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Amygdala processing of the formation and retrieval of cue-reward associations

by

Kay M. Tye

DISSERTATION

Submitted in partial satisfaction of the requirements for the degree of

DOCTOR OF PHILOSOPHY

in

Neuroscience

in the

GRADUATE DIVISION

of the

UNIVERSITY OF CALIFORNIA, SAN FRANCISCO

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by

Kay M. Tye

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This thesis is dedicated to my parents for their unconditional love and their undoubting faith in me. Both of them came to this country with nothing except their hope for a better life, and both of them have built successful careers in science that they are passionately in love with while raising two rambunctious daughters. I want to thank my father, S. Henry Tye, the most humble genius that I have ever met, for teaching me how to enjoy life. My father is a truly great man who walks softly but changes our view of the universe and is a constant inspiration for me to be a better person. I want to thank my mother, Bik K. Tye, who has never let anyone tell her that something couldn't be done, and has taught me to do the same. My mother is the picture of intelligence, resilience and courage – she always says exactly what she thinks, and knows when I need comfort and when I need to stop whining and start working harder. Words cannot express how grateful I am to them for all that they have made possible for me, and I hope to make them proud. I also want to thank my sister, Lynne D. Tye, who is and always will be a part of me.

This thesis reflects the patience, wisdom and mentoring ability of my advisor and mentor, Patricia H. Janak. I am forever indebted to Tricia for taking me in when I was a discouraged and lost rotation student on the verge of dropping out of graduate school, and reviving my belief in myself as well as my love for science. In a field where you are measured by your achievements, Tricia is an oasis of unconditional compassion and warmth. I have never met a person as kind and thoughtful as Tricia, and without her unwavering support and encouragement, I doubt I would have survived graduate school. Tricia as an advisor is everything I could ever have hoped for: she is an exceptional

scientist, a caring mentor, and a beautiful person. Few advisors would have given me the amount of support that I needed, especially in the beginning, and fewer still would have supported me even when I was chasing down my tangential ideas using techniques outside her expertise. Instead of reigning me in, she expanded her expertise so she could help me crystallize my nebulous vision. In the years that I have been in her lab, I have discovered what research really is, found myself as a scientist and developed a picture of who I hope to be someday. Until I knew Tricia, I did not think it was humanly possible to be a constant source of positive energy, but whether we are celebrating a great success or weathering a complete failure, I always feel better after talking to her. Thank you so much for everything you've done for me, I don't know where I would be without you.

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Appreciation, gratitude and admiration are words too weak to describe how I feel about my unsung mentor and role model, Garret D. Stuber. Without any obligation to me whatsoever, he took me under his wing and guided me along every step and stumble of

my journey throughout the past two years. Garret is not only a talented scientist, a creative thinker, and a technically gifted experimentalist, but also a phenomenal teacher, caring mentor and fantastic friend. Being given a seat next to Garret was one of the luckiest things to happen to me, because his enthusiasm and passion for science are contagious and because without his day to day guidance I would have been completely lost. Conversations with Garret infuse all participants with excitement and hope for future projects and insight towards current projects. I feel very lucky to have Garret as such a big part of my life, as he has been a powerful influence on my growth as a scientist, as a creative thinker, and as a person. I hope that someday I will be able to repay him for everything that he has done for me.

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esteemed colleague, and whose opinion I greatly value. Finally, I thank Jim Wagner who has been my strongest supporter, my best friend and my perfect match.

ABSTRACT

This thesis examines the neural changes that contribute to the formation, storage, retrieval and extinction of a learned association between a stimulus and a reward. A number of questions were answered in this thesis to provide insight upon the neural substrates of several goal-directed behaviors: What neural changes mediate the initial formation of an associative memory between a stimulus and a reward? What are the synaptic changes that correspond to the development of a change in task-relevant neuronal firing? What is the mechanism of these synaptic changes, and do they have a causal relationship? How are complex emotions such as frustration represented in the brain? How are reward-associated cues endowed with the power to guide goal-directed behaviors in the absence of primary rewards? Here I show that behavior improves with the rapid recruitment of amygdala neurons to the ensemble encoding a reward-predictive cue, and that this change is mediated by the rapid strengthening of thalamic synapses onto amygdala neurons by a postsynaptic increase of AMPAR-mediated currents. These synaptic changes, in addition to the acquisition of the task, depend on NMDAR activation. Amygdala neurons that store the memory of a reward are activated when an animal compares the expected reward with the unexpected omission of that reward. Finally, distinct populations of amygdala neurons reflect the motivating and reinforcing properties of a cue endowed with the emotional significance to guide behavior.

TABLE OF CONTENTS

Title page ……..……. i
Copyright page ……..……. ii
Acknowledgements ……..…….. iii
Abstract ……..…… viii
Table of Contents ……..……. ix
List of Tables ……..……... xi
List of Figures ……..…….. xii
Abbreviations ……..… xiii
Chapter 1: Introduction ……..……... 1
Overview……..……... 1
Emotion: the basis of motivated behavior ……..…… 4
Behavior: a window into learning and memory ……...…. 6
Quantifying behavior ……....….. 6
The neural basis of memory ……..…..… 7
Multiple memory systems ……..……..… 8
Amygdala anatomy ……..…….. 9
Amygdala circuitry ……..…..… 10
Neuronal composition of the amygdala ……..…..... 13
Implications of amygdala anatomy on amygdala function …..….. 13
Amygdala function: evolving emphasis ……. 14
Amygdala involvement in general affect ……...… 15
Early research of amygdala in instrumental conditioning ……..... 16
Amygdala as the ‘Fear Module’ ……..….. 17
LTP in the amygdala as a cellular basis of learning ……..…… 19
The role of the amygdala in goal-oriented behavior ……..…. 20
Goals of this dissertation ……..... 22
Summary ……..…….. 23
Chapter 2: Amygdala activity and synaptic strength increase with learning ….... 26
Abstract …….. 26
Introduction ……..…….. 27
Results ……..…….. 30
Discussion ……. 49
Methods ……..…… 54
Author contributions and acknowledgements ……..…….. 59

Chapter 3: Amygdala neurons encode motivation and reinforcement …….. 60
Abstract ……. 60
Introduction ……..…….. 61
Results ……...……. 63
Discussion …….. 77
Methods …….……. 84
Author contributions and acknowledgements ……...……..… 90
Chapter 4: Discussion ……...……. 91
Conclusions and significance of this dissertation …….. 91
Conclusions …… 92
Significance …….…... 92
Emotions and memories: influence of the amygdala ……..... 94
The emotional memory system ……...……..… 94
Competition among multiple memory systems ……..…. 95
Bottom-up processing versus top-down modulation …….. 96
The impact of emotions on declarative memories …….. 97
Arousal and emotion: which comes first? ……...… 99
The amygdala as the ‘Connectivity Hub’ ……...…. 100
Neural circuitry of emotions: Where do they diverge? ……..…. 101
Does the amygdala encode valence or intensity? …….. 101
Emotional processing: my personal sketch ……. 104
Motivational significance rather than hedonic valence …….. 106
References ……..….. 108
Library Release Statement …….. 150

LIST OF TABLES

Table 1 35

Table 2 35

Table 3 42

Table 4 70

Table 5 74

LIST OF FIGURES

Figure 1 – Emotion plotted by intensity and valence …….. 3
Figure 2 – Amygdala connectivity …….. 12
Figure 3 – Histology for Figure 4…….…….. 30
Figure 4 – Reward-related learning correlates with increases in cue-related firing 31
Figure 5 – Single unit example …….…….. 32
Figure 6 – Population histograms of excitatory and inhibitory neurons ……..….. 34
Figure 7 – Control experiment data summary ..…….. 36
Figure 8 – Control experiment histology …….…….. 37
Figure 9 – *Ex vivo* methodology ……...…….. 38
Figure 10 – Learner and Non-Learner categorization ……...…….. 39
Figure 11 – AMPAR/NMDAR enhancement predicts learning …….. 41
Figure 12 – mEPSCs and paired-pulse experiments……..…….. 43
Figure 13 – Histology for bilateral aCSF cannulae ….……..…….. 45
Figure 14 – Histology for bilateral AP5 cannulae …….....…. 45
Figure 15 – Histology for unilateral aCSF / AP5 cannulae…...…….. 45
Figure 16 – Histology for bilateral AP5 in CeN ….....…….. 45
Figure 17 – Synaptic changes and learning are NMDAR-dependent ..…….. 46
Figure 18 – CeN control for drug leakage .....…….. 47
Figure 19 – Reinstatement paradigm …….…….. 64
Figure 20 – Histology and waveform example …….. 65
Figure 21 – Behavioral comparison of Paired and Unpaired group ……..... 66
Figure 22 – Microanalysis of cue-guided behavior ……...…….. 67
Figure 23 – Example of a selectively cue-responsive neuron ……...….. 68
Figure 24 – Behavioral and neural changes from early to late reinstatement ...….. 69
Figure 25 – Neural responses to port entry or cue presentation …….. 70
Figure 26 – Examples of motivation and reinforcement encoding neurons ..…….. 72
Figure 27 – Sub-populations of cue-responsive neurons ……...…….. 73
Figure 28 – Firing rate frequency ……..…….. 75
Figure 29 – Putative interneurons do not show task-relevant responses ……..... 76

ABBREVIATIONS

aCSF (artificial cerebrospinal fluid)

AMPAR (α-amino-3-hydroxy-5-methyl-isoxazole propionic acid receptor)

ANOVA (analysis of variance)

AP5 (D(-)-2-Amino-5-phosphonopentanoic acid)

BLA (basolateral complex of the amygdala)

CeA (central nucleus of the amygdala)

CeN (central nucleus of the amygdala)

dB (decibels)

FR1 (Fixed-ratio 1)

GABA (γ-aminobutyric acid)

KHz (Kilahertz)

LA (lateral amygdala)

mV (millivolts)

NMDAR (N-methyl-D-aspartate receptor)

NP (nosepoke)

OFC (orbitofrontal cortex)

pA (picoamperes)

PE (port entry)

PET (positron emission tomography)

PFC (prefrontal cortex)

mPFC (medial prefrontal cortex)

RR2 (random ratio 2)

SEM (standard error of the mean)

CHAPTER 1

Introduction

OVERVIEW

The ability to learn and apply associations between environmental stimuli and the outcomes that they predict are essential for survival. However, the formation, consolidation, storage and retrieval of memories are, in many cases, widely distributed throughout a larger neural circuit, which may be difficult to isolate and study. One brain region that is of particular interest due to its multifunctional capabilities, is the amygdala which is known to be critically involved in the formation, storage and retrieval of emotional, or motivationally significant, associative memories. While it has been known for over a century that the amygdala is important for both positive and negative affect, a much greater emphasis has been placed on fear-related learning. Though extensive research regarding the cellular and neurophysiological bases of fear memories has been performed, relatively little is known about the neural activity involved in motivated and goal-oriented behavior.

By integrating techniques designed to quantify changes in behavior, neural activity and synaptic physiology, this thesis examines how reward-related emotional memories are encoded and translated into goal-oriented behaviors. The data presented here

demonstrate that amygdala neuron activity and synaptic strength predict the success of learning a reward-related task. Importantly, these data show that assigning motivational significance to an environmental cue requires an increase in postsynaptic AMPAR (α-amino-3-hydroxy-5-methyl-isoxazole propionic acid receptor)-mediated currents in amygdala neurons that occur via an NMDAR (N-methyl-D-aspartate receptor)-dependent mechanism. Furthermore, neurons that encode the persisting memory of an expected reward are active when frustration is expressed in response to reward omission. Finally, these data show that environmental cues endowed with the power to guide goal-directed behaviors are encoded by subpopulations of amygdala neurons that differentially encode the motivating and reinforcing properties of the emotionally significant cue.

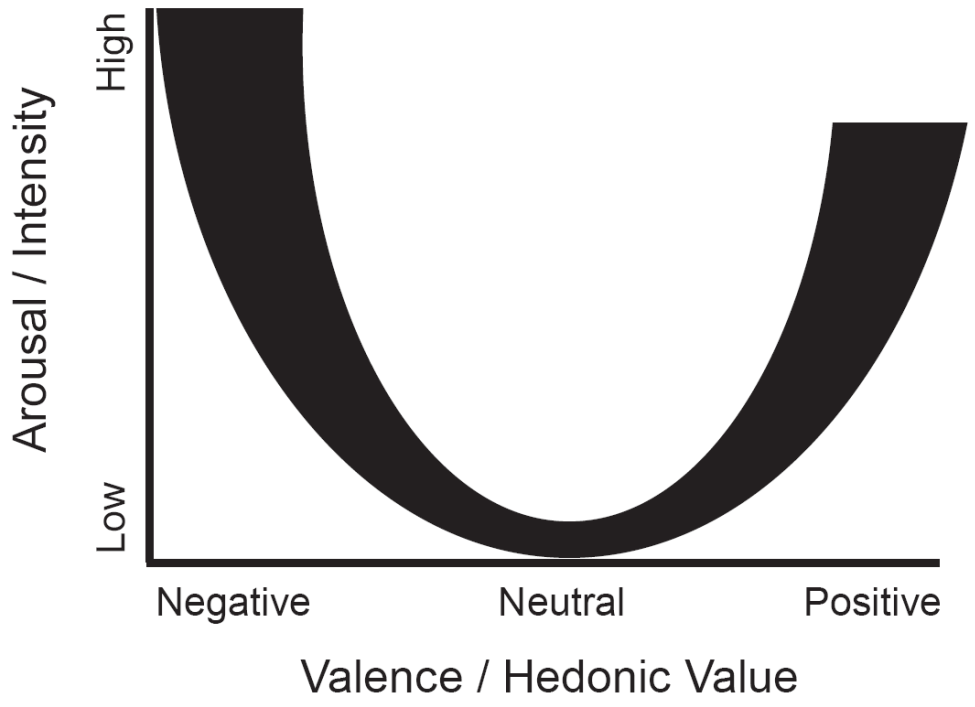


Figure 1: Schematic representation of the two dimensions that comprise emotion, as based on the dimensional theory of emotion. Affect can be described in terms of either physiological arousal or intensity, which can range from low to high arousal or intensity. Affect can also be characterized by valence or hedonic value. For example, fear is an emotion that has a negative valence and elicits a high level of physiological arousal. In contrast, joy is an emotion that has a positive valence and elicits a relatively high level of physiological arousal, though recent evidence (see discussion) suggests that the maximal intensity of emotions with a negative valence is greater than that of emotions with a positive valence, which is likely to serve a practical advantage with respect to survival. Emotions that are neither positive nor negative typically yield a very low intensity or arousal state. Intensity and valence may not be dissociable parameters of emotion (Lang, 1995; adapted from Merzlyak, 2006).

EMOTION: THE BASIS OF MOTIVATED BEHAVIOR[1]

Cognitive psychologists, behavioral neuroscientists and other emotion researchers have engaged in a semantic battle to define what emotions are. While most people would agree that emotions exist, without clearly defining what emotions are, we cannot begin experimentation to determine how our brain processes them. According to the dimensional theory of emotion, all emotions can be plotted as coordinates along the dimensions of valence and intensity (P.J. Lang, 1995; Larsen et al., 1987; Larsen et al., 1996; Diener et al., 1985). Valence refers to the hedonic aspects of the emotion, ranging from positive (pleasant) to negative (unpleasant), where as intensity refers to the level of arousal, ranging from high (excited) to low (calm or bored). Typically, emotions tend to fall along a U-shaped curve wherein emotions that have a large absolute value of valence (very positive or very negative) are high in intensity or arousal (Figure 1).

There is substantial evidence that memories are stored as physiological changes in the brain, specifically in the strength and distribution of synapses. However, if the brain functioned like a computer (void of emotion) then each additional memory would result in an additional or longer program and a slower processing time. Additionally, our brains have numerous tasks to orchestrate; from basic functions such as breathing, sleeping, and processing sensory information to complicated tasks such as engaging in courtship behaviors, escaping from a predator or planning a goal-oriented behavior. Whether

[1] While this section discusses issues beyond the immediate scope of my thesis work, I felt that this conceptual digression was necessary for several reasons. The amygdala is a brain region that is commonly thought to be important for emotional processing, and its function is specific to "emotional" memories. It is one of very few brain areas that are capable of performing multiple functions of memories. However, this capability extends only to memories that are "emotionally relevant." Additionally, I believe that understanding how emotions are processed is the crux of understanding the neural basis of consciousness. While emotion is a difficult topic to study – or even discuss – I wanted to include a section to frame the conceptual motivation of my thesis work.

processing multiple functions in series or in parallel, the brain requires a means of prioritizing among these many tasks. An animal needs to process sensory information and an animal needs to sleep, but doesn't need to process sensory information while sleeping. Similarly, an animal should not be engaging in courtship behaviors while trying to escape from a predator. How does an animal decide that it needs to stop whatever it is doing to escape from a predator? The brain requires a system to rapidly observe, evaluate, prioritize and act.

I speculate that emotions provide this system. As opposed to thoughts, which frequently fail to produce an immediate behavioral output, emotions may be evolutionary adaptations that enable an animal to coordinate competing processes and to direct the animal's attention to the most relevant environmental stimuli to respond quickly and appropriately, essentially providing a solution to the problem of mechanism orchestration (Tooby and Cosmides, 1990; Cosmides and Tooby, 2000). If emotions are an evolutionary adaptation, then it is likely that at least basic emotions (fear, happiness, etc.) are conserved in many other species.

Even in human subjects, emotion is a subjective experience that is difficult to quantify. In non-human subjects, an emotional experience may best be studied by careful examination of the animal’s behavior.

BEHAVIOR: A WINDOW INTO LEARNING AND MEMORY

During the early 1800's, the study of brain function was primarily based on speculation[2]. The lasting impact of fields such as phrenology was limited to generating interest in the physiological bases of behavioral differences between individuals. However, upon the birth of behaviorism, cognitive functions such as learning, memory, attention and voluntary action could now be systematically studied. It was the development of behavioral psychology that laid the foundation for cognitive neuroscience.

Quantifying Behavior

Until the late 1800's, the study of cognition consisted primarily of introspection. In 1885, Hermann Ebbinghaus first employed simple experimental methods of studying learning and memory in humans. Shortly thereafter, Pavlov and Thorndike joined Ebbinghaus as pioneers in the development of an empirical school of psychology which was to be called behaviorism. In an effort to establish the study of behavior as a rigorous science, behaviorists such as Watson and Skinner abandoned speculation about what the mind and brain were doing and focused on only the observable aspects of behavior. Unobservable mental processes, especially abstractions such as selective attention, memory and emotion, were deemed inaccessible to scientific study. This division between subjective experience and objectively quantifiable outputs, such as behavior, was pivotal in putting forth behaviorism as the foundation for many other fields of science, including behavioral neuroscience.

[2] For example, the now rejected field of phrenology was once considered a science, by which the personality traits of a person were determined by "reading" bumps and fissures in the skull. Developed by German physician Franz Joseph Gall around 1800, the discipline was very popular in the 19th century, as it was one of the first fields founded on the belief that behavioral traits had physiological bases.

The Neural Basis of Memory

In 1949, Donald Hebb forged the path of a field of research dedicated to identifying the neural basis of cognition. His ideas were far-reaching and insightful beyond his time. The Hebbian model of synaptic plasticity, summarized in layman's terms as "neurons that fire together wire together" is one of the central tenets in neuroscience. By synthesizing existing biological facts, he proposed that networks of neurons worked together to represent information, and that these representations were distributed over large areas of the brain.

Under the influence of Hebb, in 1957 Milner and colleagues described the now-famous patient, H.M. (Scoville and Milner, 1957)[3]. Following a bilateral resection of his medial temporal lobe structures in 1953, H. M. lost the ability to form new long-term declarative memories, but retained many other cognitive functions (Scoville, 1954). Specifically, H. M. was found to have retained the ability to form long-lasting procedural memories, shown in his improvement over days in a mirror drawing task, without any recollection of having practiced this task before (Milner, 1962). These findings led to the idea that there

[3] As an undergraduate at MIT, my first research position involved assisting in the cognitive testing of patient H. M. I was granted the rare opportunity to interact directly with H. M. and was able to experience first hand his memory deficits, as well as the functions that he retained. Although Henry was quite old by the time I met him, he was surprisingly alert and self-aware. For example, while Henry would repeatedly tell the same stories to us, for example, about how he wanted to be a doctor before his temporal lobectomy. However, he would also say that, ever since, his memory "wasn't so good." He was conscious of the fact that he was being studied because of his memory loss, and said, "I'm glad I can help people" repeatedly. This demonstrated that *some* aspect of declarative memory was retained despite the gross lobectomies performed. Additionally, given a multiple choice of who was president, Henry was able to choose correctly. This experience secured my fascination with learning and memory, and has greatly influenced my path as a neuroscientist.

may be multiple memory systems in the brain, represented by distinct neural circuitry, for different types of memories.

Multiple Memory Systems

Experience-dependent plasticity can occur in every brain region that has been extensively tested for this capability, and may be a property intrinsic to all neurons. However, the type of experience that mediates plasticity varies from region to region. In the 1980s, the idea that the hippocampus mediates 'cognitive' or 'declarative' memory and the caudate nucleus mediates stimulus-response 'habit' formation was introduced (Mishkin et al., 1984). Packard, White, McGaugh and colleagues studied this idea in the rat using the dissociation method of brain lesions and intra-cranial drug infusions, and found that these memory systems could be anatomically dissociated (Packard et al., 1992; Packard and McGaugh, 1992, 1996; Packard and Teather, 1997). In addition, it has been suggested that there is a 'perceptual' memory system, which is likely to be mediated by sensory cortices (Bussey and Saksida, 2007; Dijkerman and de Haan, 2007). The amygdala has been identified as being important for the 'emotional' memory system in animals (LeDoux, 1995; Davis et al., 1997) and humans (Damasio 1995; Cahill et al., 1996). The amygdala is a brain region of particular interest when studying learning and memory because, unlike other memory systems where memories may be encoded in one region and stored elsewhere, the amygdala is a multi-functional region wherein memory formation, consolidation, storage, extinction and retrieval can occur (Lamprecht and Dudai, 1996; Wilensky et al., 1999; Schafe et al., 2000; Hall et al., 2001; Repa et al., 2001; Stork et al., 2001; Moita et al., 2002; Pape and Stork, 2003; Zinebi et al., 2003;

Richter-Levin, 2004; Maren, 2005). Furthermore, there is substantial evidence that emotional arousal and amygdala activity can modulate memory formation (Reisberg and Hertel, 2004; Richter-Levin, 2004), see Discussion for more details. Overall, the amygdala represents an exceptional constellation of functions and capabilities, which are made possible by its unique anatomy.

AMYGDALA ANATOMY

The term "amygdala," derived from the Greek word for "almond," was first used by Burdach (1819) in reference to the almond shaped structure of the basolateral complex of the amygdala (BLA), which are the more recent nuclei by phylogeny relative to the evolutionarily primitive central nucleus (Johnston 1923; Alheid and Heimer 1988). In the past two centuries, the term "amygdala" has come to refer to a number of structurally and functionally heterogeneous nuclei (Swanson and Petrovich, 1998; Pitkanen, 2000). Swanson and Petrovich (1998) characterize these subnuclei into four distinct categories: Accessory olfactory system, which includes the medial nucleus; Main olfactory system, which includes the cortical and basomedial nuclei; Autonomic system, which includes the central nucleus; and the Frontotemporal cortical system, which includes the lateral and basolateral nuclei, or basolateral complex. This thesis will focus on the basolateral complex of the amygdala (BLA), which possesses "cortical-like" characteristics, including glutamatergic pyramidal neurons (Carlsen, 1988; Smith and Pare, 1994), and includes the lateral, basal and accessory basal nuclei (Pitkanen et al., 2000).

Amygdala circuitry

While the basal and accessory basal nuclei do project to the lateral amygdala (LA), sensory information from the thalamus and sensory cortices typically enters the amygdala via the LA (Turner et al. 1980; Ottersen, 1982; Amaral, 1987; LeDoux et al., 1990; Turner and Herkenham, 1991; Romanski and LeDoux 1992; Mascagni et al., 1993; McDonald, 1998; Pitkanen, 2000), which has strong intra-amygdala projections to the rest of the BLA (Alheid et. al, 1995; Alheid, 2003). The BLA is commonly thought to project to the central nucleus of the amygdala (CeA) (Krettek and Price, 1978; Nitecka et al., 1981; Ottersen, 1982; Roberts et al., 1982; Millhouse and DeOlmos, 1983; Aggleton, 1985; Shi and Cassel, 1998). Recently, controversy has been stirred surrounding this subject, as evidence suggests that LA neurons do not directly project to the CeA neurons that innervate the brainstem (Pare et al., 2004). Specifically, it has been suggested that the LA relays information via the dense intercalated GABAergic neuronal sheath (Marowsky et al., 2005) between nuclei and thus disinhibit brain stem projecting CeA neurons (McDonald and Augustine, 1993; Nitecka and Ben Ari, 1987; Pare and Smith, 1993; Royer et al., 1999; Pare et al., 2004). Importantly, the thalamus has strong uni-directional projections to LA (Pitkanen, 1997), and only relatively light projections to the rest of the BLA from the thalamus (Linke et al., 2000, 2004).

Because the BLA receives numerous region-specific projections not only from the thalamus and cortex (Krettek and Price, 1977; LeDoux et al., 1990; McDonald, 1998; Shi and Cassell, 1998; Vertes, 2004), but also the hippocampus (Pitkanen et al., 1997;

Pitkanen et al., 2000; Kemppainen et al., 2002), hypothalamus (Renaud and Hopkins, 1977; Ottersen, 1980; Kawai et al., 1982; Touzani et al., 1996), striatum (Kelley et al., 1982; Kita and Kitai, 1990), and midbrain (Swanson, 1982; Loughlin and Fallon, 1983), it suggests that either neurons that are structurally similar have heterogeneous functions, or that these individual neurons have multiple functions. See Figure 2 for a summary of amygdala connectivity (Knapska et al., 2007). Furthermore, while the BLA has many reciprocal connections, it also has uni-directional projections from the thalamus (Turner and Herkenham, 1991; Doron and Ledoux, 1999; Woodson et al., 2000) and midbrain (Swanson, 1982), and to the accumbens (Kita and Kitai, 1990; Wright et al., 1996), which are likely to represent the functional integration of the BLA into overlapping and non-overlapping neural circuits.

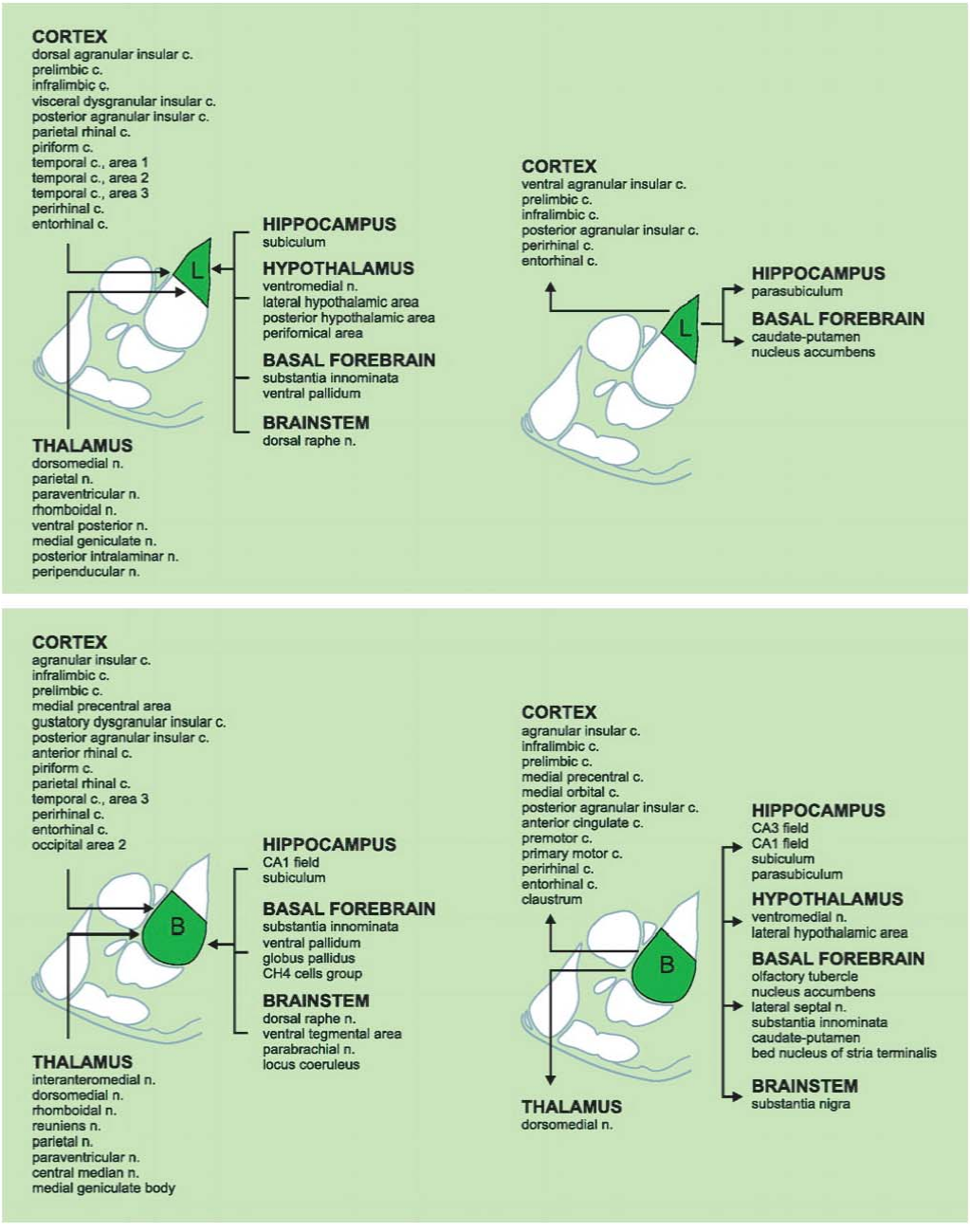


Figure 2: Summary of BLA connectivity. Top panel shows afferents and efferents of the lateral amygdala. Bottom panel shows afferents and efferents of basolateral nucleus of the amygdala. This figure was reproduced with permission from: Knapska, E. et al. Physiol. Rev. 87: 1113-1173, 2007;doi:10.1152/physrev.00037.2006.

Neuronal Composition of the Amygdala

The BLA complex is predominantly comprised of pyramidal glutamatergic neurons encapsulated in a γ-aminobutyric acid (GABA)ergic neuronal sheath (Marowsky et al., 2005). Intracellular recordings of BLA neurons *in vitro* enabled parallel electrophysiological and morphological investigation by a number of different researchers who identified several populations of neurons in the BLA (Washburn and Moises 1992; Davis et al., 1994; Rainnie et al., 1993), which can be summarized into two main groups. First, the vast majority of neurons (greater than 90%) were classified as "accomodating," which were characterized by their accommodation to a depolarizing step, and typically had large pyramidal cell bodies. Second, there are the "non-accomodating" neurons, which were described as "late-firing" or "fast-firing" neurons upon depolarization. The cell bodies of "late-firing" neurons were also pyramidal, but smaller than those of "accommodating" neurons, and "fast-firing" neurons had small spherical or multipolar somata (Chapman et al., 1990; Washburn and Moises 1992; Davis et al., 1994; Rainnie et al., 1993).

Implications of Amygdala Anatomy on Amygdala Function

The widespread projections from the amygdala to numerous other brain regions parallels the notion that emotion being important for influencing other brain functions. Furthermore, the amygdala also receives robust projections from neocortical areas known to be important for higher cognitive functions, suggesting that it also plays a role in the integration of emotion and cognition. The anatomical connections support the recent concept that the amygdala is a functional connectivity hub that integrates emotion,

cognition, and motivation to encode the discrete aspects of an animal's environment in terms of value and significance.

AMYGDALA FUNCTION: EVOLVING EMPHASIS[4]

The amygdala is best known for its role in mediating negative emotions, particularly fear, as confirmed by theories referring to the amygdala as a 'fear module' (Ohman and Mineka, 2001) or 'protection device' (Mason et al., 2006). Fear conditioning, first used by Watson and Rayner (1920) is one of the most commonly used behavioral paradigms, largely due to the simplicity of implementation and the robustness of the behavioral phenomenon. Fear conditioning typically involves the pairing of a neutral stimulus with an aversive stimulus, such as a shock, and has been shown to form lasting memories after a single trial. Rodents may express fear by freezing or with a startle response, both of which can be easily quantified. For this reason, fear is by far the most extensively-studied emotion. However, the notion that the amygdala only mediates negative affect is a common misconception that is finally being overturned. Although amygdala function related to positive emotions has been demonstrated numerous times (Baxter and Murray, 2002), this literature has been dwarfed by the proliferation of studies based on fear conditioning (Davis, 1992; Adolphs et al., 1995; LeDoux, 1998; Ledoux, 2000; Ohman and Mineka, 2001; LeDoux, 2003; Maren and Quirk, 2004; Mason, 2006). Finally, while the amygdala has primarily been conceptualized as mediating affect, it has recently been

[4] Importantly, while the amygdala is thought to be important for processing emotion and affect, for all experiments involving non-human primates, experimenters are measuring the subject's behavior and the emotion or affect that the subject is experiencing is extrapolated.

proposed that the amygdala acts as a ‘connectivity hub’ mediating cognitive-emotional interactions (Pessoa, 2008).

Amygdala Involvement in General Affect

As early as 1888, Brown and Schafer discovered that bilateral ablation of the temporal lobe has caused a dramatic change in behavior. Monkeys with these lesions displayed a general placidity, and an overall loss of affect. When a monkey with temporal lobe lesions was put into a cage with a wild monkey, it approached the wild monkey only to be violently attacked. Immediately, the experimental monkey approached the wild monkey again without any sign of fear. However, the implications of these findings were not fully appreciated until 1937 when a similar finding was reported by Heinrich Kluver and Paul Bucy. In an attempt to investigate the effects of mescaline on specific brain areas, Kluver and Bucy serendipitously discovered that the bilateral removal of the temporal lobe, including the amygdala, caused a profound change in primate behavior. Specifically, they observed that monkeys became increasingly "tame" and lost learned fear responses to stimuli, such as a hissing snake, that previously elicited a dramatic fear response. Following temporal lobectomy, these monkeys would calmly approach and investigate such fearful stimuli. In addition, these monkeys had difficulty recognizing stimuli which normally evoked a positive affect. For example, monkeys given a number of objects would devote equal amounts of attention to a lightbulb, feces, the tongue of a hissing snake and a piece of food. Neutral objects such as an iron pipe might be re-examined numerous times before the monkey examines a piece of food. Once examined, the food would be immediately consumed. This phenomenon is now referred to as the

Kluver-Bucy syndrome, which is characterized by the loss of emotional significance for sensory stimuli. At the time, Kluver and Bucy termed this phenomenon "psychic blindness" because while the animals' vision was unaltered, the objects they saw were psychologically meaningless.

However, the work done by Kluver and Bucy involved multiple brain regions. The amygdala was not singly identified as the critical brain region mediating emotional processing until 1956 when Lawrence Weiskrantz combined amygdala lesions with experimental tasks designed to parse the facets of emotional responsiveness that were impaired, such as avoidance conditioning. Monkeys with amygdala lesions were impaired in their ability to learn to perform a response that prevented or stopped an aversive stimulus, such as an electric shock. Weiskrantz also reported that monkeys with amygdala lesions displayed deficits in discriminating reinforcers from other stimuli, for example, these monkeys would eat inedible foods. These experiments and other observations enabled Weiskrantz to assert that the amygdala is necessary for associating emotional properties with their sensory representations.

Early Research of Amygdala in Instrumental Conditioning

In the subsequent decades, avoidance conditioning, an instrumental task in which the animal can perform a behavior that allows it to prevent or terminate an aversive stimulus, became one of the most common behavioral tasks used to investigate amygdala function. During this time, a number of researchers were working in parallel to explore the role of the amygdala in evaluating the reinforcing properties of sensory stimuli (Jones and

Mishkin, 1972; Horel et al., 1975; Mishkin and Aggleton, 1981). These researchers also used appetitive instrumental conditioning paradigms. However, the results for both the avoidance conditioning and appetitive conditioning studies were inconsistent and inconclusive (Goddard, 1964; Isaacson 1982; LeDoux 2000). In some studies, amygdala damage produced an avoidance deficit, but not in others. The variability of these results were likely due to the differences in the instrumental response measured, or to differences in the method or timing of amygdala damage. Regardless of the reasons for these inconsistencies, these studies failed to produce a cohesive picture of amygdala function, and were therefore largely disregarded.

The Amygdala as the 'Fear Module'

In the 1970's, Pavlovian fear conditioning offered a simple and robust behavioral paradigm that provided a clear, consistent model of how the brain mediates fear. Amygdala damage consistently impaired Pavlovian fear conditioning (Blanchard and Blanchard, 1972; Cohen, 1975; Pribram et al., 1979). Pavlovian fear conditioning involves the pairing of a neutral stimulus (usually a tone) with an aversive stimulus (usually a shock). With a single pairing, the CS acquires the capacity to elicit defensive behaviors or autonomic nervous system responses. Pavlovian fear conditioning was a behavioral paradigm with many benefits; the task was easily established, was rapidly learned, formed prolonged memories, was reliably measured by stereotyped behaviors, and was consistently reproducible (LeDoux et al., 1990b; Campeau and Davis, 1995b). The popularization of Pavlovian fear conditioning revolutionized amygdala research, as it

became the "gold standard" for a neural basis of associative learning acquired and expressed *in vivo*.

Joseph LeDoux, among others, pioneered the fear conditioning revolution by identifying the relevant neural pathways delivering information to the amygdala, the cellular mechanisms underlying fear learning within the amygdala, and the manner fear resposes were controlled by outputs from the amygdala. Afferents carrying information about the conditioned stimulus arriving from thalamic nuclei and sensory cortices synapse primarily on to neurons in the LA (LeDoux et al. 1990a; Romanski and LeDoux, 1993; Mascagni et al., 1993; McDonald, 1998). Additionally, it has been found that fear conditioning to a simple auditory stimulus could be mediated by either the thalamic or cortical inputs (Romanski and LeDoux, 1992). The notion that the thalamic pathway could mediate fear conditioning was challenged by lesion studies using a similar paradigm measuring fear-potentiated startle (Campeau and Davis, 1995a; Shi and Davis, 1999).

Electrophysiological recordings show that neurons in the LA change their phasic responses to an auditory conditioned stimulus upon fear conditioning (Quirk et al., 1995, 1997), as do neurons in the auditory cortex and thalamus (Quirk et al., 1995, 1997; Weinberger, 1995, 1998; Laviolette et al., 2005; Komura et al., 2001, 2005). Importantly, the thalamic pathway conditions more rapidly both within and across trials than the cortical pathway (Quirk et al., 1995, 1997), suggesting that plasticity in the amygdala occurs initially in the thalamic pathway. Changes in firing have also been

observed in the BLA (Maren et al., 1991; Uwano et al., 1995) and CeA (Pascoe and Kapp, 1985) during aversive conditioning, but with acoustic response latencies that are longer than in the LA. The CeA is thought to act as junction between sensory and motor systems (LeDoux, 2000). Recently, it has been argued that sensory projections bypassing the LA, arriving directly at the CeA can mediate conditioning to an auditory conditioned stimulus (Killcross et al., 1997). If this were true, it would lend support to William James' theory that emotions are the mind's interpretation of physiological conditions evoked by environmental cues (James, 1884).

LTP in the Amygdala as a Cellular Basis of Learning

Long-term potentiation (LTP), first described in the hippocampus (Bliss and Lomo, 1973), is thought to represent the cellular mechanism underlying some forms of learning *in vivo* (Lynch, 1986; Bliss and Collingridge, 1993). While the vast majority of LTP studies have involved the hippocampus, hippocampal involvement in learning and memory tasks has been difficult to isolate at the circuit level. Therefore, some of the strongest evidence of a relationship between behavioral learning and LTP in specific synapses has been found in the amygdala, which has more recently been well-characterized in terms of its thalamic and cortical afferents, and associative LTP (Shin et al., 2006). Specifically, LTP has been observed during fear conditioning in the thalamoamygdala pathway using extracellular recordings of field potentials *in vivo* (Clugnet and LeDoux, 1990; Rogan and LeDoux, 1995; Rogan et al., 1997). Animals that have undergone fear conditioning exhibit an enhancement in synaptic responses of the thalamic pathway to the LA, as has been seen by whole-cell patch-clamp recordings

made *in vitro* (McKernan and Schinnick-Gallagher, 1997). Fear conditioning also results in LTP occlusion in the cortical pathway to the LA (Tsvetkov et al. 2002). Recently, fear conditioning was shown to drive AMPARs into the synapse of postsynaptic LA neurons, and blockade of AMPAR insertion reduced the behavioral expression of the fear memory (Rumpel et al., 2005).

The Role of the Amygdala in Goal-Oriented Behavior

Efficient goal-oriented behavior is essential for survival in an environment with limited resources. To maximize efficiency, the amount of attention, time and energy devoted towards different environmental stimuli should be proportional to the importance or value of those stimuli to the animal's survival. Learned associations between environmental stimuli and rewards critically involve the BLA in many different behavioral paradigms. For example, a behavioral phenomenon called conditioned place preference, in which animals typically learn to spend more time in environments associated with reward, is impaired in animals with amygdala damage (McDonald and White, 1995). Numerous studies have implicated the BLA in the modulation of instrumental responses in appetitive instrumental tasks (Everitt et al., 1989; Everitt et al., 2000; Everitt et al., 2001; Everitt et al., 2003; Balleine, 2005). While animals with BLA lesions can acquire conditioned responses (Kilcross et al., 1997; Parkinson et al., 2000; Kilcross et al., 1998), they do show an impairment in responding to a subsequent change in reinforcer value, as shown by reinforcer-specific devaluation (Hatfield et al., 1996; Malkova et al., 1997), suggesting that the reinforcer-specific sensory properties are represented in the BLA during instrumental tasks (Balleine et al., 2003; Corbit and Balleine, 2005). These and

other studies demonstrating that the amygdala is part of the reward circuit (Berridge and Robinson, 2003) will be discussed in greater detail in the following chapters and discussion.

GOALS OF THIS DISSERTATION

While the cellular and circuit mechanisms underlying Pavlovian fear conditioning have been well-characterized by extensive research, the literature investigating the cellular mechanisms of goal-directed behavior is relatively sparse. The experimental and behavioral simplicity of fear conditioning has facilitated the analysis of its underlying circuit. However, though robust and easy to implement, Pavlovian fear conditioning has many limitations. In contrast, the study of instrumental behaviors provides the advantage of greater behavioral flexibility and numerous measurable behavioral outputs which may offer more insight as to the specificity of the memory that has been formed on a subject by subject basis.

This is not an attempt to trivialize the monumental advances afforded by fear conditioning which provide the platform for this research. On the contrary, it is an attempt to broaden the penetrating insights of the neural substrates of fear conditioning, and to bring the understanding of the neural basis of complex goal-oriented behaviors to a similar standard. While the BLA is now accepted as a critical brain region for reward-related learning, relatively little is known about the associated neural changes. The goal of this dissertation is to delineate the molecular, cellular and circuit underpinnings of the formation and storage of memories associated with goal-oriented behavior. Although this work is primarily based on behavioral neuroscience, the experimentation comprising this dissertation involves the integration of multiple techniques, including *in vivo* electrophysiology in awake behaving rats, *ex vivo* whole-cell patch-clamp electrophysiology in previously trained rats and *in vivo* intra-cranial pharmacology. The

subsequent chapters encompass the neural changes of the amygdala associated with the acquisition of cue-reward and response-outcome associations, the extinction of response-outcome associations, the reinstatement of responding induced by a reward-associated cue and the extinction of a cue-reward association. The data shown in Chapter 2 has been published by Tye, Stuber, de Ridder, Bonci and Janak in *Nature*, 2008. The data shown in Chapter 3 has been published by Tye and Janak in the *Journal of Neuroscience*, 2007.

Summary

This dissertation examines the neural activity of the amygdala, a brain area thought to be important for the formation and retrieval of emotional memories, during the acquisition and reinstatement of a reward-directed operant conditioning paradigm.

The aim of the line of experimentation detailed in Chapter 2 is to provide a foundation for understanding the neural changes underlying the acquisition of a cue-reward association during an instrumental appetitive conditioning task for a natural reward. Elucidating the neural mechanisms of acquiring this task was the overarching first aim, however, addressing this aim involved several lines of experimentation which are detailed in the sub-aims below.

> How is the learning process reflected in the firing of individual amygdala neurons? Amygdala neurons suddenly and dramatically increase their responsivity to a cue upon 'the moment of realization' that the cue predicts the

delivery of a reward. With continued training and improved performance, additional neurons are recruited to encode the reward-predictive cue.

How is the acquisition of a response-outcome association different than the acquisition of a cue-reward association? While the amygdala does have subpopulations that encode each of these associations, the amygdala is specifically tuned to encoding the significance of sensory stimuli, as a significantly higher proportion of neurons encode a reward-predictive cue than an operant response for the same reward.

What synaptic changes mediate the changes in neuronal firing during learning? Following a single session of successful task acquisition, increased glutamatergic synaptic transmission is selectively enhanced in thalamic synapses in the amygdala.

What molecular or cellular change mediates the enhancement of synaptic transmission? The increase in synaptic strength is mediated by a postsynaptic increase in AMPAR number or function.

Does this learning phenomenon share the same characteristics as associative LTP (NMDAR-dependent mechanism)? As seen in hippocampal LTP, these synaptic changes require the depolarization of NMDARs. Not only does NMDAR

blockade impair the ability to learn the task, but it also attenuates the associated increase in glutamatergic tone.

The amygdala has been shown to be important for cue-induced reward-seeking, a phenomenon seen for natural rewards, and a model for drug addiction relapse. The aim of Chapter 3 was to test the hypothesis that amygdala neurons encode a cue endowed with power to elicit operant responding in the absence of reward availability. A cue that previously predicted reward delivery can be endowed with many properties, and may act as a conditioned incentive, or a conditioned reinforcer, or both. We show that not only do amygdala neurons encode reward-associated cues, but also report that distinct subpopulations of amygdala neurons code for the motivating and reinforcing properties of these cues.

The significance of these findings in the context of the existing literature will be discussed in Chapter 4.

CHAPTER 2

Amygdala activity and synaptic strength increase with the acquisition of a cue-reward association

ABSTRACT

What neural changes underlie individual differences in goal-oriented learning? The lateral amygdala (LA) is important for assigning emotional and motivational significance to discrete environmental cues, including those that signal rewarding events. Recognizing that a cue predicts a reward enhances an animal's ability to acquire that reward; however, the cellular and synaptic mechanisms that underlie cue-reward learning are unclear. Here, we performed both *in vivo* and *ex vivo* electrophysiological recordings in the LA of rats trained to self-administer sucrose. We observed that reward learning success increased in proportion to the number of amygdala neurons that responded phasically to a reward-predictive cue. Furthermore, cue-reward learning induced an AMPA (α-amino-3-hydroxy-5-methyl-isoxazole propionic acid)-receptor-mediated increase in the strength of thalamic, but not cortical, synapses in the LA that was apparent immediately after the first training session. The level of learning attained by individual subjects highly correlated with the degree of synaptic strength enhancement.

Importantly, intra-LA NMDA (N-methyl-D-aspartate)-receptor blockade impaired reward learning performance and attenuated the associated increase in synaptic strength. These results show that dramatic changes in both cue-induced neuronal firing and input-specific synaptic strength occur upon successful acquisition of a cue-reward association within a single training session. These findings provide evidence of a connection between LA synaptic plasticity and cue-reward learning, potentially representing a key mechanism underlying goal-oriented behavior.

INTRODUCTION

Varying abilities to learn goal-oriented tasks can lead to lifelong tendencies towards success or failure. Identifying the neural differences that account for the variability in goal-oriented learning among individuals will provide tremendous insight in several important areas. First, we can determine the degree to which learning is based on neural changes. Second, we will test our hypothesis that the anatomical basis and synaptic mechanism of reward-related learning is the same or similar to fear conditioning. Third, we can apply this knowledge to the development of therapeutic interventions for some types of learning disabilities.

The basolateral amygdala complex (BLA) imparts motivational significance to sensory cues (Davis, 1992; Quirk et al., 1995; Quirk et al., 1997; Repa et al., 2001; Rosenkranz and Grace, 2002; LeDoux, 2003; Maren and Quirk, 2004), including those that signal rewarding events through associative learning (Cador et al., 1989; Everitt et al., 1989;

Uwano et al., 1995; Schoenbaum et al., 1999; Cardinal et al., 2002; Balleine and Killcross, 2006; Paton et al., 2006; Tye and Janak, 2007). Impairments of the BLA impair the ability to learn cue-reward associations, such as Pavlovian approach behaviors, as well as the expression of cue-reward associations, such as cue-induced reinstatement or cue-evoked potentiation of feeding.

Furthermore, neurons within the BLA are phasically responsive to reward-predictive cues (Uwano et al., 1995; Schoenbaum et al., 1999; Carelli et al., 2003; Tye and Janak, 2007), consistent with the idea that cue-evoked neuronal firing emerges as a consequence of cue-reward associations. It has been shown that the BLA has strong, reciprocal projections to the prefrontal and orbitofrontal cortices (Krettek and Price, 1977; Kita and Kitai, 1990; Shi and Cassell, 1998). The medial prefrontal cortex (mPFC) is thought to impose an inhibitory suppression on LA neuron responding to a fear-conditioned odorant cue that may be attenuated by dopaminergic modulation (Rosenkranz and Grace, 2002; Rosenkranz et al., 2003). The orbitofrontal cortex (OFC) is important for cue-outcome associations as seen in reversal tasks, where two odor cues are paired with either sucrose or quinine and then the pairings are reversed (Schoenbaum et al., 1999; Schoenbaum et al., 2003). These studies also show that the changes in phasic activity of BLA neurons encoding the odor cue upon a reversal precede the change in the phasic activity of OFC neurons or the behavioral shift.

The BLA is comprised of multiple distinct nuclei, including the lateral amygdala (LA) (Figure 2), which is the first site of convergence for sensory inputs carrying information

about both conditioned and unconditioned stimuli to the amygdala (LeDoux et al., 1984; LeDoux et al., 1990; Romanski et al., 1993). Numerous studies have proven the LA to be important for fear conditioning, a well-studied paradigm in rodents due to the robust behavioral phenomenon of CS-induced freezing (Davis, 1992; LeDoux, 1996; Ledoux, 2000). Fear conditioning is presumably mediated by the trafficking of AMPARs to the surface of LA neurons via an associative LTP mechanism when somatosensory information about the shock and auditory information about the conditioned stimulus are paired. However, with many pairings, CS-induced freezing can approach a maximal or near-maximal level at which some might argue creates a "ceiling" effect. The LA also receives information from the gustatory thalamus about reinforcers such as sucrose or food (Azuma et al., 1984; McDonald, 1998; Shi and Cassell, 1998; Nakashima et al., 2000). Thus, the LA is a likely initial site for the formation of cue-reward associations that endow the cue with motivational significance that impacts reward-seeking behavior.

Understanding complex reward-seeking behaviors and the behavioral changes associated with the extinction and reinstatement of cue-reward associations requires a solid foundation of knowledge about the mechanism underlying the initial acquisition of a cue-reward association. In this chapter, I will investigate how neurons are recruited to an ensemble that encodes a reward-predictive cue during acquisition, and how these changes in phasic activity are mediated by the strengthening of thalamic synapses on LA neurons in an NMDAR-dependent manner.

RESULTS

To test the hypothesis that successful acquisition of cue-directed reward-seeking behavior is dependent upon neuronal plasticity in the LA, we examined LA neuronal firing in response to a reward-predictive cue during training on a sucrose self-administration task (Figure 3). To control for the neural activity associated with the motor output of operant responding, and to ensure that the sensory cue predicted reward delivery, and not the operant response alone, responses at a nosepoke operandum were reinforced with a cue and sucrose reward after approximately 50% of nosepokes (Figure 4a,b). In rats that successfully acquired this task (see Methods), approximately half of recorded neurons (49%; 60 of 122 neurons from 7 rats during the first session in which each rat met the acquisition criterion) that did not respond to the cue prior to acquisition developed a robust phasic response to cue onset upon acquisition (Figure 4c,d; Figure 5). Cue encoding increased across sessions: the cue-evoked population

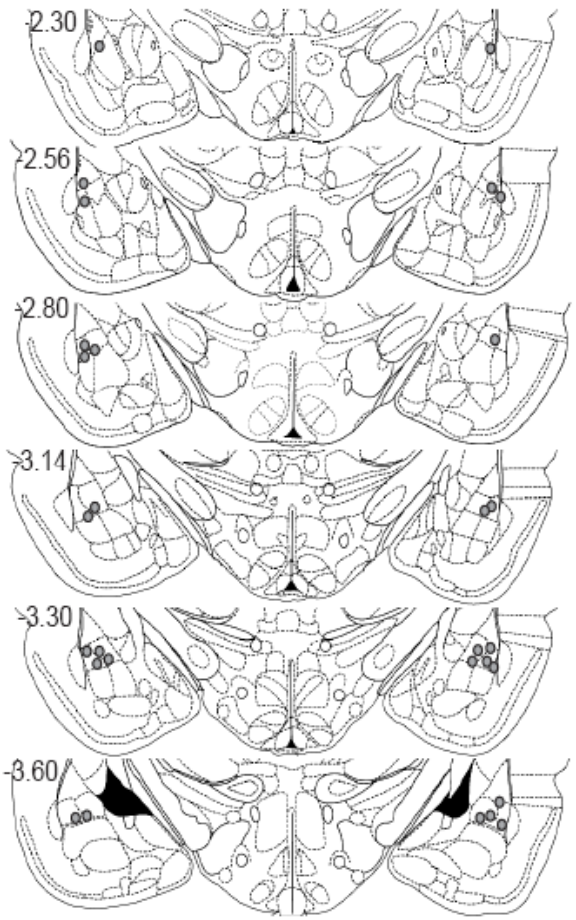


Figure 3: **Histological verification of electrode tip placements for rats shown in Figure 4.** Coronal diagrams showing chronic recording electrode tip placements (grey circles). Numbers on left indicate the anteroposterior coordinates caudal to bregma (Paxinos, G. and Watson, C. The rat brain in stereotaxic coordinates. Academic Press, 1998).

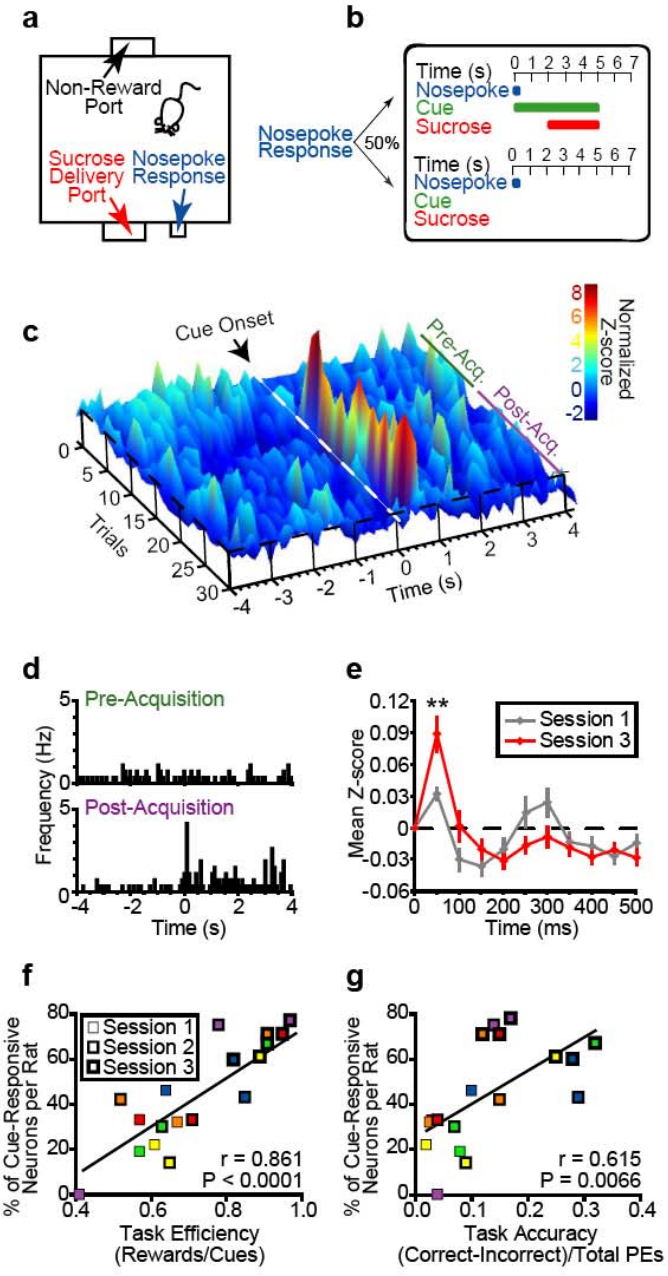


Figure 4: Reward-related learning success is correlated with rapid increases in cue-related firing. **a**, Cartoon of operant chamber from above. **b**, Schematic of behavioral paradigm. **c**, Temporal dynamics of neuronal population response to the reward-predictive cue. Spike activity of all simultaneously recorded LA units (n=13) from a rat which successfully acquired the task during the first session; 100 ms bins. This population of neurons develops a response to the onset of the reward-predictive cue upon task acquisition. **d**, Peri-event histogram (PEH) of a single LA neuron from a different rat which successfully acquired the task in the first session; 29 trials each epoch. **e**, Population histograms (50 ms bins, error bars indicate ±SEM) of the mean Z-score for all neurons recorded during session 1 (n = 95 neurons) and session 3 (n = 123 neurons) for all rats (n = 7). **P < 0.004. **f**, **g**, Correlation between proportion of cue-responsive neurons and (f) Task Efficiency and (g) Task Accuracy across 3 sessions. Port Entries (PEs). Colors indicate the same rat on different sessions. Only rats with > 6 neurons per session were included in scatterplots (n = 6). For all PEHs, time = 0 indicates cue onset.

response of all neurons recorded in the third session was enhanced relative to the first session (Session x Time interaction, $F_{9,1944} = 4.15$, $P < 0.0001$), specifically within the 50

ms after cue onset (P < 0.003, Figure 4e; Figure 6; Table 1). These changes over sessions were predictive of behavior; increasing proportions of neurons were recruited to encode the reward-predictive cue as individual rats improved reward learning performance (Figure 4f,g).

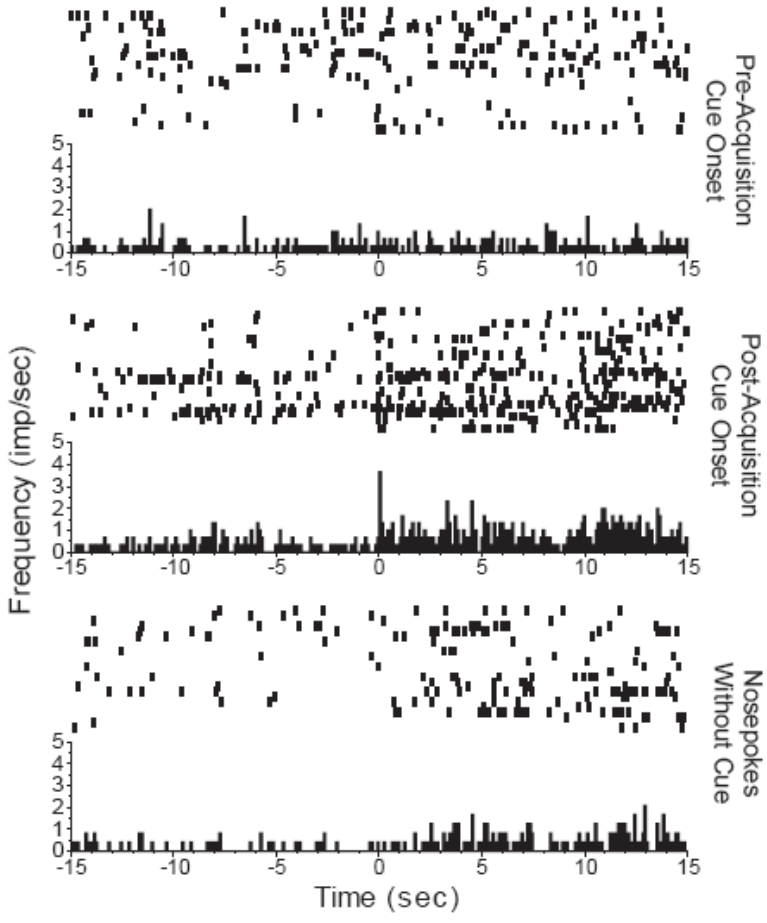


Figure 5: Perievent rasters and histograms (PERHs) from a representative LA neuron recorded during the first training session in a rat that successfully acquired the task within a single session. This neuron does not have a phasic response to the cue prior to task acquisition **(top)**, develops a phasic excitation to the cue onset after task acquisition **(middle)**, and never shows a phasic response to nosepokes alone **(bottom)**. Each mark in the raster indicates a spike. Histograms show firing rate in 100 msec bins. Reference events occur at time = 0; and are indicated to the right of each PERH.

Task Efficiency, a behavioral index defined as the number of rewards earned per number of cues presented, and Task Accuracy, a behavioral index defined as the difference in the number of correct and incorrect port entries divided by the total number of port entries, were significantly correlated (P < 0.0001, P = 0.0066, respectively; Table 2) with the percentage of neurons per rat that showed phasic responses to the reward-predictive cue (Figure 4f,g).

Control studies confirmed that the increase in cue encoding is specific to acquisition of the cue-reward association and not due to non-associative factors, such as sensitization (Figures 7 and 8). These data demonstrate that development of cue-evoked responses in the LA depends on the acquired reward-predictive nature of the cue. Further, the greater the proportion of neurons recruited to encode the reward-predictive cue, the better the rat learned the cue-reward association, and the more successful the rat was at earning rewards.

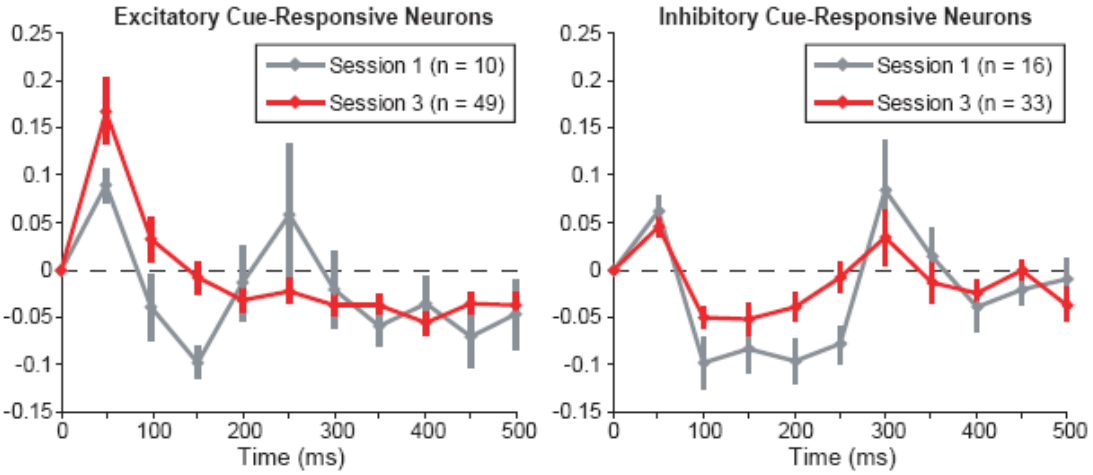


Figure 6: Population histograms of two distinct subpopulations of cue-responsive neurons displaying excitatory or inhibitory phasic responses to the onset of the cue during Session 1 and Session 3. The excitatory population response **(Left panel)** on Session 3 is significantly different from that observed on Session 1, indicated by a significant Time X Session interaction ($F_{9,513} = 1.97$, $P < 0.042$). There was also a main effect of Time ($F_{9,513} = 8.50$, $P < 0.0001$) but not Session ($F_{1,57} = 2.02$, $P = 0.16$). The interaction is accounted for by significantly greater magnitude response in the first 150 ms after cue onset ($F_{1,57} = 8.73$, $P < 0.006$) and a significant decrease in response magnitude at the 250 ms time bin ($F_{1,57} = 4.25$, $P < 0.046$). Note that the proportion of neurons with a significant excitatory response to the cue also increased from Session 1 and Session 3 (10 of 95 neurons (11%) on Session 1, relative to 49 of 123 neurons (40%) on Session 3; $P < 0.001$, Chi-square test). The inhibitory population response **(Right Panel)** on Session 3 is also significantly different from that observed on Session 1, as indicated by a significant Time X Session interaction ($F_{9,423} = 1.96$, $P < 0.043$). There was also a main effect of Time ($F_{9,423} = 10.58$, $P < 0.0001$) but not Session ($F_{1,47} = 3.61$, $P = 0.07$). The interaction is accounted for by a reduction in the response on Session 3 relative to Session 1 from 100-250 ms after cue onset ($F_{1,47} = 18.8$, $P < 0.0001$). In contrast to excitatory cells, the proportion of neurons with a predominantly inhibitory response to the cue did not change from Session 1 to Session 3 (16 of 95 neurons (17%) on Session 1 relative to 33 of 123 neurons (27%) on Session 3; $P = 0.11$, Chi-square test). Units shown are only those with significant modulation in firing rate within one or more 100 ms bins in the 500 ms after cue onset (determined as in Methods). Firing rates for each unit were then normalized and all units of that classification for that session were averaged. Because many units were bimodal, classification was determined by the direction of the response within the bin with the maximum response, measured by Z-score, within the first 250 ms after cue onset. No unit was assigned to both excitatory and inhibitory categories. Depicted are the mean ± SEM; Session 1 (grey, n = 6 rats) and Session 3 (red, n = 7 rats). Number of neurons indicated in legends.

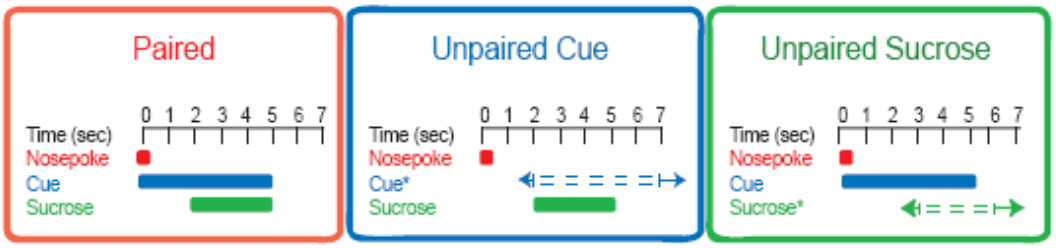
Rat ID	Session	Number of Cue-Responsive Neurons	Total Neurons Recorded	% of Cue-Responsive Neurons
A	1	3	9	33%
B	1	3	16	19%
C	1	No Data due to System Noise		
D	1	4	18	22%
E	1	6	13	46%
F	1	10	31	32%
G	1	0	8	0%
Total	1	26	95	27%
A	2	2	6	33%
B	2	3	10	30%
C	2	2	3	67%
D	2	4	29	14%
E	2	6	14	43%
F	2	16	38	42%
G	2	6	8	75%
Total	2	39	108	36%
A	3	5	7	71%
B	3	10	15	67%
C	3	4	7	57%
D	3	18	30	60%
E	3	11	18	61%
F	3	20	28	71%
G	3	14	18	78%
Total	3	82	123	67%

Table 1: Proportions of lateral amygdala units with significant phasic response to cue onset across first 3 sessions of training. Tabulation of LA units with significant changes in activity to cue onset ($P < 0.01$, Wilcoxon matched pairs test) during the first 3 sessions of training. The proportion of cue-responsive units changed across sessions ($P < 0.001$, Chi-square), accounted for by a significant increase in the proportion of cue-responsive units on Session 3 as compared with both Session 1 and Session 2 ($P < 0.001$ for each, Chi-square).

Variable	Correlation Coefficient (r)	P-value
Task Efficiency	0.8449	**0.0342**
Task Accuracy	0.3445	0.5036
Number of Sucrose Deliveries	0.5134	0.2975
Number of Port Entries	0.0598	0.9104
Number of Cue Presentations	0.3276	0.5262
Number of Nosepokes Emitted	0.2497	0.6332

Table 2: Correlations between the percent of neurons that were phasically responsive to the cue during the first session of training and learning indices (Task Efficiency and Task Accuracy), various behaviors or events. The behavioral index, Task Efficiency, is correlated with the recruitment of cue-responsive neurons during the early phase of task acquisition.

a



b

			Cue			Nosepoke		
	# Rats	Total neurons	% Neurons Responsive	% Neurons Developed Response	% Neurons Lost Response	% Neurons Responsive	% Neurons Developed Response	% Neurons Lost Response
Paired	n = 4	67	46%	24%	9%			
Unpaired Cue	n = 4	96	27%	5%	15%	29%	10%	4%
Unpaired Sucrose	n = 5	80	26%	8%	8%			

Figure 7: The development of neural responses to the cue depends upon its reward-predictive properties. To test the hypothesis that the acquired neuronal response to the cue depends upon the reward-predictive properties of the cue, we recorded extracellular spike activity from LA neurons during the first session of appetitive training in three separate groups of rats for which the cue-reward contingency varied. **a**, A schematic of the behavioral paradigm for each group. In the Paired group (n=4 rats, 67 neurons) each nosepoke was followed by a cue and (after a 2 sec delay) a 0.1 ml 10% sucrose reward delivered to a port adjacent to the nosepoke operandum. In the Unpaired Cue group (n=4 rats, 96 neurons), each nosepoke was reinforced with sucrose and the cue was presented randomly. In the Unpaired Sucrose group (n=5 rats, 80 neurons), each nosepoke was reinforced by the cue and the sucrose was delivered randomly. **b**, Table of percentages of significant responses of individual neurons to the cue or, in subjects with a random cue, to the performance of the nosepoke, measured over the first 500 msec after cue onset/nosepoke emission. The presence of the cue-reward contingency in the Paired group led to higher numbers of cue-responsive neurons, when the presence of a cue response was examined ($P < 0.015$, overall Chi-square test; Paired vs. Unpaired Cue, $P < 0.02$; Paired vs. Unpaired Sucrose, $P < 0.02$), as well as to significantly more neurons that developed a response to the cue upon within-session acquisition ($P < 0.001$, overall Chi square test; Paired vs. Unpaired Cue, $P < 0.002$; Paired vs. Unpaired Sucrose, $P < 0.012$). The proportions of neurons that lost responses to the cue did not vary among groups ($P = 0.27$, Chi-square test). The behavioral response of performing the nosepoke alone did not result in the same rapidly-occurring recruitment of neural responses as seen with the cue upon behavioral demonstration of acquisition of the response-outcome association: in the Unpaired Cue group, the number of neurons that developed a response to the nosepoke was significantly less than the number that developed a response to the Cue in the Paired group ($P < 0.04$, Chi-square test). The presence of significant phasic responses to cue onset or nosepoke emission were assayed using the Wilcoxon signed rank and Mann-Whitney U tests as described in Methods. Acquisition was defined as 80% correct responding in a moving 5-trial block and correct responding was defined as movement to the port within 10 sec of cue onset (Paired group), nosepoke emission (Unpaired Cue group), or sucrose delivery (Unpaired Sucrose group).

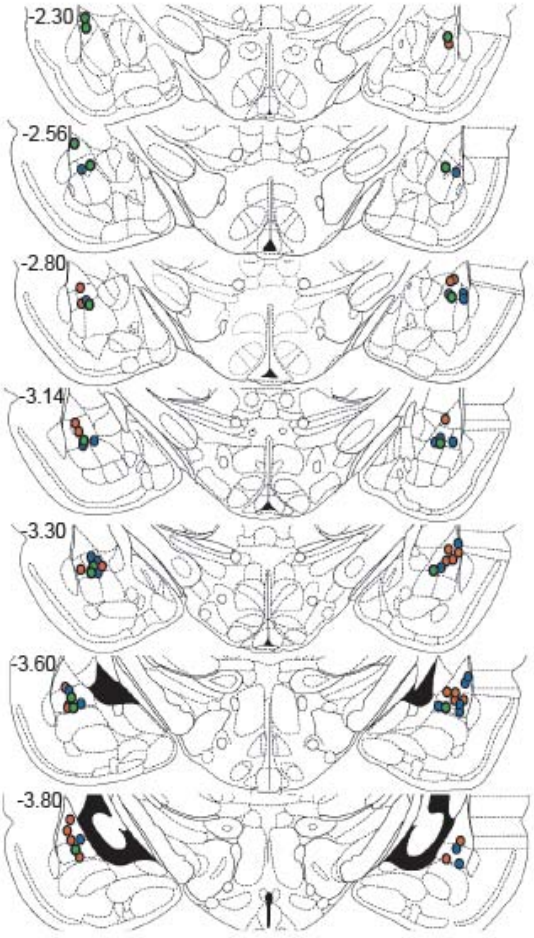


Figure 8: Schematic diagram illustrating the histological verification of electrode tip placements for the electro-physiological recording experiments described in Figure 7. Coronal diagrams showing chronic recording electrode tip placements. Colors indicate group electrode tips of group, as indicated in legend. Numbers on left indicate the anteroposterior coordinates caudal to bregma (Paxinos, G. and Watson, C. The rat brain in stereotaxic coordinates. Academic Press, 1998).

● = electrode tip of Paired Group
● = electrode tip of Unpaired Cue Group
● = electrode tip of Unpaired Sucrose Group

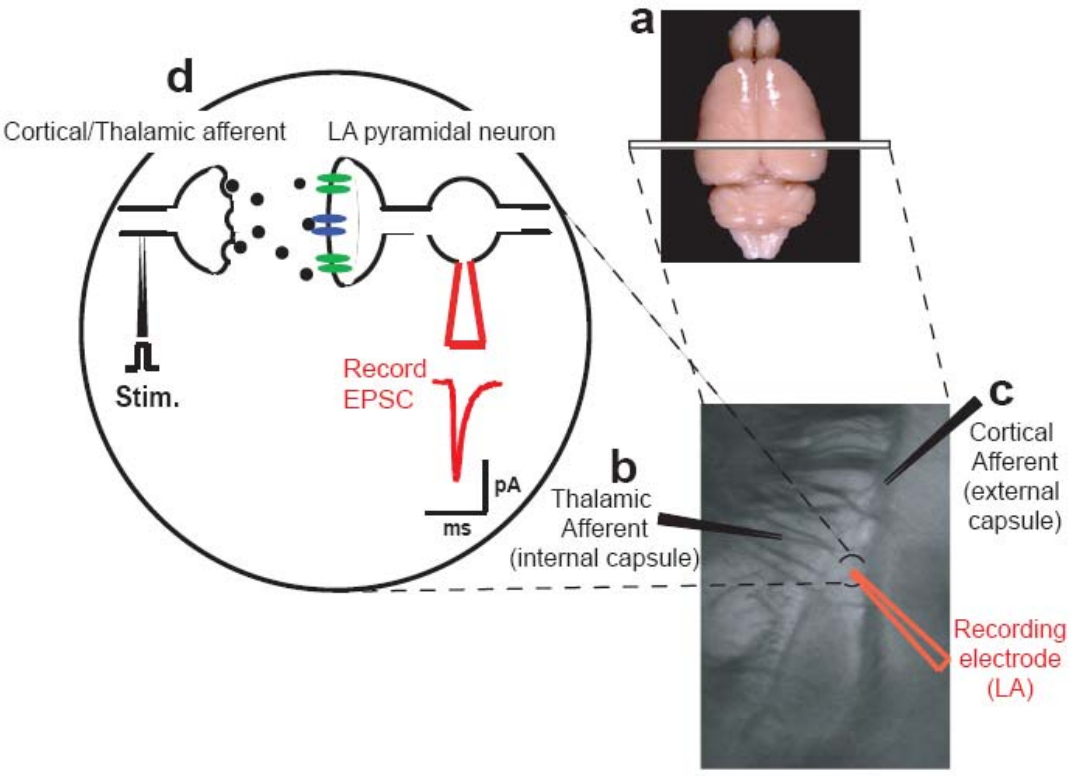


Figure 9: Schematic representation of the *ex vivo* experimental methods. For the experiments described in Figures 11 and 12, rats were trained and then sacrificed. For the experiments described in Figure 14, rats received intra-LA infusions, were trained, and subsequently sacrificed. **a**, Coronal sections containing the LA were collected and prepared for whole-cell recordings. Either the internal or external capsules, were respectively used to stimulate thalamic (**b**) or cortical (**c**) afferents to evoke EPSCs in LA neurons(**d**). Bottom right image is a photograph of a coronal section containing the LA at low-magnification, under infrared light and differential interference contrast. Darker grey lines correspond to fiber tracts.

Since our *in vivo* recordings exhibited rapidly-occurring changes in cue-related firing in the LA during successful cue-reward learning, we hypothesized that the mechanism underlying these changes was an increase in synaptic strength of thalamic or cortical sensory afferents onto LA neurons; we tested this hypothesis using *ex vivo* experimentation (Figure 9). Rats were trained on a single session of the same behavioral paradigm and classified as Learners (top 50%) or Non-learners (bottom 50%) as defined

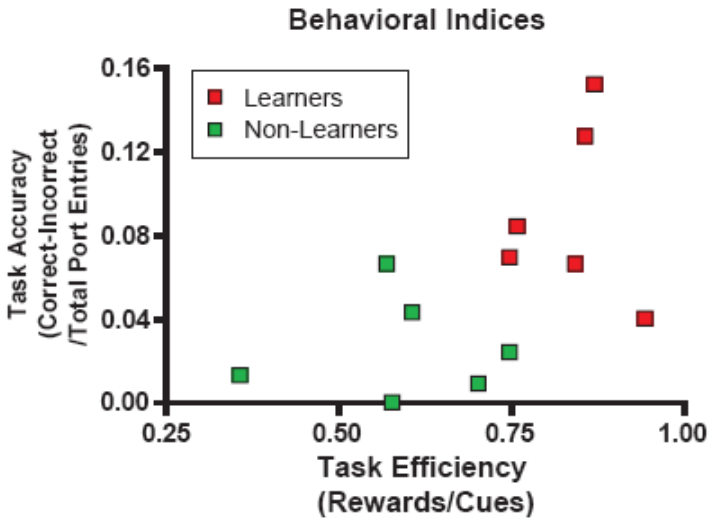


Figure 10: Scatterplot depicting individual rat performances and their classification as either Learners or Non-Learners. These rats contributed to data shown in Figure 11. In this experiment, as well as overall, Task Efficiency and Task Accuracy are highly correlated measures of learning. Rats were classified as Learners (red squares) or Non-Learners (green squares) by separating the top 50% (Learners) and the bottom 50% (Non-Learners) of performers based on the sum of the behavioural indices. To verify that Non-Learners after a single session were capable of showing the same synaptic changes seen in Learners, 2 additional Non-Learner rats were run on a second session on the following day. Both rats acquired the task on the second day and had similar AMPAR/NMDAR ratios and mEPSCs to rats that were Learners

by our learning indices of Task Efficiency and Task Accuracy (Figure 10). Any unearned sucrose was delivered in the home cage immediately after the session, ensuring that all rats received the same amount of sucrose. Rats were sacrificed within 30 minutes after session end for LA acute slice preparation. We stimulated the internal or external capsule to evoke excitatory postsynaptic currents (EPSCs) from thalamic or cortical afferents, respectively, and used whole-cell patch-clamp techniques within visually-identified

pyramidal neurons to measure EPSCs containing AMPA receptor (AMPAR)- and NMDA receptor (NMDAR)-mediated currents from pyramidal neurons. EPSCs were evoked while holding neurons in voltage-clamp at +40 mV in the absence and then the presence of the NMDAR antagonist AP5 (D(-)-2-amino-5-phosphonovaleric acid; 50 μM). We found that the AMPAR/NMDAR ratio, an index of glutamatergic synaptic strength (Perkel and Nicoll, 1993; Ungless et al., 2001), varied with task performance and afferent (main effects of Group, $F_{2,29} = 11.01$, $P < 0.001$; Afferent, $F_{1,29} = 22.13$, $P < 0.001$; Group x Afferent interaction, $F_{2,29} = 7.38$, $P < 0.004$) such that Learners exhibited a larger AMPAR/NMDAR ratio at thalamic ($P < 0.001$; Learners: 1.03 ± 0.04; Non-Learners: 0.58 ± 0.08; Naives: 0.47 ± 0.05), but not cortical (Learners: 0.45 ± 0.08; Non-Learners: 0.46 ± 0.10; Naives: 0.47 ± 0.04) synapses in the LA relative to Non-learners and Naives, which did not differ from each other ($P = 0.84$; Figure 11a,b). We determined the correlation between each rat's behavioral performance, as measured by either Task Efficiency or Task Accuracy, and AMPAR/NMDAR ratio and found a significant positive relationship at thalamic ($P = 0.0003$, $P = 0.006$, respectively), but not cortical ($P = 0.89$, $P = 0.55$, respectively), inputs (Figure 11c-f; Table 3). Hence, thalamoamygdalar synaptic strength predicted the success of individual rat's reward learning performance.

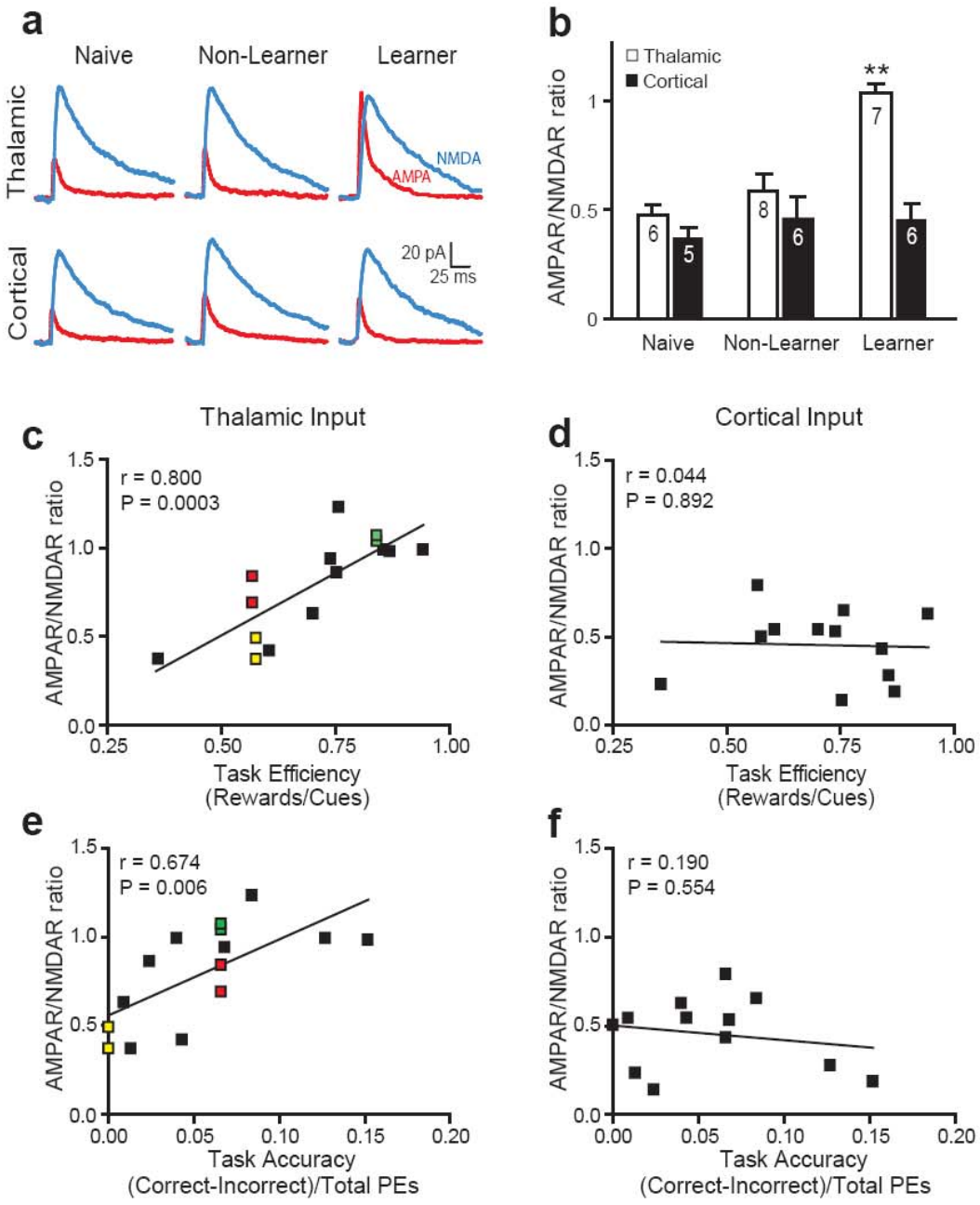


Figure 11. Degree of AMPAR/NMDAR enhancement predicts cue-reward learning. **a**, EPSCs evoked via stimulation of thalamic or cortical afferents in rats that were Naive, Non-Learners or Learners. **b**, AMPAR/NMDARs evoked from thalamic afferents were significantly increased in Learners (n = 6 rats) as compared to Non-Learners (n = 6 rats) or Naives (n = 5 rats). Number of cells indicated in bars for all figures. **P < 0.001, significant difference from other groups, as well as from cortical afferent. **c-f**, Correlation between AMPAR/NMDAR ratio and either (c, d) Task Efficiency or (e, f) Task Accuracy for EPSCs evoked from thalamic, but not cortical, pathways, same subjects as in (b). Colors indicate multiple cells recorded from same rat, black indicates single cells recorded per rat.

Variable	Thalamic		Cortical	
	r	P-value	r	P-value
Task Efficiency	0.8000	0.0003	0.0440	0.8920
Task Accuracy	0.6740	0.0060	0.1900	0.5540
Total Number of Sucrose Deliveries	0.7170	0.0026	0.0754	0.8160
Total Number of Port Entries	0.6890	0.0044	0.0978	0.7625
Total Number of Cues Presented	0.6440	0.0096	0.0241	0.9408
Total Number of Nosepokes Emitted	0.4651	0.0807	0.0848	0.7932

Table 3: **Task Efficiency is the best predictor of AMPAR/NMDAR ratio evoked from thalamic afferents to the LA.** Correlation coefficients (r) and statistical significance (P) between the AMPAR/NMDAR ratios representing the glutamatergic strength of thalamic and cortical synpases on LA neurons after a single training session and learning indices (Task Efficiency and Task Accuracy), various behaviors or events (data shown in Figure 11).

A change in the relative contribution of AMPARs and NMDARs to compound EPSCs may reflect an increase in AMPAR currents and/or a decrease in NMDAR currents at thalamoamygdalar synapses. To determine whether AMPAR currents were modified during reward learning, we examined AMPAR-mediated miniature EPSCs (mEPSCs), which reflect spontaneously released vesicles of glutamate (Malenka and Nicoll, 1999).

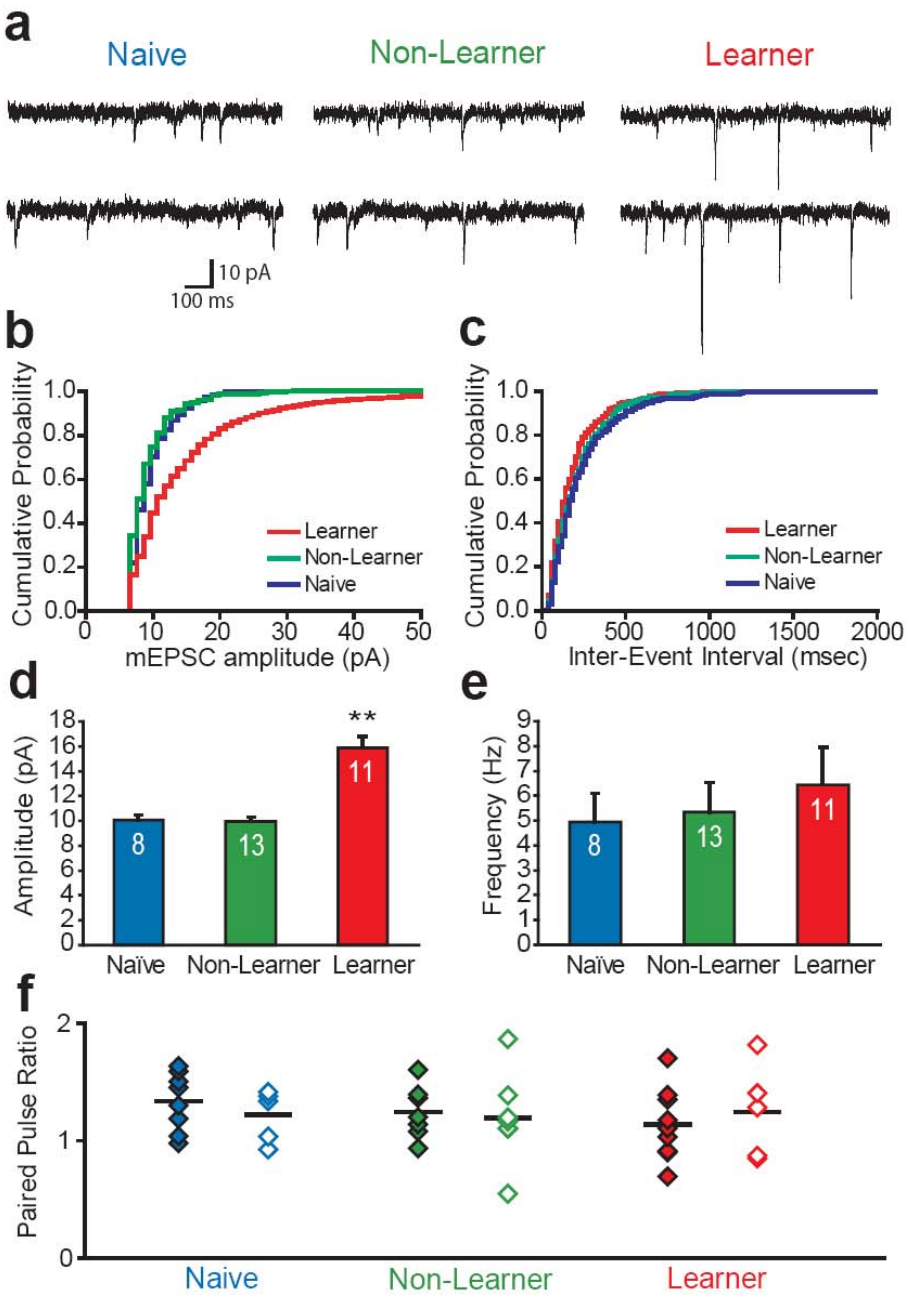


Figure 12. Successful cue-reward learning induces an increase in mEPSC amplitude but not frequency or paired pulse ratio. a, Sample mEPSCs from rats that were Naive, Non-Learners or Learners. **b, c,** Respectively, cumulative probability plots of amplitude and frequency for representative neurons from each group; 1 pA and 20 ms bins. **d, e,** Learners (n = 6 rats) had increased mEPSC amplitude relative to Non-Learners (n = 6 rats) and Naive rats (n = 5 rats), but not frequency . **P < 0.001. **f**, No change in paired-pulse ratio, filled and clear diamonds represent ratios evoked from the thalamic and cortical pathways, respectively; horizontal line is the mean.

Typically, an increase in mEPSC amplitude indicates an increase in postsynaptic AMPAR number or function, while an increase in mEPSC frequency indicates an increase in the probability of transmitter release (P_r) or number of synapses (Malenka and Nicoll, 1999). mEPSC amplitude was related to task performance ($F_{2,29} = 30.75$, $P < 0.001$), with a greater mean amplitude from LA neurons of Learners ($P < 0.001$; 15.88 ± 0.89 pA) relative to Non-learners (9.98 ± 0.29 pA) or Naives (10.05 ± 0.39 pA), which did not differ from each other ($P = 0.87$; Figure 12a,b,d). In contrast, the mean mEPSC frequency was not different ($F_{2,29} = 0.5$, $P = 0.61$) among Learners (6.45 ± 1.48 Hz), Non-Learners (5.36 ± 1.16 Hz) and Naives (4.96 ± 1.14 Hz) (Figure 12a,c,e). To further examine whether learning altered P_r, we examined the paired-pulse ratio (Hess et al., 1987; inter-stimulus interval, 50 ms; Figure 12f). There was no change in the paired-pulse ratio for either afferent ($F_{1,33} = 0.02$, $P = 0.89$) among Naives, Non-learners or Learners (main effect of Group, $F_{2,33} = 0.35$, $P = 0.71$; Group x Afferent interaction, $F_{2,33} = 0.40$, $P = 0.67$), indicating that learning does not cause an immediate change in P_r, and the rapid increase in AMPAR/NMDAR ratio is mediated postsynaptically.

The induction of associative long-term potentiation (LTP) in the LA depends on NMDAR activation (Humeau et al., 2003; Shin et al., 2006), which can lead to increases in AMPAR currents (Malenka and Nicoll, 1999). Additionally, NMDAR-blockade within the BLA impairs acquisition, but not performance, in two similar appetitive tasks (Burns et al., 1994; Baldwin et al., 2000). To test whether the learning-induced synaptic changes we observed are NMDAR-dependent, we locally infused the NMDAR-

antagonist AP5 (3μg/side) or vehicle (artificial cerebrospinal fluid; aCSF) into the LA bilaterally prior to training (Figures 13 and 14).

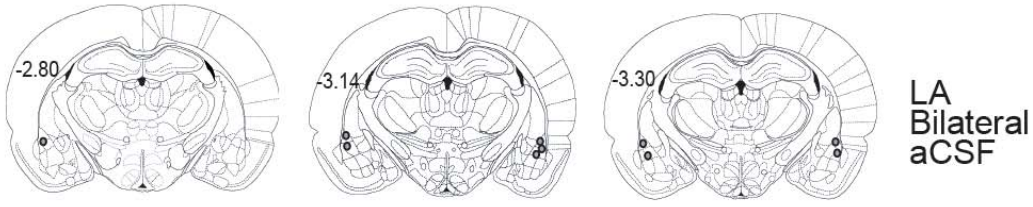


Figure 13: **Coronal diagrams showing histologically verified locations of the infusion tips for rats receiving pre-training bilateral aCSF infusions.** Numbers on left indicate the anteroposterior coordinates caudal to bregma (Paxinos and Watson, 1998).

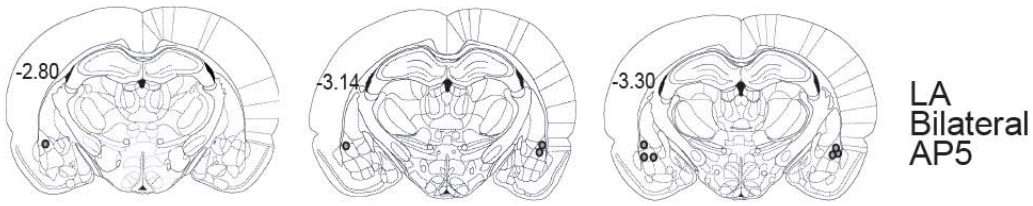


Figure 14: **Coronal diagrams showing histologically verified locations of the infusion tips for rats receiving pre-training bilateral AP5 infusions.** Numbers on left indicate the anteroposterior coordinates caudal to bregma (Paxinos and Watson, 1998).

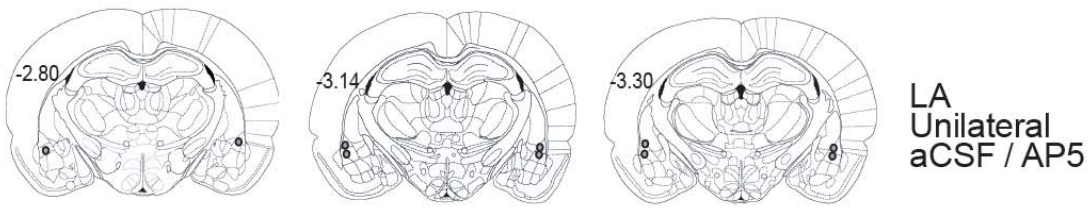


Figure 15: **Coronal diagrams showing histologically verified locations of the infusion tips for rats given pre-training unilateral infusions of AP5.** Numbers on left indicate the anteroposterior coordinates caudal to bregma (Paxinos and Watson, 1998).

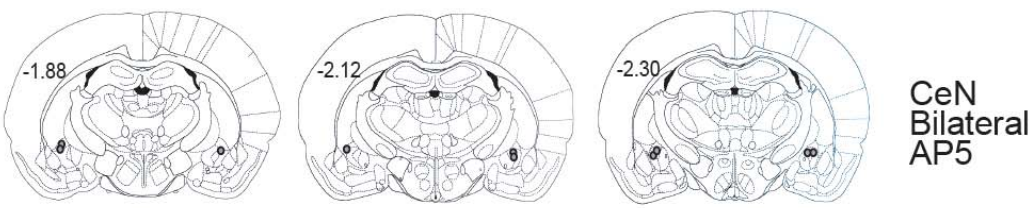


Figure 16: **Coronal diagrams showing histologically verified locations of the infusion tips for rats receiving pre-training infusions.** Numbers on left indicate the anteroposterior coordinates caudal to bregma (Paxinos and Watson, 1998).

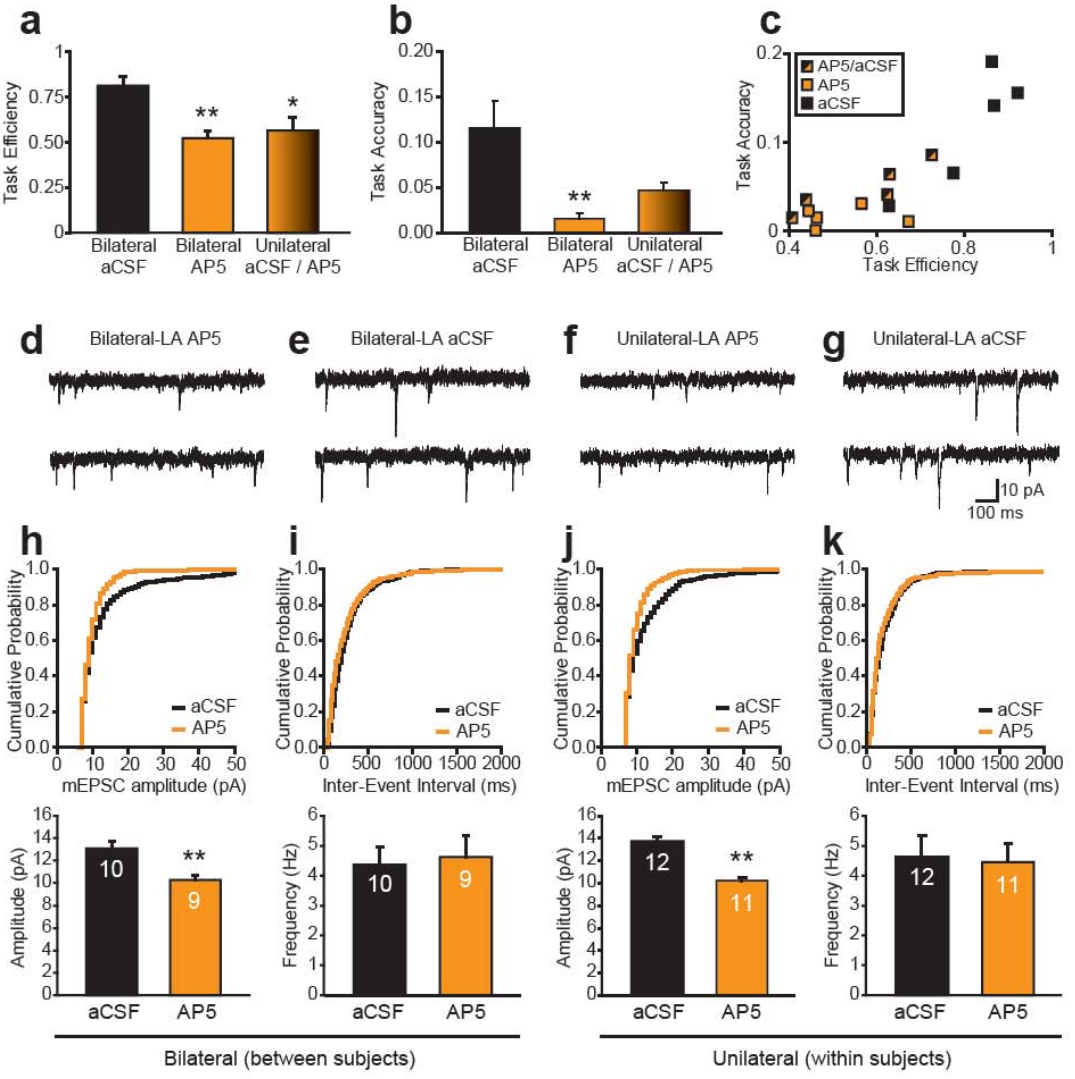


Figure 17. Local NMDAR-blockade attenuates reward-related learning and the associated increase in mEPSC amplitude. a, b, Measures of task performance among groups (n = 5 rats per group). Task Efficiency (a) is decreased following either unilateral or bilateral AP5 intra-LA infusion, while Task Accuracy (b) is decreased following bilateral AP5 (*P < 0.05, **P < 0.009, compared to aCSF). No other comparisons were significant. **c,** Individual rat performances based on Task Efficiency and Task Accuracy. **d-g,** Sample mEPSCs from rats that received pre-training infusions. **d, e,** Traces recorded from rats that received bilateral infusions of AP5 and aCSF, respectively. **f, g,** Traces recorded from a representative rat that received unilateral intra-LA infusions of AP5 and aCSF, respectively. **h-k**, Cumulative probability plots of amplitude (h, j) and frequency (i, k) for mEPSCs in representative cells from rats receiving bilateral infusions of AP5 or aCSF (h, i) or unilateral infusions of AP5 and aCSF (j, k). Below each probability plot is the corresponding bar graph indicating the group mean ± SEM. There is a difference in amplitude (h,j), but not frequency (i,k), for both bilaterally infused (h) and unilaterally infused (j) rats; **P < 0.001, compared to aCSF. **g,** No difference in mEPSC frequency between groups.

To control for the possibility that synaptic changes might be secondary to, rather than causal for, reduced behavioral performance, we included a group of rats which received unilateral intra-LA infusions of AP5 and contralateral infusions of aCSF to provide a within-animal control (Figure 15). Task Efficiency was impaired by AP5 ($F_{2,12} = 9.03$, $P < 0.005$) following both bilateral ($P < 0.007$) and unilateral ($P < 0.018$) intra-LA pre-training infusions (Figure 17a,c); bilateral, but not unilateral, intra-LA infusions of AP5 also impaired Task Accuracy ($F_{2,12} = 7.38$, $P < 0.009$; aCSF vs. bilateral AP5, $P < 0.009$; Figure 17b, c). Importantly, the effect of AP5 was not attributable to spread of drug into the neighboring central nucleus of the amygdala (CeN; Figures 16 and 18).

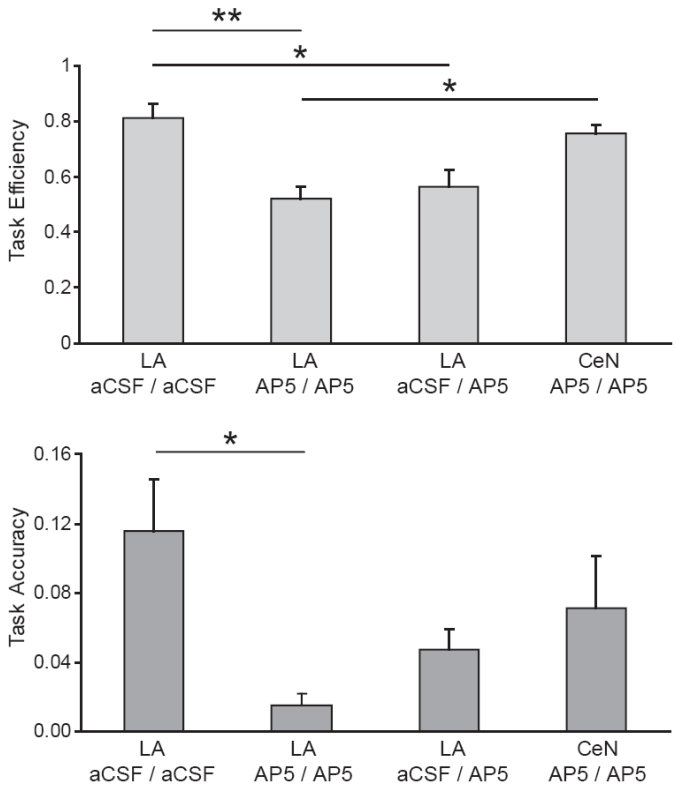


Figure 18: Bilateral intra-CeN infusions of AP5 demonstrate that the behavioral impairment seen with bilateral intra-LA infusions of AP5 are not attributable to spread of drug to CeN. Here we show the behavioral data for rats receiving pre-training bilateral intra-CeN infusions of AP5 as measured by Task Efficiency **(Top)** and Task Accuracy **(Bottom)**, and we show again the data from Figure 14a,b for comparison. For Task Efficiency ($F_{3,16} = 8.78$, $P < 0.002$), rats receiving bilateral infusions of AP5 into the CeN performed significantly differently from rats receiving bilateral AP5 intra-LA infusions ($P < 0.002$). For Task Accuracy ($F_{3,16} = 3.37$, $P < 0.05$), the main effect of group is due to the difference between the LA aCSF/aCSF and LA AP5/AP5 groups. The CeN group was not significantly different from any other group, although there is a tendency for this measure to be decreased that may attain significance with additional rats. We show that AP5 injected into the CeN does not cause a significant impairment relative to bilateral aCSF infusions into the LA, which eliminates the possibility that the behavioral impairments seen by bilateral infusion of AP5 into the LA were due to the spread of the drug to the CeN (*$P < 0.05$, **$P < 0.01$).

Following intra-LA infusions and the training session, these rats were sacrificed for acute slice preparation. Rats which received bilateral intra-LA infusions of AP5 showed a lower mean mEPSC amplitude ($P = 0.003$; 10.26 ± 0.41 pA; Figure 17d, h) than following aCSF infusions (13.09 ± 0.68 pA; Figure 17e, h), while there was no change in mEPSC frequency between groups ($P = 0.66$; Figure 17d, e, i). The decrease in Task Efficiency and the reduction in mEPSC amplitude following local infusion of an NMDAR antagonist suggest that cue-reward learning and the associated increase in AMPAR number or function are NMDAR-dependent. By comparing mEPSCs from rats with unilateral intra-LA AP5 infusions and contralateral aCSF infusions, we were able to determine with confidence that any differences between LA neurons treated with AP5 or aCSF are due to local NMDAR-blockade rather than an AP5-induced difference in task performance. Within subjects, we found that the amplitude of LA mEPSCs recorded after AP5 infusion into the LA on one side was significantly lower ($P < 0.001$; Figure 17f, j) relative to aCSF infusion on the contralateral side (Figure 17g, j), while there was no difference in frequency ($P = 0.99$; Figure 17f, g, k). Therefore, local NMDAR-blockade attenuates the learning-dependent increase in postsynaptic AMPAR currents and impairs the acquisition of reward-directed behavior.

DISCUSSION

These results show that, with cue-reward learning, cue-responsive neurons are rapidly recruited *in vivo*, thalamoamygdalar synapses are selectively strengthened, and LA neurons show NMDAR-dependent increases and associated potentiation of AMPAR number or function. This series of experiments represents the first time that *in vivo* and *ex vivo* electrophysiological techniques have been combined with *in vivo* pharmacology to dissect the neural mechanisms underlying a single behavioral phenomenon.

Remarkably, we observed a rapidly-occurring near-maximal peak of cue-evoked phasic activity in the trials immediately before some rats reached acquisition criterion (Figure 4c). I speculate that this initial peak in neural activity represents the moment of realization that the cue predicts the reward, which in layman's terms might be described as an "Aha!" moment. Prior to this realization, the contingencies between the operant response, the cue and sucrose reward may be unclear. Perhaps upon the initial recognition of the contingency between the cue and the reward, there is a burst of phasic activity followed by a high proportion of correct trials. While behavior can continue to improve with increased training, there appears to be a marked, nearly step-wise increase in cue-evoked phasic activity accompanied by an improvement in task performance. From this information, we can infer that learning occurs in three primary phases. First, "pre-acquisition" is characterized by a period of random behavior that is goal-oriented, but in a trial-and-error fashion that is not efficient. Second, "acquisition" is a transient period lasting only a few trials during which the animal realizes that there may be a contingent relationship between the cue and the reward, and during these few trials, the

rat verifies that this relationship exists. Third, "post-acquisition" is the period after the realization that the cue-reward association exists during which the rat begins to optimize its task performance to maximize the efficiency with which it earns these rewards.

We felt it was important to verify that the co-variation of task performance and synaptic strength was not confounded by genetic differences or neural predispositions. To do this, we took rats (n = 2, data not shown) that, after the initial training session, would have been characterized as Non-learners and ran them on the same paradigm the following day. Both rats successfully acquired the task on the following day and upon *ex vivo* examination of synaptic strength, found that there synaptic strength enhancement resembled those of rats that had been classified as Learners after one training day. Therefore, we can conclude that the synaptic strength enhancements that we observed were not already present and increasing the animal's ability to learn more quickly, but that these changes occurred acutely as a result of learning.

The parallel emergence of increased synaptic strength and cue-related firing in LA neurons during reward learning suggests that this excitatory synaptic increase contributes to enhanced spike activity of LA neurons in response to the conditioned stimulus, driven by auditory and visual thalamic inputs that terminate in the LA (LeDoux et al., 1990; Romanski et al., 1993; Doron and Ledoux, 1999; LeDoux, 2003). However, it is noteworthy that the proportion of cells recorded *in vivo* that developed a response to the reward-predictive cue is less than the proportion of cells that showed enhanced synaptic strength upon learning (Figure 11c). This suggests that the integration of multiple

inhibitory and excitatory synapses upon a given cell may constrain cue-related spike firing (Rosenkranz and Grace, 2002; Rosenkranz et al., 2003; Samson and Pare, 2006), even if that cell possesses enhanced thalamic inputs. This discrepancy may be explained by our experimental procedues, in which the thalamic pathway is under strong inhibitory suppression (Rosenkranz et al., 2003; Shin et al., 2006) *in vivo*, whereas our *ex vivo* recordings were performed under $GABA_AR$ antagonism to isolate EPSCs.

Consistent with our results, auditory fear conditioning, which requires an intact LA (Davis, 1992; LeDoux, 1996, 2003; Rosenkranz et al., 2003; Maren and Quirk, 2004; Shin et al., 2006) increases neuronal firing in response to a shock-predictive cue and potentiates transmission at thalamoamygdalar synapses (McKernan and Shinnick-Gallagher, 1997), via an NMDAR-dependent mechanism, likely a result of postsynaptic AMPAR trafficking (Rumpel et al., 2005). Previous work in fear conditioning suggests that plasticity also occurs at cortical (Tsvetkov et al., 2002) synapses in the LA although this enhancement was found at later time points than tested here. Single unit recordings in the LA show that the thalamic pathway conditions more rapidly than the cortical pathway during fear conditioning (Quirk et al., 1997; Schoenbaum et al., 1999; LeDoux, 2003). Additionally, studies suggest that plasticity in the LA typically precedes plasticity in the thalamus (Maren et al., 2001) or cortex (Edeline and Weinberger, 1992; Schoenbaum et al., 1999; LeDoux, 2003) .

Interestingly, while the LA has strong reciprocal projections with the medial prefrontal and orbitofrontal cortices, it only receives uni-directional projections from the thalamus

(Pitkanen, 2000). Since it has been shown that thalamic (Komura et al., 2001; Komura et al., 2005) and cortical (Mulder et al., 2003; Weinberger, 2007) neurons can encode the acquired significance of sensory stimuli, it would be interesting to explore how the early amygdalar plasticity that we observed is related to plasticity in other regions. Our findings, viewed in the context of fear conditioning, prompt further experimentation to determine whether rapidly-occurring reward-learning induced plasticity at thalamo-amygdalar synapses facilitates subsequent consolidation at other sites (McGaugh, 2002). It is likely that this plasticity allows for amygdala neurons to selectively respond to meaningful environmental stimuli and transmit this information to downstream brain regions for the expedited selection of an adaptive behavioral output.

In summary, these findings indicate that rapid synaptic changes in the LA occur during the early stages of cue-reward learning, and that the degree of neuronal recruitment and synaptic strength enhancement correspond to the degree of learning, as measured by behavioral performance. In the past three decades since the discovery of LTP, researchers have struggled to prove that synaptic plasticity is the cellular mechanism of learning and memory. These findings represent one of the strongest pieces of evidence to date that synaptic plasticity is the underlying cellular mechanism of learning. The importance and novelty of these experiments lies not only in the specific results that we present, but also in the general principles that this series of experiments helps establish, since we are the first to have combined *in vivo* and *ex vivo* electrophysiology with pharmacology to investigate a specific and acute behavioral phenomenon. For example, we are the first to show that local NMDAR-antagonism *in vivo* attenuates learning-

induced synaptic plasticity *ex vivo*. We have also established a causal relationship between synaptic plasticity and goal-oriented behavior, which broadens the extensive research pioneered in fear conditioning.

METHODS

Behavioral training

Adult male Sprague-Dawley rats (250-350 g) were food restricted to 90% of free-feeding body weight. Training session length was varied as follows: rats with chronic electrodes were trained daily for 3 hr per session, for 3 sessions; rats with cannulae were trained for one 4 hr session to allow enhanced opportunity to express learning within a single session; and rats with no prior surgery were trained for one 2 hr session, the median time required for task acquisition. Nosepoke responses were reinforced on approximately 50% of trials with a subsequent (onset 50 ms after nosepoke) light-tone cue, 3 kHz tone at 80 dB and illumination of two 5-s stimulus lights. 2 s after the nosepoke, 0.1 ml of 15% sucrose was delivered to a port adjacent to the nosepoke operandum over 3 s. Additional rewards could not be earned until the prior sucrose was consumed (as determined by port entries). Therefore, to maintain contingency between the cue and the reward, whenever sucrose was present in the reward port, all nosepoke responses were paired with the cue. Hence, early learning sessions tended to result in higher percentages of nosepokes that were co-presented with the cue (Mean = 56%; Max = 70%; Min = 39%), and in higher numbers of cue presentations than sucrose deliveries. Task acquisition was defined by >80% correct trials in a moving 5-trial block. A correct trial was defined as a nosepoke yielding a cue presentation and subsequent port entry (within 10 seconds or before performing a different behavior). Incorrect trials were defined as entering the port after a nosepoke without the cue. Behavioral indices were calculated per session. The behavioral indices, Task Efficiency and Task Accuracy, measured distinct aspects of reward learning success. Task Efficiency (Rewards earned / Cues Presented) measured

the strength of the cue-reward association, since each cue presentation signaled an opportunity for the rats to collect sucrose at the adjacent reward port. Task Accuracy (Correct-Incorrect / Total Port Entries) measured each rat's ability to accurately predict when sucrose would be present in the reward port. All procedures were approved by the Gallo Center Institutional Animal Care and Use Committee and were in accordance with National Institutes of Health guidelines.

In vivo electrophysiology

Rats were bilaterally implanted with fixed 8-wire electrode arrays (NeuroBiological Laboratories, Denison, TX) in the vLA (AP, -2.8 to -3.3 mm; ML, ±5.0 mm; DV, 7.2 mm) for chronic neural recording during learning in a custom operant conditioning chamber (MedAssociates, St. Albans, VT) as in Tye and Janak (2007). Neural activity was recorded, and unit discrimination was performed, with multichannel spike acquisition and sorting software (Plexon Inc., Dallas, TX). Responses of single units were deemed statistically significant if the firing rate within one or more 100 ms bins in the response window (0 – 0.5 s after cue onset) was significantly different ($P < 0.01$) from a 0.5 s baseline epoch (-2 to -1.5 s) using a Wilcoxon signed-rank test. To determine if single units developed a within-session cue response, the Mann-Whitney U test was used to compare trials from the pre- and post-acquisition epochs for 5, 100 ms bins in the first 500 ms after cue onset window. For all pre- vs. post-acquisition comparisons, the number of trials chosen was determined by the epoch (pre- vs. post-acquisition) with the fewest trials, and an equivalent number of trials was randomly selected from the other epoch. For the peri-event surface plot (Figure 4c), spike counts

for each unit were converted to z-scores: $[(FR_i-FR_{mb})/SD_b]$, where FR_i is the firing rate in the i^{th} bin of the peri-event period, FR_{mb} is the mean and SD_b is the standard deviation of the firing rate of a baseline period for the entire session, using the average of all trials in the session for the baseline (between 1.5 and 2 seconds prior to the event). Each unit was then smoothed by averaging each trial with its neighboring trials (± 1) and units were averaged to construct a peri-event surface plot (MATLAB, Mathworks, Natick, MA) showing the activity of all recorded units (n = 13) from one rat as the task was acquired. For the population peri-event histogram shown in Figure 4e, Z-scores were calculated in 50 ms bins for each individual neuron, using a baseline that included the response window for each trial, and averaged to reveal the population response for the first and third sessions.

***Ex vivo* electrophysiology**

Within 30 minutes after session end, rats were anesthetized with 40 mg/kg pentobarbital and transcardially perfused with 30 ml of modified aCSF (kept at a temperature of about 1°C) for perfusion containing (in mM): 225 sucrose, 126 NaCl, 2.5 KCl, 1.0 NaH_2PO_4, 4.9 $MgCl_2$, 0.1 $CaCl_2$, 26.2 $NaHCO_3$, 1.25 glucose; 3 kynurenic acid. Coronal sections containing the LA (320 μm) were collected in a holding chamber (superfusion solution, saturated with 95% O_2 and 5% CO_2, containing (in mM): 126 NaCl, 2.5 KCl, 1.0 NaH_2PO_4, 1.3 $MgCl_2$, 2.4 $CaCl_2$, 26.2 $NaHCO_3$, 11 glucose, 1 ascorbic acid at 32-34°C)), to recover for ~1 hr before recording with the same superfusion solution without ascorbic acid but with 0.1 Picrotoxin. Recordings were made from visually-identified pyramidal neurons in the ventral aspect of the LA. Recording electrodes (2.8-4.0 MΩ) were filled

with (in mM): 120 cesium methansulfonate, 20 HEPES, 0.4 EGTA, 2.8 NaCl, 5 TEA-Cl, 2.5 MgATP, and 0.25 NaGTP (pH 7.25-7.4; 280-290 mOsm). Series resistance (10-20 MΩ) and input resistance were monitored online. EPSCs were filtered at 2 kHz and collected using custom scripts written in IgorPro software (Wavemetrics, Lake Oswego, OR). AMPAR/NMDAR ratio was calculated by averaging 20-30 EPSCs at +40mV before and after application of the NMDAR blocker AP5 (50 μM) for 5 min. NMDAR responses were calculated by subtracting the average response in the presence of AP5 from that seen in its absence. Similar to previous studies (Shin et al., 2006; Rumpel et al., 2005; Tsvetkov et al., 2002), electrical stimulation was applied to the internal capsule to evoke EPSCs in LA neurons from thalamic afferents (Doron and LeDoux, 1999), and the external capsule to evoke EPSCs from cortical afferents (McDonald, 1998). In each rat from which a thalamic AMPAR/NMDAR ratio was recorded, a cortical AMPAR/NMDAR ratio was also recorded. mEPSC traces were filtered at 1 kHz, collected using Clampex (Molecular Devices, Sunnyvale, CA) and analyzed using Mini Analysis Progam (Synaptosoft, Decatur, GA). AMPAR mEPSCs were recorded in cells voltage clamped at -70 mV and in continual presence of lidocaine (500 μM) over 5 min; 300 events were analyzed per cell (detection criterion set at > 7 pA). Behavioral performance was not calculated until after whole-cell recordings were analyzed.

Intra-LA infusions

Rats were implanted with cannulae just dorsal to the LA (AP, -2.8 to -3.3 mm; ML, ±5.0 mm; DV, 7.0 mm). 1 week later, rats received sham infusions of aCSF 24 hours prior to the training session. 10-15 minutes prior to the training session, aCSF or AP5 (0.4 μl/side

aCSF or 3μg/0.4 μl/side AP5; 0.1 μl/minute) was infused bilaterally. Following training, brains were prepared for whole-cell recordings as above, after careful removal of the cannulae head stages. Cannulae placements were visualized during slice recording session with an upright microscope using infrared illumination (Figure 9). An additional group received cannulae in the CeN (AP, -1.8 to 2.3; ML, ±4.6; DV, 7.0) for AP5 infusion.

Statistical Analyses

Group values are expressed as means ± SEM. Statistical significance of multiple group data was assessed using one- or two-way ANOVAs followed by Bonferroni post-hoc tests when indicated by significant main effects or interactions; two group data were analyzed with two-tailed Student's *t*-tests. All correlations were analyzed with Pearson's correlation test.

The data shown in this chapter have been published by Tye, Stuber, de Ridder, Bonci and Janak in 2008 in *Nature.* Below we have reproduced the author contributions and acknowledgements for this publication.

AUTHOR CONTRIBUTIONS

K.M.T. performed the experiments and analyzed the data, with assistance and training in whole-cell recording from G.D.S., who performed pilot mEPSC experiments. B.R. performed cannula surgeries and trained K.M.T. in microinjection techniques. A.B. and P.H.J. provided mentorship and resources. K.M.T., G.D.S., A.B. and P.H.J. contributed to study design, results analysis, interpretation and manuscript writing.

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CHAPTER 3

Amygdala neurons differentially encode motivation and reinforcement during cue-induced reinstatement

ABSTRACT

The presentation of reward-predictive cues can provoke strong emotions, and can therefore drive reward-seeking behaviors. Lesion studies demonstrate that the basolateral amygdala complex (BLA) is important for assigning motivational significance to sensory stimuli, but little is known about how the expression of this motivation is encoded. We used *in vivo* electrophysiology procedures to investigate how the amygdala encodes motivating and reinforcing properties of cues that induce reinstatement of reward-seeking behavior. Two groups of rats were trained to respond for a sucrose reward. The Paired group was trained with a reward predictive cue while the Unpaired group was trained with a randomly presented cue. Both groups underwent identical extinction and reinstatement procedures, during which the reward was withheld. The proportion of neurons that were phasically cue-responsive during reinstatement was significantly higher in the Paired group than in the Unpaired group. Cues that induce reward-seeking behavior can do so by acting as incentives or reinforcers. Distinct populations of neurons

responded to the cue in trials where the cue acted as an incentive, triggering a motivated reward-seeking state, or as a reinforcer, supporting continued instrumental responding. The incentive motivation encoding population of neurons extinguished in temporal agreement with a decrease in the rate of instrumental responding. The conditioned reinforcement encoding population of neurons maintained their response for the duration of cue-reinforced instrumental responding. These data demonstrate that separate populations of cue-responsive neurons in the BLA encode the motivating or reinforcing properties of a cue previously associated with a reward.

INTRODUCTION

Understanding how environmental stimuli can acquire motivating and reinforcing properties when associated with a reward is essential for improving therapeutic interventions for psychopathologies such as obsession, depression, eating disorders, and drug addiction. In humans, reward-associated cues can increase reward-seeking behavior. Specifically, presentation of cocaine-associated stimuli to abstinent cocaine addicts produces intense drug craving (O'Brien et al., 1998), physiological arousal, including increased heart rate and skin conductance (Childress et al., 1988; Ehrman et al., 1992), and amygdala activation (Childress et al., 1999), all of which may lead to relapse of drug-seeking behavior.

The ability of environmental cues to control reward-seeking behavior can be explored using animal models. Rats readily lever-press to self-administer primary reinforcers such

as sucrose, cocaine, or alcohol, and subsequently, to obtain reward-paired cues in the absence of reward (Grimm et al., 2002; Davis and Smith, 1976; Meil and See, 1996; Nie and Janak, 2003). Specifically, when animals are trained to respond for a reward that is paired with a predictive cue, and responding is subsequently extinguished by the omission of the cue and reward, presentation of the cue alone increases responding. This phenomenon is called cue-induced reinstatement and is an animal model for relapse to reward-seeking behavior (Epstein et al., 2006).

A single conditioned stimulus can function as both an incentive and a reinforcer for different behavioral or environmental contingencies (Everitt and Robbins, 2005). The reward-associated cue in the cue-induced reinstatement model is typically considered to act as a conditioned reinforcer. A conditioned reinforcer is defined as a neutral stimulus that acquires reinforcing properties when associated with primary reinforcement, and conditioned reinforcement can be measured by the ability of the cue to support instrumental responding in the absence of the primary reinforcer (Arroyo et al., 1998; Everitt and Robbins, 2000, 2005). In contrast, a conditioned incentive is defined as a neutral stimulus that acquires motivating properties when associated with primary reinforcement, measured by behaviors such as conditioned approach (Berridge and Robinson, 2003).

The basolateral amygdala complex (BLA) is critically involved in the formation and expression of associations between sensory cues, and rewarding or aversive stimuli (Davis, 1992; Gallagher, 2000; LeDoux, 1996; McGaugh, 2002). Amygdala neurons

respond to sensory stimuli paired with positive or negative outcomes (Uwano et al., 1995; Quirk et al., 1995). BLA lesions attenuate responding for a cue associated with sexual reinforcement, but do not alter sexual behavior itself (Everitt et al. 1989). Furthermore, BLA lesions do not alter cocaine self-administration, but do attenuate the ability of cocaine-associated cues to reinstate extinguished responding (Meil and See, 1997). This evidence suggests that the function of the BLA is specific to the reinforcing properties of the reward-associated cue, and does not affect reinforcing properties of the reward itself (Balleine et al., 2003).

Because the BLA is critical for stimulus-reward learning and cue-induced reinstatement, we hypothesize that BLA neurons encode the cue-reward association that endows the cue with the power to motivate responding for reward. To test this hypothesis, we used *in vivo* extracellular recording of BLA neurons during cue-induced reinstatement of reward-seeking behavior.

RESULTS

Following surgical implantation of chronic recording electrodes, rats were trained to respond at a nosepoke operandum on a partial reinforcement schedule wherein a cue always predicted sucrose delivery (Paired group) or was presented at random intervals (Unpaired group), then extinguished in the absence of the cue before undergoing cue-induced reinstatement (Figure 19). All neurons from the Paired and Unpaired groups included in the analysis were recorded from electrodes located in the BLA (Figure 20a).

Individual neurons were isolated by waveform template (Figure 20b), and principal component cluster analysis.

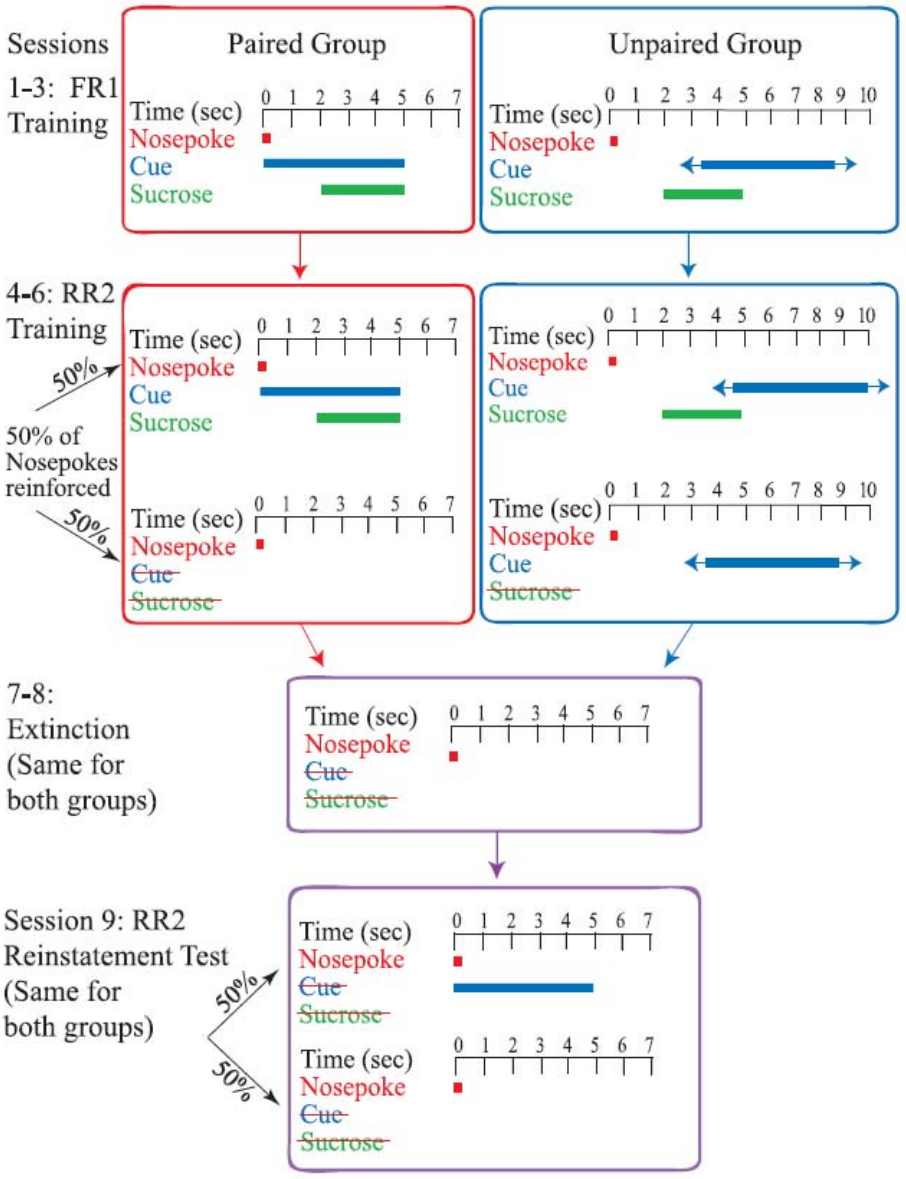

Figure 19. Diagram of behavioral paradigm. A cue-induced reinstatement paradigm was modified to allow dissociation of sensory and motor confounds. For the Paired group, sucrose delivery was always paired with the cue; for the Unpaired group, the cue was presented randomly. However, note that the cue followed 50% of responses for both groups during the reinstatement session. FR1 = Fixed Ratio 1, every response is reinforced. RR2 = Random Ratio 2, each response has a 0.5 probability of being reinforced.

A Cue-Reward Association is Required for Cue-Induced Reinstatement

The Paired and Unpaired groups demonstrated similar levels of nosepoke responding for all sessions except the reinstatement session. Here, Paired animals demonstrated reinstatement of reward-seeking behavior, indicated by a significant increase in nosepoke responses relative to extinction. In contrast, Unpaired animals failed to reinstate extinguished responding (Figure 21a). To demonstrate that animals in the Unpaired group were capable of reinstating extinguished nosepoke responding, after 80 minutes of the reinstatement session, 4 non-contingent "priming" sucrose rewards were delivered at random intervals (Figure 21b). All Unpaired animals demonstrated reinstatement of nosepoke responding following sucrose priming, indicating that they were capable of reinstating the extinguished nosepoke response that was associated with the reinforcer. In addition, the sucrose-primed reinstatement served to increase the number of trials available to compare with the Paired animals in analysis.

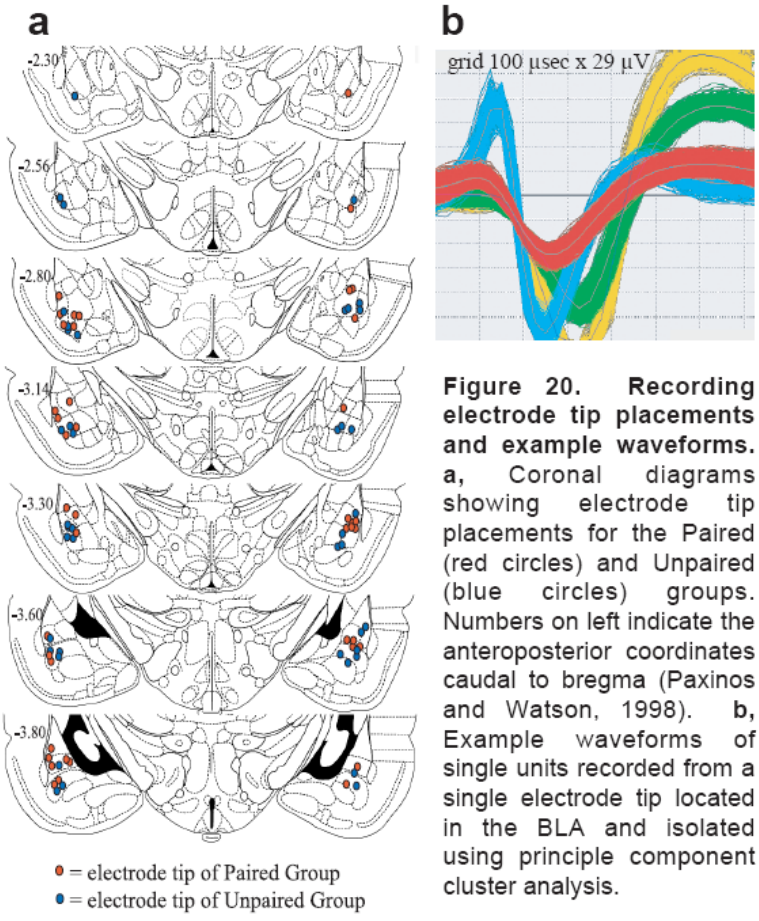

Figure 20. Recording electrode tip placements and example waveforms. **a,** Coronal diagrams showing electrode tip placements for the Paired (red circles) and Unpaired (blue circles) groups. Numbers on left indicate the anteroposterior coordinates caudal to bregma (Paxinos and Watson, 1998). **b,** Example waveforms of single units recorded from a single electrode tip located in the BLA and isolated using principle component cluster analysis.

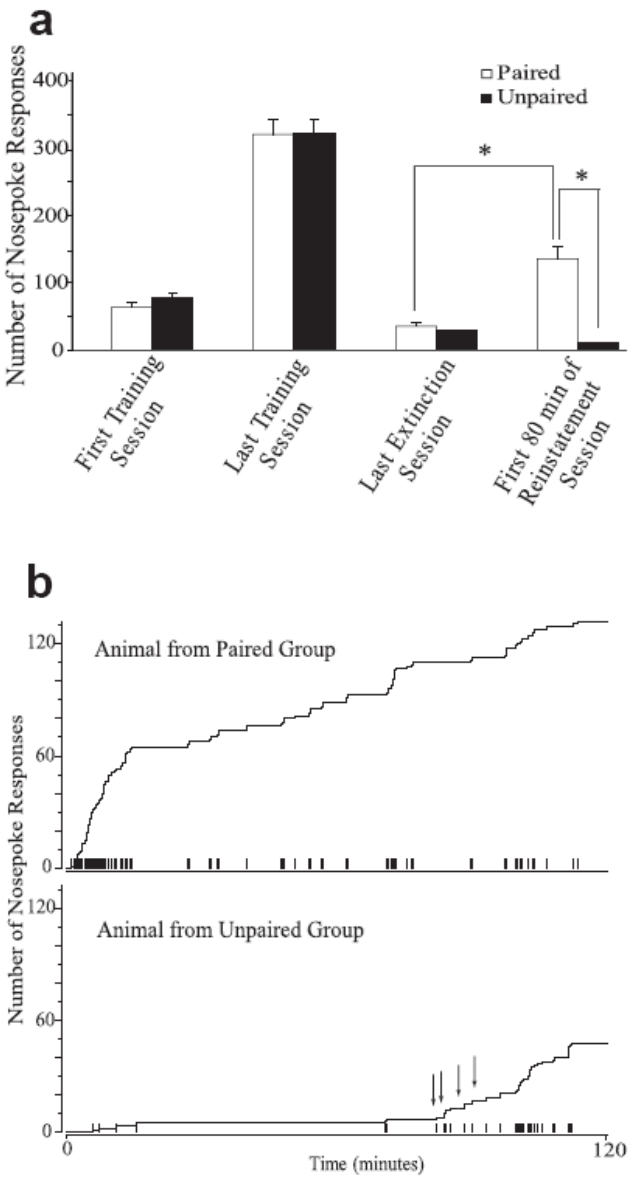

Figure 21. Behavioral differences between Paired and Unpaired groups. **a,** Mean numbers of responses (± s.e.m.) for Paired (n = 5) and Unpaired (n = 5) groups were not different during training sessions and extinction, but only Paired animals showed a cue-induced reinstatement of responding. Data analyzed with ANOVA revealed main effects of Group ($F_{1,16}$ = 16.23, P < 0.001 and Session ($F_{1,16}$ =6.57, P < 0.03), and a group x session interaction ($F_{1,16}$=13.36, P < 0.003), and were followed by pair-wise comparisons (Holm-Sidak) *P < 0.001. **b,** Cumulative activity records depicting the response pattern for representative animals of each group during the 120-min reinstatement session. Each upward inflection and corresponding tick mark represents one nosepoke response, shown on the y-axis. Note slope change for Paired animal after first ~65 nosepoke responses. Arrows represent non-contingent sucrose deliveries for the Unpaired group 80 minutes into the reinstatement session.

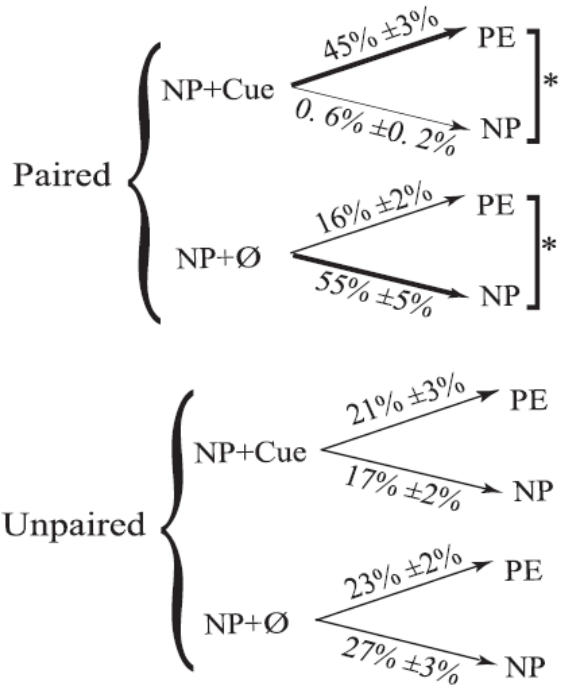

Figure 22. Microanalysis of behavior during the reinstatement session. Mean (± s.e.m.) percentages of port entries or nosepokes are given. In the 2 seconds following the nosepoke, Paired animals were more likely to enter the port following a cue (paired t-test, $P < 0.005$) and to repeat responding following the absence of a cue ($P < 0.003$), while Unpaired animals were equally likely to enter the port or to repeat nosepoking following either the presence or absence of the cue (all P's > 0.05). NP = nosepoke, Ø = absence of cue, PE = port entry. *P < 0.005.

The presence or absence of the cue guided the behavioral response pattern of Paired but not Unpaired animals (Figure 22). Specifically, within 2 seconds following the cue, Paired animals were 75 times more likely to enter the port than to nosepoke again, while Unpaired animals had a similar likelihood of port-checking or nosepoking. In the 2 seconds following a nosepoke without the cue, Paired animals were 3.4 times more likely to repeat a nosepoke response than to enter the port, while Unpaired animals had a similar likelihood of nosepoke responding or port checking.

BLA Encoding of Cue-Reward Association Guides Behavior

We recorded from a total of 212 BLA neurons during the reinstatement session, (Paired group = 100 neurons; Unpaired group = 112 neurons). While there was no significant difference between Paired and Unpaired groups in the proportion of neurons responsive to port entries, there was a significant difference between groups in the proportion of

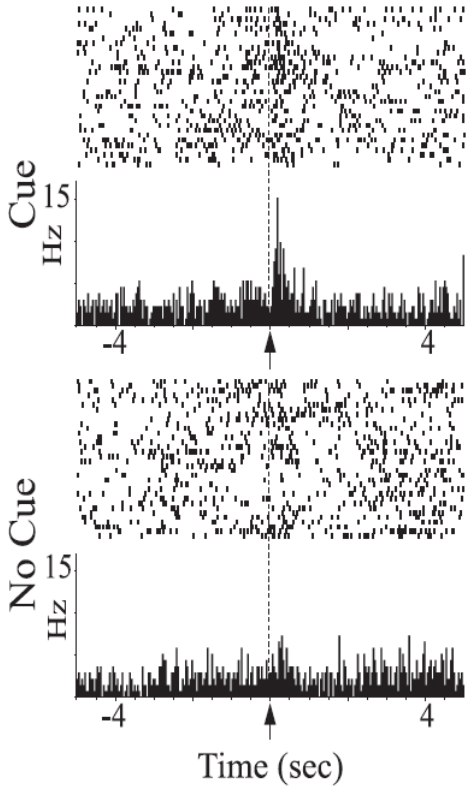

Figure 23. Example of a selectively cue-responsive neuron. The perievent raster and histogram (PERH) of a neuron which showed a significant change in phasic activity in response to nosepokes paired with a cue presentation, but did not show a significant change in phasic activity to nosepokes that were not paired with the cue. Neurons showing this response pattern are referred to as "selectively cue-responsive." Arrow indicates cue onset. Bin size = 40 ms for this and subsequent figures.

neurons that responded to the cue. 27 neurons (27%) recorded from the Paired group showed a significant ($p < 0.01$) change in phasic activity selective for the cue; an example of such a neuron is shown in Figure 23. In contrast, the Unpaired group had only 7 such neurons (6%), which was significantly different from the Paired Group (27% vs. 6%, Chi-square = 15.99, $p<0.001$). An additional population (19%; n = 19) of neurons recorded from Paired animals responded differentially to the cue presentation itself and to the interval during which the cue was anticipated, but was not presented, following nosepoke responses with no cue; an example of such a neuron is shown in Figure 24b. Only 1 such neuron was identified in the Unpaired group (0.9%), which was significantly different from the Paired group (19% vs. 0.9%, , Chi-square = 19.52, $p<0.001$). Additionally, the mean percentages of neurons in both populations per rat were significantly different between groups (Figure 25). Because rats in the Paired group were more likely to go to the port following cue presentation (Figure 22), BLA neurons with differential responses to the presence or absence of the cue could contribute to the guidance of this behavior.

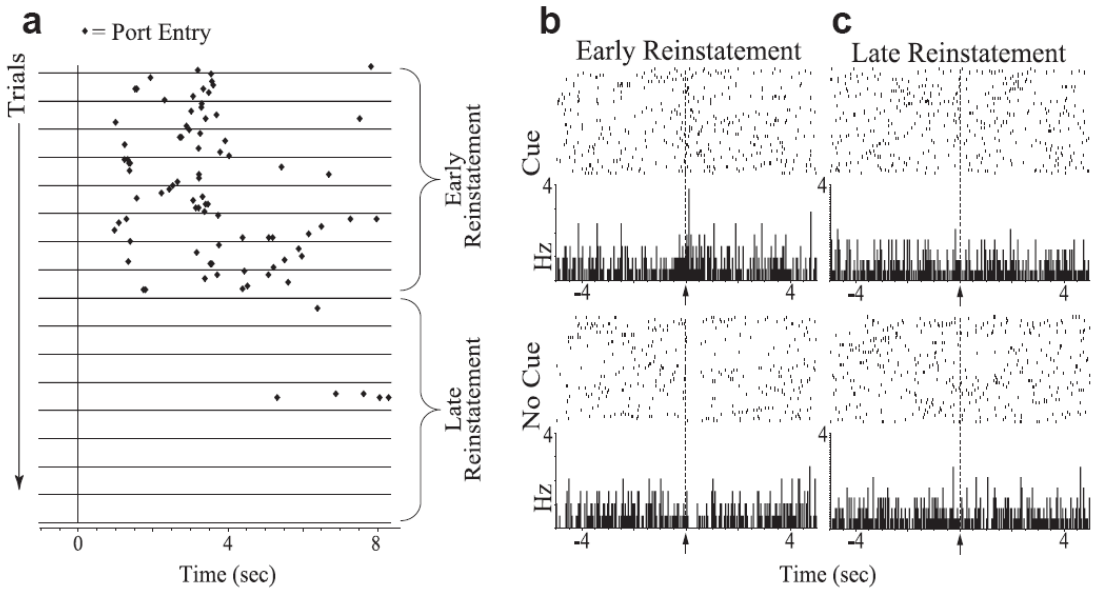

Figure 24. Corresponding changes in behavior and neural encoding of the cue from early to late reinstatement. a, Example of the pattern of port entry during the reinstatement session for a representative Paired animal shown in a PERH with response-contingent cue presentation as the reference event. Each row represents a trial, the cue presentation is the reference event. Early in the session, cue presentations were consistently followed by port entries (filled diamonds). A discrete change occurred in late reinstatement, as the Paired animal continued to respond for the cue, but no longer checked the port for sucrose. **b, c,** PERHs of a BLA single-unit with a differential response to the presence (excitation) and absence (inhibition) of the cue in Early reinstatement (b), and then a loss of these responses in Late reinstatement (c).

Recordings made during training sessions confirm the presence of these cue responses during sessions in which the cue was paired with reward. During the final training session, Paired animals showed similar proportions of neurons that were selectively cue responsive (24%) as well as differentially responsive to the presence and absence of the cue (16%), as during reinstatement, (Table 4).

	Session	
	Training	Reinstatement
Total neurons recorded	88	100
Cue responsive	35 (40%)	46 (46%)
Selectively cue responsive	21 (24%)	27 (27%)
Differentially cue responsive	14 (16%)	19 (19%)

Table 4. Neural responses from the Paired group during the final training session indicate similar proportions of cue-responsive neurons as during reinstatement.

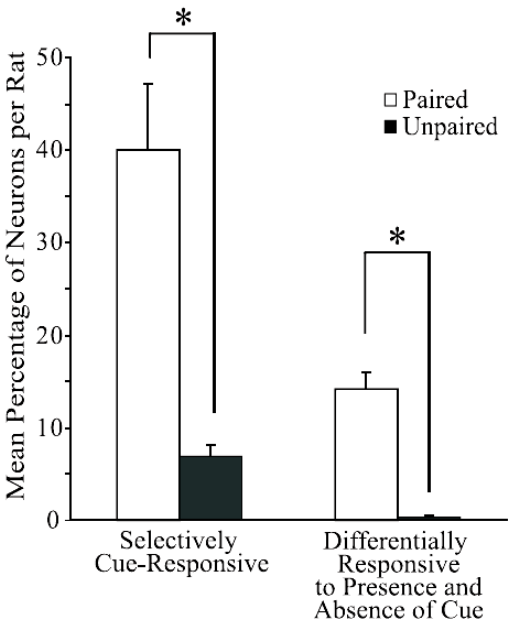

Figure 25. Neural responses to port entry or cue presentation. Two types of cue-responsive neurons include selectively cue-responsive neurons and neurons that were differentially responsive to the presence and absence of the cue. The Paired group had significantly higher mean percentages of neurons per rat selectively responding to the cue, as well as mean percentages of neurons differentially responding to the presence or absence of cue, than the Unpaired group (n = 5 rats each group; *p < 0.001, t-test).

Distinct Subpopulations of BLA Neurons Encode the Incentive Motivating and Conditioned Reinforcing Properties of the Reward-Associated Cue

At the beginning of the reinstatement session, Paired animals demonstrated a high rate of nosepoke responding (Figure 21b), followed by a steady but protracted rate of responding as the session continued. In addition, port entries immediately after the cue tended to occur early in the session, but not later in the reinstatement session (Figure 24a). Notably, Paired animals continued to respond for the cue even after they ceased checking the port for sucrose (Figure 24a), suggesting that the cue has dual effects on

behavior, acting both as a trigger for reward-directed behavior in early reinstatement, and as a conditioned reinforcer throughout reinstatement. We used the change in latency between nosepoke and subsequent port-entry as a criterion to distinguish between early and late reinstatement (see Methods for details). Also noteworthy was the step-wise manner in which animals ceased post-cue port-checking (Figure 24a) which occurred simultaneously with the discrete slope change of the rate of nosepoke responding between early and late reinstatement. The mean nosepoke response rate of Paired animals was significantly greater during early reinstatement (mean nosepokes/min= 3.6±0.15) than in late reinstatement (mean nosepokes/min= 0.31 ± 0.06) (paired t-test, *P < 0.001), an example of which is shown in Figure 21b. Interestingly, the majority of neurons that showed a differential response to the presence and absence of the cue typically lost their response to both the presence (n = 18 of 19 neurons; 94.7%) and the absence (n = 17 of 19 neurons; 89.5%) of the cue after the animal ceased port-checking in the reinstatement session (Figure 24b, c).

Additional analysis revealed that a population of cue-responsive neurons (34%; n = 34) in the Paired group responded to cues followed by port entry, but not to cues without subsequent port entry (Figure 26a, Figure 27). To determine whether this change was related to the motor activity of approaching the port, we looked at the neural response in the Unpaired group. Since the cue did not guide the behavior of the Unpaired animals, we included all trials, independent of cue presentation. Only 3 neurons (2.7%) in the Unpaired group showed a difference in activity after the nosepoke that depends upon whether the subject enters the port or not.

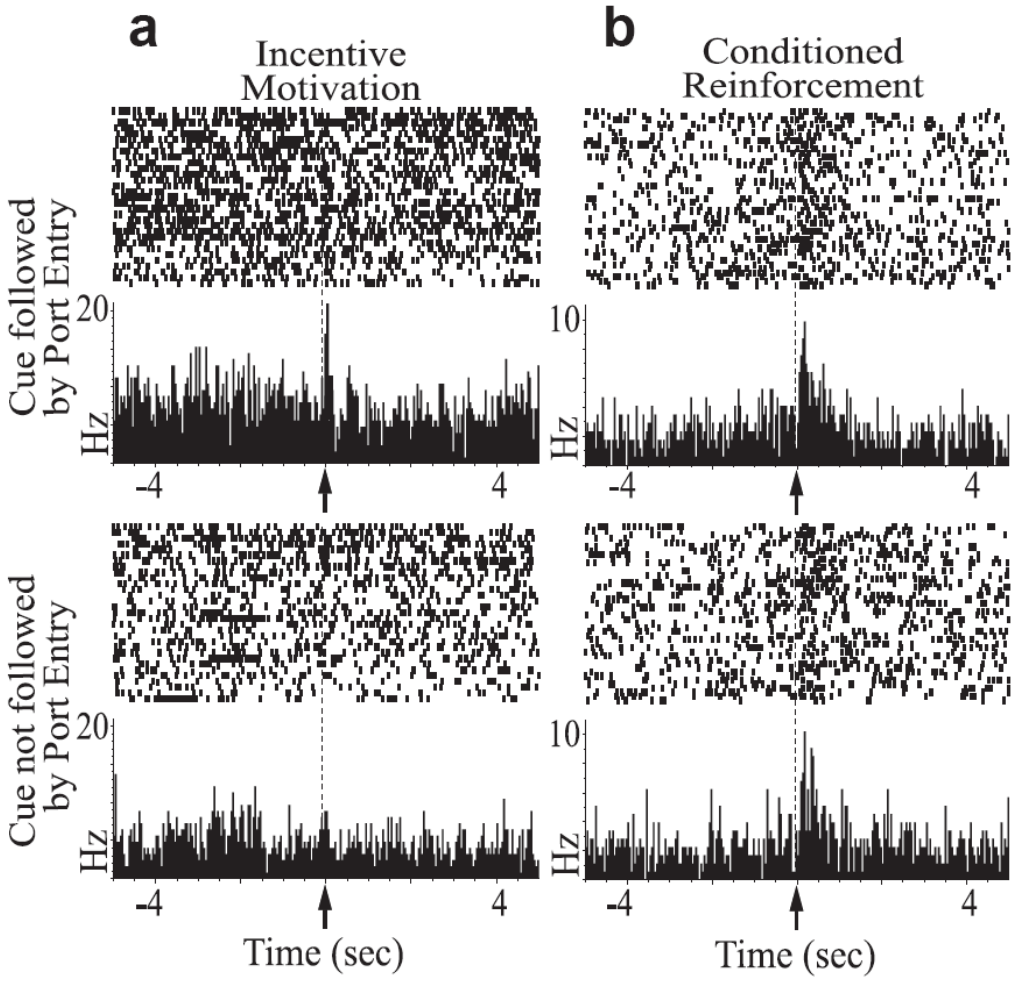

Figure 26. Cue-responsive neurons can be categorized into rapidly-extinguishing or long-lasting response types. a, PERH of BLA single unit encoding conditioned incentive; the phasic response to the cue is present only when the subject entered the port after cue presentation. Once the cue no longer provided an incentive to approach the sucrose port, the response to the cue disappeared. **b,** PERH of BLA single unit encoding conditioned reinforcement; the phasic response to the cue is present whether or not the subject entered the port after cue presentation. The cue acted as a conditioned reinforcer throughout the reinstatement session, as shown by the ability of the cue presentation to maintain a steady rate of nosepoke responding in the absence of the primary reinforcer.

Therefore, these responses in the Paired subjects are unlikely to reflect movement to the port per se. Hence, this group of neurons in the Paired subjects responded to the cue when the cue triggered the conditioned approach to the sucrose delivery port. Once the cue ceased to act as an incentive to approach the sucrose port, this population of neurons no longer showed a phasic change in activity, therefore indicating that these neurons encode conditioned incentive.

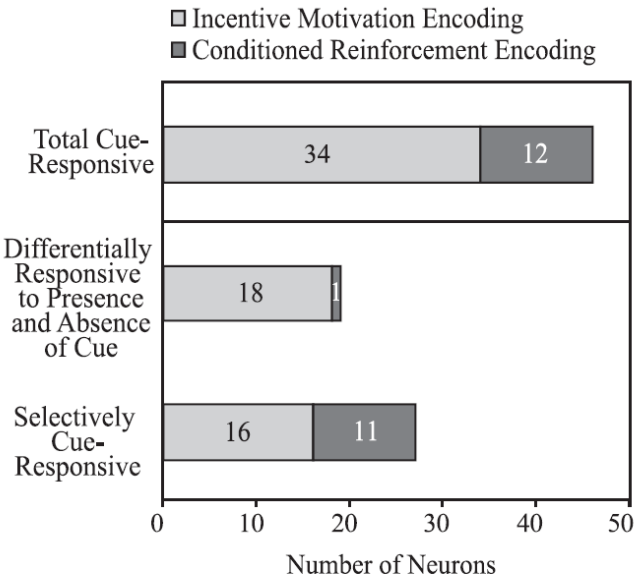

Figure 27. Sub-populations of cue-responsive neurons. Distribution of numbers of cue-responsive neurons recorded from animals in the Paired group that are conditioned incentive encoding or conditioned reinforcement encoding.

The remaining cue-responsive neurons (n=12; 12%) recorded from the Paired group maintain their cue response throughout the reinstatement session (Figure 26b; Figure 27). The response-contingent presentation of the cue acted as a conditioned reinforcer throughout the reinstatement session, as demonstrated by its ability to maintain continued responding in the absence of the primary reward. In contrast, in the Unpaired group the cue did not act as a conditioned incentive nor as a conditioned reinforcer, as demonstrated by the low level of responding in the first 80 minutes of the session.

Neurons Exhibit Increased Bursting During Reinstatement

For analysis of burst characteristics, 1000 second intervals containing comparable levels of operant responding and cue presentations were selected from the reinstatement session for each group. We found that the Paired group showed a significantly higher number of bursts, bursts per second, and percentage of spikes found in bursts relative to the Unpaired group (Table 5). However, there were no significant differences between groups in overall mean firing rate, burst duration, or mean number of spikes found in a burst (Table 5).

	Paired group		Unpaired group		
	Mean	SEM	Mean	SEM	*p* value[a]
Overall mean frequency	2.47	0.05	0.79	0.01	$p = 0.06$
Number of bursts	**350.46**	**5.11**	**112.75**	**1.91**	$\mathbf{p < 0.001}$
Bursts per second	**0.35**	**0.01**	**0.11**	**0.001**	$\mathbf{p < 0.001}$
Percentage of spikes in bursts	**37.80**	**0.24**	**26.32**	**0.13**	$\mathbf{p < 0.001}$
Mean burst duration	0.08	0.001	0.06	0.001	$p = 0.07$
Mean spikes in burst	3.12	0.04	2.31	0.001	$p = 0.06$

Significant differences are in bold.

[a]Student's *t* test.

Table 5. Increases in burst activity during reinstatement in Paired versus Unpaired subjects.

Electrophysiological Characterization of Neuronal Response Types

Pyramidal projection neurons compose approximately 95% of the neurons in the BLA, the remainder being interneurons (McDonald, 1982, 1984). Studies have shown that pyramidal neurons and interneurons can be differentiated by both morphological and electrophysiological characteristics (Rainnie et al., 1993; Washburn and Moises, 1992).

These studies have shown that pyramidal neurons have longer duration action potentials, very low spontaneous firing rates, and fewer spikes per burst. Interneurons have shorter duration action potentials, very high spontaneous firing rates, and no sign of accommodation (Washburn and Moises, 1992). Here, we show the distribution of neuronal firing rates with respect to task-relevant phasic activity (Figure 28).

	Total		SCR	DR		CI	CR	iCI
FR > 10 HZ	7		1	0		1	0	0
FR > 5 HZ	12		4	1		2	1	2
FR > 4 HZ	15		4	3		4	1	2

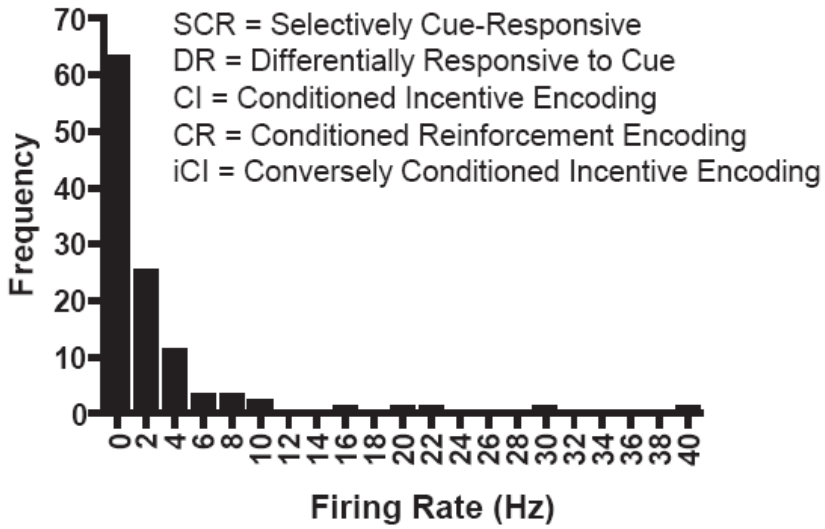

Figure 28. BLA neuron frequency histogram and response profile. Frequency histogram shows the distribution of neurons (by number) by baseline firing rate. Corresponding table shows the relationship between firing rate and response profile.

Using the electrophysiological characteristics of both firing rate and waveform duration, we identified 6 putative interneurons of 100 neurons from the Paired group (Figure 29). While a few fast-firing neurons that responded to task-relevant events, none of these

putative interneurons were phasically responsive to cue presentations or nosepokes (Figure 29).

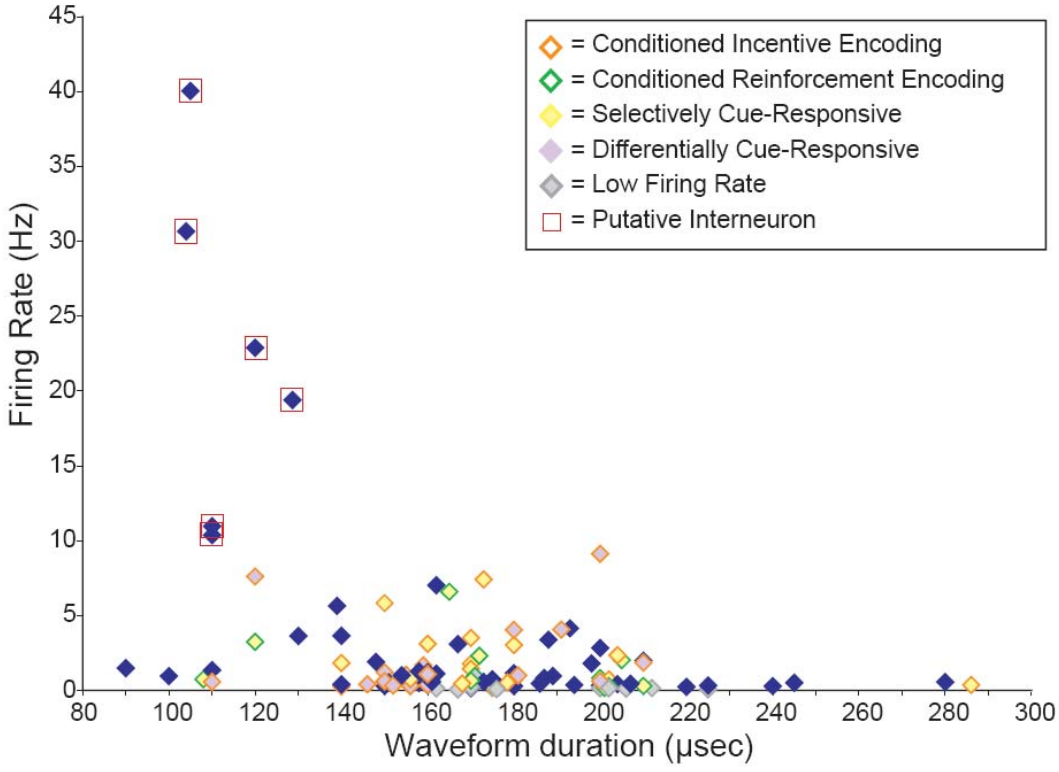

Figure 29. Putative interneurons do not show behaviorally relevant phasic responses. Diamonds represent neurons plotted with respect to firing rate and waveform duration. Dark blue diamonds indicate neurons without behaviorally relevant phasic activity.

DISCUSSION

We report that neurons in the BLA encode a cue that induces relapse to reward-seeking behavior, and we posit that these cue responses in the BLA encode two distinct behavioral functions of conditioned cues: incentive motivation and conditioned reinforcement. Neurons that responded differentially to the presence and absence of the cue were likely to contribute to the guidance of subsequent behavioral choices, and largely overlapped with incentive motivation encoding neurons.

We termed the rapidly-extinguishing population of neurons "incentive motivation encoding" because the activation of these neurons by the cue triggered a motivated behavioral state during which nosepoke responses and subsequent port entries were performed at a high rate. The slowly-extinguishing population of neurons which responded to the cue throughout reinstatement was termed "conditioned reinforcement encoding" because the activation of these neurons continued, as long as repeated nosepoking was performed, even after the incentive-triggered port-checking behavior was extinguished.

Our behavioral task gave rats the opportunity to demonstrate if they were working for sucrose or if they were working only for cue presentations. Multiple behavioral events were measured, not only the instrumental response (nosepoke), but also a measure of conditioned approach (port entry). This allowed the distinction between trials where the cue acted as a conditioned incentive, triggering a motivated reward-seeking state, and trials where the cue acted as a conditioned reinforcer, supporting continued nosepoke

responding. Hence, it was possible to track single units as different acquired properties of the cue guided the behavior of animals in the Paired group within a single session.

At the beginning of the reinstatement session, the Paired animals displayed a high rate of responding, followed by a discrete rate change (Figure 21b) which temporally corresponds with the step-wise cessation of post-cue port-checking (Figure 24). In temporal agreement with this sudden behavioral change, a sub-population of neurons ceases responding to the cue. We speculate that this change in the rate of responding occurs because, initially, both the incentive and reinforcement encoding populations of neurons are supporting the nosepoking behavior, and later, when the incentive encoding population of neurons stops responding to the cue, the response rate changes, perhaps reflecting that only one population of neurons is now supporting nosepoke responding. Our hypothesis that BLA neurons are guiding reward-seeking behavior is supported by the coincident decrease in the behavioral response rate and the proportion of cue-responsive neurons.

Both the incentive motivation encoding and conditioned reinforcement encoding populations of neurons responded to the cue while the cue-reward association was still intact, but as the meaning of the cue became ambiguous, as demonstrated by the cessation of post-cue port-checking (Figure 24a), one population of cue-responsive neurons ceased responding to the cue, which suggests these neurons are encoding incentive motivation, as measured by cue-triggered approach and cued relapse. Lesion studies show that the BLA is important for outcome-specific incentive processes (Balleine et al., 2003; Corbit

and Balleine, 2005). Interestingly, most of the conditioned incentive encoding neurons were differentially responsive to both the presence and absence of the cue. Once the animal's behavior was no longer guided by the presence or absence of the cue (when the animals ceased port-checking) this population of neurons no longer displayed the differential change in phasic activity. In agreement with a role for the BLA in conditioned reinforcement (Cador et al., 1989), the second population of cue-responsive neurons maintained their cue-response while instrumental responding continued, perhaps reflecting the conditioned reinforcing properties of the reward-paired cue which can motivate continued nosepoking even after the animals have stopped checking the reward port.

The current findings confirm that the BLA is a site for the neural representation of reward-associated cues that induce reinstatement to reward-seeking behavior, consistent with studies showing that the BLA is critically involved for cue-induced reinstatement (Meil and See, 1997; Fuchs and See, 2002; Yun and Fields, 2003). Importantly, it has been shown that varying withdrawal periods can affect extinction behavior and the degree to which cue-induced reinstatement is expressed (Myers et al., 2006; Grimm et al., 2002). While our results represent the first evidence that distinct populations of neurons encode incentive and reinforcing properties of a conditioned stimulus, additional studies are needed to dissect relevant issues. Our findings provide an impetus for further experimentation regarding BLA neural activity during distinct behavioral tests of incentive motivation and conditioned reinforcement, and the persistence of these neural responses with varying withdrawal periods.

The finding that two populations of cue-responsive BLA neurons are active during reinstatement (Figure 27) is reminiscent of studies done with extinction of cue-shock associations (Repa et al., 2001). In the extinction of a conditioned stimulus (CS)-shock association, it was shown that there were two sub-populations of neurons in the lateral amygdala with CS-evoked firing rate changes, one population that exhibited increased responses to the CS during early extinction and then fell back to baseline levels in late extinction, and one that maintained their increased responses to the CS throughout extinction (Repa et al., 2001). In our paradigm, the reinstatement session was a session in which the cue-reward association was extinguished, similar to the extinction session from the paradigm of Repa and colleagues. Our findings are consistent with the Repa et al. study, showing analogous characteristics for two populations of BLA neurons that display either transient or long-lasting responses to the CS. This suggests BLA neurons may encode appetitive and aversive stimuli similarly. Since there is evidence that distinct populations of BLA neurons encode appetitive and aversive stimuli (Paton et al., 2006), it is likely that these neurons have different projections, and that the function of the BLA lies in the absolute valence of the outcomes predicted by sensory stimuli, and that positive or negative value is processed elsewhere.

The present findings are also consistent with findings from Carelli et al. (2003) who recorded in the BLA during self-administration of cocaine. They described three typical response types, including responses that occurred during cue presentation. The present study extends this report by examining the response of BLA neurons to the reward-

associated cue during reinstatement in the absence of the primary reinforcer, and by dissociating the motor and sensory responses from the neural encoding of the cue-reward association.

The BLA works in conjunction with downstream areas in the reward circuit by supplying them with information about the motivating and/or reinforcing properties of the cue to decide upon, plan, and execute appropriate behavioral outputs. For example, the BLA has robust reciprocal projections with the medial prefrontal cortex (mPFC) (Vertes, 2004). The mPFC is also important for cue-induced reinstatement of reward-seeking behavior (McLaughlin and See 2003). Evidence suggests that the mPFC regulates the responsiveness of BLA neurons to odor cues previously associated with footshock (Rosenkranz et al., 2003). Additionally, the BLA transmits information about footshock-conditioned stimuli to a sub-population of mPFC neurons which encodes information about these emotionally salient cues with increased bursting activity (Laviolette et al., 2005). Our data showing a significant increase in the number of bursts, bursts per second, and percentage of total spikes that occur in bursts in the BLA neurons of the Paired group as compared to the Unpaired group (Table 5) suggest that the reward-paired cue may induce bursting of BLA neurons, and are consistent with the study done by Laviolette and colleagues. These results suggest that the cue-induced bursts seen in the mPFC may originate in the BLA.

The orbitofrontal cortex (OFC), which is reciprocally connected to the BLA (Kita and Kitai, 1990; Shi and Cassell, 1998) is thought to work in coordination with the BLA to

use incentive information to guide behavior (Baxter et al., 2000; Gallagher and Schoenbaum, 1999; Schoenbaum et al., 1999, 2003). It has been shown that lesions of either the BLA or OFC alter the neural encoding of information about expected outcomes and acquired value (Saddoris et al., 2005; Schoenbaum et al., 2003). In addition, the BLA sends inputs to the nucleus accumbens core (Kelley et al., 1982; Wright et al., 1996), and it has been reported that these connections are important for performing second-order conditioned responses (Setlow et al., 2002). The nucleus accumbens has been suggested as a "final common pathway" for all types of reinstatement (cue-induced, drug-induced, stress-induced) (Kalivas and McFarland, 2003). For these reasons, understanding how the BLA encodes cues that guide goal-directed behavior is crucial to understanding how our brains process sensory stimuli that induce relapse to reward-seeking behavior.

Conclusion

In conclusion, our data demonstrate that BLA neurons show changes in phasic activity to the response-contingent presentation of a cue that induces reinstatement of reward-seeking behavior. BLA neurons also respond to the absence of the anticipated cue. Importantly, distinct neuronal populations within the BLA encode incentive motivation and conditioned reinforcement.

The activity of these neural populations can motivate reward-seeking in the absence of reward, and serve to guide goal-oriented behaviors. These findings represent significant progress towards understanding the neural circuitry encoding cues that can reinstate

extinguished reward-seeking behavior. This increased understanding will aid the development of therapeutic interventions for conditioned cued relapse of reward-seeking behaviors in humans including compulsive eating disorders and drug addiction.

METHODS

In vivo electrophysiological recordings

Adult male Sprague-Dawley rats (250-350 g; Harlan, Indianapolis, IN) were bilaterally implanted with electrodes in the BLA for chronic neural recording during cue-induced reinstatement of reward-seeking behavior. Rats were anesthetized with isoflurane and stereotaxically implanted bilaterally with fixed-wire electrodes with an eight-wire 2x2x3 array, each wire insulated and 50 μm in diameter (NB Labs, Denison, TX) directed at the BLA (anteriorposterior, -2.8 to -3.6; mediolateral ±5.0; dorsoventral -7.2 to -7.5). Rats were allowed to heal for 7-10 days during which they received food and water *ad libitum*. After healing, rats were food restricted to 90% of their *ad libitum* consumption for three days prior to performing the behavioral task. All procedures were approved by the University of California Committee on Animal Research and were in accordance with National Institute of Health guidelines. Every attempt was made to minimize the number of animals required and to minimize their suffering.

Behavioral paradigms

Animals were divided into two groups, a "Paired" group and an "Unpaired" group. Animals in the Paired group were trained to perform a nosepoke response which resulted in a cue presentation and sucrose delivery to an adjacent port. To demonstrate that neural responses were a result of the cue-reward association and not unconditioned sensory responses, we included an Unpaired group of animals. Animals in the Unpaired group were also trained to respond for sucrose delivery, but the cue was presented at random intervals, independent of other experimental events. For each 2-hour session, run on

consecutive days, animals were placed into the behavioral chamber, with their headstages plugged into a cable attached to a freely rotating commutator at the top of the box to enable unrestricted movement. This behavioral chamber was equipped with a nosepoke hole and adjacent reward-delivery port. A continuous house light and white noise (60 db) signaled the onset of the behavioral program. *Training FR1, Sessions 1-3:* For both groups, animals were trained in a fixed-ratio 1 (FR1) reinforcement schedule for one session per day for three days, and during the first two days of training a palatable odor cue was presented to encourage nosepoke responding. Nosepoke responses and port entries were measured by beam breaks. For the Paired group, nosepoke responses were reinforced on a FR1 schedule with a subsequent (onset 50 msec after nosepoke) compound light-tone cue, 3 KHz tone at 80 db and illumination of two additional lights (LT-cue) lasting 5 seconds. 2 seconds after the nosepoke, 0.1 ml of a 15% sucrose solution was delivered to the port over the course of three seconds. For the Unpaired group, nosepoke responses were also reinforced on a FR1, with the same 2-second delay until onset of the 3-second sucrose solution delivery. However, the Unpaired group was presented with the LT-cue at response-independent random variable intervals. The frequency of the LT-cue presentations was matched to experimental animals so that both groups received the same approximate average number of LT-cue presentations. For both groups, animals that did not meet the learning criterion (75 nosepokes by the 3rd training session) did not move on to the next training sessions and were excluded from the study. Two rats, one from each group, were excluded from the study for failure to meet learning criterion after 3 days of training. *Training RR2, Sessions 4-6:* For the fourth session, both groups of animals were switched to a random ratio 2 (RR2) reinforcement schedule

where only %50 of randomly selected nosepokes were reinforced, while the other %50 were recorded but not reinforced. For the Paired group, reinforced nosepokes were paired with the same 50 msec delay until onset of the 5-second LT-cue in combination with the 2-second delay until onset of the 3-second sucrose delivery. For the Unpaired group, reinforced nosepokes were followed with a 2-second delay until the onset of the 3-second sucrose delivery, but 5-second LT-cues were still presented at random intervals, occurring irrespective to sucrose deliveries. *Extinction, Sessions 7-8:* Both groups were run on the same extinction paradigm. During this paradigm, nosepoke responses were recorded but not reinforced. Both the LT-cue and sucrose deliveries were completely omitted for the duration of the session. *Reinstatement Test Day, Session 9:* Both groups were run on the same reinstatement paradigm. During this paradigm, 50% of nosepokes were paired with the LT-cue, and the other 50% of nosepokes were not paired with any stimuli. All nosepoke responses and port entries were recorded. For both groups, nosepoke responses were not reinforced with sucrose deliveries. For the Paired group, the above situation was consistent for the entire session. For the Unpaired group, because the level of responding was so low (animals did not show a cue-induced reinstatement of reward-seeking behavior), after 80 minutes of the above situation, 4 non-contingent presentations of sucrose solution were passively delivered to encourage nosepoking, both to demonstrate that the animal learned the task and was able to recall the association between nosepoke responding and sucrose deliveries, and to yield more trials to allow for data analysis. All programs were written in MED-PC, and behavioral data was sent to Plexon in correspondence with neural recordings.

Histology

After the completion of the last session, animals were anesthetized with isoflurane and decapitated. A 19 μA current was passed through each recording electrode which had identifiable single units. The brain was fixed in 10% formalin, 3% potassium ferracyanide overnight. Brains were submerged in 20% sucrose and 3% potassium ferracyanide overnight. Potassium ferracyanide was used to determine the location electrode tip. Brains were sectioned (50 μm) throughout the extent of the amygdala. Alternating sections were stained with Neutral Red, Nissl staining, allowing the visualization of the blue electrode placement marking in relation to the subnuclei of the amydala. Serial sections were examined under a light microscope, and the location of each electrode tip was plotted on coronal sections taken from the rat stereotaxic atlas from Paxinos and Watson (1998).

Single-unit recording and discrimination

Neural activity was recorded through commercial hardware and software, including headstage amplifiers to programmable amplifiers, filters (0.5 and 5KHz, 3dB cutoffs), and a multichannel spike sorting software (Plexon Instruments, Dallas TX). Discrimination of individual units was performed off-line using principal component analysis of waveform shape. Single units were identified by constancy of waveform shape, cross-correlogram, autocorrelogram, and interspike interval. Action potential durations were measured from the initial inflection of the waveform to the first trough (μsec).

Analyzing neural response properties

A total of 10 rats, (n=5 rats each group) successfully recovered from surgery, met learning criterion, and were histologically confirmed to have electrode tips in the BLA. These 10 rats were included in data analyses. Sorted files were processed in NeuroExplorer to extract these unit timestamps and relevant reference event markers. Neural activity was characterized via perievent raster (PER) and perievent histogram (PEH) displays. NeuroExplorer extracted timestamps were then exported to MATLAB (Natick, MA) were analyzed for statistical significance. Responses were deemed statistically significant if any 100 msec bins in the response window (0-.5 seconds after the nosepoke) was statistically significant relative to a baseline epoch, a .5 second period after the animal had left the port on the prior trial and before the animal had initiated movement towards the nosepoke hole (-2 to -1.5 seconds).. Significance was tested using the non-parametric Wilcoxon Signed Rank Test, with a certainty of $p<0.01$. For analysis of neural responses to cues followed by port entry versus cues not followed by port entry, variables were created on NeuroExplorer. Due to animal variability in average latency from nosepoke or cue presentation to port entry, the time epoch following a nosepoke or cue presentation in which a port entry was considered to have followed the reference event was 1.5 standard deviations above the mean latency for each animal. Early reinstatement was deemed to be the initial portion of the reinstatement session, during which the animal displayed post-cue port-checking, while late reinstatement referred to trials after the time point when the latency between the nosepoke and the port-entry increased by a minimum threshold of 1.5 standard deviations in a moving 10-trial block.

Burst Analysis

Sorted neural data files were imported into NeuroExplorer. Burst analysis was performed using interval specifications such that successive spikes with an interspike interval ≤ 0.01 sec were included within a burst with a minimum of 2 spikes per burst. To compare the Paired group to the Unpaired group, a 1000 second interval, beginning after the time of operant response initiation, was selected such that both groups had similar levels of activity and cue presentations (Paired: 1000-2000 sec; Unpaired: 5600-6600 sec).

The experiments and data shown in this chapter with the exception of Figures 28 and 29 were published by Tye and Janak in 2007 in the *Journal of Neuroscience.* Figures 28 and 29 are unpublished data that are presented exclusively in this dissertation.

AUTHOR CONTRIBUTIONS

K.M.T. performed the surgeries, experiments and data analysis. P.H.J. provided guidance, mentorship and resources. K.M.T. and P.H.J. contributed to study design, results interpretation and manuscript writing.

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CHAPTER 4

Discussion

CONCLUSIONS AND SIGNIFICANCE OF THIS DISSERTATION

This thesis dissertation represents a significant advancement of our understanding of the neural circuitry mediating goal-oriented behaviors on a molecular and cellular level. Specifically, these data provide insight on the cellular and synaptic mechanisms mediating the acquisition of an association between a conditioned stimulus and a primary reinforcer, and the neural activity mediating the capability of a conditioned stimulus to reinstate extinguished reward-seeking behavior. It is noteworthy that these data strongly support distinctions made by behaviorists, by demonstrating that discrete populations of neurons encode motivation and reinforcement. These findings reinforce, and even enhance, the notion that behavior is mediated by neural activity with incredible precision. Furthermore, these data perpetuate the concept that individual brain regions are multi-functional and most brain functions require the coordinated activity of multiple brain regions in an integrated neural network.

Conclusions

The integration of *in vivo* electrophysiology, *ex vivo* electrophysiology, *in vivo* pharmacology and a novel behavioral paradigm allowed the parallel quantification of changes in neuron firing, synaptic plasticity and behavioral expressions of the formation, storage and retrieval of emotional memories applied to goal-oriented behaviors. The data presented here demonstrate that the degree of enhancement of both neural activity and synaptic strength in the amygdala predict the success of learning a reward-related task. Furthermore, these data show that assigning motivational significance to an environmental cue requires an increase in postsynaptic AMPAR-mediated currents in amygdala neurons that occur via an NMDAR-dependent mechanism. Finally, environmental cues endowed with the power to guide goal-oriented behaviors are encoded by subpopulations of amygdala neurons that distinctly encode either the motivating or reinforcing properties of the emotionally significant cue.

Significance

The finding that thalamo-amygdala synapses are rapidly and proportionally strengthened with the degree of learning of an association between a cue and a reward provides the first evidence that LTP can be quantitatively related to a behavioral measure of learning. Additionally, this finding broadens the existing literature by extending the applicability to a localized potentiation previously associated only with Pavlovian fear conditioning with an instrumental reward-directed learning task. The related finding that the proportion of neurons recruited to encode a reward-predictive cue increases commensurately with the

strength of the cue-reward association, as expressed by task performance supplies an additional vantage point of the same phenomenon. The parallel correlations between behaviorally-demonstrated learning with both *in vivo* and *ex vivo* cellular and synaptic changes provide a much needed bridge between two commonly used, but seldom integrated neurophysiological techniques. The corresponding result that the synaptic changes associated with the acute acquisition of the association between the cue and the reward occur via an NMDAR-dependent mechanism confirms a causal relationship between the synaptic changes and the behavioral expression of reward-related associative memory formation. The NMDAR-dependent characteristic of this synaptic potentiation relates this mechanism to other forms of LTP found in the amygdala, as well as the hippocampus, that have been extensively discussed in the relevant literature. Not only do the data discussed in Chapter 2 advance our understanding of how amygdala neurons mediate reward-related memory formation, but they also help to establish general principles of neuroscience, such as the relationship between learning-induced changes in neural firing *in vivo* and synaptic modifications that can only be observed *ex vivo*. While the techniques implemented in this study have been commonly used, this study was the first to integrate them with a flexible behavioral paradigm.

The finding that distinct subpopulations of amygdala neurons code for the reinforcing and motivating properties of a reward-associated cue provides the first unequivocal support for the behavioral dogma that define these terms as completely independent aspects of conditioning. This dissociation was elucidated using an animal model of relapse referred to as cue-induced reinstatement. The presentation of a reward-associated cue can induce

a relapse of an extinguished reward-seeking behavior. Understanding how amygdala neurons code for the conditioned properties assigned to the cue can advance our development of therapeutic interventions for addiction relapse, eating disorders, and other aberrant reward-seeking behaviors.

EMOTIONS AND MEMORIES: INFLUENCE OF THE AMYGDALA

The amygdala is the critical brain region for mediating the 'emotional' memory system and, importantly, it also plays a role in modulating both the 'cognitive' memory system and the 'habit' memory system (LeDoux, 1993; Cahill and McGaugh, 1996). So while the amygdala is primarily thought to be important for processing emotion, its function extends beyond emotional processing and is heavily intertwined with memory formation and retrieval.

The 'Emotional' Memory System

Irrational panic attacks and phobias are human expressions of emotional memories. These are memories that are not necessarily cognitive. For example, a person who has been bitten by a dog as a young child may not remember the event, but will experience physiological arousal (e.g. sweating, increased heart rate and blood pressure) in the presence of dogs. Thus, even though the person may be unaware of the reason they fear dogs, they behaviorally express this memory trace. The amygdala has been implicated in

both a formation and storage role for 'emotional' or 'stimulus-affect' memories, as evidenced by experiments involving the acquisition and expression of fear conditioning (Davis and Whalen, 2001; LeDoux, 2000; Fendt and Fanselow, 1999) and stimulus-reward learning (Johnsrude et al., 2000; Cador et al., 1989). In humans, it has been shown that bilateral damage to the amygdala causes an impairment in the recognition of emotions in facial expressions (Adolphs et al., 1994; Young et al., 1995), and fMRI studies show that the amygdala is active during the recognition of emotional facial expressions (Morris et al., 1996). In general, the amygdala has been strongly implicated in processing affect and the formation, storage and retrieval of affective memories.

Competition Among Multiple Memory Systems

Accumulating evidence from human and animal studies have supported the notion that not only are there multiple memory systems, but that during certain tasks, they may be in competition with each other (Poldrack and Packard, 2003). This type of competition may be demonstrated by an enhanced performance in a certain task following the lesion of a neural substrate of a competing memory system. Specifically, lesions to the hippocampal memory system prior to training have been shown to actually facilitate learning of a caudate-dependent two-way active avoidance behavior (McDonald and White, 1993; Packard et al., 1989). One explanation of this finding may be that the removal of the hippocampal memory system, and hence the processing spatial information (O'Keefe and Nadel, 1978) which might interfere with the animal returning to a place where it received an aversive stimulus, reduces any 'disagreement' between memory systems. Conversely,

lesions to the caudate-putamen memory system facilitate learning of hippcampal-dependent spatial discrimination task (Mitchell and Hall, 1988).

Bottom-Up Processing versus Top-Down Modulation

Substantial evidence implicates the cooperativity of the BLA and OFC in the performance of reversal tasks, where odor cues are paired with either sucrose and quinine and then these pairings are reversed (Schoenbaum et al., 1999, Schoenbaum et al., 2003; Saddoris et al., 2005). Recently, it has been reported that while OFC lesions alone significantly impair the ability to learn a reversal, and BLA lesions slightly impair this ability, bilateral lesions of both the BLA and OFC will actually restore the ability to acquire this reversal (Stalnaker et al., 2007).

Both the BLA and the OFC are involved in the 'emotional' memory system, though they are thought to play very different roles. While emotional responses to environmental stimuli, characterized by physiological arousal, are thought to be automatic, a declarative evaluation of emotion is a voluntary action. fMRI studies have been critical in parsing these interacting processes within the emotional memory system. The amygdala has been evidenced as a substrate of bottom-up processing (Breiter et al., 1996; Morris et al., 1996; Irwin et al., 1996; Reiman et al., 1997; Phan et al., 2002) which refers to the processing of the emotional content of a stimulus. In contrast, top-down modulation of emotional processing has been thought to involve the OFC (Rolls, 1999; Zald and Kim, 2001; Liberzon et al., 2000; Arana et al., 2003; O'Doherty, 2004) as well as the ventromedial

prefrontal cortex (vmPFC) and anterior cingulated cortex (ACC) (Lane et al., 1997; Taylor et al., 2003; Ochsner et al., 2004). Both emotional responses (Ochsner and Gross, 2005) and amygdalar responses (Liberzon et al., 2000) are subject to cognitive modulation. Recently, it has been reported that there are dissociable responses in the amygdala and OFC to respective bottom-up and top-down components of emotional evaluation (Wright et al., 2008).

This binary system of emotional processing may explain the counter-intuitive results reported by Stalnaker and colleagues (2007). Perhaps the involvement of the OFC in cognitive flexibility relies primarily on its interaction with the amygdala, and the ablation of the entire system may force the brain to utilize an alternate memory system in the absence of this one. This thread of research strongly contends that brain regions, particularly cortical ones, are not independently assigned functions, but mediate cognitive and emotional processes via communication amidst the neural circuitry within which they are deeply embedded.

The Impact of Emotions on Declarative Memories

Although emotions are thought to be relatively short-lived, they can dramatically impact the formation, storage and recall of memories (James, 1890; Ekman and Davidson, 1990; Lane and Nadel, 2000). Memories of experiences, both declarative and non-declarative, are thought to be represented in patterns of neural activity. An environmental cue can trigger the retrieval of a memory, and the activation of the network that encodes that

memory. Emotional events are often remembered with greater accuracy and vividness than events lacking an emotional component (Reisberg and Hertel, 2005). Memory storage of emotionally arousing events involve neurobiological processes including β-adrenergic receptors (McGaugh et al., 1993). In normal human subjects, β-adrenergic blockade selectively impaired the long-term memory of an emotionally arousing story (Cahill et al., 1994). Bilateral amygdala damage also results in the loss of enhanced memory for emotional stories (Cahill et al., 1995). Amygdala activity influences the encoding, consolidation and retrieval of the memory trace for an emotional event (LaBar and Cabeza, 2006; Phelps, 2004; Buchanan, 2007). The enhancement of memories for emotional events is driven by interactions between the amygdala and other brain areas such as the prefrontal cortex and hippocampus (Cahill and McGaugh, 1996), and is also modulated by stress hormones such as epinephrine and glucocorticoids (Liang and McGaugh, 1983; Gold and van Buskirk, 1975; Cahill and McGaugh, 1990).

Arousal and Emotion: Which comes first?

Among the many 'definitions' of emotion, the most commonly identified facet of an emotional state is physiological arousal. Could the elicitation of physiological responses precede the subjective experience of emotion (James, 1884)? Could the heightening of physiological functions (breathing, heart rate, perspiration, alertness) be the primary cause of the enhanced encoding of emotional events? In line with the 'Two Factor' theory of emotion, as demonstrated by the famous 'high bridge study,' arousal can precede the subjective interpretation of the emotion (Dutton and Aron, 1974). This is interesting because while the BLA is important for emotional processing, the downstream central nucleus is important for initiating the physiological arousal associated with the emotion, suggesting that the processing of emotion precedes physiological arousal.

The fact that physiological arousal itself can enhance memory retention (Castellano et al., 1993), and be mistaken as emotion (Schachter and Singer, 1962) suggests that physiological arousal precedes emotional experience. Rats demonstrated enhanced memory retention with the systemic administration of a physiologically arousing drug during the acquisition period, but this effect was abolished by pre-acquistion amygdala lesions (McGaugh et al., 1996; Roozendaal 1990a). So while physiological arousal alone can enhance memory, this phenomenon depends on the amygdala. Likewise, β-adrenergic blockade selectively prevents the enhancement of emotional memories (Cahill et al., 1994). Taken together, it seems that despite the predominantly uni-directional projections from the BLA to the CeA, that arousal may precede at least the experience of

emotion. Therefore, emotion and arousal are inextricably intertwined, and are likely to function in an Emotion – Arousal loop. Typically, environmental stimuli will trigger an emotion which results in a constellation of changes, including heightened neural activity and physiological arousal. Alternatively, physiological arousal, a function seated in the hypothalamus, may be communicated via hypothalamic projections to the LA and thusly interpreted as emotion.

The Amygdala as the 'Connectivity Hub' Integrating Emotion and Cognition

The amygdala is a prime example of a single structure that participates in multiple processes that operate both in parallel and in series. The amygdala's functional multiplicity is reflected in its promiscuous connectivity with other brain structures. Here, I have summarized the connectivity of the amygdala as described in the scientific literature in layman's terms. Thalamic nuclei directly relay low-level sensory information to the LA. Sensory cortices provide highly processed information about the stimulus to the LA. The amgydala projects back to most of the neocortex, the hippocampus, basal forebrain and basal ganglia to modulate cognition. Its projections down to hypothalamic and brainstem nuclei allow it to modulate an emotional response. A few decades ago, the purported function of the amygdala was relatively simplistic. Increasing research of the amygdala has catapulted it into the spotlight as a brain region involved in numerous cognitive processes. The amygdala has recently been pronounced a 'connectivity hub' for its versatile and prolific contributions to emotion, reward, motivation, attention, learning and memory (Murray, 2007; Pessoa, 2008).

NEURAL CIRCUITRY OF EMOTIONS: WHERE DO THEY DIVERGE?

An ongoing controversy in the field of emotion research is whether positive and negative emotions are processed in distinct neural circuits. Since the subjective experience of positive and negative emotions is palpable, it is intuitive that there is at least some phase where the processing of emotions of different valence is distinct. However, the degree to which the circuits mediating positive and negative affects overlap is unknown.

Does the Amygdala Encode Valence or Intensity?

In the past, lesion studies, electrophysiological recordings and functional imaging studies have framed the amygdala as primarily processing threatening, aversive and fearful stimuli and events (Aggleton, 2000). This viewpoint has since been challenged by findings that the amygdala is involved in positively valenced events (Cahill and McGaugh, 1990). Since accumulating evidence convincingly argues that the amygdala is important for processing both positive and negative affect, the challenge of characterizing amygdala function now requires an examination of the multidimensional nature of affective space (Lang, 1995; Scherer, 2000).

Recently, a number of findings have suggested that the amygdala may differentially encode positive and negative emotions (Morris et al., 1996; Paton et al., 2006; Berntson et al., 2007). Specifically, a study using positron-emission tomography (PET) measures of neural activity in subjects looking at photographs of either happy or fearful facial

expressions revealed a greater amygdala response to fearful expressions (Morris et al., 1996). Primate amygdala recordings provide evidence that there are distinct populations of neurons encoding the positive and negative values of visual stimuli, though they also found neurons that responded to both positive and negative stimuli, but deemphasized this finding (Paton et al, 2006).

However, there is evidence that the amygdala codes for the intensity of the emotion (Adolphs et al., 1999; Anderson et al., 2003; Small et al., 2003; Winston et al., 2005). From fMRI measures of neural activity, and careful manipulation of the intensity and valence of various tastes, the dimensions of valence and intensity were parsed and found to be represented by distinct neural substrates (Small et al., 2003). Specifically, activity in the pons, mid-insular cortex and amygdala responded proportionally to the intensity of the taste, regardless of its hedonic valence. In contrast, the orbitofrontal cortex responded only to hedonic value, regardless of the taste intensity. A similar study using olfactory cues also found that amygdala activity was driven by the intensity, rather than the valence, of odorants and that orbitalfrontal cortices differentially responded to pleasant and unpleasant odorants, independent of judged intensity (Anderson et al., 2003). The striking similarity of these two independently conducted studies bolsters the notion that dimensions of emotion are represented by distinct neural circuits.

Importantly, the interpretation of studies concluding that the amygdala is primarily important for processing negative affect, as well as the interpretation of data showing the

amygdala differentially encodes positive and negative affect may be colored by the asymmetry of intensity for positive and negative valences (see Figure 1; Merzlyak, 2006). Specifically, the intensity of extremely negative emotions is greater than the intensity of extremely positive emotions. One can imagine how this is an evolutionarily adaptive skewness, given that survival is the ultimate goal. Escaping a threat that would result immediate death needs to be a priority that overrides all other priorities that aid in survival, such as feeding or breeding. Therefore, negative emotions such as fear, which signal an immediate threat to survival, must be programmed to have a higher intensity than positive emotions, which do not signal an immediate need that compromises survival.

Based on the findings described in this thesis in the context of the existing literature, I believe that the amygdala is the first site for processing the significance, as scaled by intensity, of sensory stimuli. By comparing previous findings (Rogan et al., 1997; McKernan and Shinnick-Gallagher, 1997; Rumpel et al., 2005) with the data in Chapter 2 (Tye et al., 2008), we can glean that the mechanisms by which the amygdala encodes positive and negative emotional memories is nearly identical, and that while the emotional valence may be distributed in at least partially non-overlapping neuronal subpopulations, most of the processing of the hedonic value of a stimulus takes place in downstream brain regions.

Although there is evidence that strongly negative emotions correspond to higher intensities than strongly positive emotions, we can estimate that:

Intensity ≅ |Valence|

If the amygdala encodes positive and negative emotions similarly, then the amygdala encodes the absolute value of valence, which equals intensity. From a practical perspective, the intensity of emotion should be processed before the valence of the emotion. The rationale for this is that the first step in processing a sensory stimulus is determining the relevance of this stimulus, as graded by the intensity of the emotion evoked. A stimulus does not require further processing if it is not relevant. If it is deemed relevant, then the next level of processing involves determining *how* it is relevant, specifically, gauging the hedonic value.

Emotional Processing in an Integrated Circuit: My Personal Sketch

Environmental stimuli enter the brain via the sensory cortices which provide a detailed information and via the thalamus which acts as an express relay-station that provides a less detailed but rapidly transmitted 'summary' of information. Information about stimuli of different sensory modalities is sent to the amygdala for evaluation and judgment as to whether further processing is necessary. For stimuli that have been evaluated as lacking motivational significance, BLA neurons will not fire and the information will not be distributed. For novel salient stimuli, the BLA will initially respond and transmit the information for further processing, but if after repeated presentations the further processing produces no motivational significance, the BLA will habituate responding and

cease the encoding of this stimulus. For environmental stimuli that have been assigned motivational significance, LA activity reflects the emotional intensity of the evaluated stimulus, and confers the importance of this stimulus to the BLA and other downstream regions. The mPFC provides an inhibitory suppression at the BLA to increase selectivity and ensure that only truly relevant stimuli receive further processing, and reciprocal connections between these areas allow for fine tuning. In addition, midbrain dopamine neurons projecting to the LA bolsters neural responding for significant or salient stimuli by modulating the inhibition coming from the mPFC. Driven by LA and BLA activity, the CeA initiates the hypothalamus and brainstem to evoke a physiological state of arousal. In parallel, the BLA sends information about the significance of the stimulus to the OFC, initiating a cooperative development of a representation of the outcome that the stimulus signals. The BLA may also confer with the hippocampus to integrate contextual and discrete environmental cues. Unidirectional projections to the nucleus accumbens core from the BLA converge with inputs from the frontal cortices and the hippocampus, where information about the motivational significance and the hedonic impact are pooled for a final assessment. Then the nucleus accumbens projects to the ventral pallidum to determine an appropriate behavioral output.

The Amygdala Processes Stimuli Based on Motivational Significance Rather than Hedonic Valence

Learning the association between a stimulus and an aversive outcome is first mediated by potentiated transmission from the thalamus to the LA by associative LTP, characterized by NMDAR-dependency and post-synaptic AMPAR trafficking. The results described in Chapter 2 show that learning the association between a stimulus and a reward is also mediated by strengthening thalamo-amygdala synapses via an NMDAR-dependent mechanism that results in an increase in postsynaptic AMPAR number or function. The striking similarity of these two mechanisms is consistent with the notion that the primary function of the amygdala is to sort incoming information based on the intensity of emotional significance, though it does not preclude the possibility that valence is represented by distinct populations of neurons (Paton et al., 2006). Chapter 3 demonstrates that motivation and reinforcement are encoded by distinct subpopulations of BLA neurons, one population which extinguishes readily, and one population that resists extinction even after the animal behaviorally demonstrates that it no longer associates the cue with the sucrose reward. Studies of LA neural activity during the extinction of aversive conditioning also show that there are two subpopulations of neurons that encode a conditioned stimulus, one which extinguishes quickly, and one which resists extinction. It is likely that in both of these cases, the rapidly extinguishing population of neurons mediates memory retrieval, and is active only when the stimulus-outcome association is still relevant and 'active' in mediating the animals behavior. Following extinction, the memory trace of the association, is stored in a subpopulation of neurons that persistently encode the now irrelevant conditioned stimulus in case the

contingencies return. This thesis evidences a remarkable coherence between the processing of positive and negative affective significance on many levels, from the synapse, to the population, to the corresponding behavior. If the mechanisms underlying the processing of positive and negative emotional valence mirror each other so precisely, then perhaps evaluating the intensity, rather than valence, of sensory stimuli is the primary function of the amygdala.

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