A

Studies of The Neural Basis of Fear Learning

and Implications for Psychopathology

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TABLE OF CONTENTS

LIST OF FIGURES

CHAPTER 1

INTRODUCTION: FEAR AND PSYCHOPATHOLOGY

Fear Definition

Introduction. A patient lost her episodic memory; in other words, she was unable to remember her conscious experiences of a moment or a day ago. To explore the nature of the patient's memory loss, an investigator extended a hand in greeting; hidden in his hand was a pin: when they shook hands, the patient pulled away in pain. When the researcher later presented himself, the patient gave no sign of recognition. As he, again, extended his hand again in greeting, the patient would not shake it. When asked why, she made up a plausible but irrelevant answer. When pressed she reportedly stated that "sometimes people have pins hidden in their hands." without any awareness that this was her own memory of recent events. (Claparede, 1911)

Do we avoid things because we are afraid of them? The patient did not consciously remember her past experience with the stimuli that she wished to avoid. Was she aware of being afraid? Is consciousness of fear necessary to its definition? These questions about the patient's experience, the answer to why the patient was afraid to shake the doctor's hand, lie at the heart of the following dissertation.

When the conclusion of this dissertation is reached, support will have been developed for the idea that the patient, without awareness, acquired and retained a memory responsible for evoking fear and her avoidance of the handshake, in spite of an

evidently compromised memory for her own conscious experience. This dissertation presents evidence from animal experiments and discusses animal and human investigation of areas of the brain responsible for fear, particularly the amygdala, which is a nucleated forebrain complex lying deep in the anterior temporal lobes. Such evidence contains implications for understanding the ways in which traumatic events give rise to psychopathology.

At first glance, an understanding of the neurophysiology of fear in animals may not seem relevant to the clinical enterprise. When put in the context of the ubiquitous element of fear in most psychopathology, particularly mood and anxiety disorders, the study of the basic organization of fear is central. It will be suggested that brain systems responsible for fear and for affiliative emotion play an important role in psychopathology. A role that in significant ways is independent of the verbal and self-conscious aspects of our experience. Further investigation of animal model systems can be expected to continue to advance our understanding.

A satisfying explanation of fear must account both for the subjective and objective qualities that we understand as fear. As long as consciousness lies beyond our understanding, a full explanation of fear will be incomplete. However, neurophysiological results allow for the characterization of elements which might previously have only been identified through introspection.

Subjective aspects of fear can be pointed to by the words we use to describe it; the predominate experience of fear ranges from absolute dread to mild worry, often

thought centers on anticipated harm. The experience is notable for the absence of happiness, a feeling of unpleasantness, urgency, and a sense of pressure to relieve the state. Conscious fear experiences vary in intensity, in duration, and in awareness of the eliciting source; it is possible to further classify conscious fear by whether the eliciting source is an external stimulus or an internally generated memory or thought.

After a harmful event, salient stimuli as well as the background environmental contextual stimuli that were present along with the painful ones will by themselves elicit responses, some predominately innately shaped and some predominately learned, that increase the chances of avoiding future harm. The previously neutral stimuli now associated with harm are considered conditioned stimuli. By themselves, the conditioned stimuli can elicit a wide range of innate responses. So too, acquired activity that served to terminate or reduce the intensity of the conditioned stimuli are more likely to occur when the stimuli are present in the future. Predominately innate fear responses can be observed as a cross-species, integrated set of effects (and universal aspects across human cultures (Scherer and Wallbott, 1994)).

Physiological and behavioral effects include changes in heart rate, respiration, blood pressure, glucose metabolism, adrenaline release, piloerection, defecation, urination, decreased pain sensitivity, and selection among a range of innate behaviors including freezing, escape, and defensive aggression (Ledoux and Muller, 1997). Actions not central to threat detection and reaction are suppressed (Bouton and Bolles, 1980); suppression extends to biologically important but less urgent activities such as digestion, immune

system activity, and sexual activity. The choice of behavior is in some way predictable by the nature and proximity of the threat (Fanselow and Lester, 1988).

Fear has effects on cognition and memory as well. Central nervous system arousal increases (Davis and Whalen, 2001). Perception and awareness are redirected to facilitate threat detection and reaction. Mildly threatening percepts often result in lowered heart rate while orienting is accentuated (Kapp, Supple, et al., 1994). There is evidence to support the inference that perception is enhanced and focused on external stimuli (Dringenberg, Saber, et al., 2001; Stein, Jiang, et al., 2001). Explicit memory formation is modulated, generally increasing memory formation (Packard and Cahill, 2001). Fear can also interfere with memory formation in some paradigms (McEwen and Sapolsky, 1995; Perlstein, Elbert, et al., 2002). One explanation of the negative effects is that attended perceptions are likely to be strongly encoded, but attention directed to scan for and focus on perceived threats decreases the amount of attention available for forming memories of non-threatening aspects of the environment. Others have suggested high fear activation degrades hippocampal function, known to be implicated in explicit memory formation (Jacobs and Nadel, 1985; McEwen and Sapolsky, 1995; Kim, Lee, et al., 2001).

Finally, as has long been presumed (Freud, 1926), emerging evidence supports the view that fear can occur without conscious awareness. As detected by behavioral and physiological changes, increased heart rate and changes in skin conductance can be elicited in the absence of awareness and or subsequent explicit memory (Morris, Ohman, et al., 1998; Whalen, Rauch, et al., 1998).

No distinctions between anxiety and fear have been made in the definition above. Commonly, anxiety is considered to be less intense, or chronic, or without a consciously identifiable source (Freud, 1920), or from an internal rather than external source (Compton, 1980) when contrasted with fear (Tyrer, 1999). None of these rationales for distinction have been applied consistently. Very often the terms are used interchangeably (Wong, 1999). If fear and anxiety are overlapping if somewhat discernible conscious phenomena; aspects of fear and anxiety that lie outside of subjective experience have a great deal in common, reflecting, as will be argued further, a common neural system's activity.

Global theory of emotion deserves note in the context of a review of fear research. The distinctions among the terms emotion, affect, feeling, and mood are debated (Bucci, 2000; Damasio, 2001). Theories of emotion have been distinguished by dimensional and categorical approaches. Dimensional approaches to emotion have focused on the polarity of positive and negative emotion (Freud, 1920; Ikemoto and Panksepp 1999; Russell and Carroll 1999; Larsen, McGraw et al. 2001). Others have identified approach/avoidance as a dimension along which emotions can be located (Davidson, 2001). Gray posits three components into which emotions fall: a behavioral inhibition system, a fight/flight system, and an approach system (Gray, 1991; Corr, Pickering, et al., 1997). Categorical approaches have argued for distinction among emotions, giving rise to disputes about the number of basic emotions (Ekman, 1992). Theories about the global organization of emotion continue to influence fear research. For example, Gray's views of emotion

actively shape his identification and linking of neuroanatomical components into an emotional neural system. Similarly, Davidson views fear as part of the avoidance pole of his dimensional approach. Further review of theories of emotion lies outside the scope of this work.

As will be elaborated, the definition of fear discussed above can be accounted for by the neural acitivity of a number of central nervous system components, including the amygdala. Before reviewing the neural basis of fear conditioning, the relevance of the study of fear for clinical psychology will be briefly explored.

Fear and Psychopathology

Clinically, we know that the effects of fear can be debilitating. Successful treatment often results in reduced levels of fear. But how do we sort out the relationship between fear and the causes of psychopathology? What is the role of trauma, of early experiences with caregivers, of losses? How are these experiences represented? Of significance is the degree to which these processes are a function of explicit cognition capable of reaching awareness or reflect implicit learning, perhaps of an emotional nature. So too, how do different representations, or kinds of learning, or conscious and unconscious processes interact? The experiments discussed in this dissertation offer some introduction to current neuroscientific approaches. But what has come before; what have others theorized?

A variety of approaches to understanding emotion's role in psychopathology exist. Behaviorist, cognitive, and psychoanalytic approaches have all developed theories, which are briefly reviewed below. In addition, more recent empirically-driven approaches share with the theoretical schools a variety of different levels of analysis and methodologies. For example, at the level of the gene, investigators seek explanations of individual difference in response to environmental stressors. Sensitivity to anxiety is often explored (Kagan, 2001). As for environmental factors, one broadly grouped line of exploration suggests early trauma leads to physiological dysfunction: levels of neurotransmitters or receptors are established in childhood development, determining adult risk of pathology (Heim and Nemeroff, 2001). Some have examined the concept of affect regulation, whether it has a clear definition and if so does it correlate with psychopathology (Thompson and Calkins, 1996; Shipman and Zeman, 2001). Others have looked at personality attributes associated with psychopathology (Davidson, 1998).

Before proceeding with further examination of the role of fear in psychopathology, I want to slightly narrow the focus on psychopathology by limiting the disorders under consideration to mood and anxiety disorders. I will discuss the substantial evidence that experience, particularly early experience with caregivers, plays a predominant role in mood and anxiety disorders. While fear learning may also play a role in psychotic disorders (in for example, paranoid symptoms in schizophrenia or in the literature of expressed emotion), a strong suspected link to a predominant, genetic cause exists. I include mood disorders because of the high correlation between anxiety

symptoms and depressive symptoms. While bipolar and obsessive-compulsive disorders likely also involve acquired causal factors, these two have relatively large genetic associations in comparison to other anxiety and mood disorders. I do not mean to state that there are no innate predispositions to the remaining anxiety and mood disorders (Kagan, 2001), rather that acquired factors are predominant, and will be my focus. The remaining anxiety and mood disorders have not been distinguished or identified on the basis of understanding their causes, but on identifying observable, differentiable, and clustering symptoms so that diagnostic reliability is improved (Zachar, 2000; Levine, Cole, et al., 2001).

Explanations for anxiety and mood disorders. A number of issues in understanding mood and anxiety disorders exist. Is there a basic underlying cause with a variety of expressed symptoms giving rise to the multiplicity of diagnoses, or do the disorders arise from distinct causes? Are disorders to be understood as arising from behavioral learning paradigms, or as cognitive dysfunction, or as psychodynamic conflict? Do these distinct, and at times antagonistic, approaches share any common understanding of causes?

It is possible that distinct, independent causes for each disorder might be identified. Some investigators are currently approaching understanding human anxiety disorders by elaborating the causal distinctions among the currently identified diagnostic categories of the DSM-IV. For example, panic disorders are caused by oxygen detector

malfunctions (Gorman, Kent, et al., 2000) or misattribution of body perceptions (Chambless, Beck, et al., 2000).

Another possibility is that there is common cause, at least in part, among anxiety and mood disorders. Before the DSM-III, depression and anxiety were not separate diagnostic categories (Himmelhoch, Levine, et al., 2001). Then, evidence of a bimodal distribution of symptoms and a commitment to empirically based diagnoses lead to the two being distinguished (Zachar, 2000; Levine, Cole, et al., 2001). Lately, a number of factors have given rise to further consideration of the links between and among the anxiety and mood categories. The high prevalence of comorbidity among the anxiety and mood disorders (Brown and Barlow, 1992; Kessler, McGonagle, et al., 1994; Kaufman and Charney, 2000; Erwin, Heimberg, et al., 2002) is greater than would be expected by separate disorders randomly co-occurring (Levine, Cole, et al., 2001). The genetic factors that predict risk for anxiety disorders are the same ones as predict depression (Kendler, Neale, et al., 1992). Mood and anxiety disorders both respond to anti-depressant medications; however, anxiolytics have a mixed record of treating mood disorders (Levine, Cole, et al., 2001). The theories of both behavioral and psychodynamic treatment also lend some support to a common cause. All these findings suggest strong links, if not a single common cause. Perhaps as suggested in Brenner's work, depression is one possible result of inadequately managed anxiety. Other factors, independent of anxiety, also may be capable of causing depression.

Behaviorist Approach. Among the approaches that can offer support for a common mechanism is the behavior conditioning model. In this model the symptoms in anxiety disorders are presumed to be a result of fear conditioning which can be extinguished or de-sensitized in treatment, using among other techniques, those derived from studies of aversive conditioning. Behavioral treatment implicitly assumes a common mechanism since the model is a remedy to various anxiety disorders. In fact, behavioral treatment has had success treating a range of anxiety disorders, particularly panic attacks and phobias, including social phobia.

A number of investigators have pointed out that simple fear conditioning is an inadequate model for explaining human anxiety disorders. The link between a traumatic event and disorder is not one-to-one causality. Seligman introduced another factor, that of control. In a phenomenon termed learned helplessness, subjects exposed to inescapable shocks would not learn to avoid subsequent shocks when it became possible to do so (Overmier and Seligman, 1967; Drugan, Basile, et al., 1997). Similarly, monkeys reared in more controllable environments habituated faster after a fearful event than those reared in less controllable circumstances (Mineka, Gunnar, et al., 1986). Fear conditioning is also influenced by reactions of others. Typically, naive monkeys who observe conspecifics reacting fearfully to snakes will subsequently react fearfully to snakes. However, monkeys who observe another monkey's calm response to a snake, do not acquire fear of snakes when subsequently shown other monkeys reacting fearfully to snakes (Mineka and Cook, 1986). Another issue with simple fear conditioning as a model for anxiety

disorders is that larger cumulative aversive experience would be expected to increase the likelihood of the disorder. Phobics are likely to have fewer traumatic episodes than non-phobics because they actively avoid risky circumstances (Mineka and Ohman, 2002). For example, a water phobic will have report fewer incidents with trouble in water than a water skier, but still report much larger fear of water. All stimuli are not equal, either. The classic concept of stimulus makes little distinction between inanimate, animal, and conspecific stimuli. More importantly, there are few if any models of how a thought could be equally as evocative as an external stimulus (but see Dollard and Miller 1950). Stimuli that are historically relevant to survival are known to condition more readily or elicit stronger responses than others. Sudden, high intensity CS's, predators, heights, darkness, conspecifics (members of the same species) are all examples of CS's that condition readily. It is speculated that there is some genetic encoding of relevant feature detection and ready access of those features to fear conditioning pathways (Ohman and Mineka, 2001). While the notion of a conditioned stimulus subsequently eliciting fear continues to play an important role in explaining pathological anxiety, there is substantial evidence that disorders are a product of fear's modulation and interaction with other factors.

Cognitive approach. Cognitive psychologists have examined patterns of cognition that predispose an individual to psychopathology when stressors occur (Beck, 1987; Mazure, Bruce, et al., 2000; Just, Abramson, et al., 2001). Cognitive psychologists have looked at anxiety disorders for signs of cognitive processing faults: mood bias in memory;

attention deficit from preoccupation with specific content; hypervigilance for threats; sustained attending to threats; and disruption of attention due to low threshold for threat detection (McNally, 1998; Chambless, Beck, et al., 2000; Craske and Pontillo, 2001). Social phobics show a bias to interpret interactions negatively (Clark and McManus, 2002; Heimberg, 2002). Beck holds that depressed people are likely to have a cognitive bias in which they attend to and remember negative events about themselves, their environment, and negative expectations for their future. Abramson and colleagues' view (Just, Abramson, et al., 2001), based on Seligman's work, is that there are two dimensions of thinking that contribute to depression. One is the global/case specific axis; another is stable versus fluctuating. As such global and stable view of negative events have been shown to be correlated with depression (McGinn, 2000).

One problem with understanding psychopathology through studying cognitive patterns is understanding the patterns' causal role. Just (2001) points out that it remains unclear whether the cognitive patterns observed in depression are a cause or consequence of the depression. The observed cognitive patterns could be effects of other causal factors. For example, the attention biases toward threats observed in anxiety disorders could be a distorted over-reaction, or they could be normal reactions in the case where strong or widely generalized fear conditioning has occurred. Some have investigated the efficacy of treating the cognitive biases in anxiety disorders in lieu of or in addition to behavioral treatments for extinguishing fear; results remain unclear (Craske and Pontillo, 2001).

The importance and primacy of social interactions is not emphasized in much of the behavioral and some of the cognitive approaches. There is considerable evidence that interpersonal factors, such as emotional discord or any kind of abuse, contribute to psychopathology (Margolin and Gordis, 2000; Mazure, Bruce, et al., 2000). Increased risk for adult mental health problems following childhood abuse is also demonstrated (Saunders, Villoponteaux, et al., 1992; Mullen, Martin, et al., 1996; Stein, Walker, et al., 1996; Heim and Nemeroff, 2001). Risk of adult mental health problems associated with childhood abuse is risk over and above the higher risk of mental illness associated with troubled families and low SES (Mullen, Martin, et al., 1996; Kaplan, Pelcovitz, et al., 1998). The relative contribution and interactions among environmental factors, including abuse, single parents, divorce, family discord, parent psychopathology, lack of parental support, and genetic factors have been examined (Kaplan, Pelcovitz, et al., 1998).

The risk factors identified above do not uniformly lead to pathology. Nor does all pathology include a history of the specific risk factors identified above. The mechanism through which adverse life events come to elicit psychopathology and the correlated question of how harmful events are incorporated in the brain are the subjects of intensive investigation. Different schools propose various kinds of mediating factors.

Psychoanalytic views of psychopathology. Psychoanalytic writers such as Freud and Sullivan have alluded to the role interpersonal interactions play in psychopathology. One central tenet is that interactions between caregivers and the developing child early in life establish patterns that predispose an individual to cope adequately or not with

subsequent life stressors. Hotly debated is the extent to which adult patterns of behavior or thought are linked actively to past interactions, particularly with early caregivers (Wachtel, 1989).

The early psychoanalytic view of anxiety's role in psychopathology was that damned up libido eventually exceeded the mind's capacity to contain it; the libido was converted to and expressed as anxiety. Freud later recognized his idea of the conversion of sexual wishes to anxiety was in error. He came to see anxiety's function in anticipating future danger. In his later view, unacceptable libido-driven wishes were associated with danger, giving rise to anxiety. Anxiety served as a signal instigating a defense mechanism to prevent the conflictual thoughts along with their affective component from reaching consciousness (Freud, 1920; Freud, 1926; Brenner, 1957).

Other psychoanalytic writers have argued that there are disorders more profound than those modeled by impulse, conflict, anxiety, and defense. Object relations theorists stress the primacy of relationships, and how they are represented within individuals (Balint, 1968; Guntrip, 1968). Motivation for emotional relatedness with others (caring and being cared for) is as primary an instinct or impulse as is sexual impulse, and thereby implicitly revises Freud's view of a broadly categorized positive impulse, libido. Lack of a solid, loving relationship or severe threats to the relationship early in life undermine the very core sense of oneself. Such a person will have difficulty trusting others, engaging in mutually beneficial interactions, and in making rational, socially reinforced choices (Guntrip, 1968). Kohut, developing some strands in Freud's thinking, has theorized about

the earliest conscious experiences of positive feeling (love), which are inevitably frustrated. In healthy development, the representation of these states is gradually modulated and transformed into realistic expectations of self and other. In more traumatic relationships, idealized others and aggrandized self are clung to in the face of such painful contrast to reality. Such overwhelmingly positive representations manifest in brittle overconfidence and/or over-idealizing of others. In either case, setting up further disappointments, giving rise to anger and seemingly thoughtless and selfish efforts to bring attention to oneself (Kohut, 1966).

A particularly productive research tradition has grown out of psychoanalytically influenced observations about the consequences of early childhood deprivation. Anna Freud, Spitz, and Bowlby identified that severe deprivation in infancy leads to profound problems in psychological functioning (Spitz, 1945; Freud and Burlingham, 1973; Bowlby, 1977). An outgrowth of these early efforts was the active field of attachment research. Under observation, infants and toddlers displayed a range of reactions to separation from their mother and her subsequent return. The children's reactions were categorized into three groups: secure, anxious, and avoidant. Upon the mother's return, the secure group was happy to see and briefly sought out physical contact; the anxious sought contact and remained clinging to mother, and the avoidant group showed indifference to her absence and return (although subsequent data would show this group had physiological signs of high levels of anxiety). Later a fourth group, disorganized, was added for those who did not show a stable pattern. Attachment work continues to

contribute to our ability to predict future behavior in relationships, to identify cognitive styles associated with attachment, and to understand its transmission across generations (Main, 1996; Slade, Belsky, et al., 1999). Progress has also been made in demonstrating associations between attachment style and psychopathology (Main, 1996; Sroufe, Carlson, et al., 1999)

Animal models have offered support for the attachment paradigm. A seminal step was the work of Harlow who showed that monkeys separated from their mothers for extended periods show profound deficits, including high levels of anxiety and inability to socialize (1965). More recent models have found fear effects with less severe deprivation. Monkeys whose mothers faced a relatively high degree of unpredictability in food availability when the subjects were infants show increased fear as adolescents (Rosenblum, Forger, et al., 2001). Hofer has analyzed the components of maternal separation effects, demonstrating some components are due to touch, others to hunger, and others to temperature changes (Hofer, 1996). Early work with rats showed that handling the rats as infants resulted in adult rats that showed less fear in novel situations (Levine, 1962; Levine, Haltmeyer, et al., 1967; Ader and Grota, 1969). Rats who have received relatively high levels of grooming and nursing behaviors as pups are less fearful as adults (Caldji, Tannenbaum, et al., 1998). Caldji suggests that the handling effects are a result of increased grooming and nursing, which follows handling episodes (Caldji, Tannenbaum, et al., 1998). The increased fearfulness from maternal deprivation can be reversed by artificially mimicking grooming and licking actions during deprivation

episodes (Van Oers, de Kloet, et al., 1999). GABA receptors and their benzodiazepine binding sites show increased density in amygdala and noradrenergic nuclei of less-fearful, handled rats (Caldji, Francis, et al., 2000).

Another implication of the attachment work for understanding psychopathology comes from its ties to the developmental perspective. Cicchetti's view is that relationship problems give rise to adaptations that have negative consequences later (Cicchetti and Cannon, 1999). For example, developing an avoidant style with a rejecting caregiver leads to avoiding peers and missed opportunities for learning and developing social interaction skills. This cycle continues into adulthood. Sroufe, in reporting on a longitudinal study, showed independent and cumulative effects of early attachment style, and adolescent attachment style on predicting measures of anxiety. He emphasizes that past experience, rather than determining future pathology, interacts with current experience such that past troubles can be overcome or deepened. Thus, an anxiety disorder ought to be seen more as the final manifestation of a lifetime, rather than, as viewed in the medical model, an acute disease that has a discrete infectious agent as cause.

Memory, Emotion, and Psychopathology. The psychoanalytic work reviewed so far emphasizes experiences with caregivers as a cause of health or pathology. Some argue that genetic dispositions of both child and parents can interact in some cases to produce problems in early relationships. Others point out that parents' own upbringing patterns their parenting (Fraiberg, Adelson, et al., 1975). If some causal factors of

psychopathology are acquired ones, the question remains how are damaging events able to effect changes in memory, thoughts, and actions.

Freud's identification and explanation of transference phenomena contains a theory of representation and emotion. He observed that a patient selectively perceives or distorts perceptions with others in a manner that evokes a particular pattern of thoughts and feelings in him or herself, as well as evoking thoughts and feelings in others. Freud's presumption is that transference contains information about earlier history of interactions and their emotional content.

Other psychoanalytic theorists have also advanced views of emotion and representation. In Kernberg's view, cognitive representations of self and others were always associated with an affective valence. In borderline psychopathology, a defense mechanism called splitting is predominant, that is the active separation of positive cognitions and their attendant affects from negative ones, undermining realistic views of self and others (Kernberg, 1985). At its limit, a number of psychodynamic researchers have identified guidelines for arriving at individually identified and tailored patterns of conscious and unconscious thought, emotion, and behavior underlying pathology (Silberschatz and Curtis, 1993; Barber, Luborsky, et al., 1995).

Influenced by the attachment tradition, Bowlby's view was that early caregiving experiences come to be generalized and stored in what he called working models, of expectations for self and other in caregiving relationships. Others have extended these ideas. Smaller scale generalizations and abstractions of all kinds of emotional interactions,

not just attachment ones, are made and eventually are grouped into the overarching working models representing average expected interactions with a specific other, of which Bowlby's attachment kind are one type (Stern, 1985). Later, such representations come to hold value for the individual who then seeks to reinforce these views through subsequent intentional interactions.

The idea that interactions with significant people are represented in the mind, and that such representations contain associations to emotion and can contain conflicts with regard to both positive and negative emotions is a consistent notion across the approaches.

Psychopathology and Fear: Summary

This review of causes of mood and anxiety disorders has encountered a wide range of alternatives: simple fear conditioning; genetic variation among individuals in propensity for fear; trauma-altered brain chemistry; cognitive biases; fear associated with unacceptable instinctual impulses; disruption of attachment; and, disruption of identity formation due to diminished or compromised love. What synthesis, if any, is possible of this variety of theoretical and methodological approaches? Undoubtedly, there are multiple and interacting causes of mood and anxiety disorders. This sketch of psychopathology has emphasized that increased fear, anxiety, and depression result from problematic social interaction. In particular, abuse, neglect, or increased maternal stress often lead to sustained fearfulness in the adult children. Response to aversive events can

be moderated by a foundation of secure and autonomous functioning. When the foundation is compromised, fear is unchecked. It is possible that sustained high levels of fear generate many of the physiological effects that some observers have identified as causes of disorders. In sum, there is increasing attention to emotion and its role in psychopathology, particularly the way that early deprivation and trauma are mediated in the brain to result in adult anxiety and depression. Advancing our understanding of the neural basis of fear can allow for further investigation of its modulation by factors related to social interactions.

CHAPTER 2

CLASSICAL FEAR CONDITIONING AND ITS NEURAL BASIS

Associative Learning Theory

Associative learning is a fundamental capacity of animals, recognized at least as early as Aristotle. Pavlov's famous work established the classical conditioning paradigm. Classical conditioning consists of the forming of an association between a neutral stimulus and an unconditioned stimulus (US). The US has motivational value, eliciting behavior not produced by presentations of the innocuous stimulus alone. After the pairing of the stimuli, the innocuous stimulus alone comes to elicit a motivationally relevant response. (See Figure 2.1) Hence, the innocuous stimulus is called the conditioned stimulus (CS). Learning is inferred as a result of the change in the animal's response to CS before and after CS-US pairings. Operant conditioning focuses on the association of a response with reinforcement. An organism spontaneously produces a number of behaviors (responses); frequency of responses that are paired with reinforcement can either be increased or decreased depending on the nature of the reinforcement. Operant conditioning served as a model from which early principles of associative learning were developed (Thorndike, 1898; Hull, 1943). Modern associative learning theories (Rescorla and Wagner, 1972; Mackintosh, 1975; Pearce and Bouton, 2001) attempt to reflect empirical data that suggest associative learning is due not just to contiguity of stimuli, but to contingency of the pairing of stimuli. Evidence suggests that rather than one principle or law of learning, a

variety of approaches can and are used by animals to predict future associations (Pearce and Bouton, 2001). Gallistel suggests that rather than looking for a set of universal learning principles, neural learning systems should be understood as adapted to solve particular, evolutionarily-shaped problems (Gallistel, 1995).

Developments In Understanding The Neural Basis Of Fear Conditioning

With the rise of cognitive psychology, a number of important concepts have been elaborated, including the computational modeling approach to brain function. The complexity and dissociability of memory systems, including distinctions between implicit and explicit memory have been recognized; among explicit memory, short and long term components, working memory, declarative versus semantic have been distinguished (Tulving, 2001). Cognitive psychology also gave rise to the ongoing debate regarding understanding cognition as a serial, logical reasoning faculty coupled to categorical and hierarchical memory systems versus a parallel processing, output-error correcting model (Rumelhart, 1986).

Another fundamental advance has taken place in the emerging discipline of neuroscience. Powerful advances have occurred in our understanding of the basic functioning of neurons including action potential propagation and quantized neurotransmitter release, in understanding the development of the neural system, and in understanding the integration of neural functioning in sensory, motor, and association modules (Kandel, Schwartz, et al., 1991). The molecular basis of fundamental examples of

learning were identified in model systems, notably in Aplysia (Kandel, 1991). An experimental analogue of learning, long term potentiation (LTP) has been identified and has had great success at elucidating molecular mechanisms of associative learning (Bliss and Lomo, 1973; Cotman, Monaghan, et al., 1988).

It is in this rich context that another model learning system, the amygdala's function in fear conditioning, has grown. The role of the amygdala in fear was suspected from effects in monkeys of anterior temporal lobectomy (Kluver and Bucy, 1939), and more specifically destruction of the amygdala (Weiskrantz, 1956). In the 60's the amygdala was implicated in classic fear conditioning (Cohen, 1974). Recent work in a number of labs have added dramatic evidence supporting the amygdala's role in fear conditioning (Fendt and Fanselow, 1999; LeDoux, 2000; Davis and Whalen, 2001).

Amygdala's Role In Fear Conditioning

This section includes an updated version of a published paper (Ledoux and Muller, 1997).

Fear behavior output. The central nucleus of the amygdala (Ce) is a key structure in the control of a variety of conditioned fear responses. Thus, lesions of the Ce interfere with behavioral (freezing) reactions, autonomic (sympathetic and parasympathetic) responses, stress hormone (ACTH and glucocorticoid) release, potentiation of somatic reflexes (startle and eyeblink), and changes in pain reactivity elicited by a CS (see Davis, 1992; Kapp, Whalen, et al., 1992; Fanselow, 1994; LeDoux,

1995). The CE also projects to the periaqueductal grey responsible for freezing, running, and defensive aggression behaviors (Amorapanth, Nader, et al., 1999). Each of these responses is controlled by a separate set of output connections of Ce.

Fear behavior sensory input. The lateral nucleus (LA) is the sensory input region of the amygdala, as has been shown by anatomical, behavioral, and physiological studies (see LeDoux, Cicchetti et al. 1990; LeDoux, Farb et al. 1990; Bordi and LeDoux 1992; Romanski and LeDoux 1992; Campeau and Davis 1995). In addition to receiving afferents from non-aversive sensory modalities, LA receives afferents from subcortical and cortical areas responsible for pain sensation (Shi and Davis, 1999). At least two paths from sensory areas of the thalamus to the amygdala have been identified: one direct and one by way of the sensory cortex, which in turn projects to LA. In addition, the sensory areas of the cortex projects to the perirhinal cortex which projects to LA.

Lesion and anatomy studies. Much of our understanding of the sensory pathways involved in conditioning has involved paradigms in which an auditory CS is used. Lesion experiments coupled with anatomical tract tracing studies have established brain regions necessary for fear conditioning (See Figure 2.3). For simple classical conditioning (one stimulus paired with shock), lesions of the auditory cortex do not interfere with conditioning, but lesions of the auditory thalamus (the medial geniculate body, MG) or auditory midbrain (the inferior colliculus) do (LeDoux, Sakaguchi, et al., 1984). These data suggest that the acoustic CS is transmitted through the auditory system to the MG and from there to some region in addition to the auditory cortex. Antereograde

labeling studies demonstrate that the MG and adjacent nuclei receiving auditory input, in addition to projecting to the auditory cortex, also sends efferents to LA (LeDoux, Sakaguchi, et al., 1984; LeDoux, Farb, et al., 1990). Conditioning of fear reactions to simple acoustic stimuli can be mediated by direct projections to LA from the thalamus, and lesions of LA block fear conditioning.

Inactivation studies. Whether the amygdala has a role in the acquisition, in contrast to the expression, of fear learning is difficult to assess using permanent lesions. Temporary disruption of the amygdala's functioning can address the acquisition question. Infusions before training into the lateral and basal nuclei of the amygdala of a glutamate antagonist specific for the NMDA receptor disrupt acquisition but not expression of previous learning (Miserendino, Sananes, et al., 1990; Fanselow and Kim, 1994). Similarly, inactivation of the lateral and basal nuclei with a GABAa agonist disrupts acquisition without abolishing later acquisition, after the amygdala's function has recovered (Muller, Corodimas, et al., 1997). (See Figure 2.3)

Post-training intra-amygdala infusions of muscimol (GABAa agonist) do not affect fear conditioning in classical conditioning paradigm (Wilensky, Schafe, et al., 1999). This evidence argues against the view that the amygdala's only role in classical conditioning is as a post-acquisition modulatory system for memory formation elsewhere in the brain, continuing the debate (Cahill, Weinberger, et al., 1999; Fanselow and LeDoux, 1999) as to whether the amygdala's role in fear related learning is exclusively one of

modulation of memory formation elsewhere, or whether the amygdala also forms and stores memory.

Role of auditory cortex. Although the auditory cortex need not be intact for simple fear conditioning to occur, neural activity is modified in the auditory cortex during such conditioning (Quirk, Repa, et al., 1995; Weinberger, 1995; Quirk, Armony, et al., 1997). Further, auditory cortex lesions do disrupt fear conditioning when the direct connections from the MG to the amygdala have been destroyed (Romanski and LeDoux, 1993). While not necessary, cortico-amygdala projections are certainly sufficient in mediating simple fear conditioning.

Intra-Amygdala functional pathways. Since inputs come into the amygdala by way of LA and outputs leave by way of Ce, there must be communication between these regions. Indeed, LA projects to Ce both directly and by way of the basal (basolateral) and accessory basal (basomedial) nuclei (Price, Russchen, et al., 1987; Pitkanen, Stefanacci, et al., 1995; Savander, Go, et al., 1996). Amorapanth et al. demonstrated a role for the basal nucleus. The basal nucleus is necessary for instrumental fear learning (Amorapanth, LeDoux, et al., 2000). When the nucleus was lesioned, rats were unable to learn to avoid the CS by escape, yet still froze to the CS. Beyond this finding, the contribution of specific intraamygdala pathways is not well understood.

Context conditioning. In addition to developing fear reactions to the specific stimulus paired with the shock, animals also learn to fear the various stimuli present in the background (context). This is readily demonstrated by placing a rat back into a

chamber in which it previously received tone-shock pairings. The rat will often begin to freeze when placed in the chamber, suggesting that it has been conditioned to the apparatus where the tone and shock were paired, as well as to the tone itself. Lesions of the hippocampus have no effect on simple or discrimination fear conditioning but disrupt contextual conditioning (Selden, Everitt, et al., 1991; Kim and Fanselow, 1992; Phillips and LeDoux, 1992). This is consistent with the long held belief that the hippocampus plays an important role in situations in which the interrelation of various stimuli is important (O'Keefe and Nadel, 1978; Nadel and Willner, 1980; Sutherland and Rudy, 1989; Eichenbaum, Otto, et al., 1992). Damage to the amygdala abolishes conditioning to both a discrete CS and to contextual stimuli, and projections from the hippocampus to the amygdala may be involved.

Summary. These observations of neural connectivity, electrophysiological activity of neurons, and behavioral effects of lesions provide a description, from sensory to motor neurons, of the structures and pathways underlying auditory fear conditioning. The circuitry involves transmission of inputs through the early stages of the auditory system to the acoustic thalamus. Projections from the auditory thalamus to LA directly or by way of auditory cortex transmits CS information to the amygdala in simple conditioning but projections through cortex are required for differential conditioning. Projections to LA and possibly other amygdala regions from the hippocampus may be involved in contextual conditioning. LA projects to Ce, both directly and by way of intraamygdala connections. Efferent to Ce, the pathway diverges, with different

projections mediating different responses. These findings contribute to a circuit description of specific brain nuclei and pathways, complete with input, output and integrative processing sites. However, other brain areas certainly also contribute, which will be reviewed after the cellular mechanisms of fear learning are discussed.

Cellular and Synaptic Mechanisms of Fear Conditioning

One model of the synaptic changes underlying learning is long term potentiation (LTP), in which a post-synaptic response to a given pre-synaptic input is enhanced after a train of high frequency stimuli. LTP can be induced in LA by stimulation in the auditory thalamus (Clugnet and LeDoux, 1990; Rogan and LeDoux, 1995) or by stimulation of cortical projections to LA (Chapman, Kairiss, et al., 1990). LTP has also been demonstrated in the amygdala by stimulation of projections from the hippocampal formation (Maren and Fanselow, 1995). In addition, the evoked responses recorded in LA from an auditory tone are enhanced after LTP induction by the same method of MG stimulation (Rogan and LeDoux, 1995). Presumably the auditory stimulus is transmitted through a subset of the fibers that were potentiated. Natural information processing is thus affected by LTP (See Figure 2.4). If something like LTP occurs naturally during learning, clearly the brain can make use of it to respond more effectively to external events. In fact, after pairing of tone and shock in fear conditioning, enhanced tone evoked potentials are found in LA (Rogan, Staubli, et al., 1997), similar in time and amplitude to those induced by stimulation that induces LTP.

Findings from the classic model systems (especially the hippocampal formation) in which LTP is studied have implicated the neurotransmitter glutamate and two of the major classes of glutamate receptors, NMDA and AMPA, in LTP induction and maintenance (Cotman, Monaghan, et al., 1988; Madison, Malenka, et al., 1991; Bliss and Collingridge, 1993; Malenka and Nicoll, 1993). AMPA receptor agonist accelerates acquisition in a standard classical fear conditioning paradigm (Rogan, Staubli, et al., 1997). Blockade of NMDA receptors in the amygdala prevents fear conditioning (Miserendino, Sananes, et al., 1990; Fanselow and Kim, 1994), suggesting that an NMDA-dependent form of synaptic plasticity in the amygdala might contribute to fear conditioning. Although NMDA receptors have not yet been implicated in LTP in the sensory input pathways to LA, studies of the anatomy and physiology of synaptic transmission in the input pathways to LA provide the foundation for understanding the role of NMDA receptors in synaptic plasticity in this region in both LTP and natural learning (fear conditioning).

Since glutamate and its receptors are suspected to be responsible for both LTP and natural fear conditioning, it is important to demonstrate that synapses involved in fear conditioning are glutamatergic. This has been shown in the pathway from the medial geniculate body to the amygdala, a pathway that exhibits LTP. The cells of origin of this pathway in the thalamus, as determined by retrograde transport from LA, can also be labeled by a glutamate marker (LeDoux and Farb, 1991). In addition, these thalamo-amygdala projections mainly form asymmetric synapses (which indicates excitatory

transmission) on dendritic spines in LA (LeDoux, Farb, et al., 1991). Many terminals in LA that originate in the auditory thalamus contact spines that are immunoreactive for NMDA and AMPA receptors (Farb and LeDoux, 1994; Farb, Aoki, et al., 1995). Finally, physiological studies have shown that blockade of either NMDA or AMPA receptors interferes with transmission through this pathway (Li, Phillips, et al., 1995), suggesting that information flow in this pathway depends on both types of receptors (Li, Phillips, et al., 1995). Stimulation of the thalamic pathway evokes EPSC with a 60% larger NMDA component than the EPSC from stimulation of the cortical inputs (Weisskopf and LeDoux, 1999). These quantified results suggest NMDA receptors play a larger role in thalamic than cortical transmission. This differs somewhat from the classic picture of NMDA receptors that has emerged from studies of hippocampal circuits, where NMDA receptors have been shown to be crucial for synaptic plasticity but not for routine synaptic transmission (e.g. Bliss and Collingridge 1993). In contrast to the thalamo-amygdala pathway, and like hippocampal circuits, transmission from the auditory cortex to LA is interfered with by AMPA but not NMDA blockade (Li, Stutzmann, et al., 1996). Glutamate and its receptors thus play somewhat different roles in the transmission of auditory signals to LA from thalamic versus cortical areas.

As in model LTP systems, plasticity underlying fear conditioning in the lateral nucleus of the amygdala has a short and long term component, dissociable by disrupting signaling pathways leading to RNA and protein synthesis, which are necessary for the long term component (Schafe and LeDoux, 2000).

Unit Recording Physiology

Single unit recording studies have shown that neurons in several amygdala regions undergo changes in physiological responsivity during fear conditioning (see Kapp, Whalen et al. 1992; Quirk, Repa et al. 1995; Uwano, Nishijo et al. 1995). While it is important to understand the changes occurring in these and other areas (Weinberger, 1995), efforts have focused on LA and its sensory inputs, as LA is the first nucleus where processing occurs within the amygdala for auditory stimuli. Recent studies have shown cells in LA increased their responses to a tone after the tone had been paired with a shock (Quirk, Repa, et al., 1995). This is a demonstration that physiological response properties of cells in LA are modified by conditioning. Interestingly, the greatest change in responsivity was in the shortest latency responses (10-15 ms after tone onset). These short latency changes are consistent with direct transmission from the auditory thalamus, suggesting that the thalamo-amygdala pathway is potentiated to a greater extent than the cortico-amygdala pathway. Using a statistical technique to test the correlation between the firing of pairs of cells at various temporal intervals, putative functional coupling was also examined. Increased correlation in the firing of LA cells during the time when no tone was present (spontaneous firing) was found. Thus, LA cells express their learning experiences by responding as quickly as possible and by firing more synchronously than they did before training (See Figure 2.5).

Plasticity in auditory cortex neurons was observed during fear conditioning, as well as in LA (Quirk, Armony, et al., 1997). The cortical plasticity was significantly

diminished and did not outlast extinction training in amygdala lesioned animals (Armony, Quirk, et al., 1998).

Other recent work has begun to characterize the role of modulatory neurotransmitter systems' interaction with the lateral nucleus of the amygdala. Serotonin inhibits action potentials in LA, but not in the absence of corticosterone (Stutzmann, McEwen, et al., 1998). Systemic administration of a dopamine receptor (D2) agonist, quinpirole, impairs both acquisition and extinction in second order fear conditioning, presumably by decreasing dopamine levels systemically through autoreceptor activation (Nader and LeDoux, 1999). Consistent with the idea that decreased dopamine receptor activation impairs fear conditioning, local infusion in LA of a D1 receptor antagonist, impaired fear acquisition in a second order paradigm (Nader and LeDoux, 1999).

These studies thus begin to characterize the morphological and physiological bases of neurotransmission in the sensory input pathways to the amygdala. Such information provides initial clues to the local circuit organization of the projection and suggests hypotheses for additional physiological and behavioral studies aimed at uncovering the cellular foundations of emotional learning.

Other Regions Role In Fear

Prefrontal cortex. One candidate brain region where information about past experience can be brought together with current perceptions to make decisions based on emotional input is the medial prefrontal cortex. Damasio has developed a theory of the

prefrontal cortex's role in emotion in part based on anatomical evidence: the medial and orbital prefrontal cortex receive input from internal senses: respiration, heart rate, kinesthetic, and pain, as well as receiving input from the amygdala (Damasio, 2001). The area appears to be involved in use of fear to assess risks from impending decisions pertaining to personal gain and social interactions (Damasio, Grabowski, et al., 1994; Adolphs, Tranel, et al., 1998; Bechara, Damasio, et al., 1999). In the famous case of Phineas Gage (Damasio, Grabowski, et al., 1994) damage to this area resulted in debilitating social and emotional deficits. This medial prefrontal cortex also projects to the amygdala and to various amygdala target areas in the brainstem. The area has also been implicated in modulation of processing within the amygdala and the control of responses by the amygdala. In one animal experiment, conditioned fear reactions in rats can be made highly resistant to extinction when the medial prefrontal cortex is damaged (Morgan, Romanski, et al., 1993). It is thus possible that medial prefrontal cortex activity plays a role in pathological fear. In particular it is possible that early alterations in the medial prefrontal cortex predispose some humans to develop pathological fear and anxiety under conditions that leave less enduring emotional scars on others.

Hippocampal formation. Conscious recall of some past experience requires that the temporal lobe memory system be intact (Squire, Knowlton, et al., 1993). This system involves the hippocampus and related cortical areas. When this system is damaged, new conscious memories cannot be formed, but other kinds of learning that do not involve conscious recollection, so-called implicit forms of learning, are undisturbed. Fear

conditioning is an implicit form of learning. It can take place in the absence of the hippocampal memory system. Normally, in a traumatic situation, we form both implicit and explicit memories through these two systems. However, if for some reason the hippocampus is not fully functional, it is possible to form unconscious emotional memories without any conscious content. Claparede's patient likely is an example of someone whose implicit fear memories remained intact while her conscious memory was compromised. Memory formed from trauma early in life may be an example of implicit-only memory. The absence of explicit memories from infancy may be explained by insufficient development of the hippocampal system. There is evidence that the hippocampus develops somewhat later than the amygdala (Jacobs and Nadel, 1985). So, it is conceivable that trauma in infancy results in the formation of emotional memories for experiences that can not be recalled consciously. In addition, it is known that intense stress can alter the normal functions of the hippocampus (Diamond and Rose, 1994; McEwen and Sapolsky, 1995). As a result, it is possible that even adults with an otherwise intact hippocampus could fail to form conscious memories of a trauma while at the same time forming unconscious emotional memories. It is important to point out that these unconscious emotional memories formed by the amygdala and related brain areas can never be converted into conscious memories. Conscious memories depend on the hippocampal memory system. If this system does not form a conscious memory of some situation it is not possible to later retrieve a conscious memory.

Monoaminergic diffusely projecting systems. The amygdala also projects to the basal forebrain responsible for cholinergic innervation of the neocortex and known to be involved in learning (Weinberger, 1993; Kilgard, Pandya, et al., 2001), and attention as well as cholinergic cells providing innervation of the thalamus (Davis and Whalen, 2001). The amygdala also projects to areas that activate the locus coeruleus, providing noradrenergic innervation of the forebrain, and dopaminergic midbrain areas each implicated in arousal (Davis and Whalen, 2001).

Cingulate. Others have shown the anterior cingulate involvement in emotional facial and vocal expression (Allman, Hakeem, et al., 2001).

Variety Of Approaches To The Neurophysiological Basis Of Fear

Developing neurophysiological understanding of fear has proceeded using a number of models. Psychopharmacological studies have examined the effect of various agents on classes of behavior commonly associated with fear. These studies primarily focus more on the role of the specific agents than on the locus of action in the brain. Another body of work, reviewed above, has examined the role of the amygdala in fear related classical conditioning and instrumental learning (Fendt and Fanselow, 1999; LeDoux, 2000; Davis and Whalen, 2001). Davis distinguishes two components of the amygdala complex: one generates the acute effects which Davis terms fear, the other generates slower, longer effects which he considers anxiety (Davis, 1998). Other work has examined the role of various endogenous agents that are concomitants of fear in memory

modulation, without specificity to brain locus of the memory formation (McGaugh, Introini-Collison, et al., 1993). EEG studies and brain lesions in humans have been used to find correlations between emotional activity and brain locus (Davidson, Jackson, et al., 2000); FMRI studies have looked at which areas are activated during tasks associated with fear, which will be examined in the final discussion (LaBar, Gatenby, et al., 1998; Buchel and Dolan, 2000; Damasio, 2001). Gray advanced the position that the septum and hippocampus through their projections to the brain stem act as a behavioral inhibition system giving rise to anxiety (Gray, 1991).

In sum, there is growing evidence to support the notion of an anatomically connected set of neural systems that give rise to fear.

Three Studies Of Amygdala Plasticity's Role In Fear Conditioning

The following three experimental projects all address the role of the amygdala in fear learning. Much of what has been discovered has been based on lesions of this region. This approach, though useful, suffers from the fact that the brain is permanently altered by lesions and also that function must be inferred from dysfunction. In the present series of studies, I therefore first examined the effects of temporary inactivation of the basolateral amygdala in rats using local infusions of a drug (muscimol) that stimulates inhibitory GABAa receptors and thereby disrupts neural activity only as long as the drug is active. When muscimol was infused immediately before training, and the animals tested drug free the next day, they showed a dose-dependent impairment of learning. Because

the amygdala was intact and functional at the time of the test, the effects must be due to the disruption of neural activity during learning. To characterize the neural changes occurring in the basolateral amygdala during fear learning, I used the technique of single unit recordings. In the second study, I focused on the recordings from one component of the basolateral amygdala, the lateral nucleus, which serves as the sensory gateway into the amygdala. The results showed that neural activity changes in the lateral nucleus in anticipation of the expression of conditioned fear behavior, suggesting that this activity may account for the behavior. The third study examined neural activity in the basal nucleus, another component of the basolateral complex and an output target of the lateral nucleus. Basal nucleus cells had longer latencies and more complex responses than those of the lateral nucleus, a finding consistent with this region being a channel through which processing in the lateral nucleus might be directed to the central amygdala for the control of automatic fear reactions and to the striatum for the control of instrumental actions.

CHAPTER 3

STUDY 1 TEMPORARY INACTIVATION

This section (Study 1) is from a published paper (Muller, Corodimas, et al., 1997).

A large and growing body of evidence has implicated the amygdala in classical fear conditioning. Lesion, unit recording, and pharmacological studies all strongly suggest that the amygdala is crucially involved in the learning and expression of conditioned fear (Kapp, Whalen, et al., 1992; Davis, Rainnie, et al., 1994; Fanselow, 1994; Maren and Fanselow, 1996; Rogan and LeDoux, 1996). In particular, it appears that the lateral and/or basal nuclei of the amygdala are sites of plasticity that may be crucial for fear conditioning (Davis, Rainnie, et al., 1994; Maren and Fanselow, 1996; Rogan and LeDoux, 1996).

One useful test of the contribution of a particular brain region to learning involves the temporary interference of the function of that region during acquisition and then the testing of the effects of the manipulation once recovery is complete. In this regard, a number of studies have used drugs that interfere with neuronal functioning, such as local anesthetics or receptor agonists for the inhibitory transmitter GABA, to temporarily inactivate localized brain regions during learning to assess whether neural processes occurring in the region are crucial to learning (Albert and Madryga, 1980; Hikosaka and Wurtz, 1985; Knudsen, Knudsen, et al., 1993; Krupa, Thompson, et al., 1993). Two recent studies have applied these techniques to ask whether the amygdala, especially the region of the lateral and basal nuclei, is crucially involved in the learning of conditioned

fear (Helmstetter, 1992; Helmstetter and Bellgowan, 1994). Contrary to the effects of permanent lesions made before acquisition or before testing of conditioned fear, temporary inactivation of the amygdala prior to conditioning did not significantly interfere with learning, in spite of the fact that the same treatment performed prior to testing prevented the expression of previously conditioned fear responses. Although there was a trend toward an effect on acquisition, the contrast between the effects on expression and acquisition suggested that the amygdala may be more involved in the expression than the acquisition of conditioned fear.

The inactivation findings are inconsistent with the results of other studies, particularly studies that have blocked acquisition by infusing antagonists of NMDA receptors in the lateral and basal amygdala (Miserendino, Sananes, et al., 1990; Fanselow and Kim, 1994). NMDA receptors are known to play a key role long term potentiation, a well characterized example of use-dependent synaptic plasticity believed to underlie learning (Cotman, Monaghan, et al., 1988; Madison, Malenka, et al., 1991; Bliss and Collingridge, 1993; Malenka and Nicoll, 1993). Thus, interference with learning by blocking NMDA receptors in the amygdala is often interpreted to mean that the amygdala plays an essential role in learning.

Whether processes occurring in the amygdala during fear conditioning are crucial to learning is obviously an important issue to resolve. In the present study, we therefore reexamined whether inactivation of the lateral and basal amygdala during fear conditioning interferes with learning. We followed procedures similar to those used in Helmstetter and

Bellgowan's (Cotman, Monaghan, et al., 1988; Madison, Malenka, et al., 1991; Bliss and Collingridge, 1993; Malenka and Nicoll, 1993; Helmstetter and Bellgowan, 1994) muscimol inactivation study, with the exception that we used both a discrete conditioned stimulus (CS, a tone) and diffuse contextual stimuli to test fear, as opposed to just contextual stimuli.

Methods

Animals. The subjects were male Sprague-Dawley rats housed in pairs in Plexiglas cages (19 in by 10.5 in by 8 in) on a 12 h light-dark cycle with free access to food and water. They weighed 300-475g at time of surgery.

Surgery. The rats were anesthetized with an i.p. injection (0.12 ml per 100 g of body weight) of a solution containing ketamine (Ketaset, 100 mg per ml physiological saline, - Fort Dodge Laboratories) and xylazine (Rompun, 5 mg per ml. - Miles) and placed in a stereotaxic frame (Kopf- Tujunga, CA). Body temperature was maintained using a heating pad. The skull was exposed and small holes were drilled above the amygdala. Guide cannulae (26g, Plastics One, Roanoke, VA), temporarily fitted with internal cannulae (33g Plastics One), were implanted. To secure the guide cannulae chronically, a mixture of powdered cement and its solvent ("Cranioplastic Powder and Liquid"- Plastics One) was applied around the cannulae and three self-tapping screws ("Q-TX1-3"- Small Parts, Miami, FL) affixed to the skull. When the cement hardened, the internal cannulae were replaced with solid dummy cannulae which were left in place

chronically. The target coordinates, relative to Bregma (AP -2.5 ML ±5.2 DV -8.7), for infusion cannulae tips were chosen using Paxinos and Watson's atlas (Paxinos and Watson, 1986) and previous experience (LeDoux, Cicchetti, et al., 1990). After completion of the surgery the rats were removed from the stereotaxic frame, topical antibiotic was applied, and they were allowed to recover under a heat lamp. The rats were then returned to the housing area. During a two week recovery period before behavioral procedures were started, the rats were handled, dummy cannulae were loosened and re-tightened, and topical antibiotic was applied to the healing wounds.

Fear Conditioning. The rats were placed individually in a conditioning chamber with a metal grid floor (Model E10-10, Colbourn Instruments- Lehigh Valley, PA). The chamber had two Plexiglas and two brushed aluminum walls. The cage was enclosed in a sound dampening chamber (Model E10-20, Colbourn). Five conditioning trials were given. Each consisted of the presentation of a tone (10 kHz, 10 sec, 75 dB) accompanied by an electric shock delivered through the grid floor (1.0 mA, 500 ms) during the last 500 ms of tone (for details, see (LeDoux, Cicchetti, et al., 1990; Phillips and LeDoux, 1992). Each tone presentation was separated by an interval randomly chosen to fall between 1 and 2 minutes. The rats were returned to their cages following conditioning.

Measurement of Conditioned Fear Behavior. Freezing behavior, a species-typical defense response expressed in a characteristic posture, was used as a measure of conditioned fear (Bouton and Bolles, 1980; Fanselow, 1980; LeDoux, Sakaguchi, et al., 1984). Operationally, freezing was defined as the cessation of all movement (except for

respiratory related movements). Following Helmstetter's method (Helmstetter and Bellgowan, 1994), the rat's activity was observed for 1 sec periods every 5 sec, providing 12 observations per minute. Observations were continued for 3 minutes.

Freezing was assessed in either a novel context that differed from the conditioning context or was assessed in the conditioning chamber. The former was used to assess freezing elicited by the CS, and the latter was used to assess freezing to contextual stimuli. The novel context was located in a different room and consisted of a conditioning box of the same basic design but altered in the following manner: a black formica sheet was used to cover the floor grid and a similar black sheet with light-tone, diagonal (45 degree) stripes (2 cm wide and 2 cm apart) was placed behind the rear Plexiglas wall of the cage. In the novel chamber, tone presentation did not start until there was a 15 s period free of freezing.

Drug Injection. Rats received 0.5 µl of vehicle (0.9% saline) or vehicle plus muscimol (0.5 µg) bilaterally through infusion cannulae (described above) inserted into the surgically implanted guide cannula. Polyethylene tubing connected the infusion cannulae to 10 µl syringes (Hamilton- Reno, NV). The injections were delivered continuously over a 2 minute period by a pump, (ATI-Orion "Sage" model 1361) which automatically depressed the syringe plungers. The cannulae were left in place for 1 minute after the infusion. Behavioral testing began about 40 min following the injection.

Design. All rats received injections of either saline or muscimol just before fear conditioning. About 24 h later, they received another infusion and freezing in response to

the tone CS was tested in the novel context. Approximately a day later, they received another infusion before freezing in the original conditioning context was tested.

The infusion of saline and muscimol in the training and testing situations resulted a four groups: saline before training and before testing (*saline-saline*), saline before training and muscimol before testing (*saline-muscimol*), muscimol before training and saline before testing (*muscimol-saline*), and muscimol before training and testing (*muscimol-muscimol*). The drug condition (saline or muscimol) at the time of testing was the same for both the CS and context test. With this design, the effects of muscimol inactivation during training are tested in the absence of muscimol (saline given during the test), and the effects of muscimol inactivation during testing involve animals trained with saline infusions. By comparing these two groups (*muscimol-saline* and *saline-muscimol*) with the group given saline before both training and testing (*saline-saline*), it is possible to determine the effects of muscimol inactivation on both acquisition and expression of conditioned fear. The final group (*muscimol-muscimol*), which receives muscimol before both training and testing, is a control for state-dependent learning (Castellano and McGaugh, 1990).

Several days after completing the procedures described above, some animals underwent additional testing and/or training procedures to address specific issues. Animals from the *muscimol-saline* group were reconditioned and retested, and received saline infusions before both. This was done to determine whether any effect of muscimol during acquisition was indeed a temporary effect. In addition, animals in the *saline-muscimol*

group were given saline infusions and retested (without any additional training). This was done to establish that any effects of muscimol during testing were temporary.

Histology. When the behavioral work was completed, the animals were given an overdose of pentobarbital (65 mg in 1.0 ml) and perfused through the heart with saline followed by 10% formalin. Brains were removed and stored in a formalin-sucrose solution (3.7% formaldehyde and 10% sucrose). Brains were then frozen and sectioned (at 60 μm) in a cryostat. Sections containing the cannulae tracks were mounted on subbed slides. After dehydration and defatting (chloroform/alcohol solution), tissue was stained with cresyl violet or thionine. Cannulae tracks were reconstructed using a magnifying projector (Bausch & Lomb).

Statistics. An analysis of variance was used, with freezing (to tone and to context) as a within group repeated measure, and drug infusion assignment as a between group variable. Follow up group comparisons using Scheffe's method were used to look for differences among the drug assignment groups, if an overall effect of drug group was found. Separate analyses were used for the retraining and retesting data. Data from the *muscimol-saline* animals that underwent retraining was compared to the *saline-saline* group's original data with an analysis of variance. Similarly, data from the *saline-muscimol* group that underwent retesting was compared to the *saline-saline* group's original data with an analysis of variance. In addition, the *saline-muscimol* group's original data was compared to its own retesting data using a repeated measure analysis of variance.

Results

Cannulae Tip Placement. Cannulae were aimed for the region of the lateral and basal amygdala. Animals in which tip placements were bilaterally located in the lateral/basal nuclei were included in the data analysis. Figure 3.1 shows the location of the cannulae tips for the rats (n = 38) with acceptable placements. Figure 3.2 is a photomicrograph showing a typical cannula tract location.

Behavior. Rats that received saline infusions before training and testing froze nearly all of the time (about 90% of the total number of observation periods) after tone presentation and about 50% of the time during exposure to the context alone (see Figure 3.3). Rats that received muscimol infusions, before training or testing or both, all had greatly reduced levels of freezing, both to the tone and to the context alone (see Figure 3.3). These findings were confirmed by the analysis of variance. There was a statistically significant effect, $F(3,34 = 31\ p < .0001$, of group assignment (*saline-saline*, *muscimol-saline*, *saline-muscimol*, *muscimol-muscimol*). There was also a significant effect, $F(1,34) = 15\ p < .0004$, of testing situation (i.e., tone or context), due to the greater extent of freezing to the tone than context. There was also a significant interaction, $F(3,34) = 4.7\ p < .008$, (see Discussion) of group and testing situation. Post-hoc analysis using Scheffé's procedure showed that the significant effects within group assignment were due to the difference between the *saline-saline* group and all other groups (in all three tests, $p = .0001$). The differences among the other groups were not significant ($p > .91, .79, .99$). The same pattern of group differences held true when the data were examined separately by

each of the tone and context conditions. For the tone data the *saline-saline* group was different than all others (in all three tests, $p < .0001$) and no other groups were different than each other ($p = .32, .13, .99$). For the context data the *saline-saline* group was different than all others ($p = .0001, .0013, .0071$) and no other groups were significantly different from each other ($p > .96, .90, .99$).

It is possible that the drug's effects were permanent, which would confound interpretation of the primary results. Several follow-up procedures were conducted to show the temporary nature of the inactivation. In addition to their usefulness in demonstrating the drug's temporary nature, the additional data provide additional information concerning the role of the lateral and basal amygdala in fear learning and expression.

To address the question of whether the drug's disruption of learning was temporary, we examined whether animals that did not condition when given pre-training muscimol infusions would freeze to the tone when retrained without drug infusions. Rats which received muscimol before training and saline before testing (the *muscimol-saline* group) were retrained with saline infusions. When then retested with saline infusions, they froze at levels not significantly different from the *saline-saline* group, $F(1,13) = .072$ $p > .79$ (see Figure 3.4).

We also examined whether the infusion of muscimol before testing permanently blocked fear expression. The group that received saline before training and muscimol infusions before testing was retested (no additional training) with saline infusions. If the

disruption of expression was temporary, this group should now freeze to the tone. This was expected because they had received saline infusions before training, and thus presumably had acquired the tone-shock association but had not been able to express the learning when given muscimol infusions before testing. The results bore out this expectation. The *saline-muscimol* group, when retested with saline, froze at levels similar to the *saline-saline* control group (see Figure 3.5- for tone, left panel of first row; for context, left panel of second row) and froze at levels significantly higher than they did during original testing (Figure 3.5- for tone, right panel of first row; for context, right panel of second row).

An analysis of variance confirmed the retesting results. There was no significant effect of group (saline-saline versus retested saline-muscimol), $F(1,13) = .39$ $p > .54$. When the saline-muscimol group's original data was compared to its retesting data, there was a significant effect of test-retest status, $F(1,5) = 16.9$ $p = .0093$. Follow-up tests determined that this finding was significant both within the tone condition, $F(1,5) = 7.6$ $p = .0398$, and context condition, $F(1,5) = 25.3$ $p = .0040$.

Discussion

The present experiments examined the contribution of the amygdala in fear conditioning. Fear conditioning has long been believed to be an amygdala-dependent learning task (see Introduction). Muscimol, a GABAa agonist, has been used to inactivate local brain regions to explore their role in various learning paradigms (see Introduction).

Muscimol was infused into the lateral and basal nuclei of the amygdala before training or before testing to separately address the region's role in learning versus expression of learning. The results demonstrate that inactivation of this region disrupted both the learning and expression of fear conditioning.

Inactivation of the lateral and basal amygdala prior to training appears to disrupt fear learning. Thus, the *muscimol-saline* group had reduced freezing to the tone and context. This contrasts with the effects of retesting in the *saline-muscimol* group. They showed greatly reduced freezing in the initial test involving muscimol infusions but showed increased levels of freezing that were not different from controls when retested with saline infusions. That the disruption of learning is due to temporary inactivation during training rather than to a permanent loss of the ability to learn is demonstrated by the *muscimol-saline* group's retraining results. These rats showed little freezing to the tone or context when given pre-training muscimol infusions, but froze to the tone and context when retested after being given saline infusions prior to retraining.

It is possible that the failure of rats given pretraining muscimol infusions and pretesting saline infusions to exhibit conditioned fear responses could be due to the fact that the amygdala was in a different drug state at the time of training and testing. However, the results from the *muscimol-muscimol* group argues against this state-dependent learning explanation. This group showed a similar degree of disruption of fear conditioning as did the group that received muscimol before training and saline before testing.

The results from the *saline-muscimol* group support the notion that the lateral and basal amygdala are also crucial in the expression of fear learning. These animals showed little evidence of fear reactivity when tested in the presence of muscimol. However, when later retested with saline infusions, it became clear that they had learned and were simply unable to express that learning due to the presence of muscimol at the time of the first test.

The finding that control animals (the *saline-saline* group) froze more to the tone than the context is consistent with previous conditioning studies (Phillips and LeDoux, 1992; Morgan, Romanski, et al., 1993). This was not evident in the other groups, most likely because of the low level of freezing present in animals that received muscimol either before testing or training or both. In these groups, freezing was so reduced to the tone that there was not room to see a further reduction in freezing to context alone. In the little freezing that did occur in these groups, the average levels were lower to the context than the tone for each group, although these differences did not approach statistical significance.

It seems unlikely that the effects on acquisition are due to alterations in pain sensitivity or reactivity as opposed to plasticity. For example, Cahill & McGaugh lesioned the entire amygdala and found no differences in behavioral reactivity to footshock compared to unlesioned rats (Cahill and McGaugh, 1990). Similarly, other studies of the role of the amygdala in fear conditioning have not found effects of local drug

administration on shock sensitivity (Miserendino, Sananes, et al., 1990; Helmstetter and Bellgowan, 1994).

We distinguished an animal's fear responses to the tone and context, but did not assess these as completely independent elements. Qualitatively, contextual freezing occurred interspersed with periods of activity often including movements throughout the environment; in contrast, freezing to the tone was sustained, with occasional brief movements in which the rat adjusted its posture and then immediately froze again. In assessing tone conditioning, waiting for freezing to the novel context to cease for 15 s provided some protection against the contribution of contextual freezing to tone responses. In addition, in data not reported, we examined freezing to the tone in the original context after we had assessed freezing to this context. The freezing in that situation was not distinguishable from freezing to the tone in the novel context. In both instances, the animal froze for nearly all of the observed periods. The fact that level of freezing increased in the original context after the tone was presented is additional evidence that rats distinguished the tone from the general conditioning environment.

An important question to consider is the extent to which the findings are due to inactivation of the lateral and basal amygdala as opposed to other regions. Given the locations of the cannulae, it seems clear that diffusion sphere of the drug included the lateral and basal nuclei. Parametric studies suggest diffusion spheres of drugs are proportional to the volume of the injection (Myers, 1974). A study of the inactivation effect of localized muscimol infusions reported a 1.66 mm diffusion sphere and a 1.0 mm

radius of a sphere of reduced metabolism for 1 μg of muscimol in a volume of 1 μl injected over four minutes into cortex and brain stem (Martin, 1991). That report provided evidence that muscimol's sphere of diffusion did not spread further at two hours than it had at 10 minutes, as assessed by tritiated muscimol autoradiography. The author speculated that this is a result of muscimol's high binding affinity, which suggests it would be bound much of the time it is present, limiting diffusion. We would expect that our volume, half that used by Martin, would diffuse over a smaller region. For cannula tip placements centered in the lateral and basal nuclei, a 1 mm radius diffusion sphere would remain substantially in with those two nuclei. Further, experience from injections of tract tracing substances in this laboratory suggests that the longitudinal association bundle (lab-see Figure 3.1) also limits diffusion medially to some degree. However, in the absence of other information that precisely delineates the inactivation sphere of 0.5 μg of muscimol in 0.5 μl, we cannot rule out the possibility that areas beyond the basal and lateral nuclei were affected. The central nucleus, for example, is adjacent to the basal. Current thinking about the role of different amygdala regions indicates that the lateral and basal areas are involved in the acquisition of conditioned fear, whereas the expression of conditioned fear involves these nuclei as well as the central (e.g. Davis, Rainnie et al. 1994; Maren and Fanselow 1996; Rogan and LeDoux 1996). Diffusion of the drug into the central amygdala would undoubtedly affect the expression of conditioned fear, but effects on acquisition are expected to a lesser degree. Thus, our interpretation of the effects of muscimol infusions as primarily involving the lateral and basal nuclei is consistent with the location

of the cannulae and with existing information concerning the hypothesized role of various amygdala regions in fear conditioning. Nevertheless, it is possible that other areas are affected in ways that are, at this point, not fully understood.

Our interpretation of the present finding, then, is that fear learning depends on neural activity in the lateral and basal amygdala. However, other interpretations are possible. For example, it is possible that pretraining infusions of muscimol also affect processes that occur after learning has occurred. A number of studies have shown that post-training infusion drugs (including muscimol) into the amygdala affect the consolidation of aversive memories (e.g. McGaugh, Introini-Collison et al. 1992; Jerusalinsky, Quillfeldt et al. 1994; Parent and McGaugh 1994)). However, these studies have involved inhibitory avoidance rather than fear conditioning. There are procedural (stimulus and response) differences between avoidance and fear conditioning, as well as differences in the effects of brain lesions (Sarter and Markowitsch, 1985; LeDoux, 1995). Thus, it is not clear to what extent the inhibitory avoidance findings apply to fear conditioning. Obviously, an important issue to pursue is whether posttraining infusions of muscimol into the lateral and basal amygdala interfere with the later performance of conditioned fear. If they do, then the issue would remain unresolved. If they do not, however, it would lead to the interpretation of our pretraining effects as due to the disruption of acquisition. This issue is currently being investigated.

Finally, it is important to consider why our results differ somewhat with the findings reported by Helmstetter and colleagues (Helmstetter, 1992; Helmstetter and

Bellgowan, 1994). They failed to find a significant effect of inactivation of the lateral and basal amygdala on fear learning. There was a trend toward an effect on learning but this did not reach significance. However, a small number of subjects was studied. The trend might have reached significance with a larger group. Helmstetter and Bellgowan suggested the possibility that muscimol did not significantly block learning because the system within the lateral and basal amygdala that is critical to learning might not be sensitive to GABA induced inhibition. However, an earlier study by the same group also failed to find an effect when a local anesthetic was infused into the amygdala. This, together with our observation that muscimol infusion interferes with learning, weakens this interpretation.

One possibility that we considered initially was that inactivation of the lateral and basal might be more devastating for conditioning procedures involving a discrete tone paired with footshock than simply pairing the shock with the general experimental context. Auditory inputs enter the amygdala by way of the lateral nucleus, whereas contextual information, presumably transmitted from the hippocampus (Phillips and LeDoux, 1992; Maren and Fanselow, 1995), arrives somewhat more ventrally (Ottersen, 1982; Phillips and LeDoux, 1994). Although the hippocampal afferents terminate in the basal nucleus, they also involve the accessory basal and several other amygdala regions. Inactivation of the lateral and basal nuclei would therefore remove the auditory processing area (the lateral nucleus) but might leave some regions involved in processing contextual information from the hippocampus intact. This would explain why Helmstetter's group failed to find an effect of lateral and basal inactivation on contextual conditioning.

However, our findings support the first part of this suggestion (that lateral and basal inactivation interferes with tone conditioning) but not the second part (that context conditioning survives this inactivation). Although there does not appear to be any major differences in the pattern of cannula placements in Helmstetter and Bellgowan's study and ours, the possibility remains that some differences in tip location or diffusion parameters might account for the different results.

In sum, the present findings show that inactivation of the lateral and basal amygdala during fear conditioning disrupts the acquisition of conditioned fear to a discrete CS and to contextual stimuli. These findings mesh well with a large body of research that has pointed to the lateral and basal amygdala as essential components of the neural system underlying the acquisition of conditioned fear and add additional weight to the notion that physiological changes occurring in the lateral and/or basal amygdala during fear conditioning are essential to fear learning.

CHAPTER 4

STUDY 2 LA SINGLE UNITS AND BEHAVIOR DURING FEAR CONDITIONING

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Pavlovian fear conditioning has been used extensively to study how the brain learns about stimuli associated with danger. In fear conditioning, an initially neutral conditioned stimulus (CS), after being paired with an aversive unconditioned stimulus (US), begins to evoke defensive fear responses. Evidence indicates that the amygdala is central in fear conditioning (Davis, 1997; Fendt and Fanselow, 1999; Maren, 1999; LeDoux, 2000), but its precise contributions have been debated (Cahill, Weinberger, et al., 1999; Fanselow and LeDoux, 1999).

Anatomical tracing, lesion and electrophysiological recording studies suggest that the lateral nucleus of the amygdala (LA), and especially the dorsal subnucleus (LAd), is the sensory gateway to amygdala circuits (LeDoux, 2000), and that the processing of the CS by LA neurons is enhanced by the co-occurrence of the US (Quirk, Repa, et al., 1995; McKernan and Shinnick-Gallagher, 1997; Rogan, Staubli, et al., 1997; Maren, 2000; Pare and Collins, 2000). Whereas these and other findings (Huang and Kandel, 1998; Weisskopf, Bauer, et al., 1999) strongly suggest that LAd might be an important site of plasticity, several questions remain unanswered about the nature of the physiological changes observed in LAd during fear conditioning.

First, no study to date has examined in detail the relationship between the acquisition rates of neuronal changes in the LAd and behavioral learning. Whether the

neural changes in LAd actually account for the conditioning of fear behavior is therefore not known. In previous electrophysiological studies, the emphasis was on the recording of unit activity rather than on behavior (Quirk, Repa, et al., 1995; Maren, 2000; Pare and Collins, 2000). This is partly because subjects exhibit behavioral fear reactions such as freezing after the first US presentation, making it difficult to accurately assess freezing conditioned to the CS. Here we bypassed this technical problem by using a procedure in which fear conditioning was superimposed on an operant bar-pressing task (Estes and Skinner, 1941; Bouton and Bolles, 1980). Although rats freeze in the presence of the CS once it is associated with the US, they press a bar during the periods when the CS is not presented. As a result, this task allows a sensitive measurement of CS-elicited behavioral fear and can be used to assess the rate of learning of behavioral fear responses in relation to the acquisition of cellular plasticity by amygdala neurons.

A second unresolved issue is whether the amygdala is a site of permanent storage of fear memories (Cahill, Weinberger, et al., 1999; Fanselow and LeDoux, 1999). Previous studies in rats found that amygdala activity, after initial increases early in training, 'resets' toward baseline levels during later training (Quirk, Armony, et al., 1997). A similar finding was obtained in imaging studies in humans undergoing fear conditioning (Buchel, Morris, et al., 1998; LaBar, Gatenby, et al., 1998). These data have been interpreted as evidence that the amygdala is not a site of storage (Cahill, Weinberger, et al., 1999). However, these studies have typically not used concurrent measurements of amygdala activity and behavior to evaluate their relationship. In addition, relatively few cells were recorded in

the previous rat study. Therefore, as the finding that amygdala changes are transient has important implications for the way amygdala contributions to fear learning should be viewed, we re-evaluated neural activity in LAd using the procedures described above.

Methods

Animals and bar-press training. Studies were done on male Sprague–Dawley rats weighing 300–350 g before behavioral training. All procedures were in accordance with Public Health Service guidelines and were approved by the animal use committee of New York University. Animals were kept on a restricted diet to maintain them at ~95% body weight. They were then placed in an operant conditioning box (24 X 31 X 35 cm, MED Associates, St. Albans, Vermont) and were trained to press a bar for food rewards (45 mg; Noyes, Lancaster, New Hampshire) until a minimum of 10 responses/min at a 60-s variable interval (VI60) reinforcement schedule was reached, which typically took about 1 week of training.

Surgery. Surgical procedures were similar to those in previous studies (Quirk, Repa, et al., 1995). Once the bar-press response was learned, subjects were pretreated with atropine (0.24 mg/kg, intraperitoneally) and were anesthetized with sodium pentobarbital (50 mg/kg, intraperitoneally). Supplemental doses of anesthetic were administered throughout surgery as needed, and body temperature was regulated by a gel heating pad. Burr holes were drilled above the amygdala (Paxinos and Watson, 1986), and above frontal cortex and cerebellum for insertion of self-tapping set screws to anchor the

implant to the cranium. An electrode assembly (Gothard, Skaggs, et al., 1996) of 8–10 independently movable bundles of wires was stereotaxically implanted so that the electrode wires were positioned in the neostriatum just dorsal to LA (2.5 mm dorsal to earbar-zero). Each individual wire bundle was sheathed in a protective stainless steel tube (33 G), and consisted of 4 or more individual nichrome wires (25 micron diameter), a tetrode, or a stereotrode (McNaughton, O'Keefe, et al., 1983) made from nickel/chromium alloy wires (13 micron). All wires were insulated except for the cut tip (impedance < 1 MΩ at 1 kHz). The overall configuration of wires had a diameter of ~0.7 mm. At the end of surgery, the drive was cemented in place and rats were allowed five days to recover.

Unit recording. After recovery, animals were given additional bar-pressing sessions to ensure performance was at least at pre-surgery levels. During this time, the electrode was connected to a head stage containing unity-gain operational amplifiers. A cable then passed the signal through a hole in the top of the conditioning chamber to multichannel differential amplifiers (LYNX, Tucson, Arizona) via a slip-ring commutator (Crist Instruments, Damascus, Maryland) that allowed the rat to move freely. Signals were amplified (10,000X gain), passively filtered (600–6,000 Hz), digitized at ~25 kHz/channel, and displayed on digital oscilloscopes and on a computer monitor using Experimenter's Workbench 32 software (DataWave Technologies, Longmont, Colorado). Spike waveforms corresponding to single cells were sorted off-line on the basis of waveform parameters using cluster isolation methods as described previously for single wires (Quirk, Repa, et al., 1995), stereotrodes and tetrodes (McNaughton, O'Keefe, et al.,

1983), using the DataWave software. Only cells that were well isolated throughout the experiment were analyzed.

Each wire bundle was advanced in 40-_m steps until discriminable single units were isolated at depths believed to be in LAd. Units were tested for auditory responses using a number of experimenter-produced stimuli such as claps, taps and vocalizations. The bundles were lowered until multiple auditory-responsive cells were isolated, at which time conditioning began.

Conditioning. Conditioning took place in an operant conditioning box similar to the one where the animal had been trained to bar-press. The operant box was enclosed in a larger sound-attenuating chamber. The conditioning protocol was modified from previous studies in our lab (Quirk, Repa, et al., 1995; Rogan, Staubli, et al., 1997).The CS was a 20-s series of acoustic white noise pips (50-ms duration, 5-ms rise/fall, 80 ± 5 dB, open field) delivered at 1 Hz, emitted from a speaker mounted near the ceiling of the operant box. The use of multiple auditory presentations as a single CS allowed greater sampling of neuronal data within each trial (Rogan, Staubli, et al., 1997). The US was a mild electric footshock (0.4 mA, 0.5 s) delivered through the grid floor of the test box. A constant background pseudo-white noise (55 dB) produced by a ventilator fan was present throughout the sessions.

The experiment consisted of three phases. During habituation, the CS was presented alone for 8 trials. These were immediately followed by 16 conditioning trials in which the CS and US co-terminated. The rat was then placed in its home cage for 1 h,

after which it was returned to the test box for 20 extinction trials, during which the CS was again presented alone. Trials were separated by a variable mean interval of 4 min (range, 3–5 min). The entire training session lasted about 5 h. To test for effects of training on spontaneous neural activity, 10 min of spontaneous activity were recorded at four times during the experiment: before habituation, immediately following conditioning, just before extinction and following extinction. The spontaneous activity was recorded while the subject rested in the conditioning box, with a wall preventing access to the barpressing lever. In addition to the paired group described above, a separate group (unpaired) received explicitly unpaired CS and US presentations during the conditioning phase of the experiment, to control for non-associative influences of conditioning.

Data analysis. Data were analyzed using a combination of NEX spike train analysis software (Plexon, Dallas, Texas), Matlab and Excel, as described below.

Bar-press suppression (Bouton and Bolles, 1980) was measured using the suppression ratio ($r$pre – $r$cs)/($r$pre + $r$cs), in which $r$pre and $r$cs indicate the mean press rates during the 60 s before the CS and during the CS, respectively. This yields a value of 1 for complete suppression, 0 for no suppression, and negative values down to –1 for facilitation elicited by the CS. Freezing was defined as cessation of all movement other than respiratory activity or slight ear twitches to the onset of auditory stimuli, and was measured by a blind observer using a stopwatch, from a videotaped recording of the experiment. Testing for changes from habituation levels, for both behavioral and neural analyses, used the final six habituation trials as baseline.

PETHs of auditory responses were constructed for each cell, using 10-ms bins. As each CS consisted of 21 auditory pip presentations (Rogan, Staubli, et al., 1997), the last of which overlapped with the US during conditioning, shock artifact made recording during the twenty-first pip of conditioning trials impossible. Therefore, analyses of auditory responses were always based on the first 20 stimuli per CS.

To investigate the effects of training on the entire population of LAd cells, PETHs for each cell were summed over all stimuli for the trials being analyzed, and normalized to the 500-ms pre-stimulus baseline using a standard *Z*-score transformation. That is, each bin of the normalized PETH expressed the number of standard deviations above or below the mean baseline firing rate. The CS response of a given cell in one phase of the experiment, expressed as an average *Z*-score, was then subtracted from the cell's CS response during another phase. The distribution of the resulting '*Z* difference scores' across the population of cells yielded an index of plasticity for the region.

In addition, neurons were analyzed on a cell-by-cell basis to determine whether they showed statistical evidence of training-induced enhanced responding. First, the envelope of the CS-evoked response was determined for each cell by finding the earliest and latest bins that showed elevated CS-evoked activity. Specifically, a PETH was constructed using all trials from all phases of the experiment. All PETH bins following stimulus onset that exceeded the average firing rate during the 500-ms prestimulus period by 1.65 standard deviations or more were included, until 2 consecutive bins failed to reach this criteria, or until 25 post-CS bins (250 ms) had been considered. In addition, at least

one of the bins was required to be three standard deviations above pre-stimulus levels, or the cell was classified as not CS-responsive. The resulting response time window, calculated separately for each cell, was then analyzed further for increases in CS-elicited firing (the firing rate during the cell's response time window minus the rate during the 500-ms pre-stimulus period) over the course of the experiment.

Histology. At the end of the experiment, small lesions were made by passing current (4 μA, 8 s) through recording wires from most of the wire bundles from which cells were recorded. Animals were transcardially perfused with buffered formalin. The brains were removed and stored in a formalin-sucrose solution, with 2% nitroferrocyanide added to visualize iron deposits left by the lesioned wires (Prussian blue reaction). Frozen sections (40 μm thick) were cut on a sliding microtome or cryostat, and sections were stained for Nissl bodies. Lesion sites were used to locate the regions of the recorded cells. The known configuration of the electrode wire bundles allowed the reconstruction of all recording sites (Gothard, Skaggs, et al., 1996).

Results

Behavior. Fear conditioning was assessed using two measures, freezing and suppression of bar-pressing. Although both sensitively assess fear conditioning (Bouton and Bolles, 1980), they reflect dissociable aspects of behavioral fear mediated by different neural systems (Amorapanth, Nader, et al., 1999). The use of the bar-pressing procedure makes it possible to assess both CS-elicited freezing and suppression, as the motivation

to press the lever for food overcomes the tendency to remain immobile after shock presentation.

The 13 rats receiving paired CS–US trials exhibited increased fear levels, as measured by both conditioned suppression and freezing, during conditioning trials and early extinction trials (Figure 4.1a and b). In contrast, the nine rats receiving unpaired CS and US presentations showed no evidence of CS-elicited fear at any point.

Unit activity. A total of 170 LAd cells from 22 rats were included in the analyses. Of these, 100 cells were from 13 rats in the paired group, and 70 were from 9 rats in the unpaired group (example of recording site, Figure 4.2).

Consistent with previous studies (Quirk, Repa, et al., 1995), the spontaneous firing rates of LAd neurons were low. The average firing rate was 2.7 Hz; however, 61% of the cells had rates less than 1 Hz, and the geometric mean (± s.e.m.) was 0.4 ± 1.2 Hz. The preponderance of low rate and wide spike-width cells suggests that, like in the hippocampus (Fox and Ranck, 1981), many of the cells in our sample were pyramidal-type projection cells as opposed to interneurons (Pare and Gaudreau, 1996). Conditioning did not have any systematic effect on spontaneous firing rates ($p > 0.25$, repeated-measures analysis of variance), measured at various time points throughout the experiment (see Methods).

Seventy-three of the 100 cells in the conditioned group were classified as CS-responsive. This was determined by combining peri-event time histograms (PETHs) for all phases of the experiment (see Methods). The average response latency, defined as the

first 10-ms bin following stimulus onset that had significantly greater firing than pre-CS levels, was 42 ± 6 ms; this average was skewed by 7 cells with response latencies that exceeded 100 ms. Of the 66 cells with latencies under 100 ms, the average was 28 ± 2 ms. Nineteen cells responded with latencies under 20 ms.

Conditioned responding. CS-evoked activity an hour after conditioning (during early extinction trials) was compared to pre-conditioning (habituation) levels to determine if training-induced changes in neuronal responsivity were evident an hour after training for the entire neuronal population. For each cell, the CS-evoked activity from 0–200 ms following CS-onset was quantified as an average *Z*score (see Methods) during extinction and habituation. The difference between these two *Z*-scores provided a measure of the change in a cell's responsivity, and is depicted for each of the 100 LAd cells in the paired group in Figure 4.3 (left). The population was skewed toward positive *Z* difference scores, indicating a tendency for LAd cells to exhibit greater CS-elicited responses during early extinction trials than during habituation trials. In contrast, the *Z* difference scores in the unpaired group (Figure 4.3, right) were relatively flat, and centered on a difference of zero, suggesting little to no change from pre-training levels. In support of this observation, the mean *Z* difference score for the paired group was 0.66 ± 0.15, significantly greater than the unpaired group's mean of 0.05 ± 0.06 ($p < 0.001$, one-tailed *t*-test), which did not differ significantly from zero.

To test for changes in neuronal responsivity occurring during the conditioning phase of the experiment, the above analysis was also applied to data recorded during

conditioning. For this analysis, the 16 conditioning trials were divided into four 4-trial blocks, and the *Z* difference score was calculated for each block relative to habituation response levels. To capture any increased responsivity that may have been transient in some cells, the maximum *Z* difference score of the four conditioning blocks was determined for each cell (Figure 4.4). This analysis revealed that the paired group (mean ± s.e.m., 0.90 ± 0.11) had significantly greater *Z* difference scores than the unpaired group (0.48 ± 0.10; $p < 0.01$).

To further explore the acquisition of conditioned responding relative to behavior on a cell-by-cell basis in the paired group, individual cells showing plasticity at various time points during training were identified as follows: for each cell, neuronal CS-elicited firing during conditioning trials was compared, in 4-trial blocks, to habituation levels ($p < 0.05$, *t*-test). Twenty-four cells from this paired group passed this criterion for cell-by-cell plasticity during at least one of the conditioning blocks. With respect to CS-onset times, increases in firing were seen as early as 10–20 ms after the onset of the stimulus (Figure 4.5a). The change in neural response approached its maximum level during the first two blocks of conditioning trials (Figure 4.5b). In contrast, conditioned fear behavior was more gradually acquired, reaching maximal levels toward the end of conditioning (Figure 4.1).

To better determine whether the changes in neural response preceded changes in behavioral response, a single-trial analysis was performed on the plastic cells. For each of these cells, the first conditioning trial during which the CS response significantly exceeded

habituation levels ($p < 0.05$, one-tailed) was compared to the first trial on which behavioral evidence of fear conditioning was observed. To compare neural changes to the earliest possible evidence of behavioral learning in our protocol, the first trial of behavioral learning was defined as the first trial on which either significant freezing or suppression occurred ($p < 0.05$, one tailed), whichever came first. For a significant majority of the cells (17 of 24; $p < 0.05$, binomial probability test), the changes in the neural response occurred earlier or on the same trial as the changes in the behavior (Figure 4.6a and b). This observation— enhanced LAd neural activity preceding fear behavior—is also evident in the group data, as shown in Figure 4.6c, where the average CS response is plotted on a trial-by-trial basis relative to the trial on which behavior was learned. Note that the averaged neural response began to increase before fear behavior was evident (that is, it increased from trials –5 to –1), and, in fact, peaked at the trial at which fear behavior was first detected (trial 0).

Even for the seven cells that first showed evidence of increased neural responsivity on the same trial as behavioral learning (Figure 4.6a and b), the increased firing of these cells likely preceded the behavioral response. That is, the mean latency of the increased firing of these 7 cells was within 20–30 ms of the onset (the first auditory pip) of the CS on which behavioral fear was first evident. However, the shortest latency behavioral response elicited by a CS itself is electromyographic (EMG) neck muscle activity, which is not expressed until 150–200 ms following CS onset (Hennevin, Maho, et al., 1998). In general, behavioral reaction times in response to a warning stimulus, even

for practiced human subjects, are of a similar latency (Fitts and Posner, 1967). Therefore, neural responses occurring 20–30 ms after CS onset very likely precede behavioral conditioned fear responses such as freezing.

Persistence of conditioned response. The averaged neural response of plastic cells reached maximum levels during the first two 4-trial blocks of conditioning, and then tended to diminish during later conditioning trials (Figure 4.5b). To determine how individual cells contributed to this pattern, a 'persistence' value was quantified for each cell by dividing the increase in CS responses (over habituation levels) during late conditioning (final 8 trials) by the increased CS response during early conditioning (first 8 trials). Therefore, a persistence value of zero indicates that responses returned to baseline (pre-conditioning) levels, whereas values of one or greater indicate that responses remained elevated in late conditioning. The distribution of persistence values suggests that there are two types of cells (Figure 4.7a). One group has persistence values clustered around 0, indicating that their increased responses returned to baseline levels, whereas a second group of cells has values clustered above 1.0, indicating that their increased responses were maintained throughout later conditioning trials. Therefore, a cutoff persistence level of 0.75, which bisects this dual distribution, was used to classify these cells as either 'transiently plastic' (12 cells) or 'long-lasting plastic' (12 cells). As expected based on their classification, the response of transiently plastic cells fell back to pre-training levels during later conditioning trials, whereas long-lasting plastic cells maintained their elevated firing levels throughout conditioning (Figure 4.7b). Both cell

types (9 of 12 transiently plastic and 8 of 12 long-lasting plastic cells) tended to exhibit conditioned changes before behavioral fear was evident, though the long-lasting plastic cells took longer to reach their maximal firing rates (Figure 4.7b).

These two classes of cells could also be distinguished based on other characteristics. First, the long-lasting plastic cells had a smaller CS response before training, during habituation (for example, Figures 4.7b, and 4.8a and b). In addition, auditory response latencies of 20 ms or less, which suggest direct activation from thalamic efferents (Quirk, Armony, et al., 1996), were only seen in the transiently plastic group (Figure 4.8a and b). Furthermore, plasticity was greatest at these short latencies in the transiently plastic cells, whereas increased firing in very long latency bins (over 100 ms) was much more common in the long-lasting plastic cells. Finally, the transiently plastic cells were exclusively found near the dorsal tip of LAd, whereas the long-lasting plastic cells were found throughout LAd, and were most prevalent in the ventral half of LAd (Figure 4.8c).

Extinction of conditioned responding. Both transiently plastic and long-lasting plastic cells showed enhanced CS-evoked activity an hour after the conditioning phase, that is, during early extinction trials (Figure 4.7b). The transiently plastic cells, after returning to pre-training response levels during late conditioning, again exhibited increased responses during early extinction trials ($p < 0.05$, one-tailed t-test), only to fall back to baseline levels late in extinction. Long-lasting plastic cells, on the other hand, maintain their increased CS-evoked firing rates not only in late conditioning trials, but also in both

early and late extinction trials (all such time points are significantly elevated from habituation levels, $p < 0.02$).

Discussion

We recorded from LAd neurons while simultaneously monitoring behavior during fear conditioning, primarily to address two previously unresolved issues regarding learning-induced neural activity. Specifically, do the changes in LAd neural activity precede behavioral changes during learning, and do the neural changes persist?

Anatomical considerations. Recordings were made in LA because this region has been implicated in fear conditioning using a variety of different experimental approaches (Fanselow and LeDoux, 1999; Maren, 1999; LeDoux, 2000). However, LA is composed of several subdivisions. We focused on LAd because tract tracing and physiological studies show that this region is the primary target of pathways that transmit the auditory CS to the amygdala (Bordi and LeDoux, 1992; Doron and Ledoux, 1999; Doron and Ledoux, 2000) and is a site of CS and US convergence (Romanski, Clugnet, et al., 1993), and because previous studies have shown short latency conditioned changes in neural activity in this region (Quirk, Repa, et al., 1995; Maren, 2000; Pare and Collins, 2000).

Distinct neural responses were found in the dorsal versus the ventral portions of LAd—dorsal LAd cells were transiently plastic whereas plasticity in ventral LAd cells persisted even through extinction. This anatomical distinction is supported by the results of tract tracing studies (Doron and Ledoux, 1999; Doron and Ledoux, 2000) as well as

studies that have mapped the localization of certain molecular changes in LA during fear conditioning (Schafe, Atkins, et al., 2000). These findings pinpoint the locus of plasticity during fear conditioning to relatively small populations of neurons that may differentially account for the initial learning and subsequent maintenance of the long-term memory of the training experience, as discussed in more detail below.

LAd neural plasticity is associative. One hour after conditioning, only cells from animals receiving paired CS–US presentations showed evidence of enhanced responding to the CS. Cells from animals receiving explicitly unpaired CS–US presentations (which resulted in no observable behavioral fear response to the CS itself) exhibited little to no change in responsivity to the CS. Similarly, during the conditioning phase, neuronal CS-elicited firing in the paired group showed greater increases than in the unpaired group. Therefore, changes in the paired group are likely due to the coding of the CS–US association rather than to sensitization or other non-associative effects caused by exposure to the US.

LAd neural changes predict behavior. A previous study of LAd plasticity during fear conditioning found that the changes in neural activity occur within the first three or four training trials (Quirk, Armony, et al., 1997). However, that study did not have a concurrent measure of behavioral learning. Because fear conditioning can be learned in as little as one trial (Wilensky, Schafe, et al., 2000), the possibility remains that behavioral changes may in fact have preceded the changes noted in LAd activity, which

would suggest that changes in LAd processing are not critical to the generation of behavioral fear responses.

In the present study, three factors allowed the comparison of neural and behavioral conditioning rates on a trial-by-trial basis. First, we used a relatively low shock intensity (0.4 mA) for the US to slow down the rate of behavioral learning. Second, greater statistical reliability of neuronal data was achieved by presenting 20 separate auditory presentations as a single CS during each trial (Rogan, Staubli, et al., 1997). Third, the use of the food-motivated bar-pressing task ensured that the animals would be active rather than immobile in the intertrial period, and thus allowed a sensitive measurement of CS-elicited behavioral fear responses on each trial. With this approach, we demonstrated that the neural changes preceded the behavioral changes for most of the LAd cells that showed evidence of plasticity in their CS-evoked activity during the conditioning phase. These results support the hypothesis that increased LAd neural activity leads to the initiation of behavioral fear responses.

One possible caveat is that learning may normally take place earlier than it was observed via freezing or suppression, as these behaviors may have been masked early in training by the conflicting drive to bar-press for food rewards. However, it cannot be argued that the increased neural responsivity in the LAd is merely a consequence of the behavioral conditioned responses observed here, as the neural changes are observed before the onset of behavioral changes. Furthermore, the strong tie between LA neural activity and behavioral fear conditioning is supported both by the present data (the neural activity

peaks on the same trial as the onset of the behavior), and by many previous studies that implicate the LA as critical for fear conditioning (discussed in more detail below) (Fanselow and LeDoux, 1999). Thus, the most parsimonious interpretation of the present results is that the increased LAd activity contributes to the generation of the learned fear behavior.

Persistence of LAd plasticity. Half the LAd cells that increased their CS-evoked firing rates during conditioning maintained those increased response levels throughout the later trials of conditioning. Because behavioral fear levels, as measured by both suppression and freezing, were also elevated throughout the later conditioning trials, these elevated LAd response levels may reflect aspects of fear memory stored within the LAd.

At first glance, the current results are at odds with some previous studies of amygdala physiology in both rats and humans. In rats, cells that increased their responses early during training showed a general trend toward diminished responses late in training (Quirk, Armony, et al., 1997). In human fMRI studies, amygdala activation decreased with additional training trials (Buchel, Morris, et al., 1998; LaBar, Gatenby, et al., 1998), though this decrease was not significant in one of the studies (LaBar, Gatenby, et al., 1998).

A number of differences between these previous studies and the present design may help explain the conflicting results. First, the conclusions from the study in rats (Quirk, Armony, et al., 1997) were based on a smaller sample of cells showing conditioned responding, which may have hampered detection of the long-lasting plastic

cells. Second, the analyses in that study focused on only the first 50 ms of the CS-elicited responses. Because in the present study the shortest latency responses were found in the transiently plastic cells, which were the main cells reported in the previous study (Quirk, Armony, et al., 1997), the analytic or recording methods used in the previous study may have biased the sample. In contrast, in the present study, the entire envelope of the CS response was analyzed, up to 250 ms after the CS onset.

With regard to the failure of the human studies to detect increased amygdala activity late in training, one possibility is that amygdala activity during later trials may be restricted to the persistently responding cells that store the trace. Because fewer cells are involved at the end than at the beginning of training, the later activity might go undetected in fMRI studies, especially because learning-induced amygdala activation is near the detection threshold for this technique (Buchel, Morris, et al., 1998). A second possibility is that fear levels of human subjects may actually decrease during later conditioning phases, especially in light of the low intensity shock used as a US. Consistent with this possibility, when humans' fear responses were measured using skin conductance changes, they virtually disappeared during later fear conditioning trials (Buchel, Morris, et al., 1998).

Two different cell populations in LAd. A number of differences between the transiently plastic and longlasting plastic cells, in addition to the differential persistence levels of their enhanced auditory responding, further suggest that they may represent two different populations of LAd cells. The transiently plastic cells tended to have shorter

latency and more robust auditory responses before training. Furthermore, the transiently plastic cells tended to be located more dorsally, near the dorsal tip of the LAd, whereas the long-lasting plastic cells were most prevalent in the ventral regions of this subnucleus. One possibility consistent with these characteristics is that the transiently plastic cells receive more direct projections from the thalamus, whereas the long-lasting plastic cells instead are the targets of intra-amygdala, and/or cortico–amygdala projections. This is suggested by the short latency responses of the transiently plastic cells, as latencies less than 20 ms are possible only through direct thalamic projections (Quirk, Armony, et al., 1996), whereas the longer latencies of the long-lasting plastic cells are consistent with additional synapses being involved.

Further support that two cell types exist in LAd comes from recent experiments in our lab. For instance, one study of the biochemical mechanisms of fear conditioning found that cells exhibiting activation of mitogen-activated protein kinases after learning are much more prevalent in ventral rather than dorsal LAd, and that such changes are critical for the consolidation of fear conditioned memories that last six hours or longer (Schafe, Atkins, et al., 2000). In addition, tract-tracing studies reveal that the regions of LAd containing the two types of cells may receive slightly different afferent information from the auditory thalamus (Doron and Ledoux, 1999; Doron and Ledoux, 2000). As the functional roles of the thalamic regions in question are poorly understood, the significance of these projections, and whether they contribute to the transient versus long-lasting properties found in LAd cells, remains to be determined.

Given that LAd has approximately 20,000 excitatory neurons (based on unpublished stereological cell counts) and that the dorsal and ventral LA are roughly the same size, plasticity may be triggered and stored within two populations of about 10,000 cells in LAd (Figure 4.8d). If so, the problem of identifying cell biological correlates of fear conditioning in the mammalian brain would become a tractable pursuit.

The CS responses of the two cell types conform well to the changes in CS processing predicted by a classical learning theory, the Pearce–Hall model (Pearce and Hall, 1980). In this theory, attentional processes raise the associability of a CS when the discrepancy between the expected and received US is high on recent trials. This describes the firing behavior of the transiently plastic cells, which respond most to the CS during early conditioning and early extinction trials. Conditioned responding in the model is controlled by the strength of the previous conditioning to the CS, which is fairly well represented by the firing of the long-lasting cells. The finding that these cells maintain an elevated responsivity late into extinction may reflect the maintenance of a memory trace of the training experience, which is known to persist even after behavioral fear has been extinguished (LeDoux, Romanski, et al., 1989).

Locus of synaptic plasticity. The present data leave open the possibility that the measured changes in neuronal responsivity may be due to plasticity that occurs outside the LAd, in afferent structures such as the thalamus (Edeline and Weinberger, 1992; McEchron, McCabe, et al., 1995) and/or auditory cortex (Weinberger, 1993; McGaugh, 2000), both of which contain cells with responses that can be modified by fear

conditioning. However, certain aspects of thalamic and cortical plasticity are amygdala-dependent (Armony, Quirk, et al., 1998; Poremba and Gabriel, 2001). Although further study will help better resolve the contributions of thalamic and cortical plasticity to LAd activity, it is likely that some if not most of the plasticity in LAd is due to local integration of the CS and US. For example, the LAd is a site of massive CS–US convergence (Romanski, Clugnet, et al., 1993), more so than afferent areas in the thalamus (Bordi and LeDoux, 1994). Studies of long-term potentiation (LTP) indicate that LA synapses are capable of plasticity (Huang and Kandel, 1998; Maren, 1999; Weisskopf, Bauer, et al., 1999), and evidence suggests that fear conditioning induces LTP in LA (McKernan and Shinnick-Gallagher, 1997; Rogan, Staubli, et al., 1997; Schafe, Atkins, et al., 2000). Fear learning is blocked by temporary disruption of amygdala function during training, even when the amygdala is intact during later testing (Helmstetter and Bellgowan, 1994; Maren, Aharonov, et al., 1996; Muller, Corodimas, et al., 1997; Lee and Kim, 1998; Wilensky, Schafe, et al., 2000). Furthermore, fear learning is impeded by local disruption of putative learning mechanisms in the amygdala, including macromolecular synthesis, as well as the intracellular cascades mediated by mitogen-activated protein kinases and cyclic AMP-dependent protein kinase (Bailey, Kim, et al., 1999; Schafe, Atkins, et al., 2000; Schafe and LeDoux, 2000). Collectively, these and other findings strongly implicate LAd as a site of plasticity during fear conditioning (Fanselow and LeDoux, 1999; Maren, 1999; LeDoux, 2000). The present findings suggest it may also be involved in long-term storage.

Summary. The present data add a previously missing foundation to the hypothesis that fear conditioning may in part be subserved by increases in LAd responsivity to the CS after it is paired with the US. The results show that LAd responsivity increases rapidly but incrementally during early training trials until presumably some threshold is reached, at which time behavioral learning is expressed. Furthermore, it seems that the initiation of plasticity and storage of long-term memories may be differentially encoded by increased CS-responsivity of cells in the dorsal versus ventral parts of the LAd.

CHAPTER 5

SINGLE UNIT RECORDING IN THE REGION OF THE BASAL NUCLEUS DURING FEAR CONDITIONING

Introduction

There is a significant and growing body of experimental data showing that fear conditioning is dependent on plasticity of neural responses in the amygdala (Davis, 1997; Fendt and Fanselow, 1999; Maren, 1999; LeDoux, 2000). In classical fear conditioning an innocuous stimulus is paired with a painful one, know as the unconditioned stimulus (US). The innocuous stimulus then elicits fear behaviors, and so is identified as the conditioned stimulus (CS). Lesion (Kapp, Frysinger, et al., 1979; LeDoux, Sakaguchi, et al., 1986; Romanski and LeDoux, 1992; Kim and Davis, 1993; Maren, 1998) and temporary inactivation (Miserendino, Sananes, et al., 1990; Kim, DeCola, et al., 1991; Helmstetter and Bellgowan, 1994; Muller, Corodimas, et al., 1997; Wilensky, Schafe, et al., 1999) results point to the basolateral region of the amygdala as necessary for fear conditioning. Anatomical and electrophysiological recording experiments provide evidence that the dorsal subdivision of the lateral nucleus of the amygdala (LA) is the site of CS-US convergence (LeDoux, Cicchetti, et al., 1990; Romanski, Clugnet, et al., 1993; Bordi and LeDoux, 1994). The central nucleus (Ce) of the amygdala is recognized as an output nucleus whose efferents elicit a wide range of behavior and physiological responses associated with fear (LeDoux, 2000).

What has been less well understood is the role of the more ventral regions of the basolateral complex in fear learning. The basolateral complex is composed of the lateral, basal, and accessory basal nuclei. The dorsal subdivision of LA (LAd) has been the focus of much unit recording work (Quirk, Repa, et al., 1995; Repa, Muller, et al., 2001), while the more ventral medial subdivision (LAm) has not been specifically characterized. The basal nucleus (B) receives afferents from LA and projects to Ce as well as cholinergic forebrain and hippocampal formation (Price, Russchen, et al., 1987; Savander, Go, et al., 1995; Pitkanen, Savander, et al., 1997; Savander, LeDoux, et al., 1997; Pikkarainen, Ronkko, et al., 1999). Recent behavioral studies of lesions to the basal nucleus (B) suggest the nucleus is necessary for operant fear conditioning (Killcross, Robbins, et al., 1997; Amorapanth, LeDoux, et al., 2000). In operant conditioning behavior that results in diminution of the aversive US or CS is reinforced. The accessory basal nucleus (AB), like the basal nucleus, has projections to the cholinergic forebrain, the hippocampal formation, and the ventral striatum (Pikkarainen, Ronkko, et al., 1999; Fudge, Kunishio, et al., 2002; Jolkkonen, Miettinen, et al., 2002). To date no role in fear conditioning has been established for the AB.

Single unit recording is a useful method to observe changes in neural activity associated with learning. A number of projects have characterized the plasticity in LA associated with classical fear conditioning (Quirk, Repa, et al., 1995; McKernan and Shinnick-Gallagher, 1997; Maren, 2000; Pare and Collins, 2000; Repa, Muller, et al., 2001). Neurons in LAd showed associatively linked changes in firing to the CS (Quirk,

Repa, et al., 1995). Unit changes were observed before behavioral fear responses were seen (Repa, Muller, et al., 2001). In addition, some units showed persistent changes even after extinction. This evidence added evidence to the theory that the amygdala is involved in long term storage of the CS-US association (Repa, Muller, et al., 2001).

Neuronal activity of the ventral subdivisions of LA, as well as the basal and accessory basal nuclei, has been characterized. Affectively significant stimuli elicit single unit responses (Nishijo, Ono, et al., 1988). Description of physiological properties of neurons in the Basal nucleus, for example, suggested that a smaller percentage than in LA were tone responsive and the tone response latency tended to be longer in B than LA (Bordi and LeDoux, 1992; Bordi, LeDoux, et al., 1993; Romanski, Clugnet, et al., 1993). Multi-unit recording from the basolateral complex showed associative changes in an avoidance task (Maren, Poremba, et al., 1991). Single units discriminated between odors that predicted sweet versus bitter water reinforcement (Schoenbaum, Chiba, et al., 1999). The response of single units located in ventral regions of the basolateral amygdala in a classical fear conditioning paradigm has yet to be characterized.

The current project is a preliminary, pilot study of single unit recording from ventral regions of the basolateral amygdala. Using the same methods and design as Repa, et al. (2001) the study is an initial attempt to characterize the changes in neural activity there and compare them to behavioral changes associated with fear learning. We expect to find long latency tone responses that show plasticity. It may be possible to identify a

neuronal response that underlies instrumental fear learning known to be dependent on the basal nucleus.

Methods

All procedures were in accordance with Public Health Service guidelines and were approved by the animal use committee of New York University. The methods here are an adapted version of those published in Repa, et al. (2001).

Animals and bar-press training. Male Sprague–Dawley rats, weighing 300–350g before behavioral training, were kept on a restricted diet to maintain them at ~95% body weight. Animals were trained to press a bar for food rewards (45 mg; Noyes, Lancaster, New Hampshire) in an operant conditioning box (24 X 31 X 35 cm, MED Associates, St. Albans, Vermont) until a minimum of 10 responses/min at a 60-s variable interval (VI60) reinforcement schedule was reached, which typically took about 1 week of training.

Surgery. Surgical procedures were similar to those in previous studies (Quirk, Repa, et al., 1995; Repa, Muller, et al., 2001). Once the bar-press response was learned, subjects were pretreated with atropine (0.24 mg/kg, intraperitoneally) and were anesthetized with sodium pentobarbital (50 mg/kg, intraperitoneally). Supplemental doses of anesthetic were administered throughout surgery as needed, and body temperature was regulated by a gel heating pad. Burr holes were drilled above the amygdala (Paxinos and Watson, 1986), and above frontal cortex and cerebellum for insertion of self-tapping set

screws to anchor the implant to the cranium. An electrode assembly (McNaughton, O'Keefe, et al., 1983; Repa, Muller, et al., 2001) of 8–10 independently movable bundles of wires was stereotaxically implanted so that the electrode wires were positioned in the amygdala just dorsal to the basal nucleus. Each individual wire bundle was sheathed in a protective stainless steel tube (33 G), and consisted of 4 or more individual nichrome wires (25 micron diameter), a tetrode, or a stereotrode made from nickel/chromium alloy wires (13 micron). All wires were insulated except for the cut tip (impedance < 1 MΩ at 1 kHz). The overall configuration of wires had a diameter of ~0.7 mm. At the end of surgery, the drive was cemented in place and rats were allowed five days to recover.

Unit recording. After recovery, animals were given additional bar-pressing sessions to ensure performance was at least at pre-surgery levels. During this time, the electrode was connected to a head stage containing unity-gain operational amplifiers. A cable then passed the signal through a hole in the top of the conditioning chamber to multichannel differential amplifiers (LYNX, Tucson, Arizona) via a slip-ring commutator (Crist Instruments, Damascus, Maryland) that allowed the rat to move freely. Signals were amplified (10,000X gain), passively filtered (600–6,000 Hz), digitized at ~25 kHz/channel, and displayed on digital oscilloscopes and on a computer monitor using Experimenter's Workbench 32 software (DataWave Technologies, Longmont, Colorado). Spike waveforms corresponding to single cells were sorted off-line on the basis of waveform parameters using cluster isolation methods as described previously for single

wires (Quirk, Repa, et al., 1995), stereotrodes and tetrodes (McNaughton, O'Keefe, et al., 1983), using the DataWave software.

Each wire bundle was advanced in 40-μm steps until discriminable single units were isolated. Unlike in the LAd units (Repa, Muller, et al., 2001), few Basal nucleus units were readily identifiable as tone responsive by experimenter-generated sounds. When a unit was well isolated, no more adjustments were made on that bundle. After maximizing the number of isolated units (~3-10), conditioning began.

Conditioning. Conditioning took place in an operant conditioning box similar to the one where the animal had been trained to bar-press. The operant box was enclosed in a larger sound-attenuating chamber. The conditioning protocol was modified from previous studies in our lab (Quirk, Repa, et al., 1995; Rogan, Staubli, et al., 1997). The CS was a 21-s series of acoustic white noise pips (50-ms duration, 5-ms rise/fall, 80 ± 5 dB, open field) delivered at 1 Hz, emitted from a speaker mounted near the ceiling of the operant box. The US was a mild electric footshock (0.4 mA, 0.5 s) delivered through the grid floor of the test box. A constant background pseudo-white noise (55 dB) produced by a ventilator fan was present throughout the sessions.

The experiment consisted of three phases. During habituation, the CS was presented alone for 8 trials. These were immediately followed by 16 conditioning trials in which the CS and US co-terminated. The rat was then placed in its home cage for 1 h, after which it was returned to the test box for 20 extinction trials, during which the CS was again presented alone. Trials were separated by a variable mean interval of 4 min

(range, 3–5 min). The entire training session lasted about 5 h. To test for effects of training on spontaneous neural activity, 10 min of spontaneous activity were recorded at four times during the experiment: before habituation, immediately following conditioning, just before extinction and following extinction. The spontaneous activity was recorded while the subject rested in the conditioning box, with a wall preventing access to the barpressing lever.

Data analysis. Data were analyzed using a combination of NEX spike train analysis software (Plexon, Dallas, Texas), Matlab and Excel, as described below.

Bar-press suppression (Bouton and Bolles, 1980) was measured using the suppression ratio ($r$pre – $r$cs)/($r$pre + $r$cs), in which $r$pre and $r$cs indicate the mean press rates during the 60 s before the CS and during the CS, respectively. This yields a value of 1 for complete suppression, 0 for no suppression, and negative values down to –1 for facilitation elicited by the CS. Testing for changes from habituation levels, for both behavioral and neural analyses, used the final six habituation trials as baseline.

Cell firing was examined to find evidence of training-induced enhanced responding. For each cell, a peri-event time histogram (PETH) was constructed using all trials from all phases of the experiment. The total number of all cell firings in10 ms intervals relative to all pip presentations was tallied; the stimulus onset marked the beginning of the 0 to10 ms bin. The envelope of the CS-evoked response was determined individually for each cell by finding the earliest and latest bins that showed elevated activity. Specifically, all PETH bins following stimulus onset that exceeded the average firing rate during the 500-

ms prestimulus period by 1.65 standard deviations or more were included, until 2 consecutive bins failed to reach this criteria, or until 25 post-CS bins (250 ms) had been considered. In addition, at least one of the bins was required to be three standard deviations above pre-stimulus levels, or the cell was classified as not CS-responsive. The resulting CS response was then analyzed for changes over the course of the experiment. The CS responses (the firing rates during the cell's response time window minus the rate during the 500-ms pre-stimulus period) in last 4 trials of habituation were averaged and taken as a baseline. Subsequent 4 trail blocks throughout conditioning and extinction were compared to the baseline CS response with Bonferroni corrected t-tests. In addition, for each cell that showed a change in CS response, the changes in CS response over 4 trial blocks in the experiment were normalized using the baseline, and pooled changes in all cells' CS response were graphed.

Histology. At the end of the experiment, small lesions were made by passing current (4 μA, 8 s) through recording wires from most of the wire bundles from which cells were recorded. Animals were transcardially perfused with buffered formalin. The brains were removed and stored in a formalin-sucrose solution, with 2% nitroferrocyanide added to visualize iron deposits left by the lesioned wires (Prussian blue reaction). Frozen sections (40 _m thick) were cut on a sliding microtome or cryostat, and sections were stained for Nissl bodies. Every fourth section was stained for acetylcholinesterase. Lesion sites were used to locate the regions of the recorded cells. The known configuration of the

electrode wire bundles allowed the reconstruction of all recording sites (Gothard, Skaggs, et al., 1996).

Results

Behavior. The 6 rats receiving paired CS–US trials exhibited increased fear levels, as measured by conditioned suppression, during conditioning trials and early extinction trials. (See Figure 5.1)

Unit activity. A total of 30 cells were recorded from 6 rats (for an example of recording site, see Figure 5.2).

During the experiment, prior to isolating the 30 cells, 75 potential cells were identified by their waveform. Thus in 45 cases cellular activity was recorded in which a noticeable but small neural signal could not be isolated from background electrical noise, or a number of cells could not be differentiated from each other. Of these, one multicellular cluster showed pronounced and statistically significant changes in its tone response over the experiment. It is shown in Figure 5.5a. All the following results exclude the multicellular data.

The spontaneous firing rates of basal nucleus neurons were low. The average firing rate was 1.5 Hz with a standard deviation of 5.2; 31 of the cells (>80%) had rates less than 1 Hz.

18 of the 30 cells were classified as CS-responsive as described in the method section. The average response latency, defined as the first 10-ms bin following stimulus

onset that had significantly greater firing than pre-CS levels, was 113 ms with a standard deviation of 150ms; this average was affected by 8 cells with response latencies that exceeded 100 ms. Of the 11 cells with latencies under 100 ms, the average was 31 ms with a standard deviation of 24 ms. 4 cells responded with latencies under 30 ms.

Experiment related unit activity. Figure 5.3 displays all the wire tip locations where the 7 units with significant changes in activity through the experiment. 5 of these (including the 1 multi-unit cluster) had statistically significant increases in CS response. 2 of those and another had US responses. 1 additional cell showed a decrease in firing to the CS.

Conditioned responding. CS-evoked activity an hour after conditioning (during early extinction trials) was compared to pre-conditioning (habituation) levels. 3 cells from the experimental group had changes during conditioning (See Figure 5.4). With respect to CS-onset times, increases in firing were seen as early as in the 20 to 30 ms bin after the onset of the stimulus (See Figure 5.6). None of the cells categorized as CS responsive but with extremely low firing rates, excluded from the final CS onset latency analysis, showed statistically significant changes in CS response.

2 other unit clusters also showed increased firing to the CS in early conditioning (See Figure 5). One cell had CS response changes that did become statistically significant until extinction (Figure 5a). A multi-unit recording cluster showed a CS response that had statistically significant changes (Figure 5b).

To determine whether the changes in neural response preceded changes in behavioral response, a single-trial analysis was performed on the 3 plastic cells. For each of these cells, the first conditioning trial during which the CS response significantly exceeded habituation levels ($p < 0.05$, one-tailed) was compared to the first trial on which behavioral evidence of fear conditioning was observed. Two of the three cells showed changes on or before the first trial of changes in behavior.

Sustained changes in CS response. Two cells showed sustained changes in CS response (defined as at least 50% of the response seen in early acquisition). The third cell, displayed the pronounced drop in CS response during conditioning that was seen in a subset of the cells in the prior study of LAd (Repa, Muller, et al., 2001). (See Figure 5.7)

CS response versus pip response. Two cells of the three that showed conditioned CS responses responded most to the first pip of the 21 pip CS. In one case, this was particularly pronounced. (See Figure 5.8)

Inhibition. Two cells showed a marked decrease in firing during CS presentation. Decreased firing occurred on two time scales. One cell showed decreased firing to pips with an onset of about 15 ms (See Figure 5.9a). Both that cell and another also showed suppression during the first few seconds of the CS presentation. (See Figure 5.9b)

US response. Recording of unit activity was turned off during US presentation, as the US created a large artifact, which prevented identification of the cells' waveform. However, in each trial after the US terminated, recording was turned back on for 5 seconds. A number of cells showed enhanced activity during these 5 seconds in early

conditioning trials (trials 1-8). The three cells that were tone responsive and conditioned each showed a degree of shock responsivity. For example, the US response was particularly pronounced in the cell shown in the second column of Figure 5.8. One US responsive has not been discussed above because the cell showed no tone response. (See Figure 5.10) Of note in the cells with plastic CS responses (as can be seen in Figure 5.8), the US response begins immediately in the first conditioning trial, while the same cell's enhanced CS response takes several more trials to develop.

Discussion

We recorded from ventral region of the basolateral amygdala, targeting the basal nucleus, during classical fear conditioning. The experiment used the same design as our previous study of the dorsal subdivision of the lateral nucleus (LA) (Repa, Muller, et al., 2001) in order to compare unit responses in the two regions for signs of distinct information processing acitivity in the two regions. Specifically, would there be evidence of the basal amygdala's role in operant conditioning (Killcross, Robbins, et al., 1997; Amorapanth, LeDoux, et al., 2000) as seen by anticipatory shock responses or CS responses, as opposed to tone-pip responses?

Anatomical Issues. Given the exploratory nature of this pilot project, units in the accessory basal and very ventral part of the medial subdivision of LA were included, in spite of their lying outside the target region, the basal nucleus. Since there have been no

previous reports on any of these regions during a fear conditioning protocol, any information offers a preliminary picture of neural activity in these areas.

The anatomical target coordinates used in this experiment resulted in recordings from the posterior half of the basal nucleus. As a result, units were recorded from the parvocellular and intermediate subdivisions of the basal nucleus, but not the more anterior, magnocellular subdivision (See Figure 5.3).

Spontaneous activity. Low firing rates reported here are consistent with prior reports of rate in the basolateral amygdala (Bordi, LeDoux, et al., 1993; Quirk, Repa, et al., 1995; Repa, Muller, et al., 2001). Notably, the rates reported here were lower than those reported for LA (Quirk, Repa, et al., 1995; Repa, Muller, et al., 2001), which is consistent with an earlier report that found basolateral units had lower firing rates than LA units (Bordi, LeDoux, et al., 1993).

Auditory responses. Because the LA is the sensory gateway to the amygdala (LeDoux, Cicchetti, et al., 1990) and auditory information is thought to flow from LA to other amygdala nuclei, we expected basal nucleus units to show longer response-onset latencies than those of LA units. Of cells with latencies under 100 ms, the mean onset latency in LA was 28 ms while this study's measure of ventral basolateral amygdala mean latency was 31 ms, each mean within the measuring error of the other. However, what was different was the shortest latencies reported in LA ($\leq$15 ms) were not observed in the more ventrally located units reported here. The current finding differs with Bordi, et al. (1993) which found no latencies shorter than 30 ms in the ventral basolateral region.

While not statistically quantified here, it appears that for the most part the shorter latencies observed in this study emerged only after CS-US pairing. There were no CS-US presentations in the Bordi, et al. study (1993).

CS response plasticity is associative. Prior unit studies of amygdala unit plasticity during fear conditioning (Quirk, Repa, et al., 1995; Repa, Muller, et al., 2001) have used a sensitization control to show that changes in a neural response to the CS is not due to a non-specific shock induced change in neural activity. Including a sensitization control would be an essential part of further characterization of unit activity in the basal nucleus and nearby regions.

Unit activity and behavior. As in the study of LAd units and behavior (Repa, Muller, et al., 2001), in the current study some units showed a significant change in their response to the CS before behavioral signs of fear conditioning. While this evidence does not prove a causal link, establishing the presence of such units supports the theory that activity in the basolateral amygdala is responsible for fear conditioning.

Site of fear conditioning memory encoding. In this study, there was evidence of enhanced CS response among ventrally located basolateral amygdala neurons, consistent with the idea of long term storage of fear memory within the amygdala. The evidence here adds to the similar finding in the previous study of LAd units (Repa, Muller, et al., 2001).

Evidence of plasticity in the amygdala preceding fear expression, and lasting plasticity suggest the amygdala is involved in the acquisition and storage of fear

conditioning (Fanselow and LeDoux, 1999). There is also evidence of fear conditioning dependent plasticity in the thalamus (Edeline and Weinberger, 1992; McEchron, McCabe, et al., 1995) and auditory cortex (Edeline and Weinberger, 1993). As such, the demonstration of fear conditioning dependent plasticity by itself has not been able to establish the site of fear memory encoding. However, aspects of thalamic and cortical plasticity have been shown to be dependent on the amygdala (Armony, Quirk, et al., 1998; Maren, Yap, et al., 2001). Moreover, evidence from other methods adds further weight to the theory that fear conditioning memory is encoded in the amygdala. Temporary disruption of the amygdala blocks fear conditioning (Helmstetter, 1992; Helmstetter and Bellgowan, 1994; Muller, Corodimas, et al., 1997; Wilensky, Schafe, et al., 1999). Local infusion of NMDA antagonists blocks acquisition of new fear conditioning while allowing expression of prior conditioning (Miserendino, Sananes, et al., 1990; Campeau, Miserendino, et al., 1992). Fear conditioning induces enhanced CS evoked potentials in LA, similar to those generated by the long term potentiation (Rogan and LeDoux, 1995; Rogan, Staubli, et al., 1997). Disruption of cell signaling pathways thought to lead to protein synthesis, as well as inhibition of protein synthesis, locally in the basolateral amygdala blocks the consolidation of fear conditioning: in the short term fear conditioning is maintained, but gone 24 hours later (Schafe, Atkins, et al., 2000; Schafe and LeDoux, 2000). Taken together, the evidence suggests the amygdala is necessary for the acquisition and storage of fear conditioning.

Novel findings. There were three findings that have not previously been reported in amygdala unit recording studies of fear conditioning. First, two of the cells that conditioned showed pronounced shifts to earlier auditory-response latencies during conditioning. The changes began in the second or third trial of conditioning phase and shifted back to longer latencies in the second or third extinction trial. This shift in onset latency itself may be an important measure of fear conditioning learning. In an earlier study of LAd units, there was more firing at earlier latencies, but the data was aggregated across cells, so it was not clear if this represented a shift in latency, or an increase in rate in a cell that already had a short latency (Quirk, Repa, et al., 1995). A second finding was that some cells showed a shock response in early conditioning trials. Such a result is not necessarily surprising, but has not been reported in prior fear conditioning studies. The third novel finding was that several of the conditioned units showed a pronounced enhanced response to the first of the 21 pips. This finding suggests that processing in the amygdala may be abstracting information from more stimulus features to information important for fear learning. As previously mentioned, the basal nucleus is implicated in operant conditioning (Amorapanth, LeDoux, et al., 2000). In operant conditioning there must be a reinforcing signal which can be associated with random behaviors that succeed in changing the likelihood of the US (Deci, Koestner, et al., 1999). It is conceivable that the units that prefer the CS onset to pip onset are providing a reinforcement signal for operant conditioning. An experimental design that included unit recording during an operant task would potentially shed more light on this question.

The novel findings are not necessarily related to the difference in recording site in this study and prior unit recording studies; such changes may relevance to general principles of acquisition and expression of fear conditioning. It would be conceivable to re-examine LAd single unit recording during fear conditioning to see if similar phenomena are present in LAd.

Conclusion. The current study has explored the role of the ventral region of the basolateral amygdala in fear conditioning using a single unit recording technique. A limited number of cells showed CS responses. Some of the CS responses showed enhanced firing as a result of CS-US pairings. Some of the unit changes preceded behavior. In addition some unit changes persisted throughout conditioning. The changes in unit activity add evidence that plasticity in the amygdala could be responsible for fear acquisition and memory storage. The absence of very short latency ($\leq$ 15 ms) auditory responses in the ventral basolateral amygdala supports the view that auditory information flows from the LA to B nuclei during conditioning. Preliminary evidence suggests that CS information has been transformed from stimulus feature representation in LA units, to a representation of the presence of the CS. This CS signal is consistent with the possibility that the basal nucleus generates a reinforcement signal for operant fear conditioning.

CHAPTER 6

OVERALL DISCUSSION OF EXPERIMENTAL EVIDENCE

Introduction

Investigating the amygdala's role in fear conditioning in animals offers an approach to understanding human fear experiences, including mood and anxiety disorders. A large body of evidence has accumulated about the amygdala's role in fear phenomena. The three current studies add confirming data and new findings regarding fear conditioning. The findings will be briefly discussed, emphasizing evidence that the amygdala is the site of acquisition and storage of the CS-US association. A review of human amygdala studies about fear, remarkably consistent with our understanding developed from animal models will follow.

While evidence of the amygdala role in fear conditioning offers implications for understanding mood and anxiety disorders, fear conditioning can not provide a complete explanation of anxiety and mood disorders. The contribution of early interactions with caregivers as another central factor in mood and anxiety disorders will be integrated with our understanding of the neural basis of fear to suggest a course of further investigation.

Discussion of the three studies

Taken together, the three studies address the role of the basolateral amygdala in fear learning. They address three specific issues: the role of the basolateral amygdala in the acquisition of classical conditioning; the role of the lateral nucleus in the storage of

classical fear conditioning memory; and the role of the more ventral region of the basolateral amygdala in operant fear conditioning.

Acquisition. To demonstrate that the amygdala is required for the acquisition of fear conditioning, fear acquisition must be absent when amygdala functioning is disrupted just prior to CS-US pairings. The method by which the amygdala is disrupted affects the conclusions that can be drawn. Inactivation methodology offers an advance over lesion techniques, which permanently alter both the lesioned site, fibers of passage, and anatomically connected areas. Two types of confounds are introduced by lesions as a result. First, the nuclei in the lesioned site may have nothing to do with the changes observed, rather the axons of other nuclei that pass through the lesion site may be crucial, or nuclei that suffer substantial cell death from loss of projections from the lesioned cite may be the actual site of acquisition. Second, if in fact the nuclei in the site are involved, their role in acquisition versus expression is confounded. Temporary inactivation addresses both of these confounds. The inactivation method used in the first study has clarified the role of the region in acquisition of classical fear conditioning. The study of single units in LA adds independent evidence of the region's role in acquisition, showing that changes in neural responses (presumably a result of changes in synaptic efficacy) precede acquisition of fear behavior.

Storage. Demonstrating that the amygdala is necessary for fear conditioning leaves an important aspects of its role in fear conditioning unspecified. Is the amygdala

necessary for acquisition because the fear memory is established and stored there? Alternative views will be reviewed after the individual studies are discussed.

If changes in the amygdala are solely responsible for fear learning, then no changes in any other area of the brain are necessary for conditioning. Conversely, changes elsewhere in the brain must not be necessary for fear conditioning. In practice, complete testing of these conditions has been faced with two significant challenges. First, the amygdala is thought to be both the site of memory and expression of responses to that memory. It has been difficult to dissociate these functions. Second, some of the alternative brain regions where fear memory might be encoded are anatomically closely connected to the amygdala and, in the case of the medial geniculate and surrounding nuclei, must be intact for the amygdala to play its role in fear conditioning.

As will be discussed further, we know changes in regions that are alternative candidates for memory storage appear to be dependent on amygdala changes, which is encouraging for the importance of amygdala plasticity. But, the number of regions shown to have plasticity during fear conditioning suggests a more complex explanation than simply one site is the site of learning and others are not. While a full demonstration of the amygdala's role in memory storage remains elusive (Cahill, Weinberger, et al., 1999), recent work in individual neural recording (Quirk, Repa, et al., 1995; McKernan and Shinnick-Gallagher, 1997; Quirk, Armony, et al., 1997; Armony, Quirk, et al., 1998), in evoked potential (Rogan, Staubli, et al., 1997)and in learning related protein synthesis (Schafe, Nadel, et al., 1999; Schafe, Atkins, et al., 2000; Schafe and LeDoux, 2000) has

strengthened support for the view of memory encoding within the amygdala which will be reviewed below. Data presented here (Muller, Corodimas, et al., 1997; Repa, Muller, et al., 2001), in particular from the LA unit physiology study showing long lasting changes in CS response resulting from CS-US pairings, have contributed to the view that fear memory is encoded within the amygdala.

Operant fear conditioning. The final study, a pilot project recording single units in the region of the basal nucleus, uses the same design as the LAd study in order to explore ways in which the two areas' changes in neural activity resemble each other or have differences that might be associated with the basal nucleus' distinct role in operant conditioning.

Now, each study will be discussed in turn, in further depth.

Basolateral amygdala inactivation study

The muscimol study adds support to the notion that the amygdala is necessary for the acquisition and expression of classical fear conditioning. Inactivating the basolateral amygdala just prior to presentations of CS-US pairings blocks the ability of the CS to subsequently elicit fear behaviors when tested with the amygdala in a fully functional state. Similarly, inactivating the basolateral amygdala just prior to CS test presentations after fear conditioning blocks the expression of fear behaviors; subsequent testing free of inactivating agent demonstrates that the CS does elicit fear. These results are consistent with prior studies involving manipulations glutamate synaptic transmission

(Miserendino, Sananes, et al., 1990; Fanselow and Kim, 1994). Glutamate is the predominate excitatory neurotransmitter in the mammalian forebrain. Two of its receptor classes have different properties. One receptor type, identified by its selective agonist N-Methyl-D-Aspartate (NMDA), when activated can lead to changes in synaptic efficacy; another, Alpha-amino-3 hydroxy-5 methyl-4 isoxazole proprionic acid (AMPA) receptors, participate in synaptic transmission without changes in synaptic strength. (Miserendino, Sananes, et al., 1990; Fanselow and Kim, 1994). The muscimol study's findings help to resolve prior ambiguous findings using inactivating agents, which found trends toward disruption of acquisition of contextual conditioning that did not reach statistical significance with a smaller sample (Helmstetter, 1992; Helmstetter and Bellgowan, 1994).

The inactivation evidence suggests that the memory formation necessary for Pavlovian conditioning could not be taking place in an area afferent to the amygdala, independently of amygdala function. If the memory were formed in an afferent region during the CS-US pairings, but merely could not be expressed while the amygdala was inactivated, then on subsequent testing when the amygdala is recovered, the memory should then be expressed through the amygdala. Anatomical connections suggest both the medial geniculate (MG) and auditory cortex (AC) as candidate regions. Both the MG (Edeline and Weinberger, 1992; McEchron, McCabe, et al., 1995; McEchron, Green, et al., 1996; Hennevin, Maho, et al., 1998; Maren, Yap, et al., 2001) and AC (Edeline and Weinberger, 1993; Weinberger, 1998) display plasticity as a result of fear conditioning.

Subsequent study showed that MG plasticity is dependent on the amygdala's functioning during acquisition (Maren, Yap, et al., 2001; Poremba and Gabriel, 2001). Similarly, components of AC plasticity are absent in amygdala lesioned animals (Armony, Quirk, et al., 1998). These findings are consistent with the current findings that the amygdala's function must be intact for fear acquisition and related changes in neural activity. The either/or logic regarding the location of plasticity responsible for fear learning is probably misleading. Plasticity in MG, LA, and AC probably all make contributions to the sensitivity, strength, and discrimination of fear learning.

One explanation of the current finding is that the amygdala's role in fear learning is to facilitate memory formation in an area efferent to the amygdala. From this perspective, inactivating the amygdala during conditioning merely blocks such facilitation. We will return to this issue after discussing the contribution of the other studies.

The inactivation method is constrained by our knowledge of the extent and reach of inactivation of locally infused agents. The current study targeted the lateral and basal nuclei; however, we can not rule out diffusion to the central nucleus and the surrounding striatum. Some have used radioactive labeling to demonstrate spread due to diffusion (Martin, 1991), but the presence of labeled agent does not necessarily indicate inactivation which is presumably sensitive to concentration of the agent. Tagged glucose or oxygen uptake methods are better measures of inactivation, but they have not been explored in the basolateral amygdala.

The limitation on knowing the precise localization of area of inactivation is particularly relevant to attempts to understand the role in acquisition of specific nuclei within the amygdala by the local infusion method used here. The conclusion drawn from this inactivation study is that the basolateral amygdala, composed of the lateral and basal nuclei , is involved in acquisition. The neighboring central nucleus is thought to elicit fear responses (Applegate, Kapp, et al., 1983; LeDoux, Iwata, et al., 1988; Kapp, Supple, et al., 1994) when activated by basolateral projections (Pitkanen, Savander, et al., 1997). However, the question remains unresolved as to whether fear learning is encoded in the central nucleus (Kapp, Frysinger, et al., 1979; Applegate, Frysinger, et al., 1982; Pascoe and Kapp, 1985; McEchron, McCabe, et al., 1995). Most work examining learning related physiological changes in the amygdala have focused on LA or more broadly the basolateral complex (Quirk, Repa, et al., 1995; Rogan, Staubli, et al., 1997) because LA is the nucleus of sensory input for CS and US information (Romanski, Clugnet, et al., 1993). Unpublished data using the local infusion technique have suggested that the central nucleus of the amygdala could be necessary for the acquisition of classical fear conditioning, independent of the LA plasticity (A. Wilensky, personal communication). It remains to be established firmly whether the inactivation technique can be used to clarify the role of the central nucleus versus basolateral complex in acquisition of fear learning.

In sum, this inactivation study has added confirmation to evidence from anatomical, lesion, and other techniques suggesting the basolateral amygdala is necessary

for the acquisition and expression of the learning responsible for classical fear conditioning.

Lateral nucleus unit plasticity during fear learning.

Given the extensive neuroanatomical, lesion, and inactivation evidence that the basolateral amygdala is necessary for fear conditioning, it is important to characterize individual neuron's acitivity in the basolateral amygdala during fear conditioning. Such work offers further, independent support for the role of the amygdala in fear conditioning.

Indeed, changes in neural responses associated with fear conditioning have been demonstrated in the basolateral amygdala (Quirk, Repa, et al., 1995; Maren, 2000; Pare and Collins, 2000). The current LA unit study added two pieces of additional evidence. This study is the first that demonstrates changes in unit responses preceding changes in fear behavior. If no changes in unit responses had been found to precede behavior, it would be problematic to hold that the nucleus was responsible for the behavior. Second, it is the first study in LA that demonstrates changes in unit responses maintained throughout conditioning and extinction phases of the experiment, consistent with the idea that units in the basolateral amygdala permanently store CS information.

Site(s) of fear memory storage. Unit recording samples only a tiny proportion of the neurons in a region. For that reason, and for the fact that neurons all over the brain are also unobserved, it is difficult to establish a causal link between the unit recorded and the behavior observed. It is possible that other brain regions undergo plasticity that is also

needed for the establishment of fear conditioning. As has been noted for example unit changes during fear conditioning have also been observed in the medial geniculate (Edeline and Weinberger, 1992; McEchron, McCabe, et al., 1995) and the auditory cortex (Weinberger, 1993; Quirk, Armony, et al., 1997). However, both the medial geniculate plasticity (Poremba and Gabriel, 2001) and aspects of auditory cortex plasticity (Armony, Quirk, et al., 1998) require intact functioning of the basolateral amygdala. It remains difficult to assess the necessity of MG and Posterior Interlaminar Neucleus (PIN) plasticity as MG and PIN are also required for transmission of the auditory CS to the amygdala. As mentioned, there is some indication of plasticity in the central nucleus that may be necessary for fear conditioning (A. Wilensky, personal communication). It remains a possibility that other, as yet unspecified sites within and outside the amygdala could be involved. Such possibilities are significantly constrained by our knowledge from anatomical and behavioral lesion studies which have tested other anatomically relevant regions such as the auditory primary sensory cortex (Romanski and LeDoux, 1992) and hippocampal complex (Phillips and LeDoux, 1994), without finding another site required for classical fear conditioning.

LAd Neural Changes Are Associative. Observed changes in neural responses may not reflect learning but rather reflect an artifact of some aspect of the experiment's design other than the pairing of the CS and US. In particular, in a phenomenon known as sensitization, US presence can alter arousal and behavioral activity, which might produce the observed changes in unit responses. This experiment has used a group in which the CS

and US were not paired as a control for any design factors that might affect unit responses other than CS-US pairings. In the control group the identical design is used with the exception that the US's do not coterminate with the CS during conditioning; instead the US's are presented alone and interspersed and separated by a random inter-trial interval with CS presentations. Thus, both groups receive the same number of US and CS presentations. In fact the changes in the LA are due to the association between the CS and US, since enhanced responses were not observed in the control group.

LAd Unit Changes Predict Behavior. An important new contribution of this study was to demonstrate that changes in neural responses occurred before or during the trial that the onset of fear behavior was observed. This question had not been previously addressed. While such data does not prove a causal link, the unit's enhanced responses in contrast with the sensitization control group as well as accumulated evidence from other studies permits the interpretation of causality. If changes in neural activity preceding behavioral changes had not been found the claims for the basolateral amygdala's role in fear conditioning would have been weakened.

Persistence of Changes in Neurons' CS Response. Another finding in this study was the existence of units' enhanced response to the CS that remained through the end of extinction trials. Persistence had been observed in the auditory cortex (Quirk, Armony, et al., 1997), but never before in prior studies of LA (Quirk, Repa, et al., 1995). Prior unit recording studies of LA focused on the most dorsal part of the dorsal

subdivision of LA, while the current study found the persistence response in more ventrally located units within LAd.

The existence of enhanced responses after extinction suggests the amygdala may be a site of permanent storage of the memory of fear conditioning. We know from behavioral data that some memory of fear conditioning persists after extinction, and can be evoked after stress (LeDoux, Romanski, et al., 1989). To find evidence that such memory may exist within the amygdala is important as it addresses the debate about the amygdala's role in fear conditioning: whether it modulates memory formation elsewhere or itself is the site of fear conditioning memory. This topic will be returned to in further depth in the final discussion of all three studies.

The design of this study does have a drawback when considering its implications for behavioral phenomena. Studies that have reported unit plasticity are generally conducted within a period of hours. This means behavioral acquisition, testing, and extinction phases of design are compressed within that time. In contrast, a purely behavioral design typically separates acquisition, testing, and extinction over several days. It is possible that the significantly compressed time course of this study introduces complications that make it difficult to compare to designs that have acquisition on one day, testing another, extinction another. Unit recording in the amygdala remains challenging work, and the possibility of repeating such recording using a design more consistent with traditional behavioral designs is a significant challenge. Recording from the same cell over days is difficult regardless of brain region, but especially so in the

amygdala. This is due to the rarity of finding units, when compared to other brain structures which often yield more cells due to layered structures or significantly higher levels of firing rate of pyramidal neurons (large, with generally excitatory effects on neurons to whom they project).

Basal nucleus pilot study

The final study in this dissertation preliminarily explored unit plasticity in the basal nucleus (B) of the basolateral amygdala. Lesion evidence suggests the nucleus appears to be necessary for operant fear conditioning (Killcross, Robbins, et al., 1997; Amorapanth, LeDoux, et al., 2000). In operant conditioning, an arbitrary behavior by the animal is reinforced if it is associated with reinforcement. For example, in a fear avoidance task, running out of one side of the chamber stops the shock. Animals learn to escape at CS onset rather than waiting for the shock. This task was shown to be dependent on an intact basal nucleus (Amorapanth, LeDoux, et al., 2000). As such some transmission of information about the CS would be expected in unit recording in the basal nucleus. Indeed unit plasticity was observed, predominately in the experimental group in contrast to the unpaired controls. Such plasticity had two characteristics previously unobserved. In the unit recording experiments, the CS was a series of 21 50ms white noise pips. LA units were found to be responsive to each pip. In the recordings from the more ventral areas of the basolateral complex, a number of the unit responses signaled the onset of the whole

CS rather than individual auditory pips within the CS. Second, a shift in onset latency was observed in two of the units. Each of these findings will be discussed in turn.

The existence of units responsive to the CS more than the auditory elements within the CS suggests two relevant implications. The first allows for the development of our understanding of the nature of information processing within the basolateral complex, in which information is transformed from sensory stimulus-bound aspects to increasingly abstracted information. Second, is to provide some guidance in exploring the nature of the neural basis of operant conditioning. The basal nucleus sends projections to the ventral striatum. The striatum has long been a candidate region for operant conditioning (Robbins, Cador, et al., 1989). From this finding, a possible inference is that a signal generated in the B nucleus regarding the presence of the CS must be acquired . This CS signal could serve as the reinforcement for operant conditioning.

The shift in onset latency is consistent with a theory of how the fear value of a CS is represented in the basolateral amygdala. In both human and rat, the amygdala has been known to be activated more in early conditioning than in late conditioning trials (Quirk, Repa, et al., 1995; LaBar, Gatenby, et al., 1998). Such findings have had a variety of interpretations, including that the amygdala is not involved in ongoing activity related to fear conditioning, or that the animal habituates as the experiment goes on, becoming less fearful.

Another possibility has been suggested, namely, that the high level of basolateral activation early in conditioning does not merely reflect a simple heightened response to

the CS in a one-to-one relationship with increased fear of the CS, but instead the enhanced activity drives changes in synaptic strength among a network of neurons such that subsequent input elicits a synchronized, response among the network eliciting a stronger response in efferent areas (Hebb, 1949). Current investigation of this topic is predominately on oscillations across broad regions of the brain during visual tasks (Keil, Gruber, et al., 2001). For example, Keil's (2001) review focuses on gamma band oscillation synchrony in visual tasks. Two studies mentioned by Keil offer some evidence regarding synchrony's role in learning. One study found synchronized gamma band activity to CS+ but not Cs- after CS+ was paired with a shock US (Miltner, Braun, et al., 1999). In a study using an operant task, reinforcers but not other stimuli resulted in gamma band activity (Keil, Muller, et al., 2001). It is possible that the shift to earlier response onset in the two basal units of this study are consistent with learning driven synchronization. In the amygdala, a report of increased synchrony in the theta band during a fear conditioning task has been reported (Pare and Collins, 2000).

In conclusion, there is promising data from this pilot study of the single unit activity in the basal nucleus during fear conditioning. Since evidence of associative changes were found, and some transformation of the CS signal consistent with the regions role in operant conditioning was observed, further investigation is warranted. As to the three studies as a group, there is growing evidence, to which these studies have contributed, that the basolateral amygdala is necessary for the acquisition, storage, and expression of classical fear conditioning as well as the acquisition of instrumental fear conditioning.

Next, the neural basis of fear conditioning in rats will be compared to data found in primates, including humans.

Amygdala's role in fear memory: modulation and/or acquisition and storage

One theory has held that the amygdala only modulates fear memory formation which occurs in other brain locations (McGaugh, 2000). McGaugh's theory emerges from the longstanding view that memory undergoes a period of consolidation thought to be susceptible to modulatory influence. In his view, peripheral arousal, particularly of increased levels of norepinephrine (NE) and glucocorticoids, has the effect in the central nervous system of enhancing declarative memory (McGaugh and Roozendaal, 2002). Peripheral NE is believed to affect the CNS through its detection by vagal afferents of the nucleus of the solitary tract, since peripheral NE can not pass the blood brain barrier to directly enter the CNS. Glucocorticoid receptors are present throughout the brain, and glucocorticoids can cross the blood brain barrier. McGaugh further hypothesizes that projections from the nucleus of the solitary tract (NTS) to the amygdala, and glucocorticoid receptors in the amygdala lead to amygdala activation. In turn, the amygdala activation modulates (strengthens) declarative memory formation. McGaugh's group has two lines of evidence to support this theory. In humans, norepinephrine blockers disrupt the memory enhancing effects of arousal. In rats, local infusion of agonists and antagonists of NE and glucocorticoids into the basolateral amygdala, post

conditioning affect memory strength in a fear avoidance task in a manner consistent with the theory's predictions.

McGaugh's group has also challenged the alternative (though not necessarily incompatible) idea that the amygdala is the site of fear conditioning memory. They argue that the definitive experiment would show that conditioned responses are absent while unconditioned responses are preserved, thus distinguishing the issue of memory from that of performance of fear responses. If no such evidence exists, then it can be argued that lesion and inactivation results showing no responses when the amygdala is lesioned or inactivated simply means that the amygdala is required for generating responses to memory stored elsewhere. Among the other challenges they raise, is the point that evidence of unit plasticity in the amygdala is not conclusive since plasticity is also evident in the thalamus and auditory cortex (Cahill, Weinberger, et al., 1999).

One body of work that is used to support the modulation-only theory is that while post training lesions of amygdala in fear avoidance task disrupts acquisition, amygdala lesions do not disrupt avoidance performance after acquisition (Parent, Quirarte, et al., 1995). McGaugh's interpretation is that this shows the role of the amygdala as a modulator; it is needed only briefly during conditioning. However, a significant alternative exists. As lesion evidence (Amorapanth, LeDoux, et al., 2000) and preliminary unit evidence from the third study here have suggested, the amygdala is required for the formation of the association between the CS and the operant response. Once the response is acquired, it could be evoked habitually without the involvement of

fear pathways. The animal without an amygdala would habitually avoid, but not show signs of fear if returned to the shock chamber.

The modulation theory is consistent with the lesion evidence. Evidence of plasticity in the auditory cortex (Edeline and Weinberger, 1993) supports the notion of memory formation outside the amygdala. Similarly, post-training manipulations of the basolateral amygdala affect instrumental learning, consistent with the notion of amygdala modulation of memory formation elsewhere (Parent and McGaugh, 1994).

Recently, it was shown that inactivating the amygdala immediately after conditioning has no effect on subsequent Pavlovian fear expression, suggesting that expression does not require the amygdala's modulation of memory formation elsewhere immediately after acquisition (Wilensky, Schafe, et al., 1999). This finding is the opposite of that for fear avoidance tasks, in which immediate post training inactivation does disrupt avoidance acquisition (Parent and McGaugh, 1994; Wilensky, Schafe, et al., 2000). This finding still allows for modulation concurrent with the CS-US pairings and/or modulation that takes place in a flexible time period subsequent to acquisition. However, it appears to be inconsistent with the model offered by McGaugh, which has suggested the amygdala's role in memory modulation is in the immediate period after acquisition.

One of the most confounding problems is the lack of dissociability between storage and expression of conditioned fear. However, there is evidence that unconditioned fear responses and non-associative conditioned responses are not disrupted by basolateral amygdala lesions (Iwata and LeDoux, 1988; LeDoux, Cicchetti, et al., 1990). Since the

basolateral amygdala is hypothesized to be necessary for both memory storage and associative conditioned responses, it is not possible to ask the following question. If memory storage exists in the amygdala, then lesioning or inactivating the amygdala, should disrupt the memory. For example, evidence that lesioning the amygdala one month after acquisition still disrupts fear conditioning (Lee, Walker, et al., 1996; Maren, Aharonov, et al., 1996) might be taken as evidence that rather than modulating memory storage elsewhere over the short term, the amygdala stores the memory within itself for the long term. The storage hypothesis can not be established by these methods because the amygdala is also involved in expression of fear. So the alternate interpretation of the 1 month post acquisition lesion is that the effect is due not to disruption of storage, but to disruption of expression. The alternative explanation, that there is no memory storage in the amygdala, and inactivating or lesioning it only blocks expression of memory stored elsewhere remains viable when viewing solely the lesion data.

While the debate has not resolved (Cahill, Weinberger, et al., 1999; Fanselow and LeDoux, 1999), there are several lines of evidence for memory storage in the amygdala. The first has already been noted. That is, if the amygdala only modulates memory, then the CS-US association should be weakly acquired (in the absence of strengthening modulation by the amygdala) and stored elsewhere when the amygdala is inactivated during acquisition, and then the memory should be expressed when the amygdala's function is recovered, the CS is presented for testing, and fear behaviors can be expressed through the amygdala. The inactivation study here shows this is not the case (Muller,

Corodimas, et al., 1997). No fear expression is seen during testing in the case of amygdala inactivation during acquisition. Secondly, a number of studies, including the LA single unit study here, have demonstrated fear conditioned, associative changes in single unit activity in LA (Quirk, Repa, et al., 1995; Repa, Muller, et al., 2001). Perhaps the most significant recent finding which advances support for local storage is data from local infusion of agents that disrupt protein synthesis (Schafe, Atkins, et al., 2000; Schafe and LeDoux, 2000). (It is believed that synaptic changes encoded memory occur in two stages: the first is a short term phase– less than 24 hours– in which local acitivity in a synapse can temporarily alter the strength of the synapse independent of gene activation and protein synthesis; the second phase is long term– perhaps permanently– which requires gene activation and protein synthesis via a cascade of secondary messengers initiated by local synaptic signaling.) One of McGaugh's most compelling critiques of the within amygdala storage hypothesis is that there has been no way to dissociate memory storage from performance of responses generated by that activated memory. Both functions are believed to occur in the amygdala. However, protein synthesis inhibitors are able to dissociate storage and response performance. Infusing the inhibitor, over the short term (hours), the conditioning is present and fear responses to the CS are intact. However, 24 hours later, the CS no longer elicits fear responses. The inference drawn is that the fear memory was not successfully stored when protein synthesis was blocked. It might still be argued that the inhibitors cause disruption of the amygdala's modulation of memory storage elsewhere; but with evidence of intact short term memory and performance in the

amygdala, such an explanation needs to become more and more qualified to remain possible. Thus, local infusion of protein synthesis inhibitors appears to meet the challenge of dissociating responses to fear memory (preserved) from storage of fear memory (disrupted).

There is human evidence that bears on the question of the amygdala's role: modulation only versus local storage and parallel strengthening of memory elsewhere. A bilateral amygdala lesioned patient did not show signs of fear conditioning as assessed by galvanic skin conductance, but was able to explain to the experimenter that the CS predicted the US (Bechara, Tranel, et al., 1995). Subsequent studies have supported this conclusion (LaBar, LeDoux, et al., 1995; Peper, Karcher, et al., 2001). In particular, Peper (2001) reported that while their subject could report the association between the CS & US, the subject had a deficient sense of the emotional value predicted by the CS (Peper, Karcher, et al., 2001). Hippocampal lesioned subjects on the other hand, showed normal fear conditioning, but had no conscious awareness of the CS-US relationship (Bechara, Tranel, et al., 1995). This evidence supports the idea of two parallel memory systems. In one the amygdala stores the fear conditioning memory, by which subsequent CS presentations elicit fear. In the other, the hippocampus participates in declarative memory formation, which may be strengthened by amygdala activation.

In sum, there is independent evidence from a number of methods supporting the view that fear memory is encoded in the amygdala. Inactivation studies rule out storage in afferent structures. Single unit recording demonstrates associative changes in the amygdala

preceding behavior. Protein synthesis disruption dissociates the long term storage of fear memory from performance of conditioned responses. Finally, human evidence also suggests two parallel memory systems, rather than the amygdala acting solely as a modulator of declarative memory stored elsewhere. All this evidence points to the basolateral amygdala as a critical site of long term storage, as well as acquisition of fear related memory for the CS.

As a further confirmation, the following experiment could be conducted. Establish fear conditioning with one CS (CS1). (The protocol would 3 conditioning trials instead of 1 to obviate the reconsolidation effect reported by Nader, Schafe, and LeDoux, 2000.) Infuse the protein synthesis inhibitor locally in the amygdala in the experimental group, vehicle in the control. Show that expression of CS1 conditioning is not blocked. While in inhibitor remains active, condition both groups with a second CS (CS2). Show that CS2 elicits fear in short term. After recovery show that in the experimental group CS1 continues to elicit fear, but CS2 does not.

Consistency of animal models of fear with human findings

The findings in rat experiments add evidence of the amygdala's role in fear. Monkey and human evidence is generally consistent with the rat data. Efforts to replicate early findings of Kluver-Bucy and Weiskrantz continue to find deficits in fear after amygdala lesions in monkey (Meunier, Bachevalier, et al., 1999; Kalin, Shelton, et al., 2001).

The role of the amygdala in human fear has also been investigated (Phelps, LaBar, et al., 1997; LaBar, Gatenby, et al., 1998; Davidson, Jackson, et al., 2000; Calder, Lawrence, et al., 2001). A case report of an individual with bilateral amygdala lesions shows deficits in fear recognition as well as structured and unstructured report of lack of fear experiences (Sprengelmeyer, Young, et al., 1999). Consistent with other animals, humans with amygdala damage show impaired fear conditioning as assessed both by CS evoked galvanic skin response (Bechara, Tranel, et al., 1995; LaBar, LeDoux, et al., 1995; Peper, Karcher, et al., 2001) and fear-enhanced startle (Angrilli, Mauri, et al., 1996). Ability to recognize fearful facial expressions in others is impaired in humans with amygdala damage (Adolphs, Tranel, et al., 1994; Young, Aggleton, et al., 1995; Calder, Young, et al., 1996; Young, Hellawell, et al., 1996; Broks, Young, et al., 1998; Adolphs, Tranel, et al., 1999). Recognition of fear in vocal expressions is also impaired in humans with amygdala damage (Scott, Young, et al., 1997).

Normal human subjects show amygdala activation in fear related tasks. The amygdala is active during fear conditioning (Buchel, Morris, et al., 1998; LaBar, Gatenby, et al., 1998; Morris, Ohman, et al., 1998; Buchel, Dolan, et al., 1999; Morris, Buchel, et al., 2001). Amygdala is active during processing of fearful facial expressions (Breiter, Etcoff, et al., 1996; Morris, Frith, et al., 1996; Phillips, Young, et al., 1997). Masked presentations of fearful faces elicited amygdala activation without awareness of fear (Morris, Ohman, et al., 1998; Whalen, Rauch, et al., 1998).

Lateralization effects have been noted in humans. For example, Buchanan (2001) studies human temporal lobectomies (Lx) assesses verbal (narrative) and non verbal (picture) memory assessed with free recall 24 hours after learning. Left Lx showed lack of emotionally enhanced memory for both; the deficit was more pronounced for verbal than pictorial; right Lx showed a deficit in memory, and retained some emotionally enhanced memory performance (Buchanan, Denburg, et al., 2001).

There is growing evidence that amygdala activity is altered in subjects with anxiety and mood disorders. Social phobics, in contrast to control subjects, showed enhanced amygdala activity when viewing neutral faces that had been presented with aversive odors (Birbaumer, Grodd, et al., 1998). Obsessive-compulsive disorder subjects, in contrast to control subjects, showed elevated activation of the amygdala in "provoked" conditions when compared to control conditions (Breiter, Rauch, et al., 1996). Post traumatic stress disorder (PTSD) combat veterans, in contrast to non-PTSD combat veterans, showed enhanced amygdala activation when presented with combat sounds as compared to white noise (Liberzon, Taylor, et al., 1999). PTSD combat veterans showed larger amygdala activations than non-PTSD vets when masked (40ms presentation followed by interfering stimulus) fear faces were presented (Rauch, Whalen, et al., 2000). In children with generalized anxiety disorder, when compared to kids not meeting diagnostic criteria, showed exaggerated amygdala activation in response to fearful versus neutral faces; the degree of amygdala activation in test conditions was positively correlated with level of anxiety symptoms (Thomas, Drevets, et al., 2001). In mood

disorders, adults show enhanced amygdala activity, and amygdala activity is correlated with blood cortisol levels (Drevets, Price, et al., 2002).

Clinical implications

What does the growing body of evidence exploring the amygdala's role in fear conditioning tell us that is relevant clinically? The following questions can be addressed. For example, how are painful events represented in the brain? How do such representations influence future thoughts, feelings, and actions? What might an understanding of the neural pathways of fear imply for treatment of anxiety and mood disorders? Before attempting to speculate about the answers to these questions, let us reiterate what the previous experiments in conjunction with prior work, tell us about human fear.

First, we can explain and provide a foundation for the phenomena associated with fear: the constellation of bodily and mental activity associated with fear is an integrated, graded, modulated response to avoid danger elicited by learned associations among aversive stimuli, salient innocuous stimuli, and background information in the environment. Some of the responses are strongly hereditarily influenced: changes in the body in preparation for high activity level, changes in mental activity (arousal), and predominantly innately programmed behaviors (freezing, running, fighting). Others responses are acquired, as their utility in avoiding US's is reinforcing. Such learning and

future responses to conditioned stimuli are adaptive, it seems, because they increase an animal's chances of surviving future danger.

Recent research about the amygdala's functioning, including data presented here, offers a neural basis for understanding much of the phenomena of fear just mentioned. Afferent pathways for CS and US information have been identified that converge on neurons in the dorsal LA (Romanski, Clugnet, et al., 1993). The lateral nucleus appears to be responsible for the association of the CS – US pairings leading to elicitation of fear responses to the CS alone– the hallmark of classical or Pavlovian conditioning (LeDoux, Sakaguchi, et al., 1984; LeDoux, Sakaguchi, et al., 1985; Iwata, LeDoux, et al., 1986; LeDoux, Cicchetti, et al., 1990). The role of LA in fear conditioning has been reinforced by local disruption of normal neural activity (Miserendino, Sananes, et al., 1990; Campeau, Miserendino, et al., 1992; Muller, Corodimas, et al., 1997). Learning associated changes in neural activity in the amygdala have been demonstrated (Quirk, Repa, et al., 1995; Rogan, Staubli, et al., 1997; Repa, Muller, et al., 2001). Efferent projections from the central nucleus of the amygdala are responsible for the variety of fear responses including hormonal and physiological changes preparing the body for increased activity, behaviors including freezing, fleeing, or fighting, changes in consciousness: orientation toward threats, heightened alertness, memory formation (and possibly memory degradation in extremely high levels of arousal) (Iwata, LeDoux, et al., 1986; LeDoux, Iwata, et al., 1988; Amorapanth, Nader, et al., 1999; Davis and Whalen, 2001). The basal nucleus appears to be involved in linking the fear information to the striatum where

stimulus response associations are thought to be formed (Amorapanth, LeDoux, et al., 2000).

Despite our wishes to obtain simple, clear answers to clinical phenomena by examining brain systems, in fact brain systems' investigation yields a story full of complicated interactions within and among regions, some of which will now be briefly reviewed. While the experiments in this dissertation did not directly address the contribution of other forebrain areas to fear phenomena, some understanding of their roles have been referenced. The hippocampal complex plays a critical role in context conditioning. Context conditioning refers to the phenomena in which the surrounding environment also gains the capacity to elicit fear after a US is present. The surrounding environment is a rather amorphous term; it certainly is composed of perceptions of a wide range of modalities. The hippocampus' extensive interconnection with the cortical regions responsible for all sensory modalities is thought to underlie the basis for the hippocampus' role in context conditioning. In hippocampus lesioned animals, the animal can acquire fear conditioning to discrete CS's but not to the environment (Phillips and LeDoux, 1992; Phillips and LeDoux, 1994).

The prefrontal cortex plays an important role in social decision making related to fear. Phineas Gage started lying and harshly cursing, not honoring commitments, disrupting his personal and business relationships after an injury which destroyed his ventromedial prefrontal cortex (Damasio, Grabowski, et al., 1994). Other patients with ventromedial prefrontal lesions show an inability to take account of risk of negative

consequences when making decisions involving social interactions (Bechara, Damasio, et al., 1999).

The basal ganglia (in human: the caudate, putamen, and globus pallidus or collectively, the striatum) have long been thought to play a role learning a complex behavior program (Gabrieli, 1998). In a complex learned behavior, elemental motor activity must be bound into a complex, sequenced, and timed pattern of motor activity. The striatum is believed to be a site of such learning. In operant conditioning, a learned behavior is associated with reinforcement: if rewarded, the frequency of the behavior increases. It is believed that the reinforcement signal interacts with the learning of behavior in the striatum. This type of learning is considered to be procedural memory, which is thought to be independent of the conscious, declarative memory system (Gabrieli, 1998). For example, HM, who had lost the hippocampal complex bilaterally, had a declarative memory deficit, but could learn new habits and motor skills, considered procedural memory. The striatum receives projections from the amygdala and medial prefrontal cortex throughout its ventral aspect (Fudge, Kunishio, et al., 2002). It is believed that these anatomical connections allow for aversive information to act as reinforcement in instrumental learning (Everitt, Cador, et al., 1989).

The behavioral phenomenon of extinguished fears appears to involve the interaction of the amygdala and prefrontal cortex. In extinction, the CS is presented without aversive reinforcement. After a number of trials, the CS no longer elicits fear responses. The CS's elicitation of fear is suppressed not forgotten and can be re-evoked

under stress (LeDoux, Romanski, et al., 1989). Some evidence suggests that a possible mechanism for fear extinction is the ventral medial prefrontal cortex's inhibition of the amygdala (Morgan, Romanski, et al., 1993).

Another role of the amygdala is to reinforce declarative memory formation in the cortex (Adolphs, Cahill, et al., 1997). Declarative memory refers to conscious memory of experience, with awareness of time and place of the memory formation (as opposed to semantic memory, which is also conscious memory but without awareness of source of the memory.) Cahill and colleagues told subjects stories while the subjects viewed three pictures. In the middle panel, the experimental group got an evocative and disturbing story about a car accident with graphic description of injuries. The control group heard a more mundane description. A month later, the experimental group recalled more details of the pictures that the control group. These effects were blocked by ß-adrenergic antagonists (Cahill and McGaugh, 1998). Another group showed that the degree of amygdala activation during viewing of negative scenes was correlated with degree of recall of those scenes three weeks later (Canli, Zhao, et al., 2000).

We have reviewed the functional pathways of the amygdala and their interaction with other brain systems that underlie aspects of fear. Given this understanding of the neural basis of fear conditioning, we can now address two of the questions, how are painful events represented in the brain, and how do such representations influence future thoughts, feelings, and actions. In part, painful events are stored as fear conditioning. These memories shape the future by eliciting fixed and operant responses elicited by

salient CS's and background contextual information. So too, declarative memory of the events is strengthened. From an evolutionary standpoint, these capacities have likely been selected because they increase the chances of the animal's survival.

What might an understanding of two pathways in the amygdala, one for evoking hereditarily shaped response, the other for learned responses, imply for treatment? The amygdala can detect the fear value of external perceptions (and presumably thoughts as well) before they reach awareness (Whalen, Rauch, et al., 1998). Then, two sets of pathways could be activated: inherited physiological and behavioral responses via the lateral nucleus' projections to the central nucleus; and, acquired behavioral (and perhaps cognitive) patterns via the lateral's projection to the basal nucleus and onto the ventral striatum. It is the second pathway that could also be involved in the formation and reactivation of cognitive and behavioral coping patterns otherwise known as defenses. While we will examine this question further in subsequent sections, one implication can be directly drawn by focusing on the existence of the classical conditioning pathways versus the operant conditioning one. A suggestion offered by LeDoux and Gorman makes use of knowledge of the dual pathways. The classical conditioning route elicits evolutionarily preserved responses that do not require active thought. In contrast, in the operant pathway, immediate reinforcement shapes learned behaviors, which may be influenced by cognitive activity. They have noted the beneficial consequences of learning strategies to respond to fear so that an individual can have a daily social routine with its self-reinforcing consequences. In their words, "an active coping response reroutes processing

from a pathway controlling dysfunctional passivity to one controlling successful engagement with the environment (LeDoux and Gorman, 2001)."

The review so far offers the possibility of understanding phobias, PTSD, and perhaps panic disorders as fear conditioning. The cumulative experimental efforts of many labs, particularly the LeDoux lab, have illuminated in animal models and human studies a large body of evidence about the amygdala's role in fear conditioning. We now have a basic neural model of how fears are acquired, stored, evoked, and extinguished. Such models of acquisition and extinction are directly incorporated in behavioral treatment therapies. We now have animal models of how acquisition, extinction and coping strategies can be explained on a neural level. We will return to further discussion of clinical implications of this body of knowledge in the next several sections, after reviewing the limitations of the behavioral conditioning model.

Limitations of behavioral and psychoanalytic approaches to psychopathology. Theories to explain psychopathology abound. Historically, behavioral and psychoanalytic approaches have been at odds, despite efforts to bring them together (Dollard and Miller, 1950; Wachtel, 1977). More recently, the cognitive behavioral paradigm of treatment has emerged, incorporating some acknowledgement of higher mental acitivity as an agent in normal and abnormal psychology. In the meantime, psychoanalytic theories have multiplied and diversified. Interpersonal, ego, object-relations, self, and relational schools have developed, with varying degrees of incompatibility among themselves (e.g., see Mitchell 1988). A growing proportion of

psychotherapists identify their theoretical leaning as eclectic, meaning taking from a variety of schools (Bodkin, Klitzman, et al., 1995; Corrigan, Hess, et al., 1998). Some even question the contribution science can make to an endeavor sometimes characterized as not compatible with scientific investigation (Spence, 1984). Theoretical consensus remains elusive.

Given this theoretical diversity, one wonders how to understand the excitement which frequently meets neuroscientific efforts to address clinical issues. Near universal proclamation of relevance and interest greets studies with human fMRI or animal model data. While it is not clear that all audiences share the same reasons for enthusiasm. For example, some are excited that the mechanisms of psychotropic medications will be revealed; others that Freudian theory will finally be vindicated. It is clear that all schools are looking toward neuroscience to provide missing links in theory.

Unanswered questions in the behavioral model. Behavioral models have difficulty explaining why similar exposure to aversive events does not produce similar anxiety disorders in humans. Similarly, treatment failures are common and difficult to explain. These exceptions stand in contrast to the experimental model, in which there are individual differences in rate of acquisition and extinction but not absence of acquisition and extinction.

A major limitation of the behavioral model of anxiety disorders has been that it has difficulty incorporating higher cognitive activity that contributes to human pathology. Jeffrey Gray has incorporated a form of cognitive activity in a model of anxiety. In his

model the hippocampal complex and related cortices are continuously evaluating whether current plans are reaching expected goals and balancing conflicting goals. Detection of unexpected contingencies recruits the anxiety system into action. Gray's model is one attempt to understand cognitive activity and emotion on a neural level. Efforts to understand the interaction between emotion and higher cognitive functions are important. Human capacity for symbolic activity and particularly language permits us to make use of highly abstract information about our experiences. At the same time, such abstract thoughts themselves may serve as CS's in conditioning. Moreover, our intellectual reasoning capacity may modulate fear conditioning: at best helping to calm irrational fears, at worst heightening unreasonable ones.

Can thoughts serve as CS's and contextual stimuli just as well as perceptions of stimuli from the external world? Neuroscience has developed the knowledge of the neurons involved in bringing information about external stimuli, such as a tone, to the amygdala. In humans, some exploration of thoughts as CS's has been undertaken (Phelps, O'Connor, et al., 2001). The present level of knowledge does not allow us to fully answer compelling questions as whether emotional pain recruits the amygdala in the same way that somatosensory pain does. For example, does loss of a loved one, or humiliation, or embarrassment evoke fear learning. Developing an animal model for a CS that is a symbolic thought is a significant challenge. The goal of such an effort would be to approach addressing questions like, based on a history of traumatic interactions with

loved ones, does the thought of intimacy in a current relationship evoke fear of a similar disappointment and painful interactions as has been the case in the past?

Implications for a psychoanalytic theory of the unconscious. Psychoanalytic views, in contrast to behavioral models, attempt to explain a vast degree of human normal and abnormal behavior from externally observable behavior to unconscious mental activity. Case study is the predominant method of exploration of psychoanalytic ideas– a method notoriously subject to bias, unable to resolve competing theoretical constructs. Theory is rarely if ever operationalized, and if so even more rarely subject to experimentally designed testing (Fonagy and Target, 2000). Given the scope of psychoanalytic theory developed from extensive clinical practice, there are ample opportunities for testing and developing a neural basis for some of the psychoanalytic concepts.

Recent evidence from research about the amygdala's activity outside of awareness suggests a way that some psychoanalytic concepts can be operationally defined and tested. Evidence suggests the amygdala can evaluate stimuli and evoke responses on a time course that is too short for conscious awareness (Whalen, Rauch, et al., 1998). This finding offers a link to the psychoanalytic theory of signal anxiety (Freud, 1926). In this view, anxiety signals impending danger, so that defensive action can be taken to avoid a further anxiety and its associated danger. The ensuing responses are the classic defense mechanisms. Signal anxiety may be briefly experienced, but it is not thought to be reflectively recognized (Wong, 1999). Similarly, the operations of defense are thought to

take place outside of awareness (Freud, 1926; Wong 1999). Recent work has explored evidence consistent with the concept of signal anxiety. Evidence for unconscious fear responses in masked designs has been shown in a number of labs (Morris, Ohman, et al., 1998; Whalen, Rauch, et al., 1998; Wong, 1999). In a masked design, a stimulus is presented for a very brief period of time (on the order of 30-50 ms). The stimulus presentation is immediately followed by presentation of a second stimulus for a much longer duration of time, ensuring the second stimulus is consciously perceived. In such a design, subjects do not report awareness of the first stimulus. Were the first stimulus not immediately followed by the longer presentation of the second, a 40ms period is enough that the stimulus is sometimes consciously perceived. Hence the design is called masked presentation, since the second stimulus masks awareness of the first. Masked presentations of fear evoking stimuli have been shown to arouse fear responses, as well as predictable patterns of brain activity (Morris, Ohman, et al., 1998; Whalen, Rauch, et al., 1998; Wong, 1999). The implication offered here is that such unconscious activation of the fear system can in turn elicit learned responses, some of which rather than behavioral responses are cognitive activity. If the cognitive activity is successful in decreasing fear and repeated over many times, it could come to operate habitually, without awareness (Dollard and Miller, 1950). Whether this explanation of the neural basis of unconscious emotional activation and unconscious cognitive defense is an adequate model of psychoanalytic ideas about the unconscious remains to be seen. The neural basis of such habit learning will be further discussed below. Mental habits, acquired through operant

conditioning, may underlie the psychoanalytic concepts of defense mechanism. Similarly, when transference is understood as a patterned response to people or patterns of social interaction, at least a partial translation can be made to viewing such phenomena as cognitive habits, acquired through operant conditioning.

Earlier, the role of the striatum in operant conditioning was noted. In that context the striatum is understood as involved in motor programs or behavior. The striatum has been implicated in implicit cognitive function as well. Striatal activation has been shown during cognitive tasks in normal subjects (Poldrack, Prabhakaran, et al., 1999). Basal ganglia cognitive function has been inferred from its absence in Parkinson's patients with pallidotomies (York, Levin, et al., 1999). Deficits in cognitive tasks requiring timing have been reported (Jahanshahi, Rowe, et al., 2002; Malapani, Deweer, et al., 2002). So too, deficits in cognitive habit formation have been found (Hay, Moscovitch, et al., 2002). The ability of motor programs and cognitive habits to be evoked without conscious awareness thus, could have important links to psychoanalytic theory of unconscious defense mechanisms. Habitual patterns of thought and behavior need not exclude activity of which there is some awareness. For example, others have proposed a similar treatment of attachment, understanding the pattern of interaction with caregiver as a type of procedural memory (Fonagy, 1998).

We have just considered that learned responses to perceived dangers become habitual patterns of response. While this can often be adaptive, the problem arises when the response has negative as well as positive consequences. Often suggested is that the

child develops a response to anxiety which was the best s/he could come up with given limited life experience and cognitive understanding of the nature and cause of the threat. What was acceptably successful to the child becomes problematic as a life long response to anxiety. Treatment is seen as exposure to the fears that elicited the defenses in a safe relationship. When the emotion is reactivated in the transference, its less threatening nature can be recognized, awareness of the problematic responses can be developed, and/or more adaptive responses can be developed. This view of activated emotional memory underscores the importance of transference. In this view, an essential component of transference is the affect. Intellectual understanding of fears and defense mechanisms is inadequate by itself to make lasting therapeutic changes. The affective memory is a crucial aspect that must be modulated during psychotherapy (Breuer and Freud, 1895).

Fear and attachment

A second area in which knowledge of the amygdala has implications for understanding anxiety and mood pathology is in its interaction with the attachment system. A promising intersection in animal experimental research between the fear conditioning models and attachment models may allow for further experimental investigation of how early relationships modulate fear learning. Attachment theory has relevance to psychopathology and to a deep understanding of human behavior, and exists within a body of substantial experimental support. It does make operational important theoretical constructs of early relationships, with resulting success in developing a body

of empirical evidence. Second, animal models exist that allow experimental investigation, including designs that can not be ethically investigated in humans. Progress is being made in understanding the neurobiological basis of attachment phenomena (Insel and Young, 2001; Fleming, Kraemer, et al., 2002).

Earlier, we suggested that viewing anxiety and mood disorders as fear conditioning is an inadequate model. While it remains well established that fear is relevant to such disorders, clearly the role of positive affect (caring/love), its absence, inconsistency, or withholding also likely plays a part in such disorders. The fact that there are now realistic opportunities to explore such questions in experimental investigations of neural activity is an exciting advance.

Psychoanalytic theories place emphasis on the history of interactions with caregivers. Freud, for example, discussed the fear of losing an "object" (his term for someone with whom one has a significant relationship) and fear of losing the love of the "object" as predominant and crucial forms of anxiety (Freud, 1926). Attachment theory has lead to an observational paradigm in humans which has some reliability in associating fearfulness around separation with history of received care and future pathology. A large body of empirical research is now confirming this focus by demonstrating associations between pathology and a history of problematic interpersonal interactions. As discussed earlier, there is a large list of acquired factors that predict future pathology, including physical, sexual abuse; troubled emotional relationships with significant caregivers; exposure to violence by and to others, etc. All of this data, while not conclusively

demonstrating causal links with pathology, is highly suggestive. Significant hurdles remain. One is the same problem as with the behavioral model. Not all events lead uniformly to pathology.

Another issue is to explain the observed distinctions in reactions to separation in humans. Evidence does suggest that both insecure and avoidant categories of attachment have heightened levels of fear. The question remains as to how the differences in reaction to separation arise. Furthermore, a more recently recognized category, disorganized, has been shown to be most strongly correlated with future psychopathology (Main, 1996). Some have speculated that a separation between cognitive activity and emotion, termed dissociation, is related to the disorganized category. Furthermore, a theory of the neural basis of such dissociation, involving abnormal right frontal cortical activity has been advanced (Schore, 2002).

Animal models show that prolonged separation from caregiver produces more fearful adult animals. Physiologic consequences of prolonged separation have also been noted, for example elevated glucocorticoids (Hofer, 1994). Separated from their mothers for extended periods as infants, adult rats showed enhancements on several measures of fear, while differences were observed between males and females. Male and female rats showed decreased exploration in the open arms of an elevated maze, and enhanced stress hormones. Male rats showed enhanced startle responses, and ultrasonic vocalizations in response to startles (Kalinichev, Easterling, et al., 2002). Similar open maze findings were made by another group (Boccia and Pedersen, 2001). Another group found similar effects

of maternal separation on both open maze and cortisol level measures (Wigger and Neumann, 1999). Another group did not find consistent effects of maternal separation on adult fear when using less separation, but did note some male-only effects (Lehmann, Pryce, et al., 1999). In a study that looked only at male rats, maternal separation produced enhanced startle, decreased open maze exploration, and enhanced preference for ethanol; all the effects were attenuated or reversed after SSRI administration (Huot, Thrivikraman, et al., 2001). There have been findings showing changes in the amygdala. Benzodiazepine receptor levels are higher in rats that receive above normal grooming from their mothers; similarly, they are less fearful as adults (Caldji, Francis, et al., 2000). Efforts to map out the neural basis of attachment behaviors are underway (Insel and Young, 2001; Fleming, Kraemer, et al., 2002)

Mechanisms through which attachment modulates fear behavior

Representation. The attachment paradigm holds the view that interactions between caregiver and child build working models of expected interactions with significant others which shape future interactions. All psychoanalytic theory emphasizes memory constructs that affect future thoughts, feelings, and behavior. Bowlby's working models, Stern's (RIG), transference– all address the fact that past experiences, feelings, and behavior with particular emphasis on the feelings of the subject and those s/he interacts with are stored and affect future decisions or actions.

By touching on how the brain stores fear memory, the current work contributes to a growing body of theory on mental representation. Having expanded our understanding of how fear memory is encoded is one step toward understanding other forms of representation that involve more complicated cognitive activity.

Attachment as a modulator of fear. Adult animals are more fearful when some aspect of the childhood relationship with a caregiver went awry. In extreme cases of disruption, the effects go well beyond specific disorders to include profound physical, cognitive as well as psychological dimensions (Spitz, 1945; Harlow, Dodsworth, et al., 1965). As Freud (1926) and others since have pointed out, one possibility is that the caregivers absence leads to overwhelming stimulation stemming from unmet needs (hunger, warmth) (Hofer, 1994), which is an aversive state. So the animal learns overtime to fear the caregiver's absence. It is also possible that in the caregiver's absence, a higher incidence of painful external events arise (falls, encounters with predators, encounters with aggressive conspecifics) leading to a similar kind of association between caregiver's absence and danger. None of these possibilities suggests any neural activity other than fear conditioning. But, why such fear conditioning would lead to increased risk for adult psychopathology is not obvious.

Another notion that has been suggested is that the caregiver teaches the infant how to regulate its emotions (Southam-Gerow and Kendall, 2002). In this view the pains are there regardless of the mother's presence, but in her presence there is soothing that modulates the strength of fear conditioning. It seems entirely possible that the notion of

regulation may overlap with that of operant conditioning. If the caregiver can (in some unclear manner) facilitate the reinforcement of particular behaviors that reduce painful or fearful experiences this could be looked at as assisting in affect regulation or as operant conditioning.

However, another possibility exists. There may be some need for social contact, just as much as there is a need for food and warmth. Loneliness is certainly a relevant phenomena in this regard. The question of how absence is detected and what about it gives rise to sadness and increased fear has been explored in the rat (Hofer, 1994). Hunger, lack of warmth, lack of tactile and olfactory signals are dissociable triggers that generate distress responses in the mother's absence. Harlow's work suggests monkeys need to feel soft touch, as they select that psuedo mother to be near over the wire mesh one that provides food.

Early experiences may also affect expectations about the general state of the world, or about social interactions. Negative early experiences set up expectations that future experiences will be negative and should be approached warily, while positive early experiences set up expectations of security and rewarding interactions. Often the caregiver child interactions contain both episodes of mutually rewarding encounters, and episodes of conflictual, painful, unrewarding circumstances. Psychoanalytic theory has a particularly useful concept in regard to the kind of interactions that can lead to psychopathology, that of the double bind, in which the child learns of two opposing affective communications (I want you/ I reject you) in the caregiver (Tronick, Als, et al.,

1978). So too, there is a conflict in the case of unambivalent negative interaction: even if the caregiver is emotionally or physically abusive, the caregiver is also needed for food, warmth, and the like. Psychoanalytic theorists have dealt with similar material extensively in terms of representations having an associated affective valence, and how representations of the same object with different valences are integrated or fail to be in psychopathology (Kernberg, 1985). So to expectations about future interactions begins to look like an operationalized view of transference phenomena.

Future research

Together the findings in this work and larger efforts to understand the neural basis of fear of which they are a part offer promise for integrating our expanding knowledge of the neural basis of fear with that of attachment. Less is understood about the neural basis of attachment than of fear. One candidate region is the nucleus accumbens which is known to play a role in pleasure conditioning (Ikemoto and Panksepp, 1999). Alterations in the nucleus accumbens might affect dopaminergic activity in the amygdala during fear conditioning. In fact the role of dopamine in fear conditioning has been explored. For example, decreasing dopamine receptor activation in the basolateral amygdala, locally or by way of the nucleus accumbens, disrupted second order fear conditioning (Nader and LeDoux, 1999). Further investigation is warranted.

It would be a useful to characterize effects of separation in the classical conditioning paradigm. For example, does early separation increase fear responses as

tested in the typical paradigm used in the LeDoux lab, of four tone shock pairings, two presented on each of two days? In addition, it would be useful to seek benzodiazepine receptor levels differences within the amygdala correlated with such behavioral differences and associated with separation.

Conclusion: James' bear

James' dilemma restated is whether we run because we are afraid of the bear, or are we afraid as we notice that we are running from the bear (our perception of that running and heart pounding and sweating comes to be know consciously as fear). James' assertion that fear arises from feedback from body's organs, has a long history of empirically supported opposition (Davidson, Jackson, et al., 2000). However, current knowledge allows a subtler view that admits both sides of the argument. It is possible from our anatomical and functional understanding of the medial prefrontal cortex to suppose that it functions as James' latter alternative: it receives afferents from brain areas responsible for our perceptions of heart rate and other bodily sensation, and contributes to our conscious experience of fear (Damasio). As for the first alternative, the amygdala receives afferents providing information about the bear which, from prior conditioning, elicits fear activity, among which one is the escape behavior (running) generated in the periaqueductal grey of the midbrain.

The explanation of James' dilemma is offered as an argument by example for the possibility and the gain in understanding complex psychological phenomena through

neuroscience. Some would argue for practical or principled reasons that explanations across levels of abstraction are not possible. While a convincing full explanation of psychopathology remains a future goal, illuminating a psychological dilemma through advances in neuroscience evidence offers encouragement that a neural derived science of psychopathology is possible and promising.

FIGURE CAPTIONS

Chapter 2 Figure Captions

Figure 2.1 Classical Fear Conditioning. In fear conditioning an innocuous stimulus (the conditioned stimulus, or CS), is paired with an aversive stimulus (the unconditioned stimulus or US). Before conditioning (bc), CS presentation does not cause a fear response. After conditioning (ac), the CS elicits responses that are innate components of fear.

Figure 2.2 Fear conditioning pathways. Afferents converge on the lateral nucleus of the amygdala. The lateral nucleus projects to the basal and accessory basal nuclei which in turn project to the central nucleus, which influences various effector systems involved in the expression of emotional responses. Forward projections are indicated by solid arrows, and feedback projections are indicated by open arrows. BNST- bed nucleus of the stria terminalis, DMV- dorsal motor nucleus of the vagus, NA- nucleus ambiguus, RPC- nucleus reticularis pontis cauudalis, RVL Medulla- rostral ventrolateral nuclei of the medulla, PVH- paraventricular nucleus of the hypothalamus.

Figure 2.3 Effect of fear conditioning (tone-shock pairing) in rats. Observations were made following CS presentation without the US. Freezing, the cessation of all non-respiratory movement, was used as a measure of fear. Data is summarized from previous work as indicated. **A** Effects on conditioning to a tone. *Left Panel:* Effects of pre-training electrolytic lesions. Only auditory thalamus and amygdala lesions disrupted fear conditioning to the tone. *Right Panel:* Pre-training inactivation of the basolateral amygdala by muscimol, a GABAa agonist, disrupted fear conditioning. **B** Effects on conditioning to the context. *Left Panel:* Hippocampal and amygdaloid lesions disrupted context conditioning. *Right Panel:* Pre-training inactivation of the basolateral

amygdala by muscimol disrupted context conditioning. Abbreviations: Unop: Unoperated controls; ACx: Auditory Cortex; HPC: dorsal Hippocampal Formation; MG: Medial Geniculate; Amyg: Amygdala Complex.

Figure 2.4 Unit Recordings (Quirk, Repa, et al., 1995). LEFT: Time histogram of a lateral amygdala neuron's action potentials during presentation of a tone at three points during training: before pairing with footshock (pre), early extinction (test), and following 30 extinction trials (late-extinction). The horizontal bar indicates the start of a 5 kHz tone. Bin width is 5 ms. Note the increased number of early responses (< 15 ms) in the 'test' observation. CENTER: Histogram shows the latency of the earliest significant conditioned response for 10 neurons in the lateral amygdala. Note the preponderance of conditioned responses prior to 15 ms following tone onset. Line graph (bottom) shows the change in tone responses for 16 neurons in the lateral nucleus that significantly conditioned. Tone responses (from the first 70 ms of the tone) at different points in training are expressed as a percentage of sensitization responses. RIGHT: Cross-correlation between the spike trains of 2 simultaneously recorded lateral amygdala neurons at different points during training. Data taken during spontaneous activity (in the absence of the tone). The time of one neuron's firing is defined as 0; the time of the other's firing is shown in relation to time 0. Training induced a significant peak at +3 ms.

Figure 2.5 Unit Fear Response. Increased firing in auditory cortex units prior to time of shock onset emerges in early extinction (Quirk, Armony, et al., 1997). Pooled single unit firing rate data are displayed graphically. Each bin (small rectangles) represents the averaged firing rate of all units that showed a delayed-onset enhanced tone response in extinction (11 cells). To smooth the display, each bin is actually the average of its rate and the two bins preceding and following it. Darker shading indicates a higher firing rate.

Activity is shown for ~3 seconds in each of 40 trials; the duration of the CS is indicated by the black bar. The time of shock onset during conditioning is indicated by the labeled arrow. The first 10 trials occur in the sensitization phase, in which the novel CS and US are presented unpaired. The last 30 occur in extinction (CS alone). Not shown are the conditioning (paired CS-US) trials. In extinction trials 4-8 there is an elevated firing rate in the 0.5 seconds prior to the US onset in conditioning trials. The enhanced firing diminishes over the course of extinction.

Figure 2.6 LTP induction and fear conditioning increase auditory responses in the lateral nucleus of the amygdala (Rogan and LeDoux, 1995; Rogan, Staubli, et al., 1997). Field potentials were evoked in the lateral amygdala by electrical stimulation of the MGm and PIN, and by tone presentation. High frequency electrical stimulation in the MGm and PIN resulted in long lasting enhancement of auditory-evoked responses (top), and also produced long lasting enhancement of auditory-evoked responses (middle). Low frequency electrical stimulation did not change responses to auditory or electrical stimuli. Paired CS-US presentations (fear conditioning) produced similar long lasting enhancement of auditory-evoked responses, whereas unpaired controls did not show an enhanced evoked potential (bottom).

Figure 2.7 Protein synthesis is required for reconsolidation of an activated fear memory (Nader, Schafe, et al., 2000). A day after a single pairing of a tone and footshock, another single CS was presented, followed by local infusion of anisomycin or vehicle into the basolateral amygdala. 4 and 24 hours later 3 CS's were presented during which freezing was measured. Both groups froze at similar levels in the short term condition, but the experimental group froze less than controls in the long term condition, suggesting the experimental group's reconsolidation of the fear memory was impaired.

Chapter 3 Figure Captions

Figure 3.1 Location of cannula tips in the lateral and basal amygdala, mapped on a series of three coronal sections through the amygdala. Representations of the cannulae tips are coded to indicate experimental group: open rectangle- *saline-saline*, gray rectangle- *muscimol-muscimol*, top-half-gray rectangle- *muscimol-saline*; bottom-half-gray rectangle- *saline-muscimol*. Labels of fiber tracts and nuclei are based on classification of amygdala's subnuclei as identified by Pitkänen, et al. (1995): L-lateral nucleus; I-intercalated nucleus; B-basal nucleus with magnocellular(mc), parvocellular(pc), and intermediate(i) subdivisions; AB-accessory basal nucleus; CE-central nucleus with capsular(c), medial(m), and lateral(l) subdivisions; ec- external capsule; st- stria terminalis; lab- longitudinal association bundle;. Brain diagrams are from the Graphic File version of the Swanson rat brain atlas (1992, 1994). Distance in mm posterior to Bregma is indicated in the center labels.

Figure 3.2 Photomicrograph of a coronal section through the amygdala showing the guide cannula track and infusion cannula tip (tip identified by the '->' symbol). This tip location corresponds to the open rectangle in the center of the lateral nucleus in the diagram of the right amygdala at the -2.8 level of Figure 3.1. Abbreviations as in Figure 3.1 with the following additions: M- medial nucleus of the amygdala; Pir- Piriform Cortex;

PAC- periamygdaloid cortex; CP- caudoputamen. The filled diamond symbol lies in the external capsule. The outlined diamond symbol lies in the longitudinal association bundle.

Figure 3.3 Effects of muscimol injected into the lateral and basal amygdala on conditioned freezing. All rats received infusions of muscimol or saline immediately before training, and immediately before each testing session. Experimental groups are distinguished on the x-axis by infusion types: e.g., the group which received infusion of muscimol before training and saline before each day of testing is designated muscimol-saline. Percentages reflect the group's average number of observation periods (out of a possible 36) in which rats were freezing. The left panel shows the effects of the drug on freezing elicited by the tone in a novel context during the first day of testing. The right panel shows the amount of freezing observed when the rats were placed in the original conditioning chamber on the second day of testing. No tone was presented. The symbol, '*', indicates a significant effect of drug treatment relative to the saline-saline control.

Figure 3.4 Temporary effect of muscimol on acquisition: The group that received muscimol before training and saline before testing had suppressed levels of freezing (see Figure 3.3). To determine whether this was due to temporary inactivation of the amygdala, this group was retrained with saline infusions and then retested with saline infusions. In contrast to the deficit observed when they were trained with muscimol

injections, they performed similar to the saline-saline control group when retrained with saline injections (retraining data is shown as the dark bar).

Figure 3.5 Temporary effects of amygdala inactivation on the expression of conditioned fear. The saline-muscimol group was retested with saline infusions (no additional training was given). The amount of freezing in the retest (dark bar in last column of each plot) was then compared to the results of the same animals in the original test (middle column), as well as to the original saline-saline control group (first column). Freezing during the retest was significantly higher than it had been when the same animals had been tested with muscimol infusions, and was not different from the saline-saline control group. This pattern held for both tone and context tests.

Chapter 4 Figure Captions

Figure 4.1 Behavioral measures of conditioned fear. (a) Mean (± s.e.m.) suppression ratio for both the paired and unpaired groups throughout the three phases of the experiment, habituation, conditioning and extinction. Positive suppression ratios indicate bar-press suppression during the CS, whereas a suppression ratio of 0 indicates no change in press rates during the CS. The suppression ratios are derived from the raw bar-press rates during the pre-CS period (60 s) and during the CS (20 s), which are depicted on the right for the paired (top) and unpaired (bottom) groups (rates are given in bar-presses per second). (b) Mean (± s.e.m.) CS-elicited freezing levels (freezing during the 20-s CS minus freezing during the previous 20 s) for both the paired and unpaired groups throughout the three phases of the experiment. For (a) and (b), data points are four trial blocks averaged together, except the first point, which is the average of the final 6 trials of habituation.

Figure 4.2 Photomicrograph of a thionin-stained brain section from a representative rat, showing electrode tract and lesion site in LAd. LAd, dorsal subnucleus of the LA; LAvm, medial division of the ventral LA; LAvl, lateral division of the ventral LA; BL, basolateral amygdala.

Figure 4.3 Neuronal plasticity during early extinction, for all cells. The distribution of *Z* difference scores is depicted, showing the average CS-elicited response from 0–200 ms after stimulus onset, expressed as a *Z* score, during early extinction minus habituation

levels, for each of the 100 LAd cells in the paired group and 70 LAd cells in the unpaired group. Values are organized along the abscissa in rank order, from the smallest to largest *Z* difference score. Each bar represents exactly one cell. Note the shift of the paired population toward positive *Z* difference scores, indicating a tendency for these LAd cells to exhibit greater CS-elicited responses during early extinction trials than during habituation trials.

Figure 4.4 Neuronal plasticity during conditioning, for all cells. The distribution of *Z* difference scores is depicted, showing the average CS-elicited response from 0–200 ms after stimulus onset, expressed as the maximum *Z*-score of four conditioning 4-trial blocks minus habituation levels (see text for details), for each of the 100 LAd cells in the paired group and 70 LAd cells in the unpaired group. Values are organized along the abscissa in rank order, from the smallest to largest *Z* difference score, and each bar represents exactly one cell.

Figure 4.5 Latency and time course of neuronal plasticity. (a) Mean PETHs (spikes normalized to the pre-CS baseline; 10-ms bins) for two phases of the experiment (habituation trials 1–8 and average of conditioning trials 1–8 and 9–16) for all conditioned cells from the paired group (n = 24 cells). (b) Time course of the average CS response of plastic cells. Mean (± s.e.m.) CS response for all conditioned cells from the paired group (n = 24 cells) throughout the three phases of the experiment. Data points are four trial

blocks averaged together, except the first point, which is the average of the final 6 trials of habituation.

Figure 4.6 Acquisition of neuronal versus behavioral measures of learning. (a) First significant trial for conditioned responding of neuronal units (y-axis) plotted versus the first significant trial for conditioned responding of fear behavior (x-axis) as determined by bar-press suppression and freezing, whichever changed first. Each conditioned cell from the paired group is plotted once ($n = 24$ cells). The outlined 45-degree area indicates cells that first showed neuronal conditioning on the same trial on which behavioral conditioning was first detected. The circled region represents cells for which the neuronal changes were detected on the same or earlier trials than behavioral changes. (b) Data summarized from (a), showing the difference for each cell, in trials, between the first significant neuronal trial and the first significant behavioral trial. Negative numbers indicate cells for which neuronal unit changes were detected before behavioral changes, whereas zero indicates cells for which unit and behavioral changes were detected on the same trial. Dark gray bars represent all cells for which unit changes were detected on the same or earlier trials than behavioral changes, as summarized in the right panel of (b). (c) Mean CS-response for all conditioned cells from the paired group ($n = 24$ cells) throughout habituation and conditioning. Conditioning trials are aligned based on the trial of behavioral learning (trial 0).

Figure 4.7 Persistence of neuronal plasticity during conditioning. (a) Persistence values (see text for details) for all conditioned cells from the paired group ($n = 24$ cells). Dashed line, cutoff used to distinguish transiently plastic cells from long-term plastic cells. (b) Mean (± s.e.m.) CS-response for transiently plastic and long-term plastic cells ($n = 12$ cells per group) throughout the three phases of the experiment. Data points are four trial blocks averaged together, except for the first point, which is the average of the final six trials of habituation. Asterisks indicate trial blocks during which the response is significantly greater than habituation levels ($p < 0.05$).

Figure 4.8 Breakdown of transiently plastic versus long-lasting plastic cells, by latency of plasticity and anatomical location. (a, b) Mean PETHs (spikes normalized to the pre-CS baseline; 10-ms bins) for two phases of the experiment (habituation trials 1–8 and average of conditioning trials 1–8 and 9–16) for transiently plastic cells (a; $n = 12$ cells) and long-term plastic cells (b; $n = 12$ cells). (c) Anatomical recording locations for transiently plastic cells (gray circles) and long-term plastic cells (white circles). (d) Diagram of our hypothesis of how LAd cells encode fear learning. Abbreviations are as in Figure 4.2. d and v, dorsal and ventral portions of LAd, respectively; CE, central nucleus of the amygdala.

Chapter 5 Figure Captions

Figure 5.1 Behavioral measure of conditioned fear. Mean (± standard error) suppression ratio throughout the three phases of the experiment, habituation, conditioning and extinction (n=5). Positive suppression ratios indicate bar-press suppression during the CS, whereas a suppression ratio of 0 indicates no change in press rates during the CS. The suppression ratios are derived from the raw bar-press rates during the pre-CS period (60 s) and during the CS (20 s). Data points are four trial blocks averaged together, except the first point, which is the average of the final 6 trials of habituation.

Figure 5.2 Photomicrographs of successive slices from a representative rat showing electrode tract and lesion site in the basal nucleus: one, a thionin-stained brain section, the other an acetylcholinesterase stained section, which distinguishes the labeled basal nucleus from the lighter stained lateral nucleus. Two basal nucleus recording sites are evident, the medial one shows a hole at the lesion site, the lateral one is less disrupted. Abbreviations: Ce, Central Nucleus; LAd, dorsal subdivision of the lateral nucleus; LAm, medial subdivision of LA; B, basal nucleus; st Stria Terminalis; ec External Capsule.

Figure 5.3 Location of recording sites. Location of wire tips from which cells that showed changes in CS response. Abbreviations: AB, Accessory Basal Nucleus; B, Basal Nucleus (m, magnocellular; i, intermediate; pc, parvocellular); Ce, Central Nucleus (l,

lateral; m, medial; c, capsular); I, Intercalated Nuclei; L, Lateral Nucleus; ec, External Capsule; lab, Lateral Association Bundle; st, Stria Terminalis;

Figure 5.4 CS Response Plasticity. Peri event time histograms (PETH's) are displayed for the 3 cells that showed statistically significant increases in CS response during conditioning. Notice the increase in the peak after CS onset (time 0) in conditioning trials 4-8 (c 4-8) when compared to the last for trials of habituation (h 4-8). Each PETH plots data from 4 trials of the number of firings in 10 ms intervals with respect to the CS onset. The PETH's are labeled with an abbreviation to indicate the phase of the experiment (h, habituation; c, conditioning; e, extinction) and a number range indicating the trails of the experimental phase included in the block. The x-axis of the PETH provides a time scale in seconds for the raster plot, where 0 represents CS onset. The y-axis of the PETH represents the number of cell firings in a bin. All three cells show increases in post-CS-onset bins, indicating they are tone responsive. In addition, the high bin count regions show further increases over the course of conditioning. The changes in CS responses are statistically significant (method discussed in the paper text).

Figure 5.5 Other Increases in CS Response. PETH's of 2 cluster recordings. (a) A fourth cell that showed plasticity, but met criteria for statistically significant change only in extinction (b) A multi-unit cluster that showed a CS response, with statistically significant plasticity. Both showed statistically significant CS responses (as defined in the

paper text). Pronounced increases in tone response are visible. For other details, see Figure 5.4 caption.

Figure 5.6 CS Onset latency shift. Raster plot and PETH for two cells that showed pronounced shift in onset latency. For each CS presentation throughout the experiment, 250 ms of unit activity is shown in the raster plot. The raster plot shows 52 rows, one for each trial: 8 Habituation, 16 Conditioning, 20 Extinction, and 8 Reinstatement trials. Each mark indicates the time of one action potential. The time scale is identical to the scale from the PETH below the raster plot. The time interval includes 100 ms prior to CS onset and 150 ms after CS onset. The PETH bins are 1 ms intervals. The first white-noise pip of the CS is present from 0 to 50 ms of each trial. The 4 filled triangles in the raster plot indicate the first 4 trials of conditioning. The open squares indicate the first 4 trials of extinction. The filled circles indicate the first 4 trials of reinstatement. Note the decreased latencies in each cell begin by the third or fourth trial of conditioning and maintain through the first several trials of conditioning. For other details see Figure 5.4 caption.

Figure 5.7 Transient and sustained enhanced CS responses. (a) Graph of ratio values of mean CS response (see text for details) in the last 8 trials of conditioning divided by the mean CS response in the first 8 trials of conditioning. Data is taken from the conditioned cells from the paired group (n = 3 cells). (b) Mean (± standard error) CS-

response for transiently plastic (n = 1) and long-term plastic cells (n = 2) throughout the four phases of the experiment. Data points are four trial blocks averaged together, except for the first point, which is the average of the final six trials of habituation.

Figure 5.8 Enhanced response to first pip of CS. Raster plot and PETH for three cells of the 30 seconds of recorded cell firing of each of 52 CS presentations in the experiment. The PETH bins are 20 ms intervals. In all three cells a pronounced response to the first pip is noticeably larger than responses to subsequent pips in the CS. There are no data for the 0.5 seconds of US presentation in the 16 conditioning trials (evident as a blank rectangle). For other details see Figure 5.6 caption.

Figure 5.9 Decreased firing to the CS. Examples of decreased cell firing in response to CS presentations on two time scales. (a) PETH of a 250 ms interval of one cell's firing for all pips in the experiment. Bins are 1 ms. (b) For two cells, PETH's of the 30s intervals during various phases of the experiment (phase is labeled in the figure). Each PETH represents pooled cell firing for 8 trial blocks, except "late extinction" which is a 4 trial block. Bins are 20 ms. The cell on the right is the same cell as presented in (a). See Figure 5.4 caption for other details.

Figure 5.10 US Response. Raster plot and PETH of one cell that showed no CS response, but some indication of a US response. The significant region of the raster plot is the early conditioning trials just after the 21 second mark, in which a relatively high

concentration of spikes is evident. PETH bins are 20 ms. There are similar US responses in the 3 cells in Figure 5.8. For other details see Figure 5.6 caption.

FIGURES

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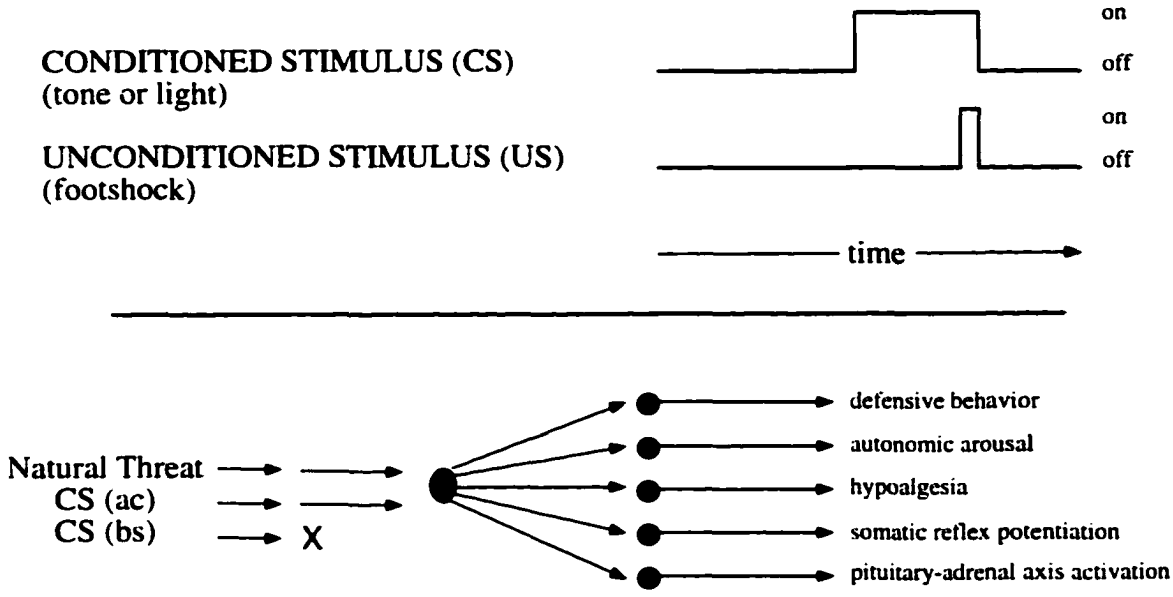
FIGURE 2.1



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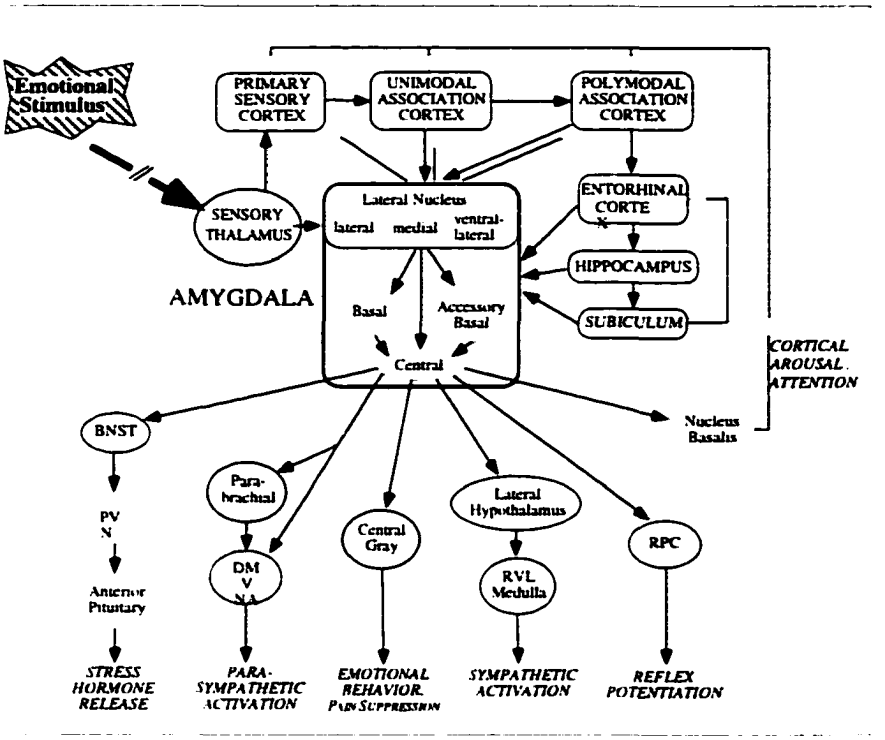

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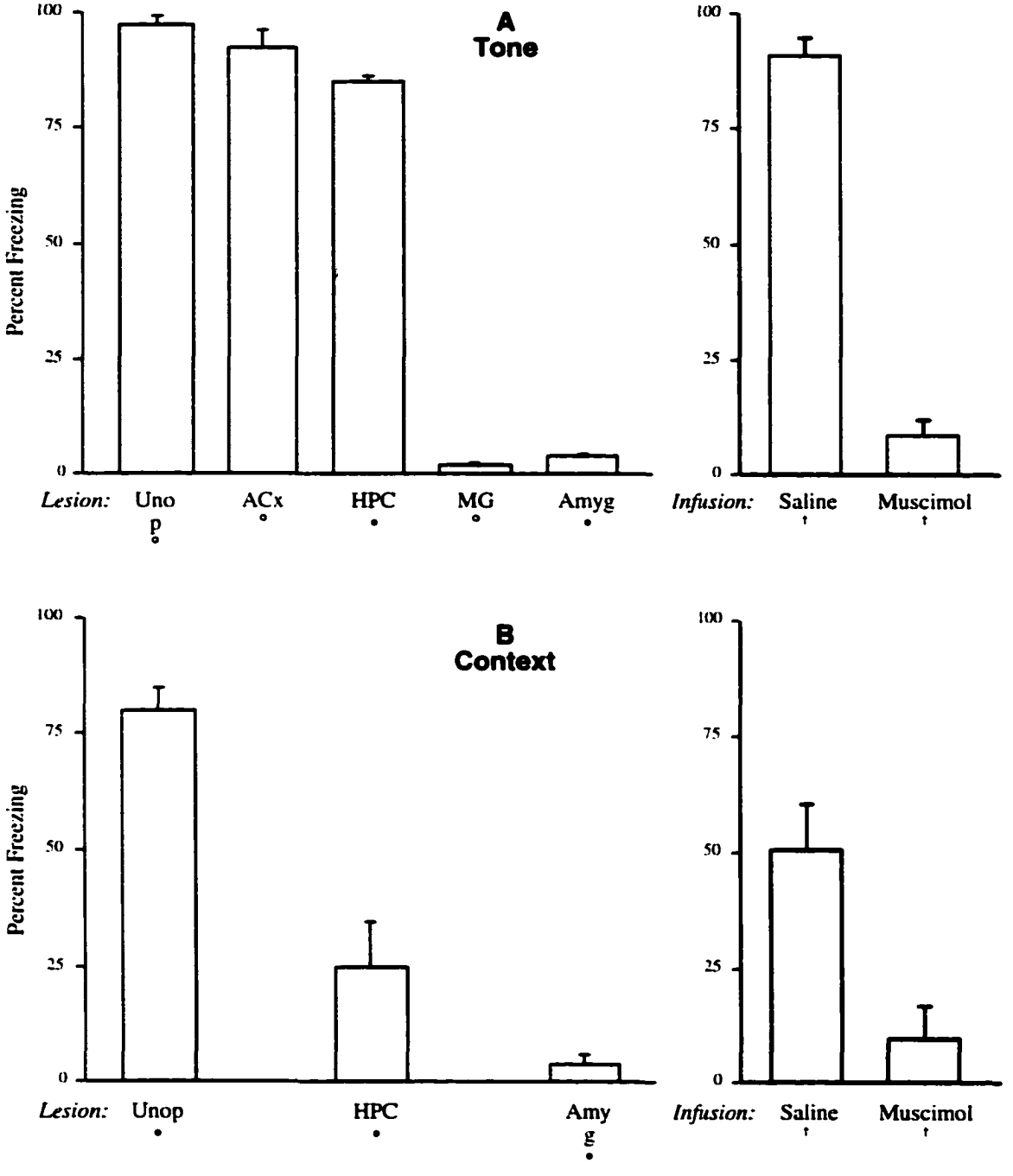
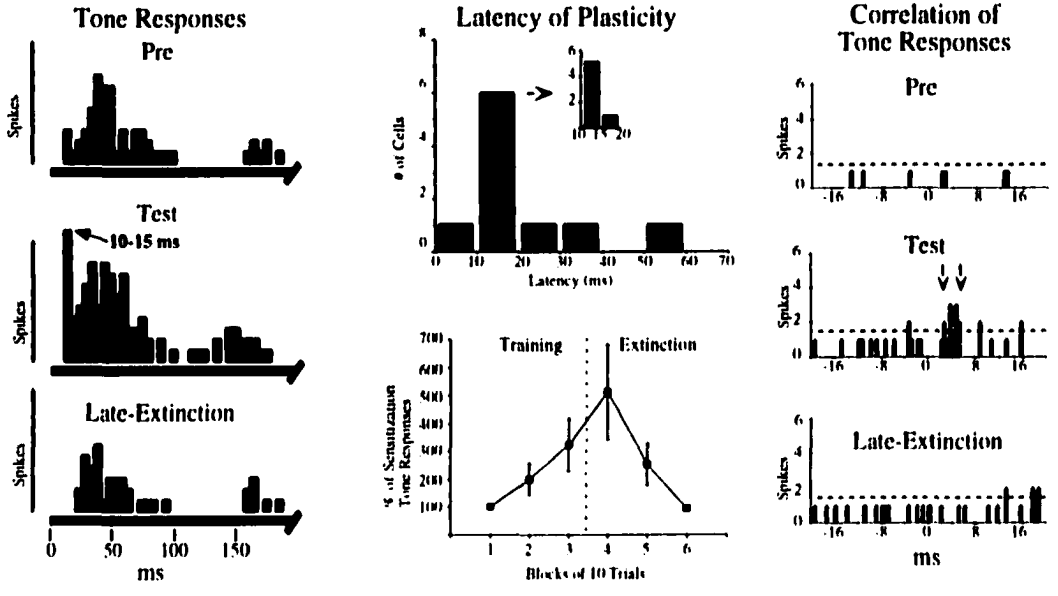


Sources: ° Romanski & LeDoux, 1992 • Phillips & LeDoux, 1992 † Muller et al., 1997

FIGURE 2.4



(Quirk et al., 1995)

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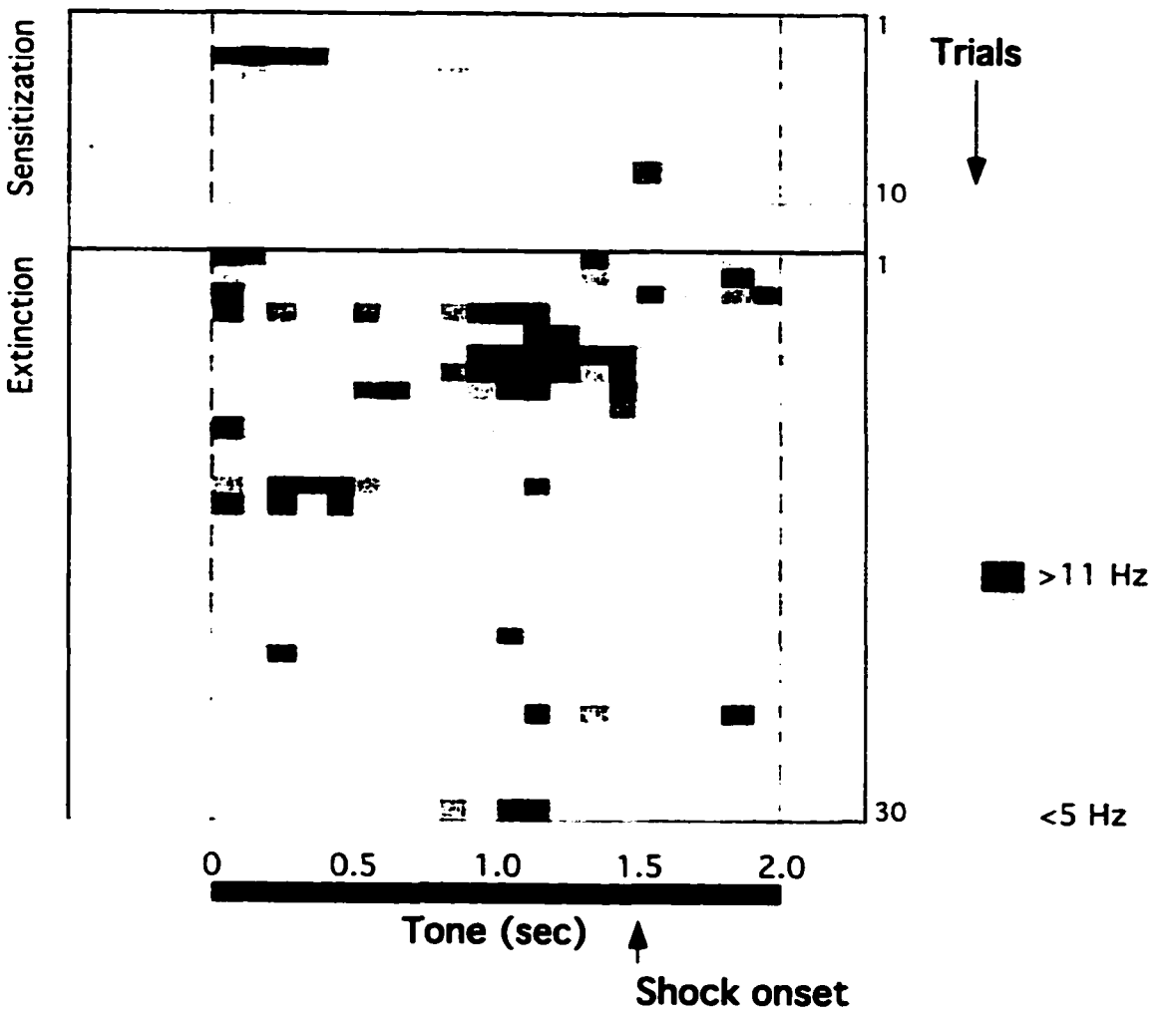

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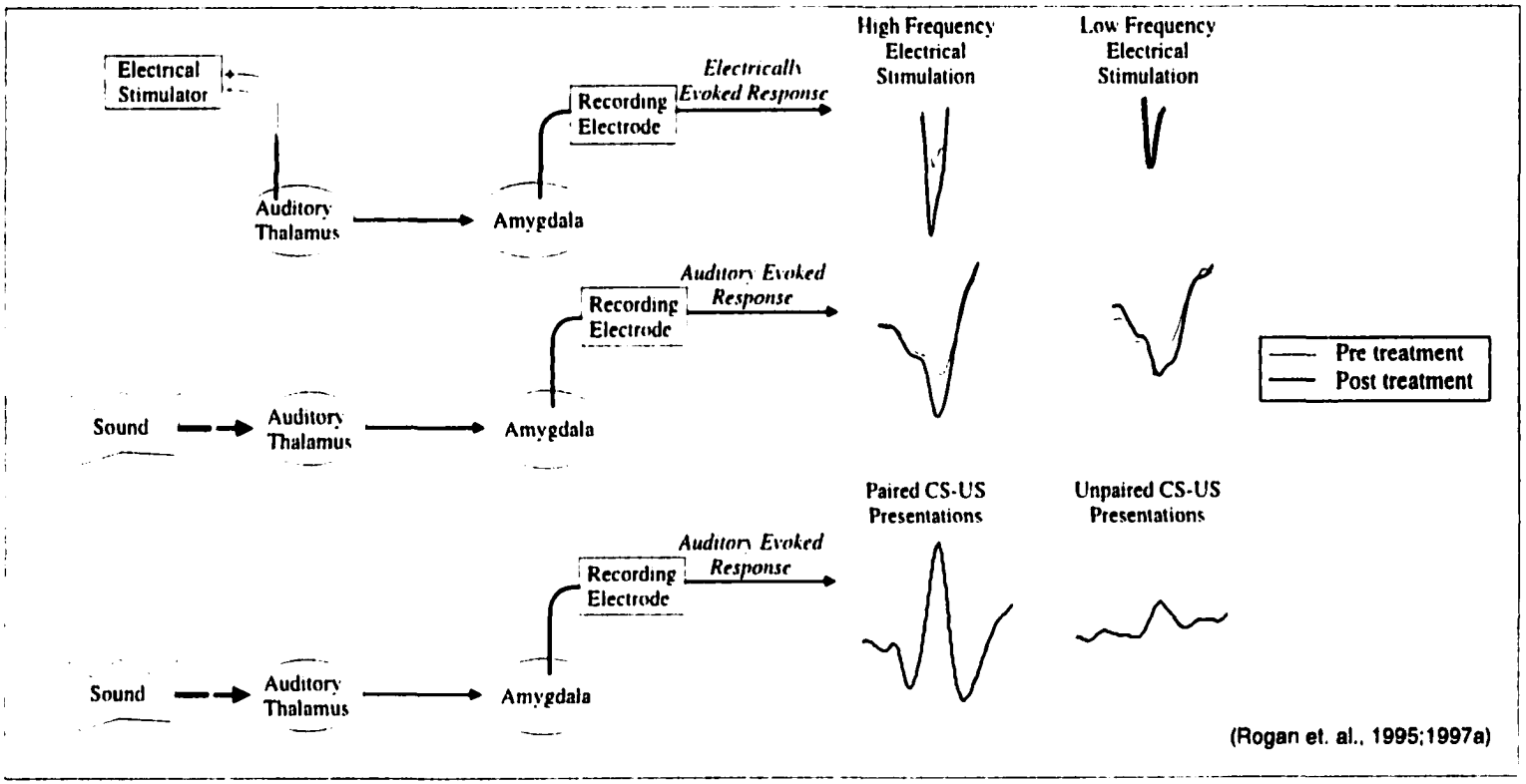

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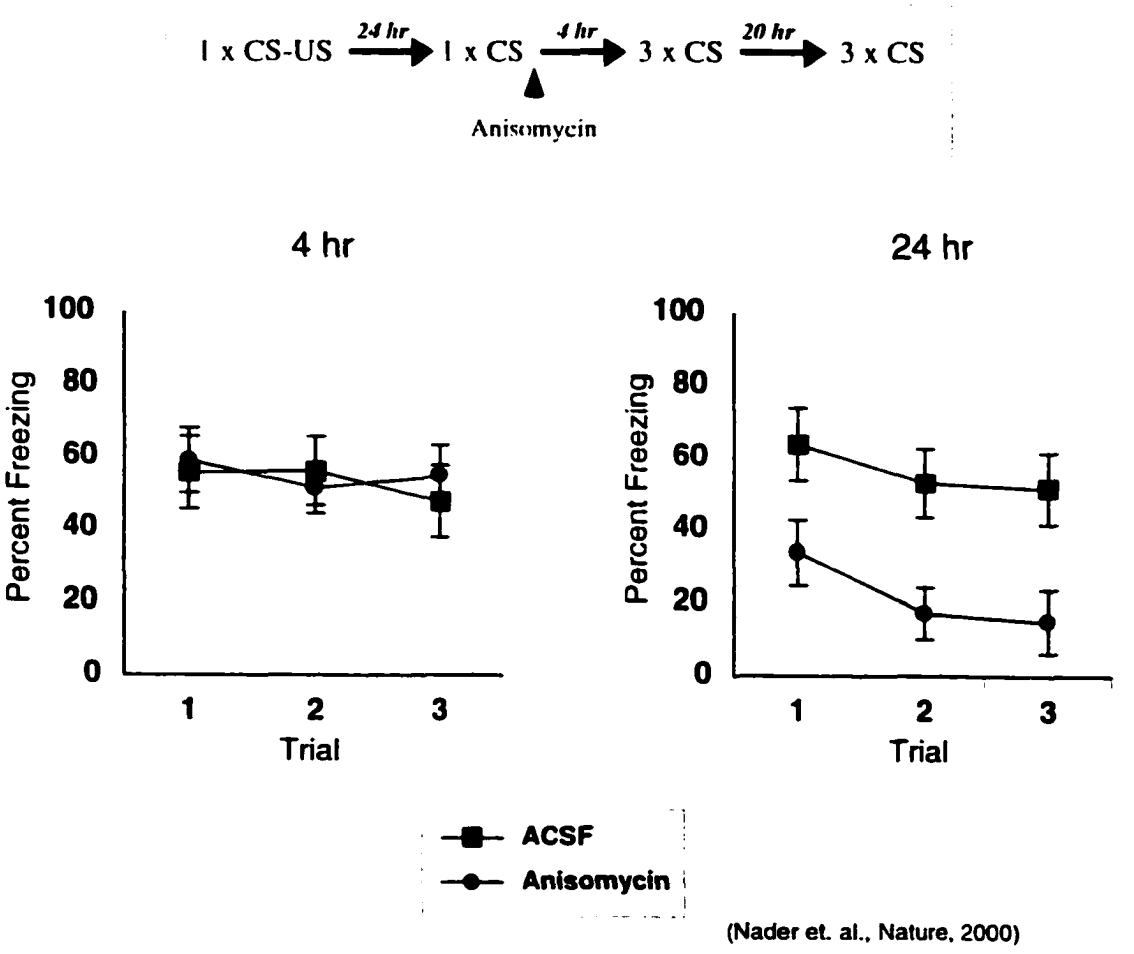

(Nader et. al., Nature, 2000)

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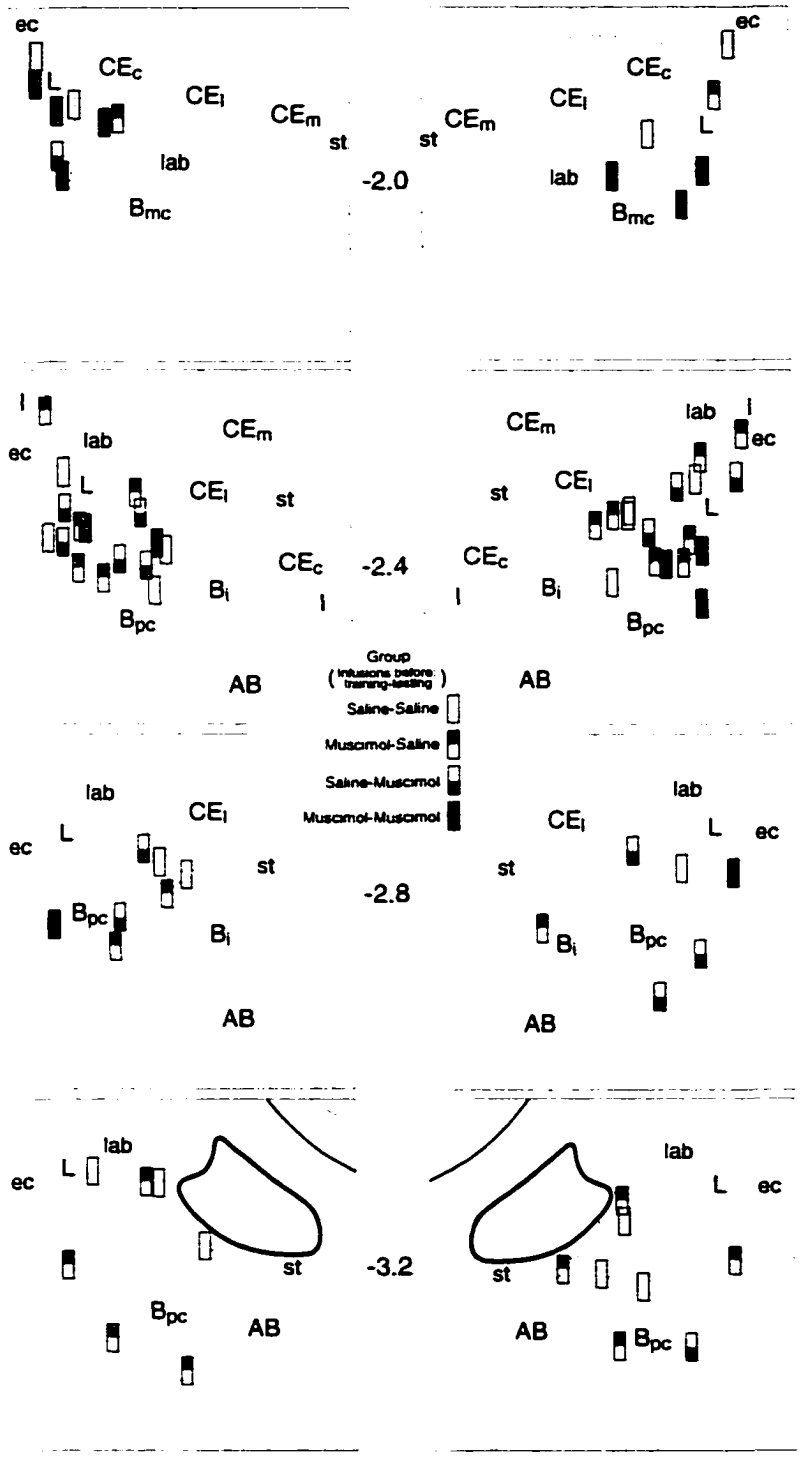

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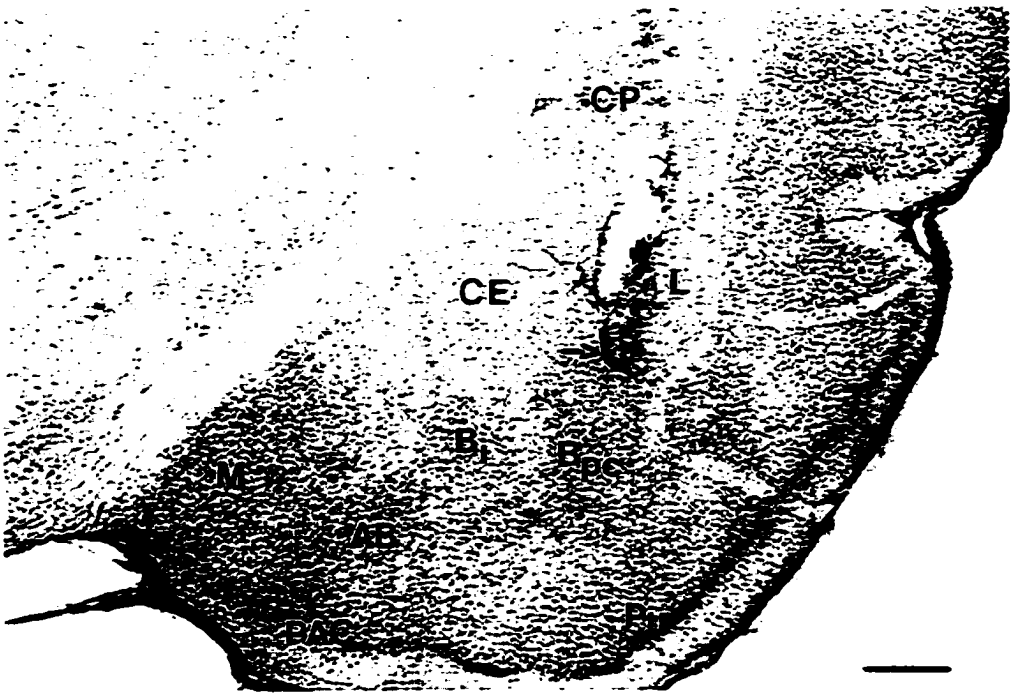

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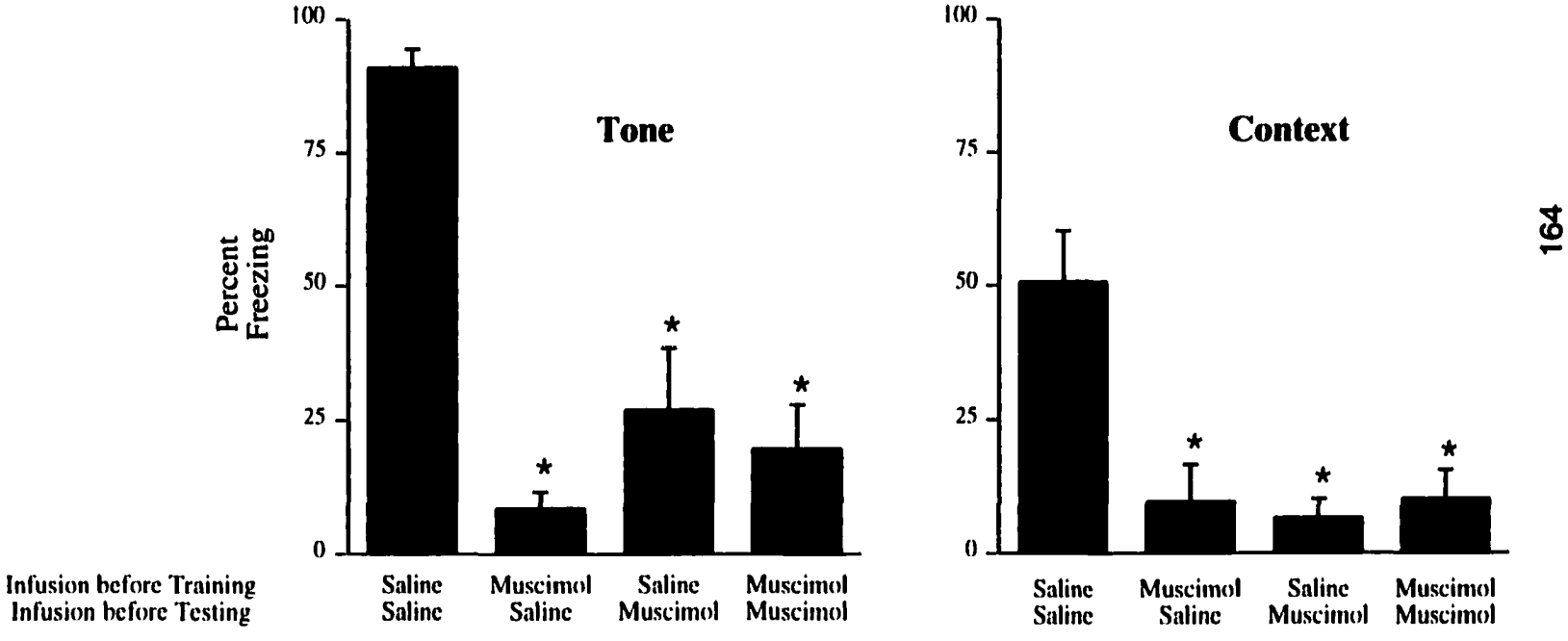

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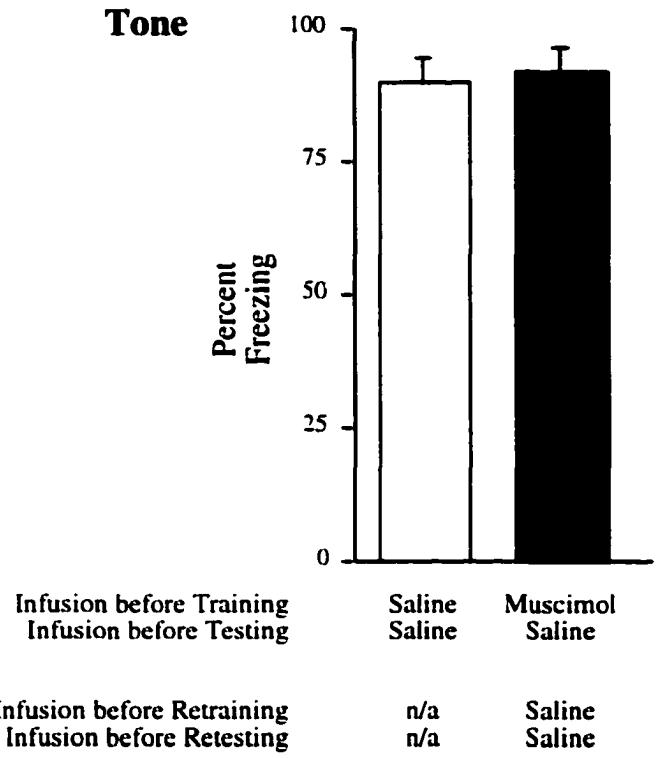

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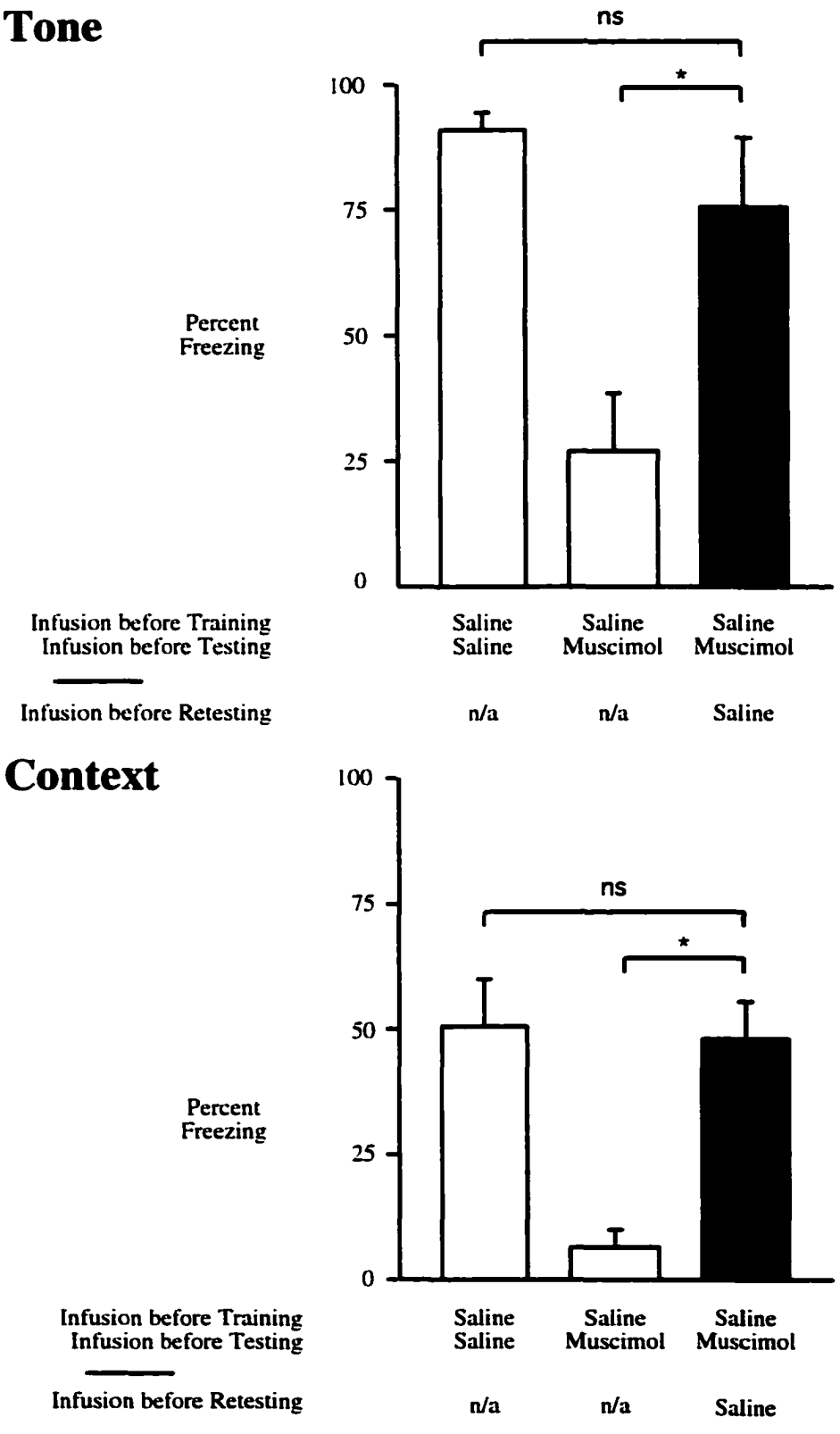

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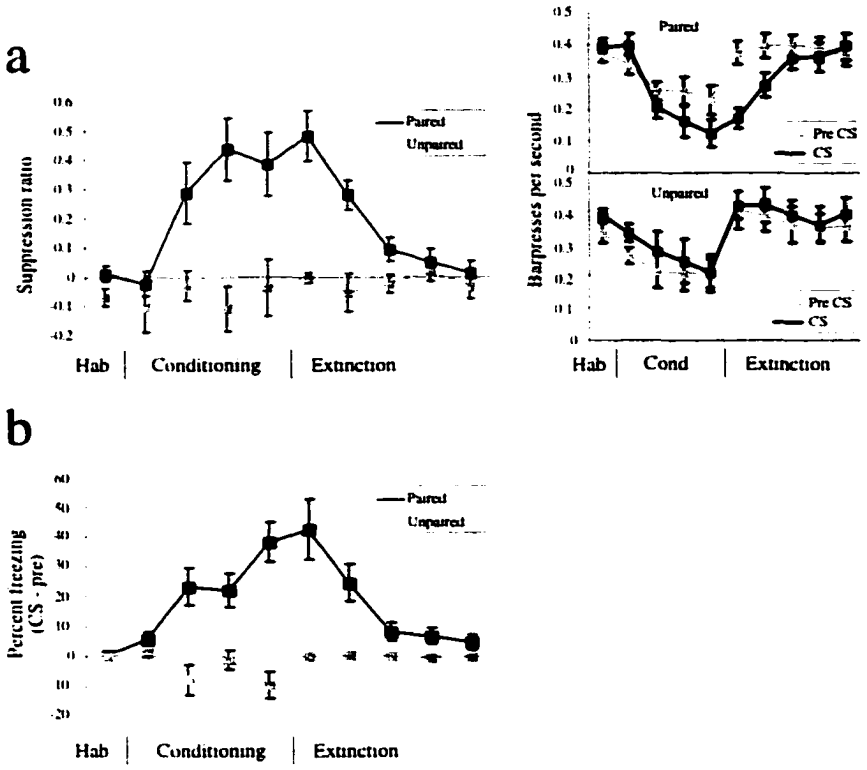

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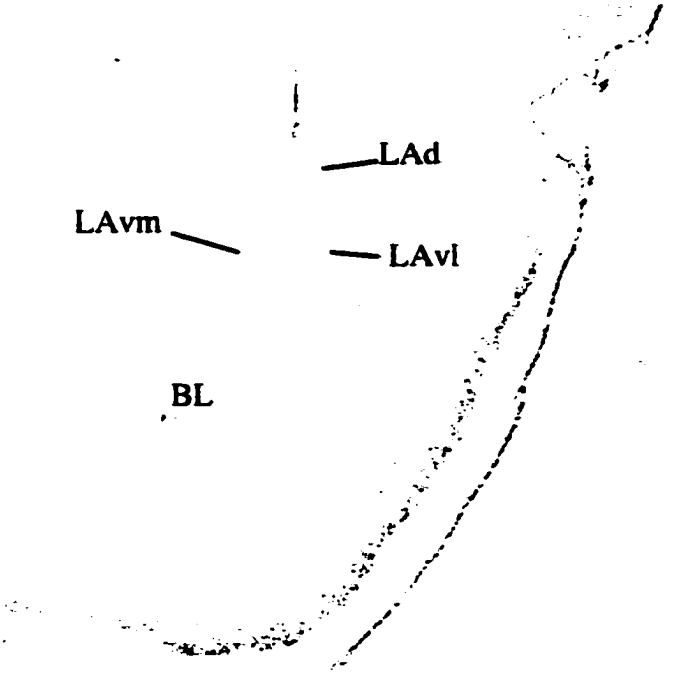

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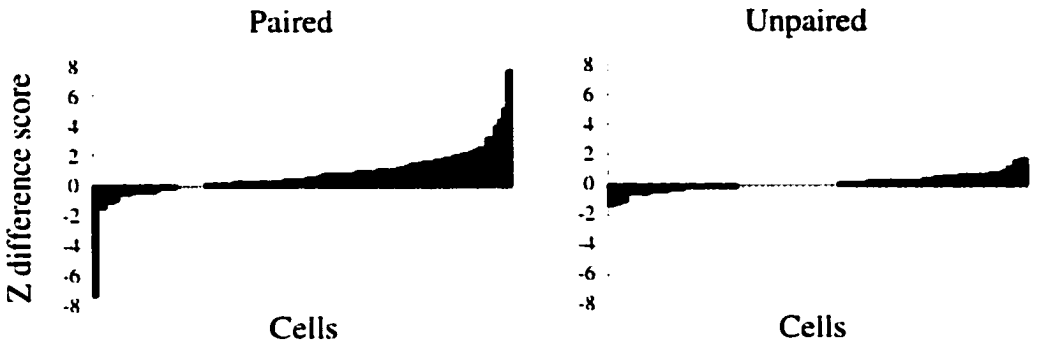

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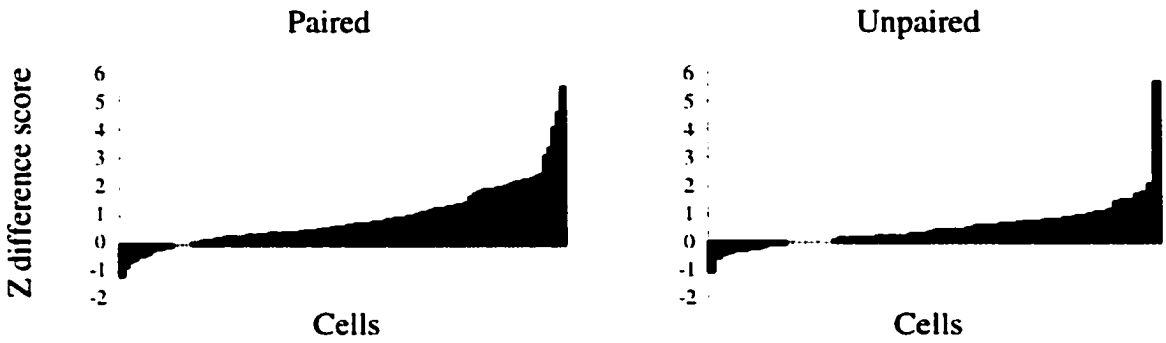

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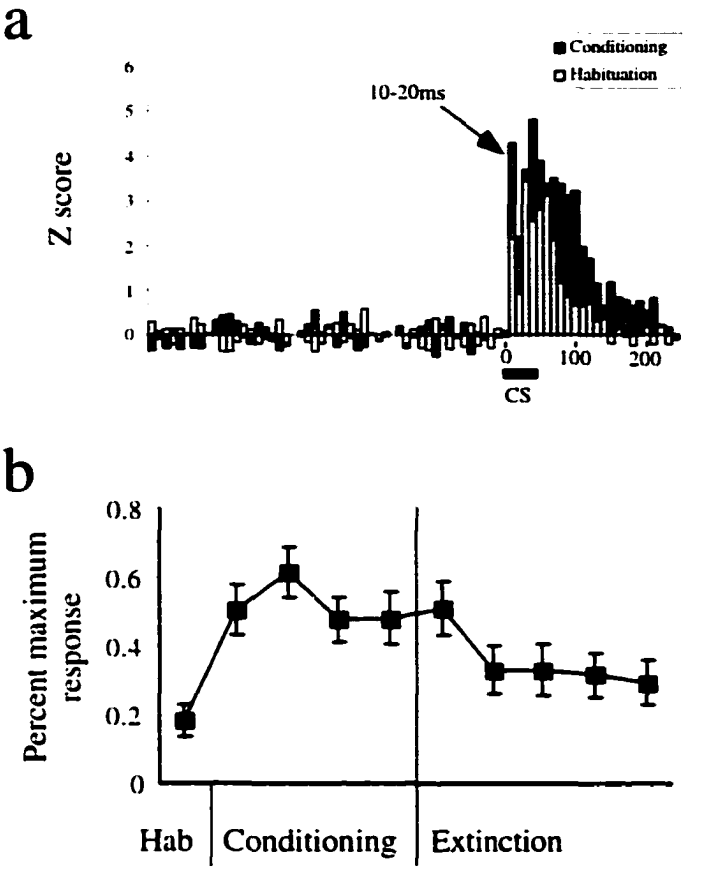

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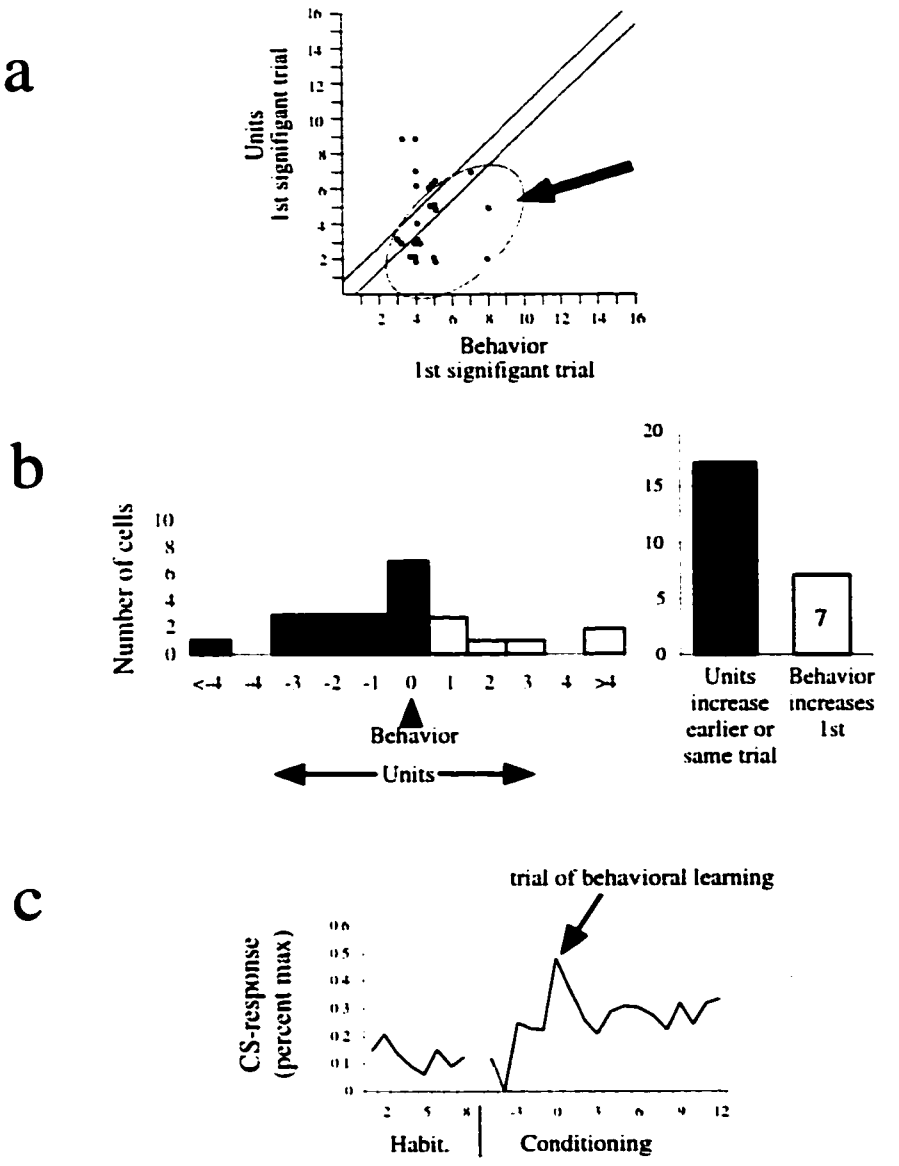

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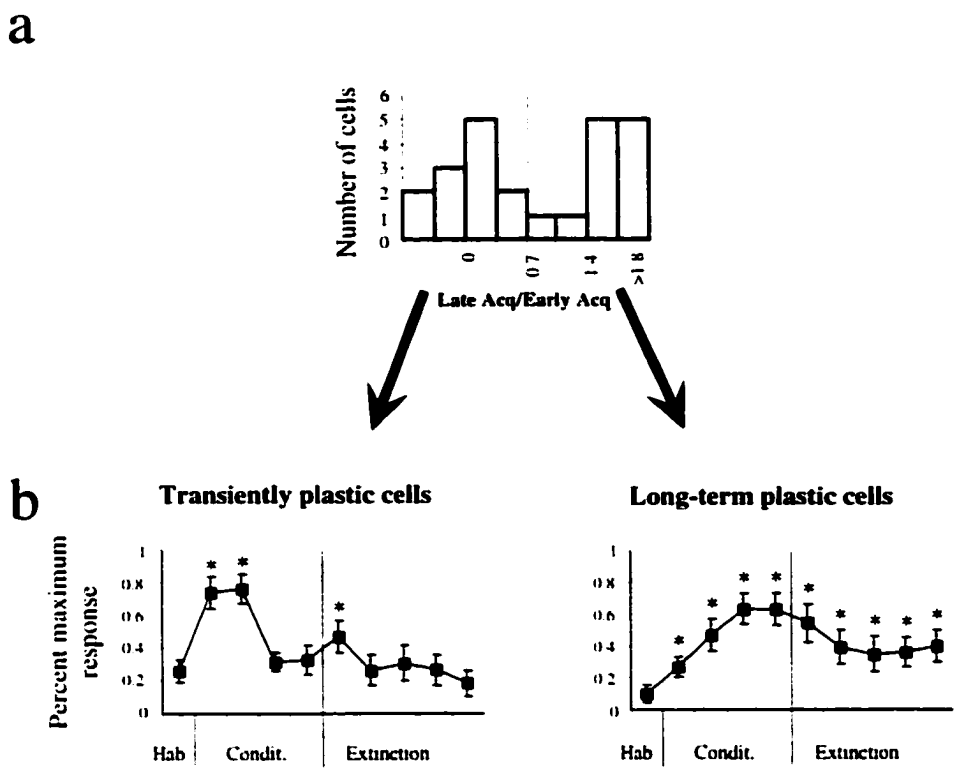

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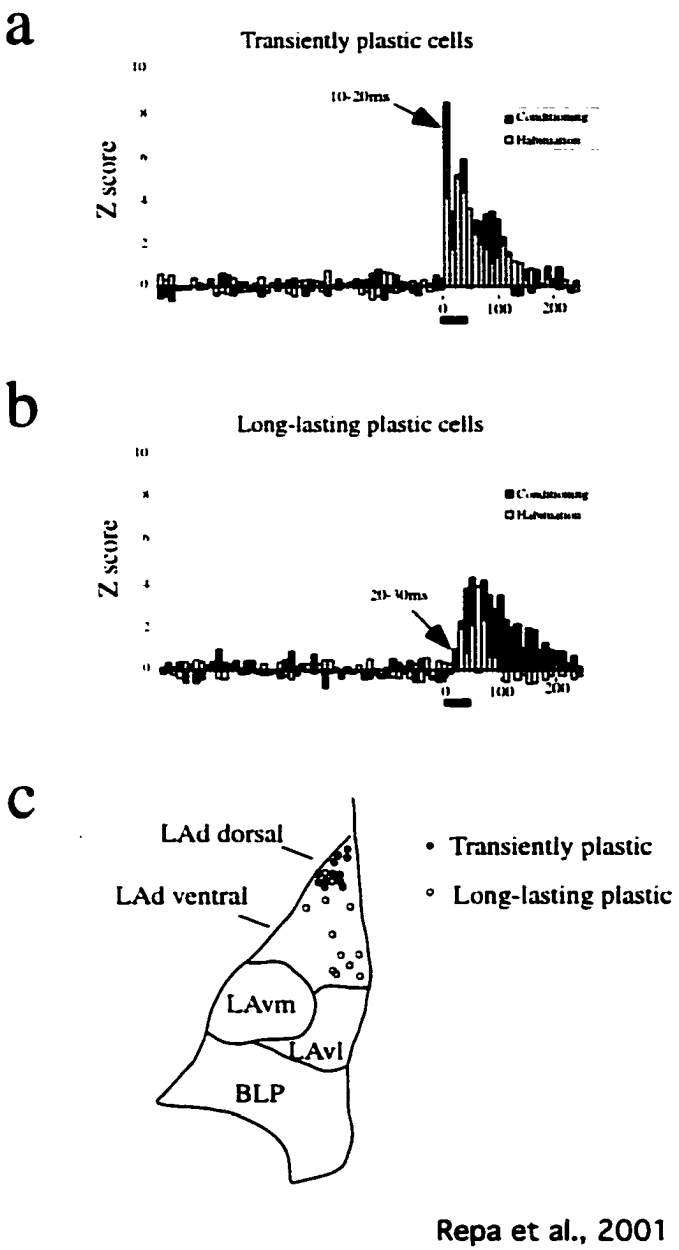

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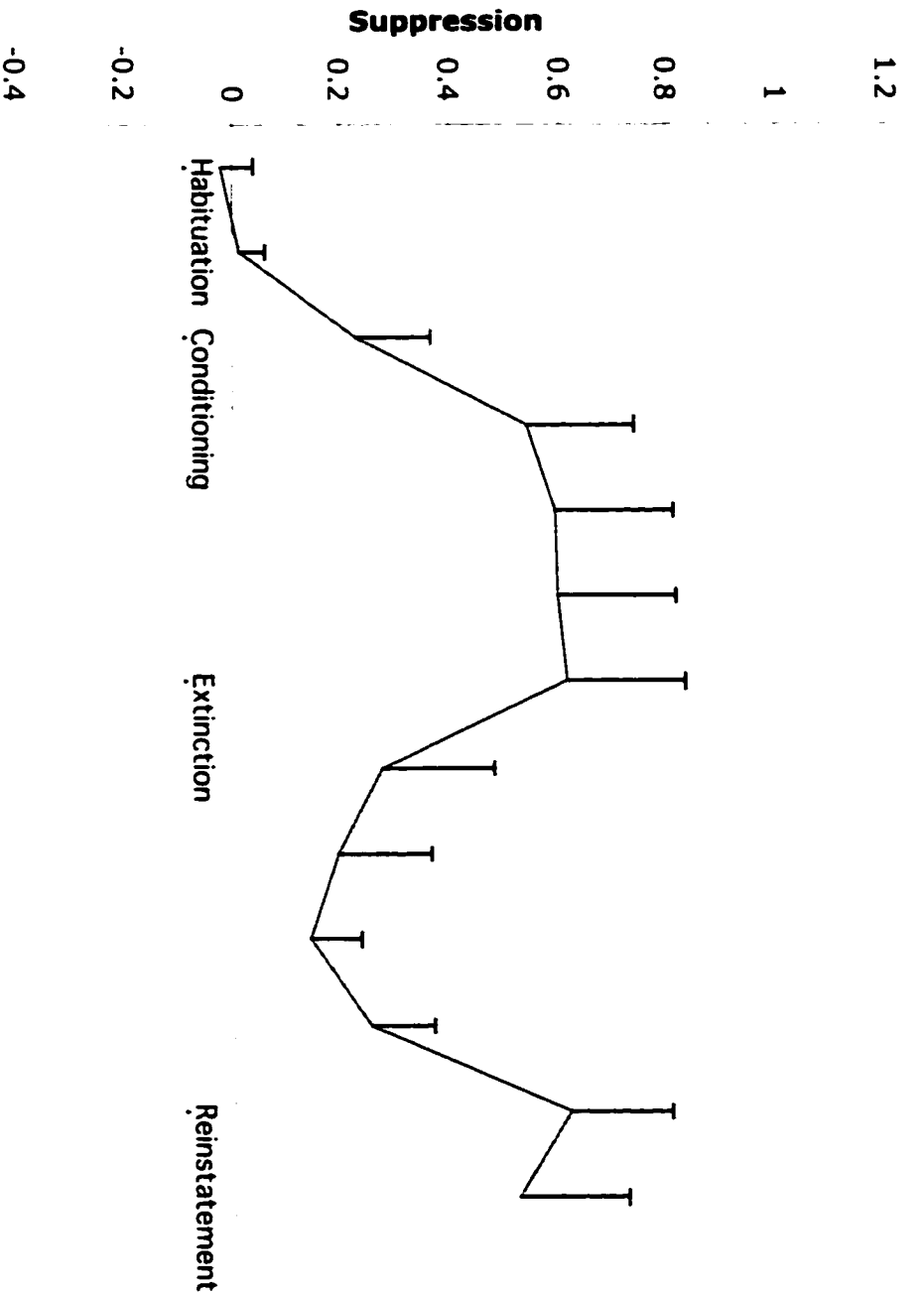

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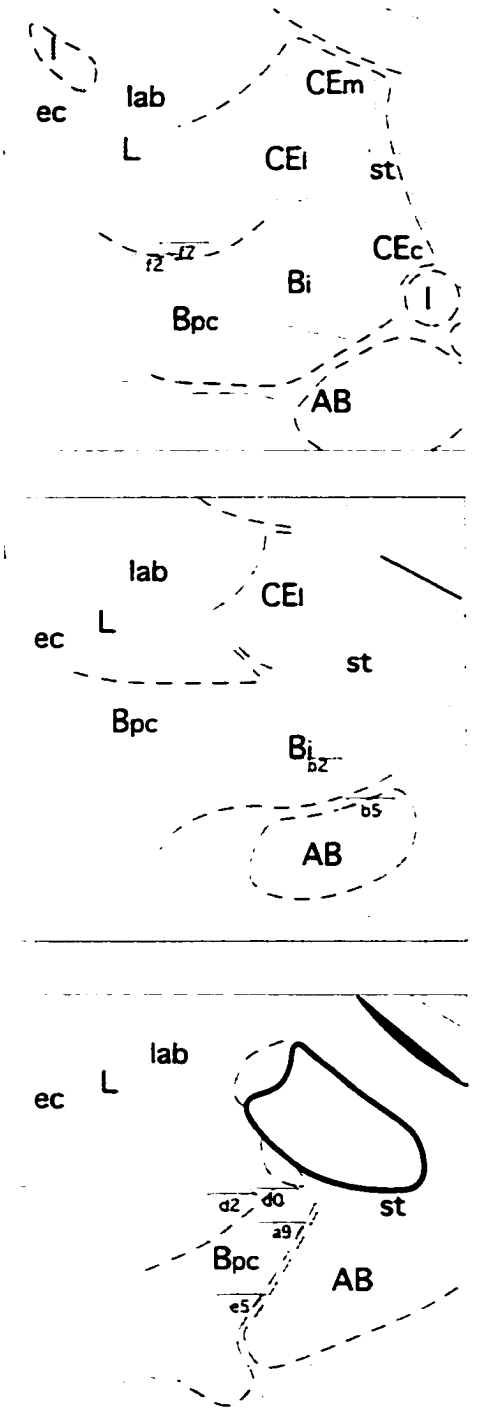

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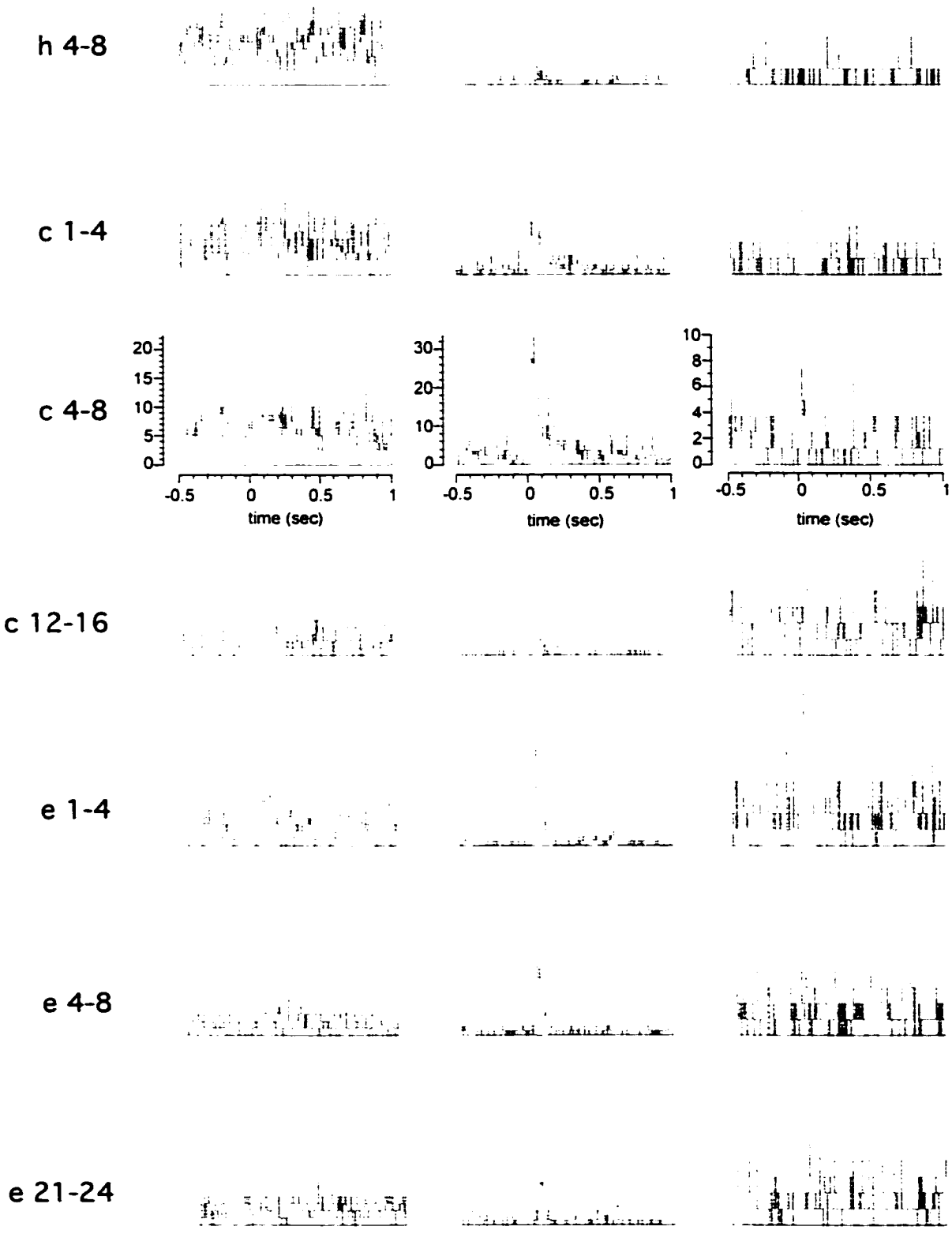

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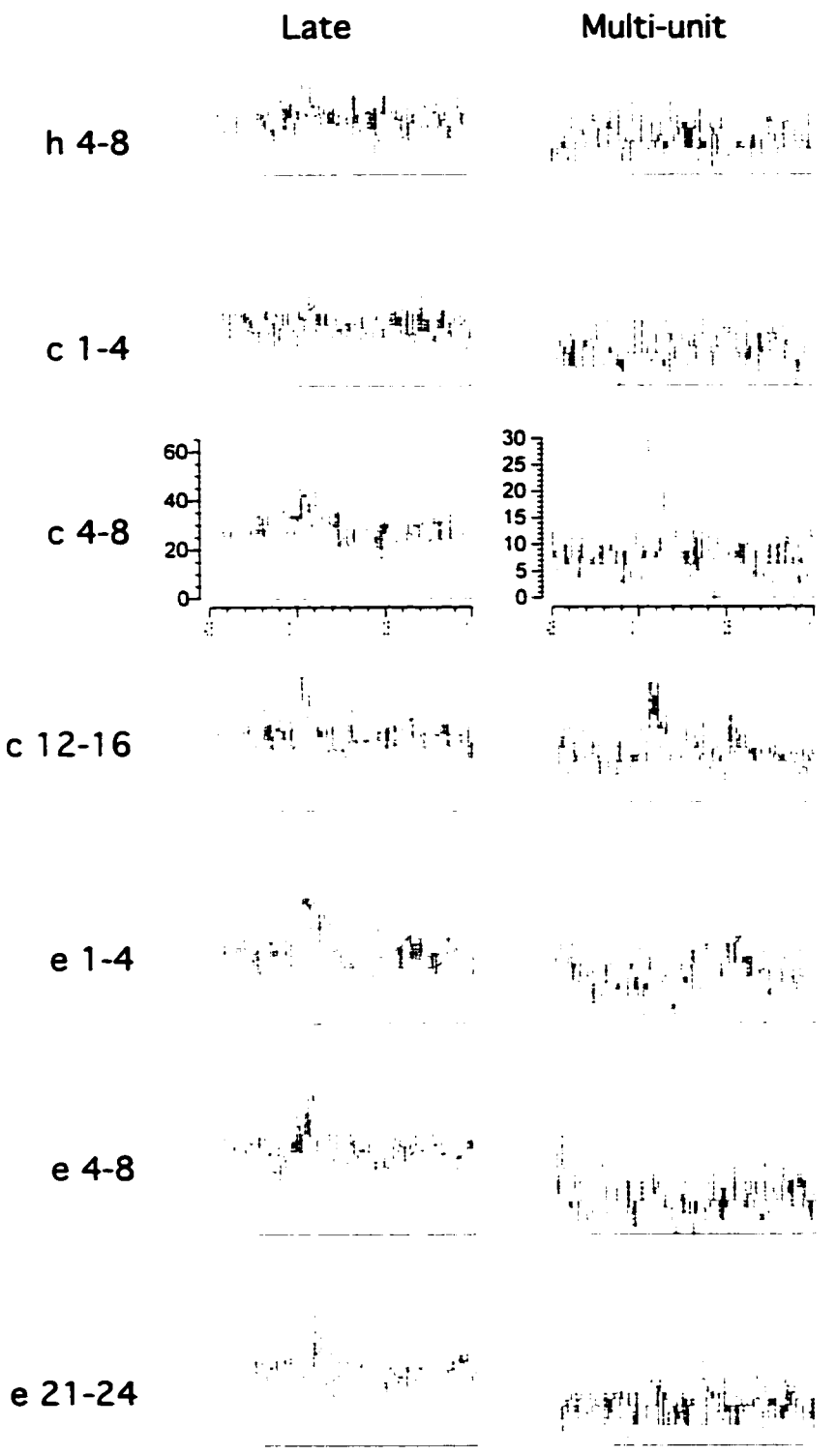


FIGURE 5.6

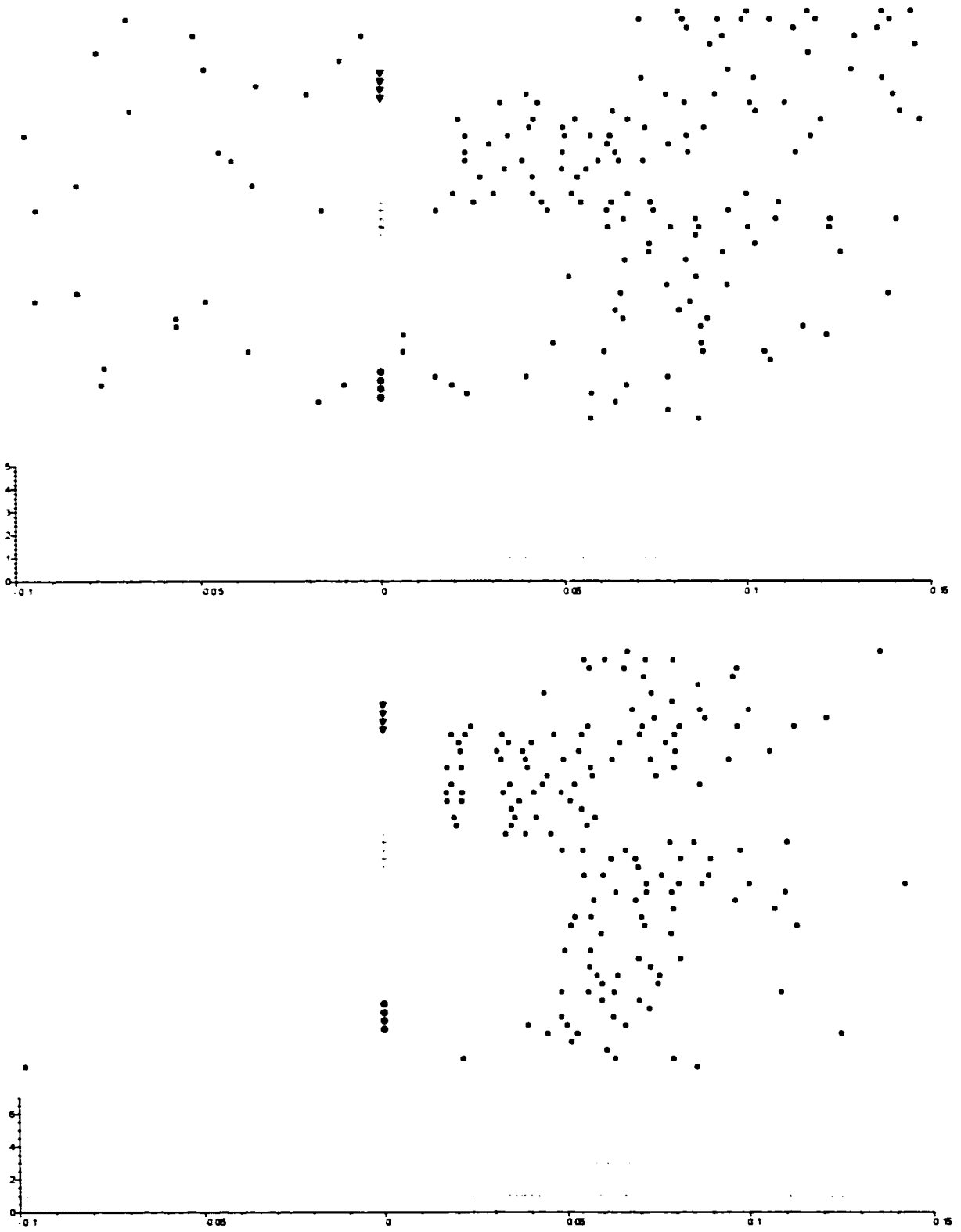
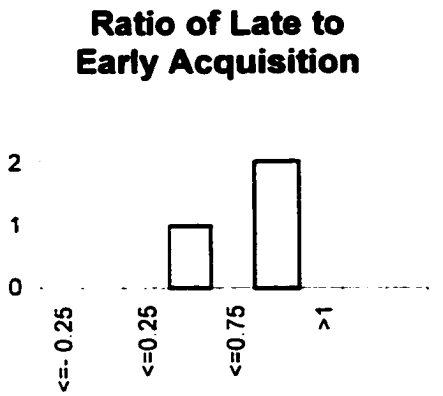


FIGURE 5.7

a



b

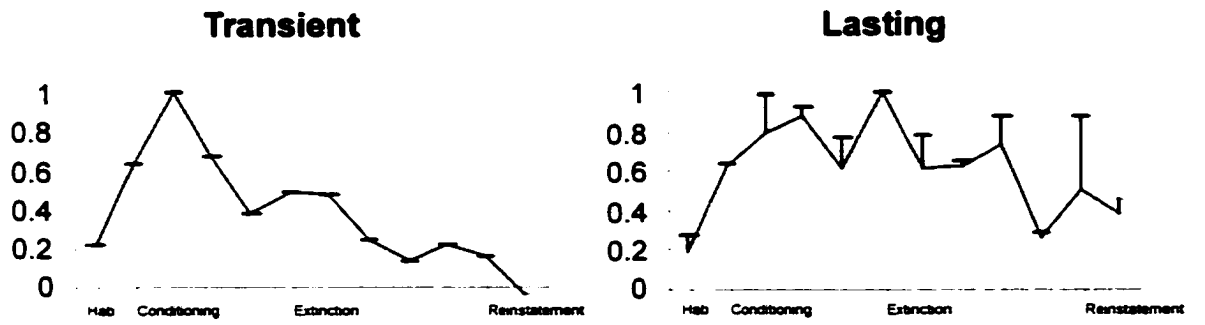


Figure 5.8

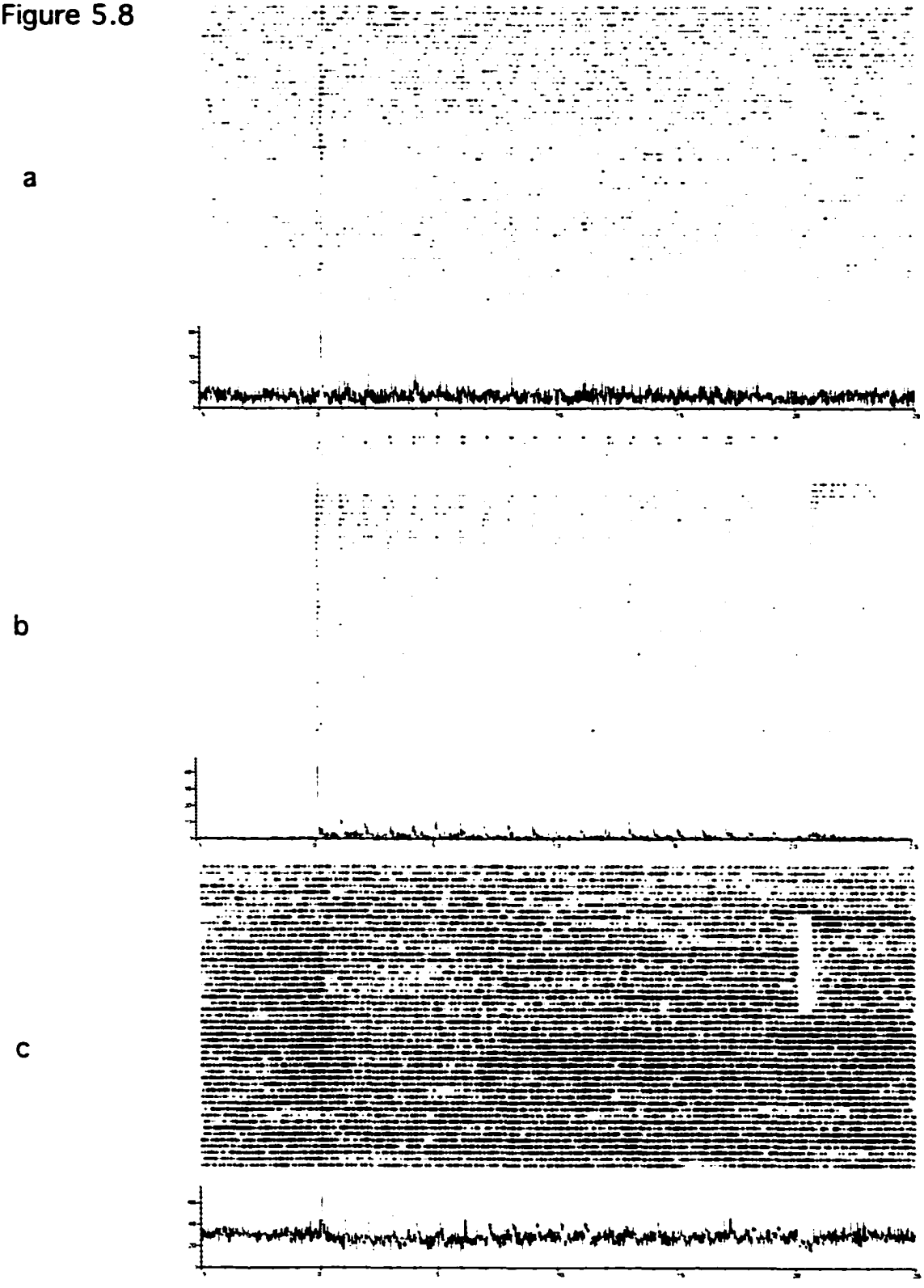
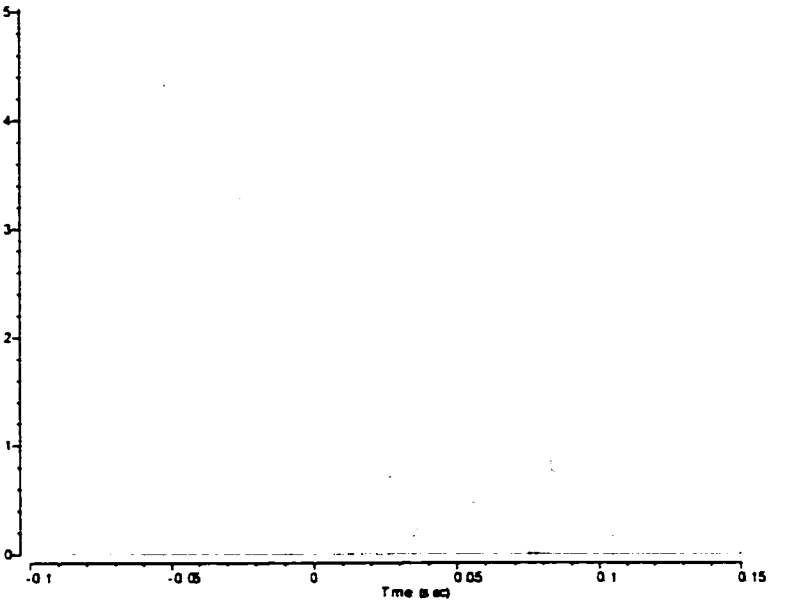


FIGURE 5.9

a



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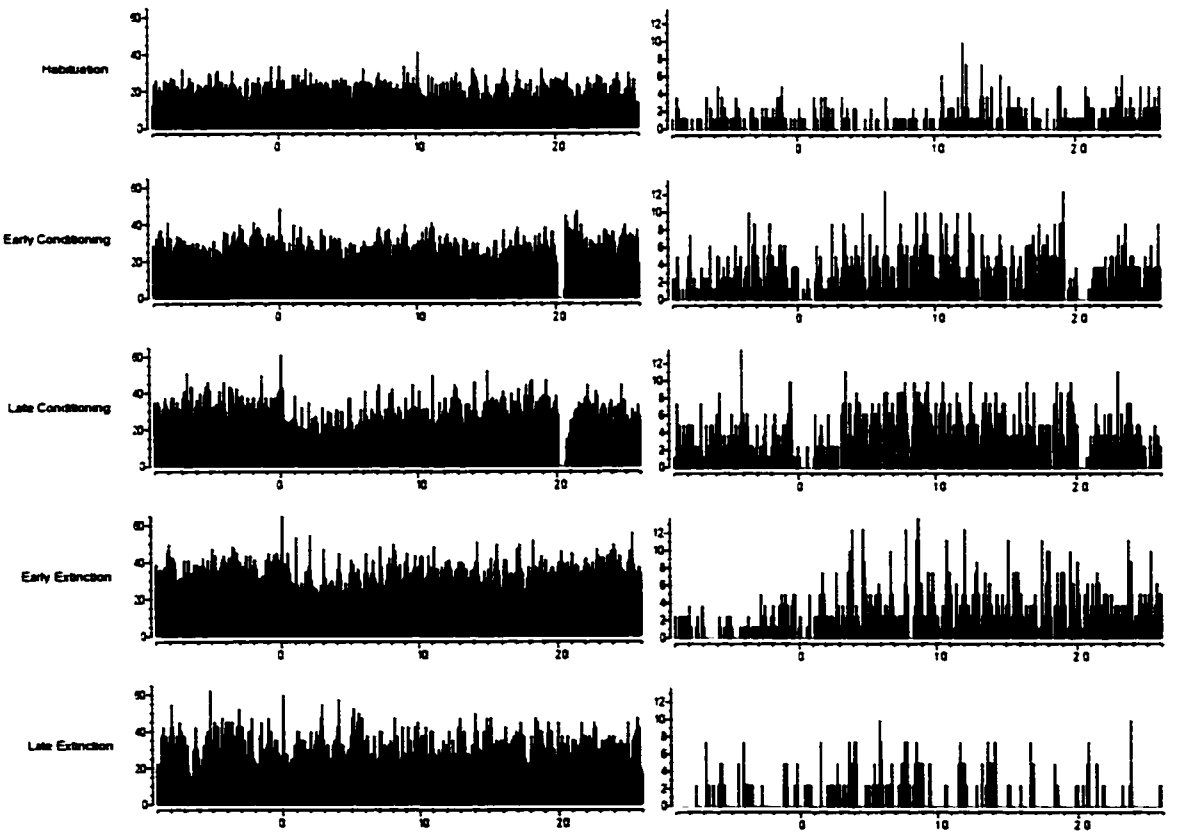
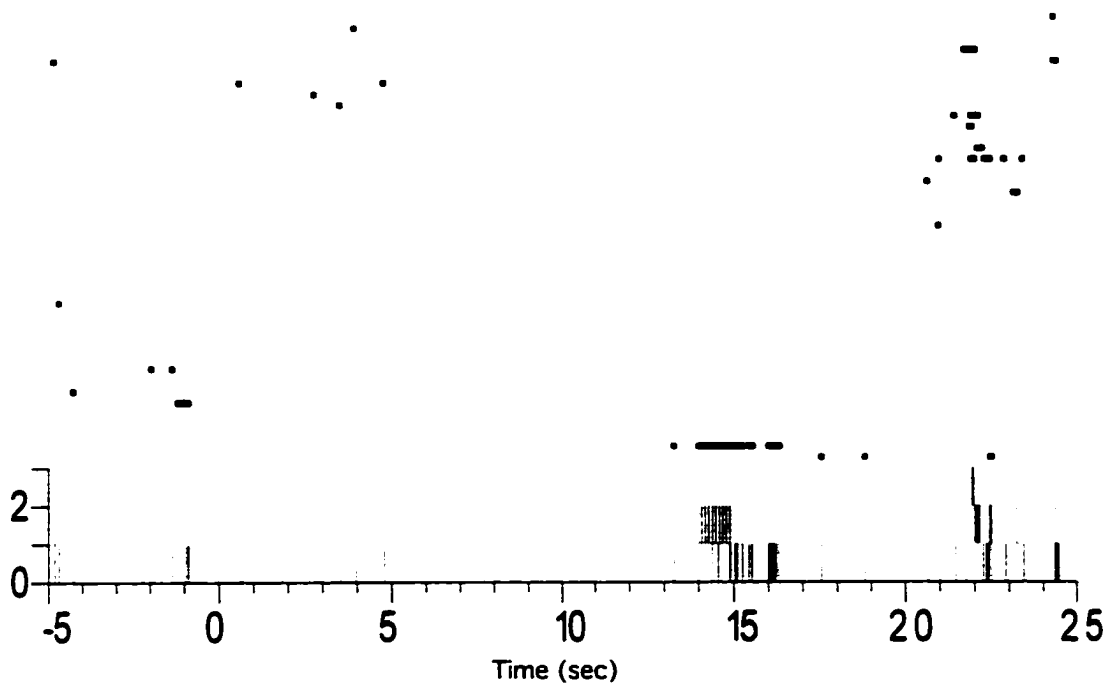


FIGURE 5.10



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