeated MB injections had no significant effects on general motor activity or fear-related behavior. Experiment 3 demonstrated that MB administration diminishes spontaneous recovery of an extinguished CR for as long as 22 days following the extinction training and the last MB injection. Additionally, since MB was administered during the extinction training, this experiment not only examined the effects of MB administration on extinction memory retention, but on extinction memory consolidation as well. The group administered 4 mg/kg MB following the first day of extinction training showed enhanced between-session extinction retention as compared to the saline-treated group. Experiment 3 revealed no meaningful differences in motor activity or anxiety between groups as measured with a light-dark exploration test.

Methylene blue improved memory retention without affecting motor activity and fearfulness

In experiment 1, there was an acquisition phase and an extinction phase before MB or saline administration, in which subjects showed similar acquisition and extinction of conditioned freezing. MB was administered in the post-extinction period to improve the retention of the extinction memory. The rationale

was that MB administered post-extinction would enhance brain metabolic processes underlying retention of extinction. Since MB was given after extinction, it could not have interfered with the acquisition trials. Instead MB enhanced the retention of extinction memory as tested in the probe trials, suggesting that MB acted primarily on the brain processes mediating the retention of extinction memory.

One alternative interpretation of experiment 1 is that repeated MB injections could simply increase levels of motor activity, and thereby reduce freezing behavior. However, post-extinction pre-CS freezing behavior was not different between saline and MB-treated rats, suggesting that the effect of repeated MB administration was specific to the CS-evoked conditioned response. To further investigate the possibility of whether motor effects could account for the observed differences between saline and MB-treated rats, experiment 2 was conducted with rats given single or repeated MB administration and tested for general motor activity in the open field. There were no significant differences in any activity measurements between saline and MB-treated rats.

Yet another possibility is that MB decreased fearfulness in the animals, as opposed to enhanced extinction memory. To test this possibility, we evaluated fear-related behavior in the open field. Increased time in the periphery (thigmotaxic time) and center avoidance (less time spent in center) is associated with fearfulness or anxiety in rats (Treit & Fundytus, 1988). Experiment 2 showed that there were no differences in time spent in center or periphery in subjects in any condition tested: before treatment, after saline, after one MB injection or after five MB injections (Figure 9). Experiment 2 served to rule out the possibility that repeated MB injections could simply increase levels of general motor activity.

Experiment 3 showed that MB's effects on extinction memory retention were long-lasting (22 days) and can be achieved with only four repeated injections of MB (as opposed to five). In this experiment, rats were tested with light-dark exploration test to obtain an additional measure of anxiety level in MB-treated rats. No differences between groups in any of the measures attained with this test were found. Therefore, the control experiments suggest that the observed MB effects on conditioned freezing are more likely the result of enhanced retention of extinction memory than non-specific motor or anxiolytic effects of MB.

The observed improvement in memory retention following post-extinction MB administration is consistent with the results of a number of previous animal MB studies supporting the conclusion that post-training administration of low-dose MB improves memory retention in different tasks (Martinez et al., 1978; Callaway et al., 2002, 2004). For example, successful memory retention is improved by post-training MB administration in an inhibitory avoidance task (Martinez et al., 1978). Repeated post-training MB administration has also enhanced spatial memory retention in probe trials in a holeboard food search task in rats without affecting general motor activity (Callaway et al., 2004). Large MB doses (50-100 mg/kg), however, impair memory because they lead to methemoglobin formation and impaired oxygen consumption (Martinez et al., 1978). Administration of low-dose MB in the post-extinction period would be expected to facilitate consolidation and retention of extinction memory.

Methylene blue increased overall brain oxidative metabolism

Measurements of cytochrome oxidase activity were used to quantify the long-term neural metabolic alterations that developed during the post-extinction phase of the experiment. As neuronal activity (and consequent need for ATP) increases or decreases, cytochrome oxidase activity adjusts accordingly to meet neuronal demand for ATP. Previous studies using different learning paradigms, such as classical conditioning (Poremba et al., 1998) and the Morris water maze (Villarreal, Gonzalez-Lima, Berndt, & Barea-Rodriguez, 2002), suggested that changes in cytochrome oxidase activity can be measured after several days of training. Therefore, measuring cytochrome oxidase activity is well-suited for evaluating the effects of MB on the brain during the post-extinction phase.

The metabolic effect of low-dose MB on memory retention may be due to increased brain oxygen consumption, because MB provides an alternate route of electron flow to oxygen (Visarius et al., 1997). This increase in brain oxidative metabolism is reflected by the overall increase in brain cytochrome oxidase activity observed in MB-treated animals. Our biochemical in vitro studies show a 25% increased rate of cytochrome oxidase activity in brain homogenates after the introduction of a low concentration of MB (Callaway et al., 2004). We also demonstrated 30% increases in absolute brain cytochrome oxidase activity 24 h following in vivo administration of MB to rats (Callaway et al., 2004). In the present study, a 4 mg/kg i.p. injection of MB given daily for five days resulted in a cumulative 38% increase in overall brain cytochrome oxidase activity measured 24 h after the last injection. MB progressively accumulates in the brain, reaching a concentration over 10 times greater in the brain than in the blood 1h after the administration (Peter et al., 2000).

If MB improves memory by increasing cytochrome oxidase activity, treatments that result in inhibition of cytochrome oxidase activity should lead to impaired memory. For example, it has been shown that sodium azide decreases cytochrome oxidase activity when chronically administered to rats (Berndt, Callaway, & Gonzalez-Lima, 2001; Cada, Gonzalez-Lima, Rose, & Bennett, 1995) and causes spatial memory deficits in rats tested in the Morris water maze (Bennett, Mlady, Fleshner, & Rose, 1996) and the holeboard maze (Callaway et al., 2002). Remarkably, when MB is administered to rats with reduced brain cytochrome oxidase activity, their memory retention scores are normalized to the same level as that of control animals (Callaway et al., 2002). Administration of low-dose MB can also enhance spatial memory retention in normal rats by increasing brain cytochrome oxidase activity (Callaway et al., 2004). These findings, together with our present results, suggest that an increase in oxidative energy metabolism in the brain is a mechanism whereby MB enhances memory retention. An increase in cytochrome oxidase activity results in increased oxidative metabolic capacity of neurons because it allows more oxygen consumption and ATP formation in the brain (Gonzalez-Lima et al., 1998).

We hypothesized that regions more engaged in the retention of extinction memory will be targeted by MB metabolic-enhancing effects based on two premises. First, in experiment 1, MB was not administered throughout the entire procedure, but only during the post-extinction period, and thus could only affect brain metabolism during that period. Second, brain regions with more metabolic demand during the post-extinction period, and the concomitant behavior, would be expected to benefit more from the metabolic-enhancing effects of MB than regions engaged in other aspects of behavior prior to MB administration. Brain

accumulation of MB during the post-extinction phase of the experiment would selectively facilitate cytochrome oxidase activity during that period, as opposed to the initial acquisition and extinction phases of the experiment. On the other hand, when MB was administered during the extinction phase in experiment 3, it was expected that MB would facilitate retention of the acquired behavior as found in previous studies (Martinez et al., 1978; Callaway et al., 2003, 2004). Which regions are more affected by MB would seem to depend on which regions have more metabolic energy demand during the period when MB is administered. Since post-extinction MB administration increased oxidative energy metabolism in the brain during the period of extinction memory consolidation and retrieval, this effect may lead to enhanced extinction memory retention.

Summary

In conclusion, low-dose MB administered post-extinction enhanced both the retention of extinction memory and the overall brain cytochrome oxidase activity. MB administered during extinction training also had an effect on retention of extinction memory, in that it reduced spontaneous recovery of CR 10 and 22 days following extinction. The observed MB effects on conditioned freezing were more likely the result of enhanced retention of extinction memory than non-specific motor or anxiolytic effects of MB, since we found no statistically significant between-group differences in thigmotaxic behavior or light-dark exploration test measures. Metabolic enhancers such as MB, which improve brain energy production, may be successful at improving memory retention under many conditions depending on the period of administration. The metabolic

enhancing effects of MB are not necessarily limited to one brain region or function; rather, MB may help meet ongoing metabolic demands for any region engaged in a particular function, such as memory retention in the post-extinction phase. MB is an FDA-approved drug that is readily available and that has already been used clinically for many years as an antidote for certain metabolic poisons (Kupfer, Aeschlimann, & Cerny, 1996). MB is also an inhibitor of oxygen free radical generation in reperfusion injury to the brain (Kelner, Bagnell, Hale, & Alexander, 1988; Salaris et al., 1991). Our results suggest that MB administered in conjunction with exposure behavioral therapy in humans may be a useful therapeutic agent to facilitate retention of extinction of conditioned fear or other traumatic memories.

Chapter 5: Discussion

Potential learning mechanisms of conditioned fear renewal

The modern view of the Pavlovian stimulus-substitution model (Domjan, 1998), in which the CR is elicited through the reactivation of the US neural representation is consistent with the pattern of stimulus-evoked neural activation in the renewal context. According to this model, eliciting a CR depends on two factors: 1) activation of the neural CS-US association, and 2) activation of the US representation. Therefore, the auditory CS elicited the CR because of its association with the somatosensory US representation. This view is supported by our findings that fear renewal in the ABC paradigm is mediated by the neural reactivation of the CS-US association involving excitatory responses to the tone CS in the auditory system, and reactivation of the US representation in somatosensory pathways in the absence of the US. The CS in the different context activates a neural circuit that links CS and US pathways with limbic and response pathways mediating CR renewal.

Bouton has ascribed the contextual influence on the extinguished CS through a theory of contextual occasion setting (Bouton, 2004). He proposes that an extinguished CS has an ambiguous meaning, in that it could signify an impending US or the absence of it. In order to determine which meaning the CS has, organisms employ contextual cues to help disambiguate the meaning of the CS. Therefore, Bouton's model can be visualized as an "AND" gate. According to this theory, in the extinction context both the ambiguous CS AND the extinction

context are necessary for the no-CR behavioral outcome. In the case of a non-extinction context AND the ambiguous CS, the CR is renewed, because to achieve the no-CR behavioral output the context needs to be inhibitory. Results of our study suggest that during ABC renewal, both the excitatory CS representation (as evidenced by auditory system activation) and the excitatory context representation (as evidenced by the perirhinal cortex and anterior lateral hypothalamic activation) contribute to CR renewal. In contrast, FDG metabolic mapping of extinction effects did not reveal an excitatory CS representation in the auditory system or excitatory contextual neural representation in the perirhinal cortex and anterior lateral hypothalamus, thus satisfying the Bouton's "AND" gate module prediction of contextual occasion setting in extinction. Bouton's occasion-setting account of extinction and renewal is not incompatible with Pavlov's stimulus-substitution theory, rather it adds to it by accounting for a possible mechanism through which contextual stimuli may come to influence the CR.

Elevated arousal level may contribute to CR renewal

During ABC renewal, animals were placed in a relatively novel context and their general arousal was likely elevated, which could be one of the mechanisms of renewal that bears a resemblance to reinstatement with a strong stimulus, in this instance the context. This explanation of fear relapse is conceptually similar to Bouton and Nelson's (1998) theory that the reoccurrence of CR, as it is observed in various conditioning phenomena (spontaneous recovery, reinstatement, renewal and reacquisition), is driven by a related mechanism. Bouton and Nelson (1998) propose that many of these phenomena are special

cases of renewal, citing context as an occasion setter that can trigger the CS-US association. Brain imaging data obtained in the present study suggest that renewal might fall under the umbrella of reinstatement, which is likely driven by an increase in arousal that a stimulating environment may provide to an animal. For example, elevated metabolic activity in ICD and IC nuclei generally reflects not only auditory information processing, but arousal level as well (Gonzalez-Lima et al., 1984a; Gonzalez-Lima et al., 1984b).

Another area that was found to be more metabolically active in the renewal group was the nucleus of the solitary tract. Elevated activity in this nucleus has been associated with increased arousal (Miyashita & Williams, 2004), supporting the hypothesis that there is increased arousal in renewal group subjects. However, some of the thalamic nuclei in which arousal-related activational changes were found in previous metabolic studies (Gonzalez-Lima & Scheich, 1985; Gonzalez-Lima, Helmstetter, & Agudo, 1993), as well as prefrontal regions thought to play a role in attention and arousal (Lecas, 1995; Smith, Stewart, & Pfaus, 1997), were not activated in the renewal group. This could be explained by a possible interplay between arousal-related activity within some of the neuronal populations in these regions and the need to alter their extinction-related activity in order to provide a more appropriate, disinhibited, fear renewal behavioral response. As noted in Gonzalez-Lima et al. (1989), learning-related changes may not be correlated with a net change in radiolabeled glucose uptake in the case of heterogeneous neuronal populations showing both increased and decreased activity as a function of learning. In addition, since Pavlovian fear conditioning most closely models a specific phobia, it is likely that during CR renewal, anxiety level and the corresponding activation of frontal

areas are not as elevated as they might be in a model of more generalized anxiety disorder (Fredrikson et al., 1993). On the other hand, differential levels of arousal and anxiety in ABC renewal may also be attributed to perceived threat imminence in the renewal context (Fanselow, 1989). During fear renewal, some subjects might perceive CS in a novel context as signaling a more distant threat, perhaps creating a more generalized anxiety-like state (Craske, 1999). Our data show that renewal group rats had strong autonomic activation (defecation and urination was increased) as well as robust freezing response, suggesting that both arousal and fear might be elevated during fear renewal. Thereby, an ambiguous CS encountered in a novel, possibly arousing context, could evoke a US neural representation even in the absence of the US.

Performance theory of conditioned fear extinction and renewal

Miller and Matzel's (1988) comparator hypothesis of performance may be applicable to fear extinction and CR renewal since it is a theory which takes contextual input as an important contributing factor for the generation of a behavioral response. According to the comparator hypothesis, three associations are learned during the course of conditioning: a CS-US association, CS-comparator contextual cues association, and a US-comparator contextual association. Once the CS is presented, the US representation can be activated directly or indirectly through these associations, and a comparison between these activations determines the degree of responding that occurs. Since novel contextual cues were never associated with the CS, the CS presentation would

activate only the direct US representation, thus leading to CR renewal. However, neural metabolic correlates of CS and US representations in the extinction group in the present study do not lend support to this account of conditioned responding in extinction and renewal. Activation of a possible US neural representation was found in the renewal group, but not in the extinction group, and according to the comparator hypothesis, direct US representation should exist in both of these groups.

Potential mechanisms of conditioned fear extinction

While the stimulus-substitution learning theory fits with metabolic measures of CS and US neural representations in the renewal group, observed neural correlates of extinction learning and recall proved less amenable for interpretation with extinction learning theories. Generally, if extinction of conditioned behavior leaves the CS-US association intact, then decline in CR can not be attributed to an S-S mechanism. Rather, nonreinforcement may produce an inhibitory stimulus-response (S-R) association in the presence of a specific stimulus (such as the extinction context). Indeed, evidence for an inhibitory S-R association (response-inhibition hypothesis) which forms during the course of extinction has been found in appetitive instrumental conditioning (Rescorla, 1993). To my knowledge, no analogous experiments have been published in the Pavlovian fear conditioning literature. However, since higher responding in extinction does not guarantee better extinction learning (Bouton, 2004), the response-inhibition hypothesis of extinction might not be valid.

Mackintosh (1974) suggested a habituation-like mechanism of extinction,

proposing that with every non-reinforced presentation of a stimulus, there is a decline in the efficiency of transmission along a S-R pathway. More recently, it has been proposed that fear extinction in rodents might be regarded as a habituation-like process which abolishes the influence of sensitization on the freezing response to the tone without affecting the expression of the associative memory component (McSweeney & Swindell, 2002; Kamprath et al., 2004). However, the habituation model of extinction cannot account for the persistence of some fears despite repeated CS presentations, or improved extinction with massed exposures to CSs which should impede habituation (Cain et al., 2003; Sandin & Chorot, 2006).

Other learning theories view extinction as a function of discrimination of reinforcement rate (Gallistel & Gibbon, 2000), generalization decrement (Capaldi, 1994) or violation of reinforcer expectation (Pearce & Hall, 1980). Although it is possible that response outcome in extinction is determined by comparing the current rate of reinforcement in the CS with its memory of the conditioning rate, there is little support for this theory in the literature (Haselgrove & Pearce, 2003; Drew, Yang, Ohyama, & Balsam, 2004). On the other hand, violation of expectation during extinction appears to be one of the main factors that causes the loss of responding in extinction (Bouton, 2004). The violation of reinforcer expectation model of extinction can be fitted to our data describing neural correlates of extinction if the sometimes-opponent-process (SOP) is used to represent this model (Wagner et al., 1989). According to the SOP, the CS and US are represented as memory nodes that become associated during conditioning. To strengthen this association, both nodes must be activated to an active state (A1) at the same time. Once the association has been formed, the

CS presentation generates the CR. When the CS is activated in A1 state and the US is activated to A2, rather than an A1 state, an inhibitory connection is formed. According to the SOP model, extinction learning will occur as long as the CS is on and no US occurs. If this model is correct, then we would have expected to see activated neural representation of the CS in our extinction group subjects. Nonetheless, this was not evident in either the FDG or CO metabolic maps, and thus our data do not support this extinction learning theory. SOP model of extinction also fails to account for the role of context in extinction, which seems to be of critical importance for extinction memory retrieval.

If conditioned fear extinction is viewed as another example of a retroactive inhibition phenomena in which new learning inhibits old, then other behavioral paradigms with links to extinction might help elucidate how context might influence behavioral processes in extinction. For example, counterconditioning shares many similarities with extinction, in that it supports renewal, spontaneous recovery and reinstatement (Bouton, 2004). Similarly, discrimination reversal learning and latent inhibition also show sensitivity to contextual manipulations. Bouton (1993) argued that a memory retrieval account for all these learning paradigms, including extinction, which accepts that both phases (e.g. acquisition/extinction, preexposure/conditioning) of learning are available and that the CR is determined by which memory is retrieved, can explain many examples of interference. Importantly, he also argues that extinction is not context-specific simply because it is a form of inhibition. Rather, the fact that extinction is the second thing that the organism has learned about the CS may also account for some of the context-specificity of extinction. In an attempt to explain how context might modulate extinction, Bouton proposes that the

extinction context "…activates or retrieves the CS's own second (inhibitory) association, much as a negative occasion setter might" (Bouton, 2004).

In order to examine Bouton's occasion-setting mechanism of extinction, one must first identify the possible candidates for the neural correlates of occasion-setting. Neural representation of context would most likely involve one of the regions of the hippocampal formation, or another area in which multiple sensory inputs converge to form a spatial representation. One such possible candidate would be caudal caudate-putamen, a region in which FDG uptake was found to be elevated during retrieval of extinction. Another element of Bouton's occasion-setting theory is the proposed CS-noUS association which is formed during extinction. However, from a neurobiological perspective, it would be difficult to quantify a neural representation of a noUS, so a more quantifiable contextual inhibition of the US presentation can be explored. The metabolic mapping data from our study are in agreement with a model of inhibited US neural representation in extinction, in that the somatosensory areas found to be activated in renewal were not activated during extinction retrieval. The neural activation of the CS was also absent in the extinction group in this study, suggesting that Bouton's occasion-setting model of extinction might not account for all associative changes that take place in extinction learning. Indeed, extinction is a highly complex phenomenon which is probably determined by multiple factors. More behavioral and neurobiological studies are needed to develop an extinction model that will be able to integrate many aspects of extinction, such as its contextual dependence and its relapse effects. The studies presented here contribute to the development of such a model by suggesting that

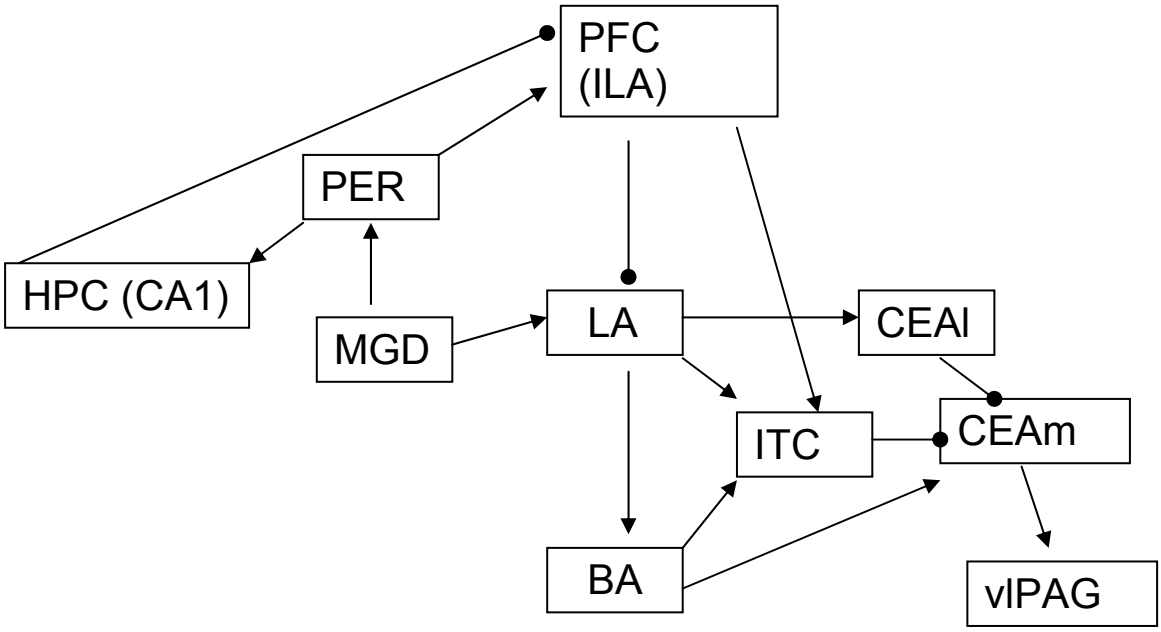
extinction of fear conditioning may be modified by both CS and US neural representations, possibly through a contextual occasion-setting mechanism.

Network analysis of fear extinction and renewal functional pathways mapped with FDG

To further consider how changes in metabolic activity might be related to changes in the neural circuitry of fear extinction, interactions among brain areas of interest were quantified beyond pairwise covariance relationships through the use of structural equation modeling (McIntosh & Gonzalez-Lima, 1994; McIntosh et al., 1995). Structural equation modeling (SEM) and path analysis quantify the impact of links between variables in a causally structured network. Directional links between elements of the network (regions of interest, ROIs) are assigned based on the anatomical pathways, and the correlation coefficients of activity between ROIs are used to identify the functional pathways. The application of SEM to neural data assumes that ROI correlations reflect common influences and direct anatomical connections between them (McIntosh et al., 1995). Thus, a neural network model combines known anatomical connections between ROIs and a functional network model in which the interregional correlations of activity are decomposed to assign path coefficients to these connections. Specific functional interactions within the same anatomical model can then be compared for the strength of path coefficients to identify task-specific functional interactions within that network. Thus, use of SEM reveals patterns of interrelations between brain ROIs within an anatomically constrained neural system, but extends beyond pairwise correlations to include direct causal influences.

The first application of SEM to brain mapping data was from a study of auditory system activity following long-term habituation, which successfully demonstrated that interactions between auditory pathways changed depending on the experience with the acoustic stimulus (Gonzalez-Lima et al., 1989). Since then, other brain mapping studies utilized SEM to analyze functional neural networks in a variety of tasks (McIntosh et al., 1994; McIntosh et al., 1995; McIntosh et al., 1998), validating the combined use of functional brain mapping and anatomically based SEM as an analytical tool for advancing our understanding of brain-behavior relations at the systems level. In the current study, SEM was applied to the cortical and hippocampal circuitry model of fear extinction proposed by Maren (2005).

Figure 16. Maren's model of fear extinction. Excitatory projections are indicated by arrowheads, and inhibitory projections by circles. CEAl = lateral division of the central amygdaloid nucleus; CEAm = medial division of the central amygdaloid nucleus; ITC = intercalated cells of amygdala.



This model was chosen as a basis to study the impact of links between regions in fear extinction and renewal neural networks because it considers the role of context, its inter-regional connections are anatomically sound, and many of the model's ROIs were identified in this study as having either mean differences in metabolic activity or having task-specific functional interactions. According to this model, a CS undergoing extinction recruits PFC and HPC circuits involved in regulating CR behavioral output through the amygdala. The context specificity of extinction is proposed to be conferred by the hippocampal formation, which detects CS and context mismatches. A detected CS-context mismatch inhibits the PFC circuit, which reduces the inhibitory influence of the PFC on the amygdala resulting in CR renewal.

Structural equation modeling (SEM)

SEM algorithms strive to account for an observed pattern of correlations based on the causal structure of the system (McIntosh et al., 1995). If a brain regional network is represented by a set of path equations where the correlations between regions are the sum of the compound paths connecting those regions, then path coefficients can be obtained through algebraic substitution (McIntosh et al., 1995). While path equations specify the components of each correlation coefficient, another representation of the causal order of the system can be obtained by a set of structural equations. These equations specify the influences on the variance for each ROI, and their solutions are obtained using matrix operations best described as simultaneous multiple regressions (McIntosh et al., 1995). SEM allows for unaccounted influences on an ROI to be incorporated in

the model as residuals (PSI), which would include the influence of ROI upon itself, as well as the combined influence of areas outside the model (McIntosh et al., 1995).

Path coefficients representing the influences through the anatomical pathways were computed using LISREL (version 8.54, Scientific software). A statistical comparison of the models was done using the multiple group stacked model approach through which functional models whose path coefficients are constrained to be equal between conditions are compared with those where the coefficients are allowed to differ (McIntosh et al., 1995). In this study, the "all paths free" alternative model reproduced a correlation matrix whose $\chi 2$ goodness-of-fit statistic was significantly different from the $\chi 2$ of the original (null model) correlation matrix, indicating that all path coefficients between ROIs were statistically different between the renewal and the extinction group.

Direct effects and residual values for each ROI obtained when our FDG data was applied to Maren's model of extinction pathways indicated that this model was unable to account for a large amount of influences on some of the critical regions (such as PFC and LA). Thus, another ROI and inter-regional connection were added to the model in an attempt to better approximate the nominal functional neural network of fear extinction. The anterior cingulate was included in the model because it was the only region that showed consistent extinction effects in both of our metabolic maps, and because of the recent work suggesting that this region might play an important role in memory for footshock (Malin & McGaugh, 2006), extinction of specific phobia (Straube et al., 2006), and gating among external sensory and internally driven stimuli and limbic structures involved in emotional and behavioral responses to CSs (Hamner,

Lorberbaum, & George, 1999). Although all the pathways indicated in Maren's extinction model are anatomically correct, they are not complete. For example, many of the ROIs in this model which are shown to have only one-way connectivity in fact have reciprocal connections, and some of the known inter-regional connections are not represented. Therefore, this model is a rather simplified representation of the actual neural network that might participate in some aspects of conditioned fear extinction. However, there are limitations as to how many regions and/or connections one can consider when doing SEM in order to have solvable structural equations, so construction of this model represents a compromise between anatomical accuracy and the ability to interpret the model.

Figure 17. Graphic representation of the direct effects for the a) Fear extinction model and b) Extinguished fear renewal model

Fig 17a.

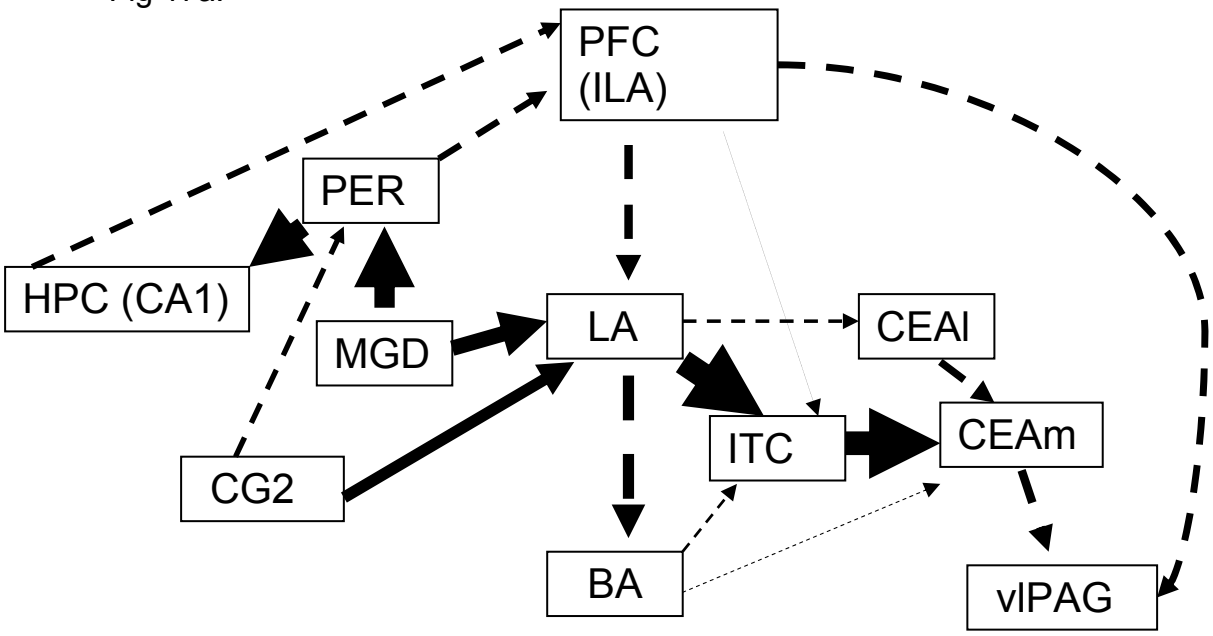
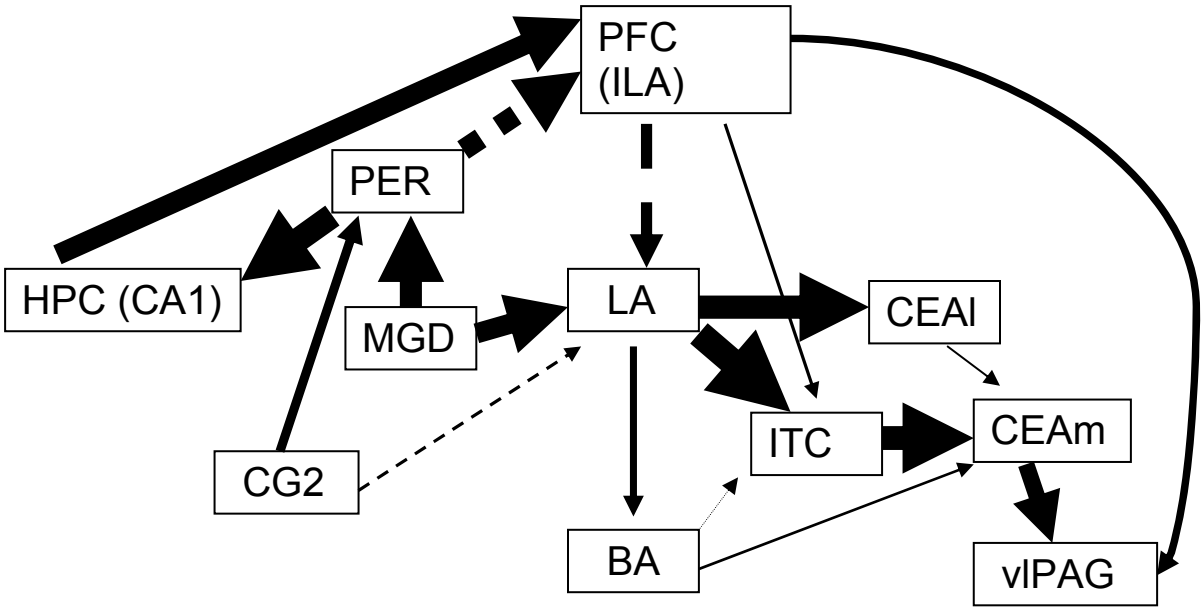


Fig17b.



In both of these graphic representations, the magnitude of the direct effect is proportional to the arrow width for each path (Fig. 17). Positive path coefficients are shown as solid arrows, while negative path coefficients are shown as segmented arrows. As seen, there are several path coefficients that showed changes not only in magnitude, but in sign as well. Perhaps the most clear example of this is the direct effect from CA1 hippocampal field to the infralimbic cortex. While this path coefficient was strongly positive (0.77) in the fear renewal condition, it was weakly negative (-0.23) in the fear extinction condition. Other path coefficients and residual PSI values for this model are listed in table 12.

Table 12. Direct effects and PSI values for the functional neural network model of fear extinction and renewal. a) Extinction recall group. The columns list origins of effect and the rows list structures being affected.

Direct Effects

ROI	ILA	CG2	PER	CA1	MGD	LA	BA	ITC	CEAl	CEAm	vlPAG
ILA			-0.24	-0.23							
CG2											
PER		-0.2			0.76						
CA1			0.74								
MGD											
LA	-0.32	0.44			0.58						
BA						-0.4					
ITC	0.01					0.93	-0.1				
CEAl						-0.13					
CEAm							-0.04	0.88	-0.28		
vlPAG										-0.32	

	ILA	CG2	PER	CA1	MGD	LA	BA	ITC	CEAl	CEAm	vlPAG
PSI	0.81	1	0.38	0.45	1	0.27	0.84	0.05	0.98	0.04	0.9

Table 12. b) Fear renewal group. The columns list origins of effect and the rows list structures being affected.

Direct Effects

ROI	ILA	CG2	PER	CA1	MGD	LA	BA	ITC	CEAl	CEAm	vlPAG
ILA			-0.79	0.77							
CG2											
PER		0.34			0.75						
CA1			0.91								
MGD											
LA	0.15	-0.12			0.79						
BA						0.26					
ITC	0.12					1	0.04				
CEAl						0.87					
CEAm							0.1	0.89	0.06		
vlPAG										0.62	

	ILA	CG2	PER	CA1	MGD	LA	BA	ITC	CEAl	CEAm	vlPAG
PSI	0.89	1	0.32	0.18	1	0.15	0.93	0.03	0.24	0.03	0.61

Overall, this model revealed many more negative direct influences on the brain regions in the fear extinction than the fear renewal condition, suggesting a reversal in the functional interactions from negative during extinction recall to positive during fear renewal. Additionally, the magnitude of direct effects was often different between groups, reflecting a change in the strength of the influences conveyed through those pathways. Path weights of regional influences are essentially linear regression coefficients with the sign implying the positive or negative influence of one region on another. The magnitude of the weight gives the strength of the regression. It has been suggested that relative suppression of activity at one region with respect to another produces path weights that are negative, implying that this represents an inhibitory process at the neuronal level (Nyberg et al., 1996). However, interpretation of path coefficient signs as representing excitatory or inhibitory influences in the electrophysiological sense is unwarranted, considering that the relationship between FDG uptake and action potential rates may differ in various brain regions (McIntosh and Gonzalez-Lima, 1994). Rather, weight of a positive path coefficient indicates the proportion of the activity in the target region which will increase given a unit increase in the source region; a negative coefficient indicates the proportion the activity in the target region will decrease given a unit increase in the source variable.

SEM analysis of the functional extinction and fear renewal neural circuitry suggests that there are several pathways through which neural representations of CS and context might come to influence behavioral output. While the proposed auditory CS input through MGD seemed to exert similar direct effects on PER and LA in both extinction and renewal groups, possibly reflecting similar learned behavioral significance of the tone in two conditions, direct effects from lateral to

central amygdala became strongly positive in renewal condition. Likewise, there was a switch in LA to BA and ILA to vlPAG path coefficient signs from negative in extinction to positive in renewal condition. The direct effect sign switch in most of the intra-amygdala path coefficients might reflect the powerful control that inhibition exerts over the activity of projecting LA principal cells (Bauer et al., 2004). Direct effects from anterior cingulate to PER and LA also displayed a reversal of sign between the two experimental conditions. Another network analysis of Pavlovian auditory conditioning completed by our laboratory showed similar trends of path coefficient sign reversal between tone-excitor and tone-inhibitor functional neural network models (MacIntosh and Gonzalez-Lima, 1994). In the tone-excitor model, similarly to our renewal model, some of the effects were transmitted through positive covariance relationships. In the tone-inhibitor model, there was a change in those relationships to negative (McIntosh et al., 1994). In our extinction model, it appears that contextual cues might have acquired some inhibitory stimulus properties, given that HPC influence on ILA was found to be negative in the extinction group.

One of the limitations of Maren's extinction model is that it does not take into account how neural representation of the US might contribute to this functional network, which might prove important when considering emergent properties of distributed brain activity and network interactions in extinction learning. In addition, several large residual values (PSI) were present, which may have occurred through a combination of influences from brain regions not included in this model and internal processes within these regions. Some residual values, such as those of the lateral central amygdaloid nucleus and ventrolateral periaqueductal grey, were lower in the functional network model of

fear renewal, as compared to extinction model. This indicates that some influences on these regions were not accounted for by the presented extinction model.

Nevertheless, the application of anatomically based SEM to metabolic brain mapping data can broaden our understanding of brain-behavior relations at the systems level. For example, one possible application of this model would be to evaluate how a functional disconnection would be reflected in the functional connectivity maps of the proposed regions of interest, such as in transgenic mice that do not show fear renewal (Waddell, Dunnett, & Falls, 2004), or in phobics who do not extinguish their fear responses. Furthermore, use of metabolic brain mapping in conjunction with structural equation modeling could be utilized to relate differences in the functional interactions between brain regions to the learned significance of stimuli and their behavioral outcomes.

Fear extinction memory retention and retrieval

In experiment two, quantitative CO histochemistry was used to assess training-related metabolic capacity changes in various brain regions thought to play a role in conditioned fear extinction. One hypothesis which was explored was that the amygdala might have only a transient role in fear conditioning and extinction (Breiter et al., 1996; Knight et al., 2004; Krolak-Salmon, Henaff, Vighetto, Bertrand, & Mauguiere, 2004), which could be measured with the FDG technique, but not be quantifiable using the CO method which measures training-evoked, rather than stimulus-evoked metabolic changes. Indeed, our data support this hypothesis, since we were able to observe significant CS-evoked

(FDG uptake) changes in amygdaloid nuclei in rats recalling the extinction memory, while no acquisition and extinction training-related changes (CO activity) were observed in the amygdala. Similarly, the hypothesis that the affective dimension of fear conditioning and extinction might be differentially processed in an intact brain was supported by data which showed changes in CO metabolic capacity, but not stimulus-evoked activity, in the lateral septum.

It was also anticipated that during the retrieval of fear extinction memory, some of the same brain regions that showed altered metabolic capacity as a result of extinction training might also show changes in FDG uptake. Specifically, we hypothesized that mPFC could be one area in which both extinction training and extinction memory recall would modify brain metabolism as measured with our chosen metabolic markers. After examination of both FDG and CO metabolic brain maps, the only two brain regions that showed common between-group differences were anterior cingulate cortex and caudal caudate putamen. Next, in order to evaluate any difference in means in the context of their variability, both FDG and CO brain regional data were standardized using averaged pseudorandom group measures from each ROI. This provided us with a relative measure of metabolism in each region, as compared to the pseudorandom group. A series of paired t-tests ($p<0.01$) was then employed to examine these relative regional metabolic differences between the two conditions (retention vs. retrieval). The results are listed in table 13.

Table 13. Brain regions found to be different between fear extinction retention and fear extinction recall condition.

ROI	CO mean		SE	FDG mean		SE
CG2	1.05	±	0.12	0.93	±	0.11
PPA	0.75	±	0.05	1.03	±	0.05
CA1	0.84	±	0.08	1.01	±	0.037
CA3	0.84	±	0.07	1.05	±	0.08
HB	0.86	±	0.1	1.04	±	0.06
PER	0.78	±	0.07	0.95	±	0.26
MM	0.9	±	0.13	1.07	±	0.09
CHM	0.99	±	0.03	1.16	±	0.04

Interestingly, out of 79 regions examined, there was only one region that showed greater relative metabolic capacity following fear acquisition followed by extinction, and smaller relative metabolic activity during extinction memory retrieval. This region was the anterior cingulate cortex, the possible role of which in fear conditioning and extinction has already been discussed. Thus, we verified our hypothesis that mPFC might be one of the regions which undergoes changes in metabolism associated with both conditioned fear extinction memory retention and fear extinction memory retrieval in rats.

Methylene blue as an adjunct therapeutic for specific phobias

In vivo exposure therapy represents the treatment of choice for patients with specific phobias (Craske, 1999), although many patients receive additional pharmacological treatment. There are six classes of drugs that are commonly used for treatment of anxiety disorders. These include: heterocyclic antidepressants (e.g. imipramine), selective serotonin reuptake inhibitors (SSRIs, e.g. paroxetine), benzodiazepines (e.g. diazepam), monoamine oxidase inhibitors (e.g. phenelzine), azaripones (e.g. buspirone), and beta blockers (e.g. propranolol). Intriguingly, despite the widespread use of these drugs in the treatment of anxiety disorders, medications generally have no effect or only slightly enhance behavioral treatment efficacy in the short term, and may even be detrimental to behavioral treatments in the long term (Craske, 1999). Then why should researchers investigate a novel pharmacological treatment to be used as an adjunct to exposure therapy? One reason is that an estimated 25 to 30% of patients with anxiety disorders reject behavioral therapy alone as the only

treatment option (Craske, 1999). Another reason is that certain medications may reduce initial negative effects associated with behavioral therapy, as well as improve compliance with behavioral treatments. Unfortunately, combining existing pharmacotherapy with behavioral treatment might lead to increased return of fear, as compared to behavioral treatment alone. This might be due to drug-induced states contributing to a particular interoceptive context which may change once the drug therapy is discontinued. Bouton's context-ambiguity model of extinction predicts such an outcome (Bouton, 2002). Brewin (1989) found evidence that treatment gains made within a specific exposure context involving medications failed to generalize to a different context (non-medicated interoceptive context) once the medication was discontinued. State-dependent retention of extinction has been found in nonprimate animal models of anxiety disorders (Bouton, Kenney, & Rosengard, 1990).

In humans, in addition to creating an interoceptive context, anxiolytics may be detrimental to behavioral therapy because they may assume the function of safety signals (Sanderson & Wetzler, 1993) and lower the perception of self-efficacy (Westral & Stewart, 2002). These effects may be particularly pronounced for medications with rapid anxiety-reducing effects (Craske, 1999), so that a slower-acting drug, such as methylene blue, might be able to circumvent some of the abovementioned problems. Another reason to test methylene blue as an adjunct treatment to behavioral therapy for specific phobias is that there are some findings that suggest a benefit of combined treatment approach for phobias (Blomhoff et al., 2001; Ressler et al., 2004). Therefore, further studies of how adjunct pharmacological therapy might contribute to treatment of specific phobias in the long term would be informative. Another question that remains to be

answered empirically is whether an integrated treatment approach with slower-acting medications would be more advantageous than combining behavior therapy with faster-acting drugs. There is some evidence that slower-acting therapeutics combined with behavior therapy might be beneficial, but attrition also may be elevated by slower-acting relative to faster-acting medications (Craske, 1999). In our study, MB-treated animals showed improved retention of extinction over a relatively long (22 days) time period, but further studies are needed to translate this research into a more clinically relevant time frame.

Another recent approach used to improve fear extinction involved use of the NMDA receptor partial agonist d-cycloserine (DCS). Rather than exert its effects on neuromodulators such as serotonin and norepinephrine, DCS enhances the stimulating effects of glutamate on the NMDA receptor. Hence, DCS is thought to facilitate new learning which occurs in extinction. Accordingly, both DCS and MB, could be potentially useful for general improvement of learning retention. This indeed appears to be the case. There are several studies which show that DCS may effectively enhance synaptic plasticity that occurs during associative learning (Port & Seybold, 1998; Lelong, Dauphin, & Boulouard, 2001; Andersen, Lindberg, & Myhrer, 2002); and our laboratory has found that MB also can enhance different forms of learning when administered following the learning episode (Callaway et al., 2002; Callaway et al., 2004; Gonzalez-Lima et al., 2004; Riha et al., 2005). However, MB's mechanism of action is quite different from that of DCS. DCS's partial agonist properties are determined by the glutamatergic activity and/or the occupancy of the glycine binding site. Furthermore, DCS has been reported to have both anxiogenic and anxiolytic effects (Ho et al., 2005; Anthony & Nevins, 1993; Karcz-Kubicha et al.,

1997). It has recently been shown that baseline level of anxiety (as determined by the elevated-plus maze test in rats) determines whether DCS will have anxiolytic or anxiogenic effects. For example, 10-30 mg/kg DCS administered to rats showing low anxiety in the plus maze exerts anxiogenic activity in these rats, but has no effect on rats that display high anxiety levels (Ho et al., 2005). Therefore, it appears that individual differences in anxiety level may play a critical role for the DCS behavioral effects, and this might complicate the interpretation of studies in which DCS is used as an adjunct to behavioral therapy. In addition, DCS acts only on glutamatergic receptors, and there is some evidence that suggests that GABA-ergic neurotransmission may play an important role in fear extinction (Akirav et al., 2006).

Unlike DCS, which acts on a particular synaptic receptor, MB might improve extinction memory retention by acting as an electron shuttle to the mitochondrial respiratory complexes. Therefore, MB might selectively enhance energy production in brain regions with an ongoing metabolic demand, such as memory consolidation and retention in the post-extinction phase, without any non-specific side effects (Riha et al., 2005; Gonzalez-Lima et al., 2004). Potential candidates for such brain areas could be selected using the above-described metabolic maps and functional neural network models of fear extinction. Future studies could examine whether addition of MB to exposure therapy could improve its effectiveness, and utilize non-invasive imaging techniques such as fMRI to determine which brain regions might benefit from MB treatment and facilitate memory retention in human subjects.

Summary

The first two experiments of this study were aimed at identifying brain regions associated with extinguished fear consolidation, renewal and retention of extinction memory. The third experiment aimed to characterize a possible novel therapeutic compound which might be used as an adjunct drug to improve extinction. In addition to identifying brain areas which were differentially involved in fear renewal and extinction, this study also described a potential functional neural network associated with fear extinction and renewal. The results obtained using FDG method suggest that renewal of conditioned fear is mediated by the neural reactivation of the CS-US association involving excitatory responses to the tone CS in the auditory system, and reactivation of the US representation in somatosensory pathways in the absence of the US. Additionally, quantitative CO histochemistry revealed that Pavlovian fear acquisition training increased metabolic capacity in several brain areas, including mPFC and septum, while acquisition and extinction training reduced CO activity in these regions back to pseudorandom group levels. A functional neural extinction network constructed based on an existing neurobiological model of extinction suggested that during fear renewal many of the direct influences on relevant brain regions become opposite in the path coefficient sign, as compared to extinction recall condition. Finally, the third experiment suggested that low-dose MB might be a useful adjunct to exposure therapy, since it improved retention of fear extinction in our animal model of specific phobias, without affecting non-specific motor or anxiety measures. Overall, these studies integrated information about the neural correlates of fear extinction training and recall obtained with two different metabolic mapping techniques, and proposed a novel metabolic enhancer, MB,

as a candidate compound to be used for enhancement of conditioned fear extinction.

Bibliography

Aitkin, L., Kenyon, C., & Philpott, P. (1981). The representation of the auditory and somatosensory systems in the external nucleus of the cat inferior colliculus. *J Comp. Neuro., 196,* 25-40.

Akirav, I., Raizel, H., & Maroun, M. (2006). Enhancement of conditioned fear extinction by infusion of the GABAA agonist muscimol into the rat prefrontal cortex and amygdala. *Eur. J Neurosci., 23,* 758-764.

An, X., Bandler, D., Ongur, J., & Price, J. (1998). Prefrontal cortical projections to longitudinal columns in the midbrain periaqueductal gray in Macaque monkeys. *J Comp. Neuro., 401,* 455-479.

Andersen, J. M., Lindberg, V., & Myhrer, T. (2002). Effects of scopolamine and d-cycloserine on non-spatial reference memory in rats. *Behav. Brain Res., 129,* 211-216.

Andersen, R. (1997). Multimodal integration for the representation of space in the posterior parietal cortex. *Philos Trans R Soc Lond B Biol Sci, 352,* 1421-1428.

Anglada-Figueroa, D. & Quirk, G. J. (2005). Lesions of the Basal Amygdala Block Expression of Conditioned Fear But Not Extinction. *J Neurosci., 25,* 9680-9685.

Anthony, E. & Nevins, M. (1993). Anxiolytic-like effects of N-methyl-D-aspartate-associated glycine receptor ligands in the rat potentiated startle test. *Eur. J Pharm., 250,* 317-324.

Asaka, Y., Seager, M., Griffin, A., & Berry, S. (2000). Medial septal microinfusion of scopolamine disrupts hippocampal activity and trace jaw movement conditioning. *Behav.Neurosci., 114,* 1068-1077.

Attwell, D. & Laughlin, S. B. (2001). An Energy Budget for Signaling in the Grey Matter of the Brain. *J Cereb Blood Flow Metab., 21,* 1133-1145.

Ballenger, J. (2004). Remission rates in patients with anxiety disorders treated with paroxetine. *J Clin Psychiatry, 65,* 1696-1707.

Barad, M. (2005). Fear extinction in rodents: basic insight to clinical promise. *Curr. Op. Neurobiology, 15,* 710-715.

Barlow, D. H., Gorman, J. M., Shear, M. K., & Woods, S. W. (2000). Cognitive-Behavioral Therapy, Imipramine, or Their Combination for Panic

Disorder: A Randomized Controlled Trial. *JAMA: J American Med. Ass., 283,* 2529-2536.

Barrett, D., Shumake, J., Jones, D., & Gonzalez-Lima, F. (2003). Metabolic mapping of mouse brain activity after extinction of a conditioned emotional response. *J Neurosci., 23,* 5740-5749.

Bauer, E. P. & Ledoux, J. E. (2004). Heterosynaptic Long-Term Potentiation of Inhibitory Interneurons in the Lateral Amygdala. *J Neurosci., 24,* 9507-9512.

Beck, C. H. M. & Fibiger, H. C. (1995). Conditioned fear-induced changes in behavior and in the expression of the immediate early gene c-fos: With and without diazepam pretreatment. *J Neurosci., 15,* 709-720.

Bennet, L., Roelfsema, V., Pathipati, P., Quaedackers, J. S., & Gunn, A. J. (2006). Relationship between evolving epileptiform activity and delayed loss of mitochondrial activity after asphyxia measured by near-infrared spectroscopy in preterm fetal sheep. *J Phys.,* 216-224.

Bennett, M. C., Mlady, G. W., Fleshner, M., & Rose, G. M. (1996). Synergy between chronic corticosterone and sodium azide treatments in producing a spatial learning deficit and inhibiting cytochrome oxidase activity. *Proc.Natl.Acad.Sci., 93,* 1330-1334.

Berlau, D. J. & McGaugh, J. L. (2006). Enhancement of extinction memory consolidation: The role of the noradrenergic and GABAergic systems within the basolateral amygdala. *Neurobiology of Learning and Memory, In Press, Corrected Proof*.

Berndt, J. D., Callaway, N. L., & Gonzalez-Lima, F. (2001). Effects of chronic sodium azide on brain and muscle cytochrome oxidase activity: A potential model to investigate environmental contributions to neurodegenerative diseases. *J Tox. and Environmental Health-Part A, 63,* 67-77.

Blomhoff, S., Tangen, H., Hellstrom, K., Holme, I., Humble, M., Madsbu, H. et al. (2001). Randomised controlled general practice trial of sertraline, exposure therapy and combined treatment in generalised social phobia. *The British J Psychiatry, 179,* 23-30.

Bontempi, B., Laurent-Demir, C., Destrade, C., & Jaffard, R. (1999). Time-dependent reorganization of brain circuitry underlying long-term memory storage. *Nature, 400,* 671-675.

Bouton, M. E. (1993). Context, time, and memory retrieval in the interference paradigms of Pavlovian learning. *Psychol.Bull., 114,* 80-99.

Bouton, M. E., Kenney, F., & Rosengard, C. (1990). State-dependent fear extinction with two benzodiazepine tranquilizers. *Behav.Neurosci., 104,* 44-55.

Bouton, M. E. & King, D. A. (1983). Contextual control of the extinction of conditioned fear: tests for the associative value of the context. *J Exp.Psychol.Anim Behav.Process, 9,* 248-265.

Bouton, M. E. & Nelson, J. B. (1998). The role of context in classical conditioning: some implications for cognitive behavior therapy. In W.O'Donohue (Ed.), *Learning and Behavior Therapy* (pp. 59-84). Boston: Allyn and Bacon.

Bouton, M. E. (2002). Context, ambiguity, and unlearning: sources of relapse after behavioral extinction. *Biological Psychiatry, 52,* 976-986.

Bouton, M. E. (2004). Context and Behavioral Processes in Extinction. *Learning & Memory, 11,* 485-494.

Bouton, M. E. & Moody, E. W. (2004). Memory processes in classical conditioning. *Neurosci. & Biobehav. Reviews, 28,* 663-674.

Brady, J. & Nauta, J. (1953). Subcortical mechanisms in emotional behavior: affective changes following septal forebrain lesions in the albino rat. *J.Comp.Physiol.Psychol., 46,* 339-346.

Breiter, H. C., Etcoff, N. L., Whalen, P. J., Kennedy, W. A., Rauch, S. L., Buckner, R. L. et al. (1996). Response and Habituation of the Human Amygdala during Visual Processing of Facial Expression. *Neuron, 17,* 875-887.

Bremner, J. D., Vermetten, E., Schmahl, C., Vaccarino, V., Vythilingam, M., Afzal, N. et al. (2005). Positron emission tomographic imaging of neural correlates of a fear acquisition and extinction paradigm in women with childhood sexual-abuse-related post-traumatic stress disorder. *Psychol Med, 35,* 791-806.

Brewin, C. (1989). Cognitive change processes in psychotherapy. *Psychol Rev, 96,* 379-394.

Burrows, G. (1984). Methylene blue: effects and disposition in sheep. *J Vet Pharmacol Ther, 7,* 225-231.

Burwell, R. D., Bucci, D. J., Sanborn, M. R., & Jutras, M. J. (2004). Perirhinal and Postrhinal Contributions to Remote Memory for Context. *J Neurosci., 24,* 11023-11028.

Bussey, T. J., Muir, J. L., & Aggleton, J. P. (1999). Functionally Dissociating Aspects of Event Memory: the Effects of Combined Perirhinal and Postrhinal Cortex Lesions on Object and Place Memory in the Rat. *J Neurosci., 19,* 495-502.

Bystritsky, A. (2004). Current Pharmacological Treatments for Obsessive-Compulsive Disorder. *Essential Psychopharmacology, 5,* 251-272.

Cada, A., Gonzalez-Lima, F., Rose, G. M., & Bennett, M. C. (1995). Regional brain effects of sodium azide treatment on cytochrome oxidase activity: a quantitative histochemical study. *Metab.Brain Dis., 10,* 303-319.

Cain, C. K., Blouin, A. M., & Barad, M. (2003). Temporally massed CS presentations generate more fear extinction than spaced presentations. *J Exp.Psychol.Anim Behav.Process, 29,* 323-333.

Callaway, N. L., Riha, P. D., Bruchey, A. K., Munshi, Z., & Gonzalez-Lima, F. (2004). Methylene blue improves brain oxidative metabolism and memory retention in rats. *Pharmacol.Biochem.Behav., 77,* 175-181.

Callaway, N. L., Riha, P. D., Wrubel, K. M., McCollum, D., & Gonzalez-Lima, F. (2002). Methylene blue restores spatial memory retention impaired by an inhibitor of cytochrome oxidase in rats. *Neurosci.Lett., 332,* 83-86.

Campeau, S., Falls, W. A., Cullinan, W. E., Helmreich, D. L., Davis, M., & Watson, S. J. (1997). Elicitation and reduction of fear: behavioural and neuroendocrine indices and brain induction of the immediate-early gene c-fos. *Neuroscience, 78,* 1087-1104.

Capaldi, E. (1994). The sequential view: From rapidly fading stimulus traces to the organization of memory and the abstract concept of number. *Psych. Bull. Rev, 1,* 156-181.

Capaldi, R. A. (1990). Structure and Function of Cytochrome c Oxidase. *Ann. Review of Biochem., 59,* 569-596.

Carrive, P., Leung, P., Harris, J., & Paxinos, G. (1997). Conditioned fear to context is associated with increased Fos expression in the caudal ventrolateral region of the midbrain periaqueductal gray. *Neuroscience, 78,* 165-177.

Cavada, C. & Goldman-Rakic, P. S. (2004). Posterior parietal cortex in rhesus monkey: I. Parcellation of areas based on distinctive limbic and sensory corticocortical connections. *J Comp. Neurology, 287,* 393-421.

Christofi, G., Nowicky, A. V., Bolsover, S. R., & Bindman, L. J. (1993). The postsynaptic induction of nonassociative long-term depression of excitatory synaptic transmission in rat hippocampal slices. *J Neurophys., 69,* 219-229.

Conde, F., Maire-Lepoivre, E., Audinat, E., & Crepel, F. (1995). Afferent connections of the medial frontal cortex of the rat. II. Cortical and subcortical afferents. *J Comp Neurol, 352,* 567-593.

Conejo, N. M., Gonzalez-Pardo, H., Vallejo, G., & Arias, J. L. (2004). Involvement of the mammillary bodies in spatial working memory revealed by cytochrome oxidase activity. *Brain Research, 1011,* 107-114.

Conejo, N. M., Lopez, M., Cantora, R., Gonzalez-Pardo, H., Lopez, L., Begega, A. et al. (2005). Effects of Pavlovian fear conditioning on septohippocampal metabolism in rats. *Neurosci. Letters, 373,* 94-98.

Cooke, S. & Bliss, T. (2005). Long-term potentiation and cognitive drug discovery. *Curr Opin Investig Drugs, 6,* 25-34.

Corcoran, K. A., Desmond, T. J., Frey, K. A., & Maren, S. (2005). Hippocampal Inactivation Disrupts the Acquisition and Contextual Encoding of Fear Extinction. *J Neurosci., 25,* 8978-8987.

Corcoran, K. A. & Maren, S. (2004). Factors Regulating the Effects of Hippocampal Inactivation on Renewal of Conditional Fear After Extinction. *Learning Memory, 11,* 598-603.

Corodimas, K. & Ledoux, J. E. (1995). Disruptive effects of posttraining perirhinal cortex lesions on conditioned fear: contributions of contextual cues. *Behav. Neurosci., 109,* 613-619.

Costa Gomez, T. M. & Behbehani, M. M. (1995). An electrophysiological characterization of the projection from the central nucleus of the amygdala to the periaqueductal gray of the rat: the role of opioid receptors. *Brain Research, 689,* 21-31.

Craske, M. (1999). *Anxiety Disorders: Psychological approaches to theory and treament*. Boulder, CO: Westview Press.

Critchley, H. (2003). Emotion and its disorders: Imaging in clinical neuroscience. *British Medical Bulletin, 65,* 35-47.

Davis, M. (2002). Role of NMDA receptors and MAP kinase in the amygdala in extinction of fear: clinical implications for exposure therapy*. *Eur. J Neurosci., 16,* 395-398.

de Oliveira, R. W., Del Bel, E. A., & Guimaraes, F. S. (2000). Behavioral and c-fos expression changes induced by nitric oxide donors microinjected into the dorsal periaqueductal gray. *Brain Res. Bull., 51,* 457-464.

Delamater, A. (2004). Experimental extinction in Pavlovian conditioning: Behavioural and neuroscience perspectives. *The Quarterly J Exp. Psychology, 57B,* 97-132.

Desmedt, A., Garcia, R., & Jaffard, R. (1999). Vasopressin in the lateral septum promotes elemental conditioning to the detriment of contextual fear conditioning in mice. *Eur. J Neurosci., 11,* 3913-3921.

Devinsky, O., Morrell, M., & Vogt, B. (1995). Contributions of anterior cingulate cortex to behaviour. *Brain, 1995,* 1-279.

Domjan, M. (1998). *The principles of learning and behavior*. (Fourth ed.) Pacific Grove, CA: Brooks/Cole Publishing Company.

Drew, M., Yang, C., Ohyama, T., & Balsam, P. (2004). Temporal specificity of extinction in autoshaping. *J Exp Psychol Anim Behav Process, 30,* 163-176.

Falls, W. A., Miserendino, M. J., & Davis, M. (1992). Extinction of fear-potentiated startle: blockade by infusion of an NMDA antagonist into the amygdala. *J Neurosci., 12,* 854-863.

Fanselow, M. S. (1989). The adaptive function of conditioned defensive behavior: an ecological apporach to Pavlovian stimulus-substitution theory. In R.J.Blanchard, Brain P.F., Blanchard D.C., & Parimigiani S. (Eds.), *Ethoexperimental approaches to the study of behavior* (pp. 151-166). Boston: Kluwer.

Fanselow, M. S. (2000). Contextual fear, gestalt memories, and the hippocampus. *Behav.I Brain Research, 110,* 73-81.

Fanselow, M. S. & Ledoux, J. E. (1999). Why We Think Plasticity Underlying Pavlovian Fear Conditioning Occurs in the Basolateral Amygdala. *Neuron, 23,* 229-232.

Federoff, I. & Taylor, S. (2001). Psychological and Pharmacological Treatments of Social Phobia: A Meta-Analysis. *Journal of Clinical Psychopharmacology, 21,* 311-324.

Ferguson, C., LaVia, M., & Crossan, P. (1999). Are serotonin selective reuptake inhibitors effective in underweight anorexia nervosa? *International J Eating Disorders, 25,* 11-17.

Ferreira, T. L., Moreira, K. M., Ikeda, D. C., Bueno, O. F. A., & Oliveira, M. G. (2003). Effects of dorsal striatum lesions in tone fear conditioning and contextual fear conditioning. *Brain Research, 987,* 17-24.

Fischer, H., Andersson, J., Furmark, T., & Fredrikson, M. (2006). Fear conditioning and brain activity: a positron emission tomography study in humans. *Behav.Neurosci., 114,* 671-680.

Fredrikson, M., Wik, G., Greitz, T., Eriksson, L., Stone-Elander, S., Ericson, K. et al. (1993). Regional cerebral blood flow during experimental phobic fear. *Psychophysiology, 30,* 126-130.

Frohardt, R. J., Guarraci, F. A., & Bouton, M. E. (2000). The effects of neurotoxic hippocampal lesions on two effects of context after fear extinction. *Behav.Neurosci., 114,* 227-240.

Gabbott, P., Warner, A., Jays, P., Salway, P., & Busby, S. (2005). Prefrontal cortex in the rat: Projections to subcortical autonomic, motor, and limbic centers. *J Comp. Neurology, 492,* 145-177.

Gallistel, C. & Gibbon, J. (2000). Time, rate, and conditioning. *Psychological Review, 107,* 289-344.

Garcia, R., Chang, C. h., & Maren, S. (2006). Electrolytic lesions of the medial prefrontal cortex do not interfere with long-term memory of extinction of conditioned fear. *Learning & Memory, 13,* 14-17.

Gewirtz, J., Falls, W. A., & Davis, M. (1997). Normal Conditioned Inhibition and Extinction of Freezing and Fear-Potentiated Startle Following Electrolytic Lesions of Medial Prefrontal Cortex in Rats. *Behav.Neurosci., 11,* 712-716.

Ghashghaei, H. T. & Barbas, H. (2002). Pathways for emotion: interactions of prefrontal and anterior temporal pathways in the amygdala of the rhesus monkey. *Neuroscience, 115,* 1261-1279.

Giza, B. K., Ackroff, K., McCaughey, S. A., Sclafani, A., & Scott, T. R. (1997). Preference conditioning alters taste responses in the nucleus of the solitary tract of the rat. *AJP - Regulatory, Integrative and Comp. Phys., 273,* R1230-R1240.

Goldman-Rakic, P. S. (1987). Circuitry of the frontal association cortex and its relevance to dementia. *Arch. of Gerontology and Geriatrics, 6,* 299-309.

Gonzalez-Lima, F. (1992). Brain imaging of auditory learning functions in rats: studies with fluorodeoxyglucose autoradiography and cytochrome oxidase histochemistry. In F.Gonzalez-Lima, t. Finkenstadt, & H. Scheich (Eds.), *Advances in Metabolic Mapping Techniqyes for Brain Imaging of Behavioral and Learning Functions* (pp. 39-109). Boston/London: Kluwer Academic Publishers.

Gonzalez-Lima, F. (1998). *Cytochrome oxidase in neuronal metabolism and Alzheimer's disease*. (1 ed.) (vols. 1) New York: Plenum Press.

Gonzalez-Lima, F. & Agudo, J. (1990). Functional reorganization of neural auditory maps by differential learning. *Neuroreport, 1,* 161-164.

Gonzalez-Lima, F. & Bruchey, A. K. (2004). Extinction Memory Improvement by the Metabolic Enhancer Methylene Blue. *Learning & Memory, 11,* 633-640.

Gonzalez-Lima, F. & Cada, A. (1998). Quantitative histochemistry of cytochrome oxidase activity. In F.Gonzalez-Lima (Ed.), *Cytochrome Oxidase in Neuronal Metabolism and Alzheimer's Disease* (pp. 55-90). New York: Plenum Press.

Gonzalez-Lima, F., Finkenstadt, t., & Ewert, J. (1989). Learning-related activation in the auditory system of the rat produced by long-term habituation: a 2-deoxyglucose study. *Brain Research, 489,* 67-79.

Gonzalez-Lima, F., Helmstetter, F., & Agudo, J. (1993). Functional mapping of the rat brain during drinking behavior: a fluorodeoxyglucose study. *Physiol Behav, 54,* 605-12.

Gonzalez-Lima, F. & Scheich, H. (1984a). Classical conditioning enhances auditory 2-Deoxyglucose patterns in the inferior colliculus. *Neurosci.Lett., 51,* 79-85.

Gonzalez-Lima, F. & Scheich, H. (1984b). Functional activation in the auditory system of the rat produced by arousing reticular stimulation: a 2-deoxyglucose study. *Brain Research, 299,* 201-214.

Gonzalez-Lima, F. & Scheich, H. (1985). Ascending reticular activating system in the rat: a 2-deoxyglucose study. *Brain Research, 344,* 70-88.

Goosens, K. A. & Maren, S. (2001). Contextual and Auditory Fear Conditioning are Mediated by the Lateral, Basal, and Central Amygdaloid Nuclei in Rats. *Learning Memory, 8,* 148-155.

Gosselin, R., Hodge, H., & Smith, R. (1976). *Clinical toxicology of commercial products.* (Fourth ed.) Baltimore: Williams and Wilkins.

Gottfried, J. A. & Dolan, R. J. (2004). Human orbitofrontal cortex mediates extinction learning while accessing conditioned representations of value. *Nat Neurosci, 7,* 1144-1152.

Grace, A. A. & Rosenkranz, J. A. (2002). Regulation of conditioned responses of basolateral amygdala neurons. *Physiology & Behavior, 77,* 489-493.

Gray, J. & McNaughton, N. (1983). Comparison between the behavioural effect of septal and hippocampal lesions: a review. *Neurosci. and Biobehav. Reviews, 7,* 119-188.

Grigson, P., Shimura, T., & Norgren, R. (1997). Brainstem lesions and gustatory function: III. The role of the nucleus of the solitary tract and the parabrachial nucleus in retention of a conditioned taste aversion in rats. *Behav. Neurosci., 111,* 180-187.

Groenewegen, H., Wright, C., Beijer, A., & Voorn, P. (1999). Convergence and Segregation of Ventral Striatal Inputs and Outputs. *Annals of the New York Academy of Sciences, 877,* 49-63.

Gunther, L., Denniston, J., & Miller, R. (1998). Conducting exposure treatment in multiple contexts can prevent relapse. *Behav. Res. and Therapy, 36,* 75-91.

Hamner, M., Lorberbaum, J., & George, M. (1999). Potential role of the anterior cingulate cortex in PTSD: Review and hypothesis. *Depression and Anxiety, 9,* 1-14.

Haselgrove, M. & Pearce, J. (2003). Facilitation of extinction by an increase or a decrease in trial duration. *J Exp Psychol Anim Behav Process, 29,* 153-166.

Hembree, E., Foa, E., Dorfan, N., Street, G., Kowalski, J., & Tu, X. (2006). Do patients drop out prematurely from exposure therapy for PTSD? *J Trauma Stress, 16,* 555-562.

Herdegen, T. & Leah, J. D. (1998). Inducible and constitutive transcription factors in the mammalian nervous system: control of gene expression by Jun, Fos and Krox, and CREB/ATF proteins. *Brain Res. Reviews, 28,* 370-490.

Herry, C. & Mons, N. (2004). Resistance to extinction is associated with impaired immediate early gene induction in medial prefrontal cortex and amygdala. *Eur.J Neurosci., 20,* 781-790.

Hevner, R., Duff, R., & Wong-Riley, M. (1992). Coordination of ATP production and consumption in brain: parallel regulation of cytochrome oxidase and Na+, K(+)-ATPase. *Neurosci.Lett., 138,* 188-192.

Ho, Y. J., Hsu, L. S., Wang, C. F., Hsu, W. Y., Lai, T. J., Hsu, C. C. et al. (2005). Behavioral effects of d-cycloserine in rats: The role of anxiety level. *Brain Research, 1043,* 179-185.

Ishikawa, A. & Nakamura, S. (2003). Convergence and Interaction of Hippocampal and Amygdalar Projections within the Prefrontal Cortex in the Rat. *J Neurosci., 23,* 9987-9995.

Jay, T. & Witter, M. (1991). Distribution of hippocampal CA1 and subicular efferents in the prefrontal cortex of the rat studied by means of anterograde transport of Phaseolus vulgaris-leucoagglutinin. *J Comp Neurol, 313,* 574-586.

Ji, J. & Maren, S. (2005). Electrolytic lesions of the dorsal hippocampus disrupt renewal of conditional fear after extinction. *Learning & Memory, 12,* 270-276.

Jones, D. & Gonzalez-Lima, F. (2001a). Associative effects of Pavlovian differential inhibition of behaviour. *Eur. J Neurosci., 14,* 1915-1927.

Jones, D. & Gonzalez-Lima, F. (2001b). Mapping pavlovian conditioning effects on the brain: Blocking, contiguity, and excitatory effects. *J Neurophys., 86,* 809-823.

Kamprath, K. & Wotjak, C. T. (2004). Nonassociative learning processes determine expression and extinction of conditioned fear in mice. *Learning Memory, 11,* 770-786.

Karcz-Kubicha, M., Jessa, M., Nazar, M., Plaznik, A., Hartmann, S., Parsons, C. G. et al. (1997). Anxiolytic activity of glycine-B antagonists and partial agonists--No relation to intrinsic activity in the patch clamp. *Neuropharmacology, 36,* 1355-1367.

Kelner, M. J., Bagnell, R., Hale, B., & Alexander, N. M. (1988). Potential of methylene blue to block oxygen radical generation in reperfusion injury. In C.Von Sonntag (Ed.), *Oxygen radicals in biology and medicine* (New York, NY: Plenum Press.

Kim, J. J. & Jung, M. W. (2006). Neural circuits and mechanisms involved in Pavlovian fear conditioning: A critical review. *Neurosci. & Biobehav. Reviews, 30,* 188-202.

Knight, D., Smith, C., Cheng, T., Stein, E., & Helmstetter, F. (2004). Amygdala and hippocampal activity during acquisition and extinction of human fear conditioning. *Cognitive, Affective, & Behav. Neurosci., 4,* 317-325.

Knight, D., Smith, C., Stein, E., & Helmstetter, F. (1999). Functional MRI of human Pavlovian fear conditioning: patterns of activation as a function of learning. *Neuroreport, 10,* 3665-3670.

Krolak-Salmon, P., Henaff, M. A., Vighetto, A., Bertrand, O., & Mauguiere, F. (2004). Early Amygdala Reaction to Fear Spreading in Occipital, Temporal, and Frontal Cortex: A Depth Electrode ERP Study in Human. *Neuron, 42,* 665-676.

Krout, K. E., Mettenleiter, T. C., Karpitskiy, V., Van Nguyen, X., & Loewy, A. D. (2005). CNS neurons with links to both mood-related cortex and sympathetic nervous system. *Brain Research, 1050,* 199-202.

Kupfer, A., Aeschlimann, C., & Cerny, T. (1996). Methylene blue and the neurotoxic mechanisms of ifosfamide encephalopathy. *European Journal of Clinical Pharmacology, 50,* 249-252.

Lacroix, L., Spinelli, S., White, W., & Feldon, J. (2000). The effects of ibotenic acid lesions of the medial and lateral prefrontal cortex on latent

inhibition, prepulse inhibition and amphetamine-induced hyperlocomotion. *Neuroscience, 97,* 459-468.

Landry, P. & Deschenes, M. (1981). Intracortical arborizations and receptive fields of identified ventrobasal thalamocortical afferents to the primary somatic sensory cortex in the cat. *J Comp. Neurology, 199,* 345-371.

Langa, P., Davish, M., & Ohmanc, A. (2000). Fear and anxiety: animal models and human cognitive psychophysiology. *Journal of Affective Disorders, 61,* 137-159.

Lecas, J. (1995). Prefrontal neurones sensitive to increased visual attention in the monkey. *Neuroreport, 7,* 305-309.

Ledoux, J. E., Farb, C., & Ruggiero, D. A. (1990). Topographic organization of neurons in the acoustic thalamus that project to the amygdala. *J Neurosci., 10,* 1043-1054.

Ledoux, J. E., Sakaguchi, A., & Reis, D. J. (1984). Subcortical efferent projections of the medial geniculate nucleus mediate emotional responses conditioned to acoustic stimuli. J *Neurosc., 4,* 683-698.

Lelong, V., Dauphin, F., & Boulouard, M. (2001). RS 67333 and D-cycloserine accelerate learning acquisition in the rat. *Neuropharmacology, 41,* 517-522.

Li, H. Y., Ericsson, A., & Sawchenko, P. E. (1996). Distinct mechanisms underlie activation of hypothalamic neurosecretory neurons and their medullary catecholaminergic afferents in categorically different stress paradigms. *Proceedings of the National Academy of Sciences, 93,* 2359-2364.

Lindahl, P. E. & Öberg, K. E. (1961). The effect of rotenone on respiration and its point of attack. *Exp.Cell Res., 23,* 237.

Linke, R. & Schwegler, H. (2000). Convergent and Complementary Projections of the Caudal Paralaminar Thalamic Nuclei to Rat Temporal and Insular Cortex. *Cerebral Cortex, 10,* 753-771.

Liu, P. & Bilkey, D. (2001). The effect of excitotoxic lesions centered on the hippocampus or perirhinal cortex in object recognition and spatial memory tasks. *Behavioral Neuroscience, 115,* 94-111.

Mackintosh, N. (1974). *The psychology of animal learning*. New York, NY: Academic Press.

Malin, E. L. & McGaugh, J. L. (2006). Differential involvement of the hippocampus, anterior cingulate cortex, and basolateral amygdala in memory for context and footshock. *Proceedings of the National Academy of Sciences, 103,* 1959-1963.

Maren, S. (2001). Neurobiology of Pavlovian fear conditioning. *Annual Review of Neuroscience, 24,* 897-931.

Maren, S. (2005). Building and Burying Fear Memories in the Brain. *The Neuroscientist, 11,* 89-99.

Maren, S., Aharonov, G., & Fanselow, M. S. (1997a). Neurotoxic lesions of the dorsal hippocampus and Pavlovian fear conditioning in rats. *Behav. Brain Research, 88,* 261-274.

Maren, S. & Fanselow, M. S. (1997b). Electrolytic Lesions of the Fimbria/Fornix, Dorsal Hippocampus, or Entorhinal Cortex Produce Anterograde Deficits in Contextual Fear Conditioning in Rats. *Neurob. of Learning and Memory, 67,* 142-149.

Maren, S. & Quirk, G. J. (2004). Neuronal signalling of fear memory. *Nature Reviews Neuroscience, 5,* 844-852.

Marsicano, G., Wotjak, C. T., Azad, S. C., Bisogno, T., Rammes, G., Cascio, M. G. et al. (2002). The endogenous cannabinoid system controls extinction of aversive memories. *Nature, 418,* 530-534.

Martinez, J. L., Jensen, R. A., Vasquez, B., McGuiness, T., & McGaugh, J. L. (1978). Methylene blue alters retention of inhibitory avoidance responses. *Physiol. Psychology, 6,* 387-390.

Mauk, M. D. & Ohyama, T. (2004). Extinction as New Learning Versus Unlearning: Considerations from a Computer Simulation of the Cerebellum. *Learning Memory, 11,* 566-571.

McCaughey, S. A., Giza, B. K., Nolan, L. J., & Scott, T. R. (1997). Extinction of a Conditioned Taste Aversion in Rats: II. Neural Effects in the Nucleus of the Solitary Tract. *Physiology & Behaviour, 61,* 373-379.

McGaugh, J. L. (2004). The amygdala modulates the consolidation of memories of emotionally arousing experiences. *Annual Review of Neuroscience, 27,* 1-28.

McGeorge, A. & Faull, R. (1989). The organization of the projection from the cerebral cortex to the striatum in the rat. *Neuroscience, 29,* 503-537.

McGregor, I. S., Hargreaves, G. A., Apfelbach, R., & Hunt, G. E. (2004). Neural Correlates of Cat Odor-Induced Anxiety in Rats: Region-Specific Effects of the Benzodiazepine Midazolam. *J Neurosci., 24,* 4134-4144.

McIntosh, A. R. & Gonzalez-Lima, F. (1994). Network interactions among limbic cortices, basal forebrain, and cerebellum differentiate a tone conditioned as a Pavlovian excitor or inhibitor: fluorodeoxyglucose mapping and covariance structural modeling. *J Neurophys., 72,* 1717-1733.

McIntosh, A. R. & Gonzalez-Lima, F. (1995). Functional network interactions between parallel auditory pathways during Pavlovian conditioned inhibition. *Brain Research, 683,* 228-241.

McIntosh, A. R. & Gonzalez-Lima, F. (1998). Large-scale functional connectivity in associative learning: Interrelations of the rat auditory, visual, and limbic systems. *J Neurophys., 80,* 3148-3162.

McNally, G. P., Pigg, M., & Weidemann, G. (2004). Opioid Receptors in the Midbrain Periaqueductal Gray Regulate Extinction of Pavlovian Fear Conditioning. *J Neurosci., 24,* 6912-6919.

McSweeney, F. & Swindell, S. (2002). Common processes may contribute to extinction and habituation. *J Gen Psychol, 129,* 364-400.

Megias, M., Emri, Z., Freund, T. F., & Gulyas, A. I. (2001). Total number and distribution of inhibitory and excitatory synapses on hippocampal CA1 pyramidal cells. *Neuroscience, 102,* 527-540.

Milad, M., Orr, S., Pitman, R., & Rauch, S. (2005). Context modulation of memory for fear extinction in humans. *Psychophysiology, 42,* 456-464.

Milad, M. & Quirk, G. (2002). Neurons in medial prefrontal cortex signal memory for fear extinction. *Nature, 420,* 70-74.

Milad, M., Vidal-Gonzalez, I., & Quirk, G. (2004). Electrical stimulation of medial prefrontal cortex reduces conditioned fear in a temporally specific manner. *Behav.Neurosci., 118,* 389-394.

Miller, E. K. & Cohen, J. D. (2001). An integrative theory of prefrontal cortex function. *Annual Review of Neuroscience, 24,* 167-202.

Miller, R. & Matzel, L. (1988). The comparator hypothesis: A response rule for the expression of associations. In G.Bower (Ed.), *The psychology of learning and motivation* (pp. 51-92). Orlando, FL: Academic Press.

Mineka, S., Mystkowski, J., Hladek, D., & Rodriguez, B. I. (1999). The effects of changing contexts on return of fear following exposure therapy for spider fear. *J Consulting and Clinical Psychology, 67,* 599-604.

Miyashita, T. & Williams, C. L. (2004). Peripheral arousal-related hormones modulate norepinephrine release in the hippocampus via influences on brainstem nuclei. *Behav. Brain Research, 153,* 87-95.

Mogenson, G. & Yang, C. (1991). The contribution of basal forebrain to limbic-motor integration and the mediation of motivation to action. *Adv Exp Med Biol., 295,* 267-290.

Mongeau, R., Miller, G. A., Chiang, E., & Anderson, D. J. (2003). Neural correlates of competing fear behaviors evoked by an innately aversive stimulus. *J Neurosc., 23,* 3855-3868.

Morgan, M. A. & Ledoux, J. E. (1995). Differential contribution of dorsal and ventral medial prefrontal cortex to the acquisition and extinction of conditioned fear in rats. *Behav.Neurosci., 109,* 681-688.

Morgan, M. A., Romanski, L., & Ledoux, J. E. (1993). Extinction of emotional learning: contribution of medial prefrontal cortex. *Neurosci.Lett., 163,* 109-113.

Morgan, M. A., Schulkin, J., & Ledoux, J. E. (2003). Ventral medial prefrontal cortex and emotional perseveration: the memory for prior extinction training. *Behav.Brain Res., 146,* 121-130.

Morgan, M. A. & Ledoux, J. E. (1999). Contribution of Ventrolateral Prefrontal Cortex to the Acquisition and Extinction of Conditioned Fear in Rats. *Neurobiology of Learning and Memory, 72,* 244-251.

Morgan, R. E. & Riccio, D. C. (1994). Extinction of An Amnestic Memory in Rats - Evidence for the Malleability of Inaccessible Information. *Learning and Motivation, 25,* 431-446.

Murray, E. A. & Richmond, B. J. (2001). Role of perirhinal cortex in object perception, memory, and associations. *Current Opinion in Neurobiology, 11,* 188-193.

Myers, K. M. & Davis, M. (2002). Behavioral and Neural Analysis of Extinction. *Neuron, 36,* 567-584.

Mystkowski, J. L., Craske, M. G., & Echiverri, A. M. (2002). Treatment context and return of fear in spider phobia. *Behavior Therapy, 33,* 399-416.

Naber, P., Witter, M., & Lopes da Silva, F. (1999). Perirhinal cortex input to the hippocampus in the rat: evidence for parallel pathways, both direct and indirect. A combined physiological and anatomical study. *Eur.J Neurosci., 11,* 4119-4235.

Nauta, W. (1971). The problem of the frontal lobe: a reinterpretation. *J Psychiatr Res, 8,* 167-187.

Naylor, G. J., Martin, B., Hopwood, S. E., & Watson, Y. (1986). A two-year double-blind crossover trial of the prophylactic effect of methylene blue in manic-depressive psychosis. *Biological Psychiatry, 21,* 915-920.

Nemeroff, C. B., Bremner, J. D., Foa, E. B., Mayberg, H. S., North, C. S., & Stein, M. B. (2006). Posttraumatic stress disorder: A state-of-the-science review. *J Psychiatric Research, 40,* 1-21.

Nerad, L. & Bilkey, D. K. (2005). Ten- to 12-Hz EEG Oscillation in the Rat Hippocampus and Rhinal Cortex That Is Modulated by Environmental Familiarity. *J Neurophys., 93,* 1246-1254.

Nickel JC (2005). Management of urinary tract infections: historical perspective and current strategies: Part 1--Before antibiotics. *J Urology, 173,* 21-26.

Nyberg, L., McIntosh, A. R., Cabeza, R., Nilsson, L. G., Houle, S., Habib, R. et al. (1996). Network Analysis of Positron Emission Tomography Regional Cerebral Blood Flow Data: Ensemble Inhibition during Episodic Memory Retrieval. *J Neurosci., 16,* 3753-3759.

O'Donohue, W. (1998). Conditioning and Third-Generation Behavior Therapy. In W.O'Donohue (Ed.), *Learning and behavior therapy* (Boston: Allyn and Bacon.

Paquette, V., Levesque, J., Mensour, B., Leroux, J. M., Beaudoin, G., Bourgouin, P. et al. (2003). "Change the mind and you change the brain": effects of cognitive-behavioral therapy on the neural correlates of spider phobia. *NeuroImage, 18,* 401-409.

Pare, D., Quirk, G. J., & Ledoux, J. E. (2004). New Vistas on Amygdala Networks in Conditioned Fear. *J Neurophys., 92,* 1-9.

Pavlov, I. (1927). *Conditioned reflexes.* London: Oxford UP.

Paxinos, G. & Watson, C. (1997). *The rat brain in stereotaxic coordinates*. San Diego, CA: Academic Press.

Pearce, J. & Hall, G. (1980). A model for Pavlovian conditioning: Variations in the effectiveness of conditioned but not unconditioned stimuli. *Psychol Rev, 87,* 332-352.

Peter, C., Hongwan, D., Küpfer, A., & Lauterburg, B. H. (2000). Pharmacokinetics and organ distribution of intravenous and oral methylene blue. *Eur.J.Clin.Pharmacol., 56,* 247-250.

Phelps, E. A., Delgado, M. R., Nearing, K. I., & Ledoux, J. E. (2004). Extinction Learning in Humans: Role of the Amygdala and vmPFC. *Neuron, 43,* 897-905.

Phelps, E. A. & Ledoux, J. E. (2005). Contributions of the Amygdala to Emotion Processing: From Animal Models to Human Behavior. *Neuron, 48,* 175-187.

Poremba, A., Jones, D., & Gonzalez-Lima, F. (1998). Classical conditioning modifies cytochrome oxidase activity in the auditory system. *Eur.J Neurosci., 10,* 3035-3043.

Port, R. L. & Seybold, K. S. (1998). Manipulation of NMDA-receptor activity alters extinction of an instrumental response in rats. *Physiology & Behavior, 64,* 391-393.

Post, S. & Mai, J. (1978). Evidence for amygdaloid projections to the contralateral hypothalamus and the ipsilateral midbrain in the rat. *Cell and Tissue Res., 191,* 183-186.

Powers, M., Smits, J., & Telch, M. (2004). Disentangling the Effects of Safety-Behavior Utilization and Safety-Behavior Availability During Exposure-Based Treatment: A Placebo-Controlled Trial. *J Consulting and Clinical Psych., 72,* 448-454.

Price, J. L. & Amaral, D. G. (1981). An autoradiographic study of the projections of the central nucleus of the monkey amygdala. *J Neurosci., 1,* 1242-1259.

Quirk, G. J. (2002). Memory for Extinction of Conditioned Fear Is Long-lasting and Persists Following Spontaneous Recovery. *Learning Memory, 9,* 402-407.

Quirk, G. J., Russo, G. K., Barron, J. L., & Lebron, K. (2000). The Role of Ventromedial Prefrontal Cortex in the Recovery of Extinguished Fear. *J Neurosci., 20,* 6225-6231.

Raj, A. B. M., Bertolone, S. J. M., & Jaffe, N. M. D. (2004). Methylene Blue Reversal of Ifosfamide-Related Encephalopathy. *J Pediatric Hematology/Oncology, 26,* 116.

Rauch, S. L., Savage, C. R., Alpert, N. M., Fischman, A. J., & Jenike, M. A. (1997). The functional neuroanatomy of anxiety: a study of three disorders using positron emission tomography and symptom provocation. *Biol. Psychiatry, 42,* 446-452.

Rescorla, R. (1979). Condition inhibition and extinction. In A.Dickinson & R. Boakes (Eds.), *Mechanisms of learning and motivation: A memorial volume to Jerzy Konorski* (pp. 83-110). Hillsdale, NJ: Erlbaum.

Rescorla, R. (1993). Inhibitory associations between S and R in extinction. *Animal Learning & Behavior, 21,* 327-336.

Rescorla, R. & Wagner, A. (1972). A Theory of Pavlovian Conditioning: variation in effectiveness of reinforcement and nonreinforecement. In A.Black & W. Prokasy (Eds.), *Classical conditioning II* (pp. 64-99). New York: Appleton-Century-Crofts.

Ressler, K. J., Rothbaum, B. O., Tannenbaum, L., Anderson, P., Graap, K., Zimand, E. et al. (2004). Cognitive Enhancers as Adjuncts to Psychotherapy: Use of D-Cycloserine in Phobic Individuals to Facilitate Extinction of Fear. *Archives of General Psychiatry, 61,* 1136-1144.

Richards, W. G., Farley, J., & Alkon, D. L. (1984). Extinction of associative learning in Hermissenda: behavior and neural correlates. *Behav.Brain Res., 14,* 161-170.

Riedel, G., Casabona, G., Platt, B., Macphail, E. M., & Nicoletti, F. (2000). Fear conditioning-induced time- and subregion-specific increase in expression of mGlu5 receptor protein in rat hippocampus. *Neuropharmacology, 39,* 1943-1951.

Riha, P. D., Bruchey, A. K., Echevarria, D. J., & Gonzalez-Lima, F. (2005). Memory facilitation by methylene blue: Dose-dependent effect on behavior and brain oxygen consumption. *European J Pharm., 511,* 151-158.

Rodriguez, B. I., Craske, M. G., Mineka, S., & Hladek, D. (1999). Context-specificity of relapse: effects of therapist and environmental context on return of fear. *Behav. Res. and Therapy, 37,* 845-862.

Rokers, B., Mercado, E., Allen MT, Myers, C., & Gluck, M. (2002). A connectionist model of septohippocampal dynamics during conditioning: closing the loop. *Behav.Neurosci., 116,* 48-62.

Rosen, J. B., Hitchcock, J. M., Miserendino, M. J., Falls, W. A., Campeau, S., & Davis, M. (1992). Lesions of the perirhinal cortex but not of the frontal, medial prefrontal, visual, or insular cortex block fear-potentiated startle using a visual conditioned stimulus. *J Neurosci., 12,* 4624-4633.

Rudy, J. W., Huff, N. C., & Matus-Amat, P. (2004). Understanding contextual fear conditioning: insights from a two-process model. *Neurosci. & Biobehav. Reviews, 28,* 675-685.

Sacchetti, B., Lorenzini, C. A., Baldi, E., Tassoni, G., & Bucherelli, C. (1999). Auditory Thalamus, Dorsal Hippocampus, Basolateral Amygdala, and Perirhinal Cortex Role in the Consolidation of Conditioned Freezing to Context and to Acoustic Conditioned Stimulus in the Rat. *J. Neurosci., 19,* 9570-9578.

Salaris, S. C., Babbs, C. F., & Voorhees III, W. D. (1991). Methylene blue as an inhibitor of superoxide generation by xanthine oxidase. *Biochem.Pharmac., 42,* 499-506.

Sanderson, W. & Wetzler, S. (1993). Observations on the cognitive behavioral treatment of panic disorder: Impact of benzodiazepines. *Psychotherapy, 30,* 125-132.

Sandin, B. & Chorot, P. (2006). Resistance to extinction of conditioned electrodermal responses: a study of the incubation fear hypothesis. *Psychol Rep, 91,* 37-46.

Santini, E., Ge, H., Ren, K., Pena, d. O., & Quirk, G. J. (2004). Consolidation of fear extinction requires protein synthesis in the medial prefrontal cortex. *J Neurosci., 24,* 5704-5710.

Schafe, G. E., Doyere, V., & Ledoux, J. E. (2005). Tracking the Fear Engram: The Lateral Amygdala Is an Essential Locus of Fear Memory Storage. *J Neurosci., 25,* 10010-10014.

Schneider, M. & Koch, M. (2005). Behavioral and morphological alterations following neonatal excitotoxic lesions of the medial prefrontal cortex in rats. *Exp Neurology, 195,* 185-198.

Schubert, K., Shaikh, M., & Siegel, A. (1996). NMDA receptors in the midbrain periaqueductal gray mediate hypothalamically evoked hissing behavior in the cat. *Brain Res., 726,* 80-90.

Seillier, A., Seillier, A., Majchrzak, M., Marchand, A., & Di Scala, G. (2005). Systemic or intra-accumbens injection of D-amphetamine delays habituation to a tone stimulus in rats. *Behavioral Pharmacology, 16,* 35-42.

Sheehan, T. P., Chambers, R. A., & Russell, D. S. (2004). Regulation of affect by the lateral septum: implications for neuropsychiatry. *Brain Res Reviews, 46,* 71-117.

Siegel, J. (1976). Effect of medial septal lesions on conditioned taste aversion in the rat. *Physiol Behav, 17,* 761-765.

Smith, W. J., Stewart, J., & Pfaus, J. G. (1997). Tail Pinch Induces Fos Immunoreactivity Within Several Regions of the Male Rat Brain: Effects of Age. *Physiology & Behaviour, 61,* 717-723.

Sokoloff, L. (1992). Imaging techniques in studies of neuronal functions. In F.Gonzalez-Lima & H. Scheich (Eds.), *Advances in metabolic mapping techniques for brain imaging of behavioral and learning functions* (pp. 1-37). Boston: Kluwer.

Solomon, P. R., Solomon, S., van der Schaaf, E., & Perry, H. (1983). Altered activity in the hippocampus is more detrimental to classical conditioning than removing the structure. *Science, 22,* 329-331.

Sotres-Bayon, F., Bush, D. E. A., & Ledoux, J. E. (2004). Emotional Perseveration: An Update on Prefrontal-Amygdala Interactions in Fear Extinction. *Learning Memory, 11,* 525-535.

Sotres-Bayon, F., Cain, C. K., & Ledoux, J. E. (2006). Brain Mechanisms of Fear Extinction: Historical Perspectives on the Contribution of Prefrontal Cortex. *Biological Psychiatry, In Press, Corrected Proof*.

Sparks, P. & Ledoux, J. E. (1995). Septal lesions potentiate freezing behavior to contextual but not to phasic conditioned stimuli in rats. *Behav Neuroscience, 109,* 184-188.

Staples, L. G., Hunt, G. E., Cornish, J. L., & McGregor, I. S. (2005). Neural activation during cat odor-induced conditioned fear and 'trial 2' fear in rats. *Neurosc & Biobehav Reviews, 29,* 1265-1277.

Straube, T., Glauer, M., Dilger, S., Mentzel, H. J., & Miltner, W. H. R. (2006). Effects of cognitive-behavioral therapy on brain activation in specific phobia. *NeuroImage, 29,* 125-135.

Suzuki, W. A. & Amaral, D. G. (1994). Topographic organization of the reciprocal connections between the monkey entorhinal cortex and the perirhinal and parahippocampal cortices. *J of Neuroscience, 14,* 1856-1877.

Swanson, L. & Cowan, W. (1977). An autoradiographic study of the organization of the efferent connections of the hippocampal formation in the rat. *J of Comp Neurology, 172,* 49-84.

Tang, J., Ko, S., Ding, H. K., Qiu, C. S., Calejesan, A., & Zhuo, M. (2005). Pavlovian fear memory induced by activation in the anterior cingulate cortex. *Molecular Pain, 1,* 6.

Teich, A. H., Mccabe, P. M., Gentile, C. C., Schneiderman, L. S., Winters, R. W., Liskowsky, D. R. et al. (1989). Auditory-Cortex Lesions Prevent the Extinction of Pavlovian Differential Heart-Rate Conditioning to Tonal Stimuli in Rabbits. *Brain Research, 480,* 210-218.

Telch, M., Agras, W., Taylor, C., Roth, W., & Gallen, C. (1985). Combined pharmacological and behavioral treatment for agoraphobia. *Behav Res and Therapy, 23,* 325-335.

Thomas, B. L., Larsen, N., & Ayres, J. J. B. (2003). Role of context similarity in ABA, ABC, and AAB renewal paradigms: Implications for theories of renewal and for treating human phobias. *Learning and Motivation, 34,* 410-436.

Thomas, E. (1988). Forebrain mechanisms in the relief of fear: the role of the lateral septum. *Psychobiology, 16,* 36-44.

Thomas, E. & Evans, G. (1983). Septal inhibition of aversive emotional states. *Physiol.Behav, 31,* 673-678.

Thomas, K. L., Hall, J., & Everitt, B. J. (2002). Cellular imaging with zif268 expression in the rat nucleus accumbens and frontal cortex further dissociates the neural pathways activated following the retrieval of contextual and cued fear memory. *European J of Neuroscience, 16,* 1789-1796.

Tierney, P. L., Degenetais, E., Thierry, A. M., Glowinski, J., & Gioanni, Y. (2004). Influence of the hippocampus on interneurons of the rat prefrontal cortex. *European J of Neuroscience, 20,* 514-524.

Treit, D. & Fundytus, M. (1988). Thigmotaxis as a test for anxiolytic activity in rats. *Pharmacol.Biochem.Behav., 31,* 959-962.

Tremere, L. & Pinaud, R. (2005). Incongruent restoration of inhibitory transmission and general metabolic activity during reorganization of somatosensory cortex. *Int J Neurosci, 115,* 1003-1115.

Turner, B. & Herkenham, M. (1991). Thalamoamygdaloid projections in the rat: a test of the amygdala's role in sensory processing. *J Comp Neurol, 313,* 295-325.

Uylings, H. B. M., Groenewegen, H. J., & Kolb, B. (2003). Do rats have a prefrontal cortex? *Behav Brain Research, 146,* 3-17.

Vansteenwegen, D., Francken, G., Vervliet, B., De Clercq, A., & Eelen, P. (2006). Resistance to extinction in evaluative conditioning. *J Exp Psychol Anim Behav Process, 32,* 71-79.

Vianna, D. M. L., Graeff, F. G., Brandao, M. L., & Landeira-Fernandez, J. (2001). Defensive freezing evoked by electrical stimulation of the periaqueductal gray: comparison between dorsolateral and ventrolateral regions. *Neuroreport, 12,* 4109-4112.

Villarreal, J. S., Gonzalez-Lima, F., Berndt, J., & Barea-Rodriguez, E. J. (2002). Water maze training in aged rats: effects on brain metabolic capacity and behavior. *Brain Res., 939,* 43-51.

Visarius, T. M., Stucki, J. W., & Lauterburg, B. H. (1997). Stimulation of respiration by methylene blue in rat liver mitochondria. *FEBS Lett., 412,* 157-160.

Vogt, B. & Miller, M. (1983). Cortical connections between rat cingulate cortex and visual, motor, and postsubicular cortices. *J of Comp Neurology, 216,* 192-210.

Vouimba, R., Garcia, R., & Jaffard, R. (1998). Opposite effects of lateral septal LTP and lateral septal lesions on contextual fear conditioning in mice. *Behav.Neurosci., 112,* 875-884.

Waddell, J., Dunnett, C., & Falls, W. A. (2004). C57BL/6J and DBA/2J mice differ in extinction and renewal of extinguished conditioned fear. *Behavl Brain Research, 154,* 567-576.

Wagner, A. & Brandon, S. (1989). Evolution of a structured connectionist model of Pavlovian conditioning (AESOP). In S.Klein & R. Mowrer (Eds.), *Contemporary Learning theories: Pavlovian conditioning and the status of traditional learning theory.* (pp. 149-189). Hillsdale, NJ: Lawrence Erlbaum Associaties, Inc.

Wainwright, M. & Crossley, K. B. (2002). Methylene Blue--a therapeutic dye for all seasons? *J of Chemotherapy, 14,* 431-443.

Westral, H. & Stewart, S. (2002). As-needed Use of Benzodiazepins in Managing Clinical Anxiety: Incidence and Implications. *Current Pharmaceutical Design, 8,* 59-74.

Wilson, A., Brooks, D. C., & Bouton, M. E. (1995). The role of the rat hippocampal system in several effects of context in extinction. *Behav.Neurosci., 109,* 828-836.

Wolpe, J. (1958). *Psychotherapy by reciprocal inhibition*. Stanford: Stanford University Press.

Wong-Riley, M. (1989). Cytochrome oxidase: an endogenous metabolic marker for neuronal activity. *Trends Neurosci., 12,* 94-101.

Wong-Riley, M., Antuono, P., Ho, K. C., Egan, R., Hevner, R., Liebl, W. et al. (1997). Cytochrome oxidase in Alzheimer's disease: biochemical, histochemical, and immunohistochemical analyses of the visual and other systems. *Vision Res., 37,* 3593-3608.

Yadin, E., Thomas, E., Grishkat, H., & Strickland, C. (1993). The role of the lateral septum in anxiolysis. *Physiology and Behavior, 53,* 1077-1083.

Yasui, Y., Itoh, K., Mizuno, N., Nomura, S., Takada, M., Konishi, A. et al. (1983). The posteromedial ventral nucleus of the thalamus (VPM) of the cat: direct ascending projections to the cytoarchitectonic subdivisions. *J Comp Neurol, 220,* 219-228.

Zagrodska, J., Romaniuk, A., Wieczorek, M., & Boguszewski, P. (2000). Bicuculline administration into ventromedial hypothalamus: effects on fear and regional brain monoamines and GABA concentrations in rats. *Acta Neurobiol.Exp, 60,* 333-343.

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