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Examination of Phasic and Sustained Fear Responses Using a Novel Sustained Fear Conditioning Paradigm

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Abstract

Examination of Phasic and Sustained Fear Responses Using a Novel Sustained Fear Conditioning Paradigm

By: Leigh Miles

Basic research has greatly improved our understanding of the neural mechanisms underlying emotional disorders such as fear and anxiety. Preclinical research has shown that while fear- and anxiety- like responses share similar physiological symptoms, they are mediated by different neural substrates. The medial division of the central nucleus of the amygdala (CeA_M) is necessary for the expression of fear-like responses to short-duration, predictable threats (operationally defined as phasic fear), and the bed nucleus of the stria terminalis (BNST) is necessary for the expression of anxiety-like responses to more long-duration, less predictable threats (operationally defined as sustained fear).

Experiments within this dissertation used fear-potentiated startle procedures to further examine the neural mechanisms mediating phasic and sustained fear responses in rats. Studies were designed to measure within-subject phasic and sustained fear, to pharmacologically dissociate fear responses using treatments that either *are* or are *not* clinically effective anxiolytics, and to evaluate if the selective serotonin reuptake inhibitor fluoxetine mediates its effects through serotonin within the BNST.

Rats received a single pre-test injection of the benzodiazepine chlordiazepoxide (10 mg/kg), the 5-HT_{1A} partial agonist buspirone (5 mg/kg), the selective serotonin reuptake inhibitor fluoxetine (10 mg/kg), or chronic (21-day) fluoxetine treatment and were tested for phasic and sustained fear. The role of serotonin within the BNST in chronic fluoxetine treatment was assessed in rats given chronic fluoxetine and then tested for sustained fear 48 hrs after bilateral BNST infusions of the serotonin lesioning agent, 5,7-dihydroxytryptamine (2μg/side).

Acute chlordiazepoxide (clinically effective treatment) blocked sustained but not phasic responses, acute buspirone (not clinically effective) did not affect sustained, but did disrupt phasic responses, chronic fluoxetine (clinically effective treatment) blocked sustained responses and unreliably reduced phasic responses, and acute fluoxetine (not clinically effective) affected neither. The results provide further evidence that phasic and sustained fear are mediated by different neural mechanisms and suggest that sustained fear may have greater predictive validity as a model of clinical anxiety as compared to phasic fear. Finally, although not statistically significant, results suggest that serotonin within the BNST may be important for the anxiolytic effects of chronic fluoxetine in this model.

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5,7-DHT	5,7-Dihydroxytryptamine
5-HT	serotonin
5-HTT	serotonin transporter
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor
BLA	basolateral amygdala nuclei
BMA	basomedial amygdala nuclei
BNST	bed nucleus of the stria terminalis
CeA_L	lateral division of the central nucleus of the amygdala
CeA_M	medial division of the central nucleus of the amygdala
CGRP	calcitonin gene-related peptide
CRF	corticotrophin releasing factor
CS	conditioned stimulus
DRN	dorsal raphe nuclei
GABA	γ-aminobutyric acid
HPA	hypothalamic-pituitary-axis
HPLC	high performance liquid chromatography
ISI	inter-stimulus interval
LA	lateral amygdala nuclei
NBQX	2,3-dihydroxy-6-nitro-7-sulfamoyl-benzo[f]quinoxaline-2,3-dione
NE	norepinephrine
PTSD	post-traumatic stress disorder

Chapter 1

General Introduction

General Introduction:

The National Institute of Health estimates that in a given year, up to 40 million adults are affected by a type of emotional disorder (e.g., generalized anxiety disorder, post-traumatic stress disorder (PTSD)), making anxiety disorders a national public health concern (Kessler et al., 2005). The Diagnostic and Statisical Manual of Mental Disorders-IV characterizes certain anxiety disorders as a slowly developing fear of diffuse threats or situations that remind the individual of a past traumatic event. Anxiety disorders are commonly treated with behavioral therapy and anti-anxiety medications that modulate an individual's neurochemical (e.g., γ-aminobutyric acid (GABA), serotonin (5-HT)) activity. However, the neurobiology underlying the regulation and maintenance of certain emotional disorders, as well as the contribution of anxiolytics within specific anxiety-related circuits are still poorly understood. A major aim within the psychiatric research field has been to develop a better understanding of the neural mechanisms underlying fear and anxiety disorders.

The purpose of this dissertation is to explore the neural mechanisms mediating fear-like (phasic fear) and anxiety-like (sustained fear) responses in an attempt to further dissociate the two emotional states. In this chapter I will review prior work on fear conditioning and the differences between phasic and sustained fear. In Chapter 2, I will discuss the modifications of two behavioral paradigms that elicit either phasic or sustained fear responses. In Chapter 3, I will discuss efforts made to pharmacologically dissociate phasic and sustained fear, as well as validation of sustained fear as a potential behavioral

model of anxiety. In Chapter 4, I will explore the role of 5-HT in sustained fear responses. Finally, in Chapter 5 I will discuss how my work fits into the larger framework of fear and anxiety research and what further questions it raises.

Fear is an evolutionarily conserved survival response, prompted by the presentation of an immediate threat. It is an adaptive state of apprehension or dread, that motivates an individual to produce active defense responses (Davis et al., 2010). Unfortunately, in some cases, the intensity and/or duration of a fear response can become inappropriate, and develop into an emotional disorder. To address this mental health concern, biomedical researchers use operational models of fear to elucidate the neural mechanisms mediating fear-motivated learning and memory.

Although in some animals there may be certain innate fears, fear generally results through associative learning processes. Pavlovian fear conditioning (a form of associative learning) is widely used as a behavioral model for examining the neurobiological mechanisms of fear (Davis, 1990; LeDoux, 2000). Conditioned fear is developed when a subject produces specific behavioral (e.g., increased startle), autonomic (e.g., increased blood pressure) and endocrine (e.g., elevated hormone release) responses--characteristically expressed in the presence of danger--to a once neutral conditioned stimulus (CS - e.g., light) that has been paired with an aversive unconditioned stimulus (US - e.g., footshock - (Blanchard and Blanchard, 1969; Davis, 2000; Fanselow, 1980; Fendt and Fanselow, 1999). Over the years, researchers have developed several methodological techniques with which to measure the expression of conditioned fear.

One of the most widely used behavioral tests is the acoustic startle reflex.

The acoustic startle reflex is a simple and effective method to indirectly measure fear memory in animals and humans (Davis and Astrachan, 1978; Grillon and Davis, 1997). In acoustic startle paradigms, fear memory is inferred from a quantifiable increase in startle amplitude to a startle-eliciting noise burst presented in the presence versus the absence of the CS. The increase in startle response in the presence of the CS is called fear-potentiated startle (Brown et al., 1951). In our laboratory, we use classical fear conditioning in combination with fear-potentiated startle to investigate the neural bases of fear memory (Davis, 1986; Davis et al., 1993; Hijzen et al., 1995). Fear-potentiated startle has served as a useful behavioral technique in human (i.e., eye-blink component) and animal (i.e., whole body component) studies. When stressed, both human and animal subjects tend to produce elevated startle responses (Brown et al., 1951; Grillon and Davis, 1997; Grillon et al., 1997; Hamm et al., 1991). These elevated responses are correspondingly reduced by similar anti-anxiety medications (Baas et al., 2002; Davis et al., 1997; Grillon et al., 2006; Grillon et al., 2009a; Miles et al., 2011; Swerdlow et al., 1986; Walker and Davis, 2002a), suggesting that preclinical fear-potentiated startle techniques are a potentially powerful translational approach for studying anxiety in healthy human subjects (Davis et al., 2010).

Using fear-potentiated startle in learning and memory research has a number of significant advantages. Briefly, it defines fear as a within-subject difference in startle amplitude (in the presence versus the absence of the CS), thereby reducing potential

between-subjects variability. Being able to measure fear in the absence of the CS allows the investigator to evaluate non-specific effects on startle amplitude per se (e.g., drug-induced reduction of startle). No footshocks are given during fear test sessions and therefore drug effects seen during testing cannot be attributed to changes in sensitivity to shocks. Additionally, there is a separation between training and testing sessions, which allows researchers to evaluate drug effects on acquisition versus expression of fear memory. Fear-potentiated startle takes advantage of an individual's innate startle reflex and thus does not involve any obvious operant behavior. This removes the potential for drug-induced effects that might alter subjects' ability to make or withhold a voluntary response. Moreover, in tests for fear or anxiety, fear-potentiated startle does not involve a suppression of on-going behavior, a major experimental benefit in that certain anxiolytic treatments themselves may alter behavior without affecting anxiety itself (Thiebot, 1983). Fear-potentiated startle is a useful experimental technique, allowing the investigator to observe both increases as well as decreases in fear expression due to pharmacological treatment (c.f.'s Davis, 1986; Davis, 1990).

In our typical rodent fear-potentiated startle paradigm we fear condition animals to a short-duration (seconds) stimulus that co-terminates with a mild 0.5-sec footshock. During behavioral testing, animals show a highly time-locked increase in startle during the CS, with a fast onset and fast offset that coincides with the timing in which the shock occurred during training (Davis et al., 1989; de Jongh et al., 2003). We operationally define this fear response to a short-duration, imminent threat as 'phasic fear'.

Over the last few decades, using a number of experimental manipulations, researchers have characterized key brain areas and anatomical connections responsible for regulation of phasic fear learning and memory (c.f. Davis, 1992b; Fendt and Fanselow, 1999; LeDoux, 2000; Maren and Fanselow, 1996). These brain areas and anatomical connections now define the standard neural circuit model of fear. Briefly, the neural model of fear involves sensory input from the thalamus and sensory cortices projecting to the amygdala complex. The amygdala is a forebrain structure found to be critical in fear memory processing (Amorapanth et al., 2000; Davis, 2000; Nader et al., 2001; Pitkanen et al., 1997). After sensory information is paired with an aversive US, output projections from the lateral division of the amygdala to the medial division of the central amygdala (CeA_M) activate target areas known to mediate specific behaviors that collectively define a state of conditioned fear (e.g., Gray and Magnuson, 1987; Hopkins and Holstege, 1978; Moga and Gray, 1985; Schwaber et al., 1982; Veening et al., 1984). Figures and a more detailed description of the neural circuit model of fear can be found in Chapter 2.

Both human and animal studies have confirmed that the amygdala regulates many aspects of the fear response. Lesions or inactivation of various amygdala nuclei produce deficits in the acquisition and expression of conditioned fear responses (e.g., fear-potentiated startle, freezing - (Davis, 1992b; Feinstein et al., 2010; LeDoux, 1992). Positron emission tomography and functional magnetic resonance imaging studies in humans show increased amygdala activation when subjects are presented with fear stimuli such as fear-conditioned cues, fearful faces, and fear-inducing images (LaBar et al., 1998; Phan et al., 2002; Whalen et al., 2001).

Somewhat more recently, an additional brain structure called the bed nucleus of the stria terminalis (BNST) has been added to the now modified neural circuit model of fear. The BNST, like the amygdala, is also a forebrain structure that receives afferent projections from cortical brain areas as well as specific amygdala nuclei, and like the CeA_M, projects to a number of the same anatomical sites known to elicit fear-like behavior (Alheid et al., 1998; Alheid et al., 1999; de Olmos and Heimer, 1999; Dong et al., 2001; Gray and Magnuson, 1987; Gray and Magnuson, 1992; Holstege et al., 1985; Ju et al., 1989; Moga and Gray, 1985; Moga et al., 1989; Pitkanen et al., 1997; Schwaber et al., 1982; Sofroniew, 1983; Veening et al., 1984). In addition, the BNST and the amygdala share a number of structural and neurochemical similarities (de Olmos and Heimer, 1999). These findings have led researchers to investigate the BNST's role in fear learning and memory. Figures and a more detailed description of the neural circuit model of fear can be found in Chapter 2.

By means of various classical fear conditioning paradigms and lesion/local inactivation techniques (detailed in Chapter 2), researchers have found that the amygdala (specifically the CeA_M) is necessary in mediating fear responses to short-duration, imminent threats (phasic fear), while the BNST seems to be necessary in mediating anxiety-like responses to more long-duration, less predictable threats, that we operationally define as 'sustained fear' (Campeau and Davis, 1995; Hitchcock and Davis, 1987; Lee et al., 1996; Resstel et al., 2008; Sullivan et al., 2004; Walker and Davis, 1997a; Walker and Davis, 1997b; Walker et al., 2009b). We view phasic fear as a more specific fear response and sustained

fear as more of a diffuse anxiety-like response (Walker et al., 2003). Efforts to more directly compare phasic and sustained fear responses are discussed in Chapter 2.

A number of localization studies have identified significant differences in morphology and peptide content between the CeA_M versus the lateral division of the central nucleus of the amygdala CeA_L and the BNST (Cassell et al., 1986; Day et al., 1999; Gray and Magnuson, 1987; Moga and Gray, 1985; Otake and Nakamura, 1995; Shimada et al., 1989; Veening et al., 1984; Wray and Hoffman, 1983). For example, high levels of the stress hormone corticotropin-releasing factor (CRF) are found in neurons in the BNST and CeA_L vs. the CeA_M (Chalmers et al., 1995; De Souza et al., 1985; Phelix et al., 1992b). CRF containing neurons in the CeA_L project to and act on CRF receptors in the BNST (De Souza et al., 1985; Phelix et al., 1992b; Sakanaka et al., 1986). Our laboratory found that excitotoxic lesions of the BNST or local infusions of a CRF antagonist into the BNST blocked the increase in startle amplitude caused by CRF given intracerebroventricularly (CRF-enhanced startle), whereas neither excitotoxic lesions of CeA or local CeA infusions of a CRF antagonist produced similar effects (Lee and Davis, 1997; Swerdlow et al., 1986). In addition, our lab also found that local infusion of calcitonin gene-related peptide (CGRP) into the BNST, which has high levels of CGRP receptors in close apposition to CRF neurons (Christopoulos et al., 1995; Kruger et al., 1988; Skofitsch and Jacobowitz, 1985), increased anxiety measures in the elevated plus maze and that intra-BNST infusions of a CGRP antagonist decrease sustained startle potentiation produced by a predator odor (Sink et al., 2011). These data support our efforts in further dissociating BNST-mediated sustained fear as a separate entity from that

of CeA_M-mediated phasic fear. Efforts to pharmacologically dissociate phasic and sustained fear responses are discussed in Chapter 3.

The BNST also plays an active role in stress/anxiety networks (Cullinan et al., 1993). Animal studies reveal a norepinephrine-induced increase in BNST Fos immunoreactivity and CRF expression following stress, as well as enhanced Fos expression within BNST-hypothalamus projecting neurons (Cullinan et al., 1996; Santibanez et al., 2006). Lesions of the BNST reduce stress-induced Fos activation in the hypothalamic-pituitary-axis (HPA) and result in a decrease in stress hormone levels (Crane et al., 2003). Human fMRI studies suggest that the BNST monitors changes in environmental threat levels, and that there is a highly active BNST threat-monitoring process in individuals with high trait anxiety that may be associated with hypervigilance (Somerville et al., 2010). These studies and others suggest a critical role for the BNST in the regulation of stress/anxiety responses (Walker and Davis, 2008).

Recently, a negative feedback loop between BNST CRF efferent projections and BNST 5-HT afferent projections has been suggested to be an important modulator of BNST-mediated anxiety-like responses (Hammack et al., 2009). Altered levels and functioning of 5-HT are hallmarks of certain mood disorders (e.g., depression, anxiety), suggesting that 5-HT is an active modulator of emotional states, but the literature remains unclear on the role of 5-HT within specific anxiety-circuits. The BNST has dense innervation by 5-HT neurons (Commons et al., 2003; Phelix et al., 1992a), expresses multiple 5-HT receptor subtypes (Cornea-Hebert et al., 1999; Hammack et al., 2009; Heidmann et al.,

1998; Kia et al., 1996; Mengod et al., 1990; Waeber et al., 1994; Wright et al., 1995), and is rich in 5-HT transporters (Commons et al., 2003; Hammack et al., 2009; Phelix et al., 1992b), the site of action of the commonly used anxiolytic, selective serotonin reuptake inhibitors (SSRI). Hence, the BNST may serve as a potential site of action of SSRI treatment; efforts to explore this notion are detailed in Chapter 4.

In this dissertation, I present experiments designed to further dissociate phasic and sustained fear. In Chapter 2, I discuss my efforts in the modification of phasic and sustained fear paradigms. In Chapter 3, I use the newly modified behavioral paradigms to pharmacologically dissociate phasic versus sustained fear using clinically-relevant drug treatments that either *are* or are *not* clinically effective anxiolytics. In Chapter 4, I examine the neurobiological underpinnings of sustained fear, investigating the role of 5-HT (within the BNST) in the expression of sustained fear. I believe these experiments will contribute to the field of neuroscience to broaden our understanding of the complex story of fear and anxiety.

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Chapter 2

Modification of phasic and sustained fear paradigms

Abstract:

A major aim within the psychiatric research field has been to develop a better understanding of the neural mechanisms underlying fear and anxiety disorders. Interestingly, while there is great overlap in the physiological symptoms of fear and anxiety, pre-clinical studies suggest that the two aversive emotional states are mediated by different neural substrates. Research has shown that the medial division of the central nucleus of the amygdala (CeA_M) is necessary in the expression of fear-like responses to short-duration, predictable threats (i.e., phasic fear), and that the bed nucleus of the stria terminalis (BNST) is necessary in the expression of anxiety-like responses to more long-duration, less predictable threats (i.e., sustained fear). The present chapter describes our attempts to develop reliable procedures to model phasic and sustained fear using identical training procedures. These behavioral models will allow us to more directly compare the two fear states in the hopes of further dissociating their unique neuronal processes.

Introduction:

Fear is a highly adaptive emotional response that serves as an internal alarm system to prepare an individual for an impending, potentially life-threatening danger within the immediate environment. Unfortunately, in some cases the intensity and/or duration of this fear response can become inappropriate and develop into an emotional disorder, which may compromise an individual's quality of life.

Excessive fear is thought be a key component underlying certain anxiety disorders. Not surprisingly, the emotional states of both fear and anxiety share many of the same physiological symptoms (Shin and Liberzon, 2010). Nevertheless, as discussed in the General Introduction, a number of recent findings suggest that although similar, fear and anxiety-like responses are mediated by different brain areas, using unique neuronal processes (c.f., Walker et al., 2009b).

Over the last few decades, a number of experimental manipulations have characterized key brain areas and anatomical connections responsible for regulation of fear learning and memory (c.f.'s Davis, 1992b; Fendt and Fanselow, 1999; LeDoux, 2000; Maren and Fanselow, 1996). These studies and many others have assisted in the development of the standard neural circuit model of fear (Figure 2.1; bold black lines); a model that involves sensory input from the thalamus, sensory cortex, and association areas being projected down to the basolateral complex of the amygdala, which includes the lateral (LA), basolateral (BLA), and the basomedial (BMA) nuclei

Figure 2.1

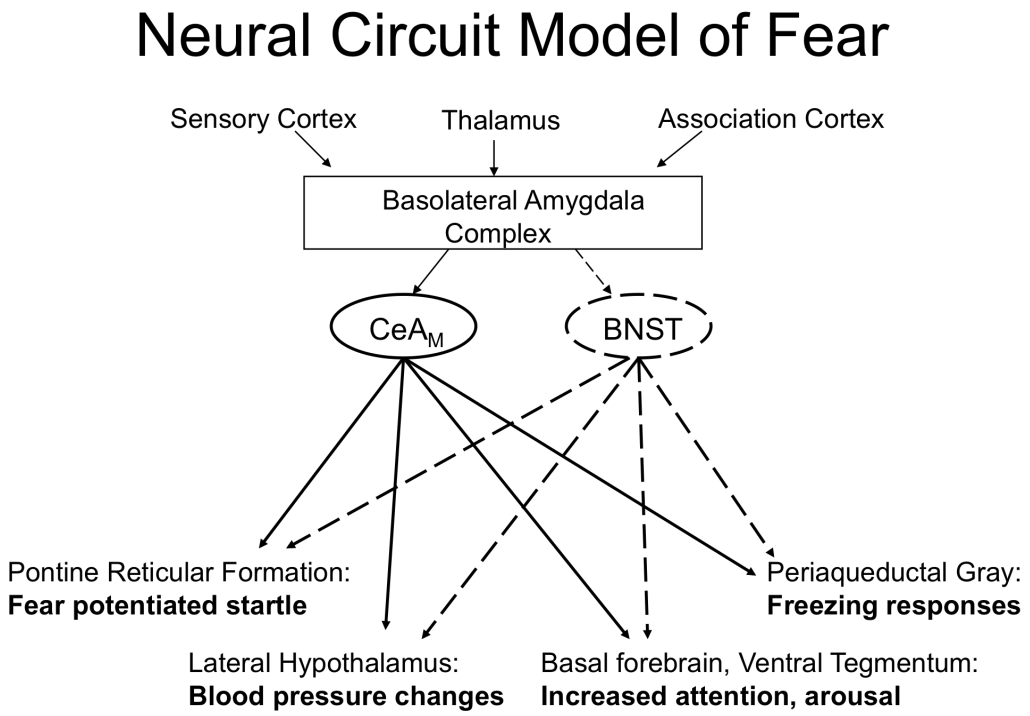

Figure 2.1. The neural circuit model of fear.

Following an emotional event, sensory inputs from afferent areas (e.g., the cortices, thalamus) project to the basolateral complex of the amygdala. If the informational input is processed as a threat signal, the information is relayed to the medial division of the central nucleus of the amygdala as well as the bed nucleus of the stria terminalis. These areas in turn project to a set of common target areas known to be key in mediating fear-associated behavior.

(McDonald et al., 1999; Turner and Herkenham, 1991). If these sensory inputs have been paired with an aversive event, outputs from the basolateral amygdala exceed some threshold so as to activate the medial division of the central amygdala (CeA_M), which then projects to a set of target areas known to mediate specific behaviors that underlie fear expression (e.g., Gray and Magnuson, 1987; Hopkins and Holstege, 1978; Moga and Gray, 1985; Schwaber et al., 1982; Veening et al., 1984).

More recently, a modified version of this neural circuit model of fear (Figure 2.1; dashed black lines) has been developed (c.f.'s Davis, 1992b; Fendt and Fanselow, 1999; LeDoux, 2000; Maren and Fanselow, 1996). It incorporates the bed nucleus of stria terminalis (BNST) (Dong et al., 2001; Pitkanen et al., 2000), a region often referred to as part of the 'extended amygdala', (Alheid et al., 1998; Ju et al., 1989) which projects to many of the same target areas as the CeA_M (Gray and Magnuson, 1987; Gray and Magnuson, 1992; Holstege et al., 1985; Moga and Gray, 1985; Moga et al., 1989; Schwaber et al., 1982; Sofroniew, 1983; Veening et al., 1984). The BNST shares both structural and neurochemical similarities, as well as reciprocal connections to that of the amygdala (specifically to the lateral division of the CeA and the BLA) (de Olmos and Heimer, 1999). Based on the similar afferent and efferent projections (Alheid et al., 1999) one might assume that experimental manipulations to the CeA would elicit comparable effects on fear learning and memory as would the same manipulations to the BNST. However, as described below, this is not always the case.

In rat studies, fear responses to a *short-duration conditioned* stimulus (CS) (i.e., phasic fear) were blocked by post-training lesions of the CeA or by pre-test infusions of the AMPA receptor antagonist, NBQX into the CeA (Campeau and Davis, 1995; Hitchcock and Davis, 1987; Lee et al., 1996; Walker and Davis, 1997b). However, these fear responses were not disrupted by post-training lesions of the BNST or by pre-test intra-BNST NBQX infusions (Sullivan et al., 2004; Walker and Davis, 1997b) (Table 2.1; Column 2). In contrast, inactivation of the BNST blocks slowly-developing, *long-duration unconditioned* increases in startle produced by sustained exposure to bright light, or infusion of the stress hormone corticotropin-releasing factor (CRF) into the lateral ventricle (i.e., light-enhanced and CRF-enhanced startle: unconditioned anxiogenic responses), while inactivation of the CeA did not (Table 2.1; Columns 3 and 4) (Swerdlow et al., 1986; Walker and Davis, 1997a; Walker and Davis, 1997b; Walker et al., 2009b). These observations provide direct support for the argument that phasic fear responses can be successfully dissociated from those that are more sustained.

Based on the above findings, there are two possibilities, 1) the CeA is necessary for producing fear responses to short-duration (seconds), discrete stimuli, while the BNST mediates fear responses to long-duration (minutes), more diffuse stimuli or 2) the CeA is necessary for producing conditioned fear responses, while the BNST is responsible for unconditioned responses. To disentangle these possibilities, Dr. David Walker from our laboratory developed a behavioral paradigm that could produce a fear-potentiated startle response to a stimulus that was both *conditioned* and *long in duration*. His studies (using a 8-min filtered white noise CS) found that inactivation of the CeA using NBQX

Table 2.1

Summary of lesions and/or NBQX infusions on fear-potentiated startle	Fear-Potentiated Startle (3.7-s noise CS)	Light-Enhanced Startle (20-min)	CRF-Enhanced Startle (2-hr)	Fear-Potentiated Startle (8-min noise CS)
Lesioned or inactivated nuclei	SHORT-DURATION and CONDITIONED	LONG-DURATION and UNCONDITIONED		LONG-DURATION and CONDITIONED
CeA	Blocked	No Effect	No Effect	Blocked early portions of CS presentation
BNST	No Effect	Blocked	Blocked	Blocked later portions of CS presentation

Table 2.1. Summary of lesion and/or NBQX infusion effects on fear-potentiated startle. Data suggests that the CeA mediates fear responses to short-duration stimuli and the BNST mediates fear responses to longer-duration stimuli.

reduced fear-potentiated startle at the very beginning, but not the later portions of the 8-min CS presentation, and that the inactivation of the BNST blocked fear-potentiated startle during the later, but not earlier portions of the 8-min CS presentation (Table 2.1; column 5 - (Walker et al., 2009b). These data supported the view that the CeA contributes to emotional responses following short-duration stimuli, and that the BNST controls emotional responses to more sustained fear stimuli, but it fails to support the other view that the CeA mediates conditioned fear responses and the BNST mediates unconditioned fear responses. Furthermore, these studies suggest that the neural basis of sustained fear could be experimentally evaluated using this novel sustained fear conditioning paradigm.

Although Dr. Walker's preliminary sustained fear paradigm yielded some very exciting findings, which elucidated important distinctions in the role of the CeA and the BNST in fear expression, it did not reliably produce robust sustained fear responses. Hence, more research was needed to strengthen this model.

In this project, we attempted to modify Dr. Walker's phasic and sustained fear paradigms, in an effort to reliably produce two different types of fear responses to the same fear stimulus. Our goal was to more directly compare and contrast the unique neuronal processes mediating phasic and sustained fear.

Materials and Methods:

Animals:

Male Sprague-Dawley rats (200–250 g at arrival; Charles River, Raleigh, NC) were housed 4/cage on a 12-hr light-dark cycle in a temperature- and humidity-controlled room with food and water freely available. Behavioral procedures began approximately one week after arrival, and were conducted in accordance with USDA, NIH, and Emory University guidelines.

Apparatus:

Rats were trained and tested in 8 x 15 x 15-cm Plexiglas and wire mesh cages with four 6.0-mm diameter stainless steel floorbars, located within a sound-attenuated behavior chamber. Startle responses were evoked by 50-ms (95 dB) white-noise bursts generated by a computer sound file, amplified by a Radio Shack amplifier (Tandy, Fort Worth, TX, USA), and delivered through Radio Shack Supertweeter speakers located ~12 cm in front of the cage. The same speakers delivered background noise (60 dB, 1-20 kHz) provided by an ACO Pacific, Inc. (Belmont, CA, USA) noise generator. All sound level measurements were made from the center of the cage.

Startle amplitude and shock reactivity were quantified using a PCB Piezotronics (Depew, NY, USA) accelerometer affixed to the bottom of the cage. The accelerometer produces a voltage output proportional to the velocity of cage movement (e.g., produced by the rats' startle response), which is integrated by a PCB Piezotronics signal conditioner and digitized by a GW Instruments (Somerville, MA, USA) InstruNet device. Startle

amplitude was defined as the maximum peak-to-peak voltage during the first 200 ms after each noise burst. Shock responses were similarly quantified, using a 500-ms sampling window concurrent with shock delivery.

The conditioned stimulus (CS) was either a 70-dB filtered noise or 60-Hz clicker stimulus (dependent on the experiment) delivered through speakers located 25 cm behind each chamber. The unconditioned stimulus was a 0.5-sec, 0.25 mA, 0.3 mA, 0.35 mA, or 0.4 mA footshock (dependent on the experiment) delivered through the floor bars. The sequencing of all stimuli was controlled by a desktop computer using custom-designed software (The Experimenter; Glass Bead Software, New Haven, CT, USA).

Common Behavioral Procedures:

Experimental Sequence: Rats received two acclimation sessions followed by a pre-conditioning test for sustained or phasic fear, followed by conditioning sessions, followed 48 hrs later by a post-conditioning test.

Acclimation: Rats were placed into the test cage and, after 5 minutes, presented with the first of 48 startle-eliciting white-noise bursts (inter-stimulus interval (ISI) = 30 seconds).

Pre-Conditioning Sustained Fear Test: Rats were placed into the test cage and, after 5 minutes, presented with the first of 32 startle-eliciting noise bursts (ISI = 30 sec). The first 16 were presented in the absence, and the next 16 in the presence of the CS.

Pre-Conditioning Phasic Fear Test: Rats were placed into the test cage and, after 5 minutes, presented with the first of 75 startle-eliciting noise bursts (ISI = 30 sec). Thirty of the final 60 were presented 3.2 seconds after onset of a 3.7-sec CS and another 30 were presented in its absence in a balanced irregular sequence across the session.

Fear Conditioning: The varying details of these procedures for each experiment are described below.

Post-Conditioning Phasic and Sustained Fear Tests: Rats were tested after conditioning, using procedures identical to those described for the pre-conditioning tests.

Context: During conditioning, a cotton gauze pad wetted with 0.4 ml of 70% ethanol solution was placed in front of the test cage. A fluorescent light placed on the back of the cage (150-lux as measured from the middle of the cage) provided constant illumination. During testing, to minimize context-potentiated startle, the cage was dark and no explicit olfactory stimuli were introduced. In addition, two 5-cm chains hung from the top of the test cage and a sandpaper insert was placed over the floorbars.

Statistical analyses:

Sustained Fear: Each rat tested for sustained fear received a sustained fear-potentiated startle score. Because our analysis of the control dataset indicated that the first startle response after CS onset was markedly higher than all those that followed, and that

sustained fear diminished with time, becoming unreliable after approximately the 4th minute of CS presentation, we calculated a sustained fear-potentiated startle score by dividing mean startle amplitude during the first 4 minutes of the CS (beginning with the 2nd CS test trial) by the mean startle amplitude during the last 4 minutes of the pre-CS period (see Figure 2.2). For presentation purposes, these ratios were converted to percent change scores.

Phasic Fear Measure: Each rat tested for phasic fear received a phasic fear-potentiated startle score, defined as the ratio between the mean startle amplitude of all CS test trials and the mean startle amplitude of all intermixed non-CS test trials. For presentation purposes, these ratios were converted to percent change scores.

Exclusion Criteria: Fear conditioning using these relatively weak footshocks depends on the rats actually receiving the shock through their footpads. Some rats largely avoid this by lying on the bars or putting their legs through them. Based on reactivity to footshock measured by cage output during the 500-msec shocks we excluded from further analysis the data obtained from rats with footshock reactions of 1.0 or less on 12 or more of the 24 conditioning trials. Because meaningful ratios cannot be calculated for rats that do not show a baseline startle response, the data from rats with a mean accelerometer output of

Figure 2.2

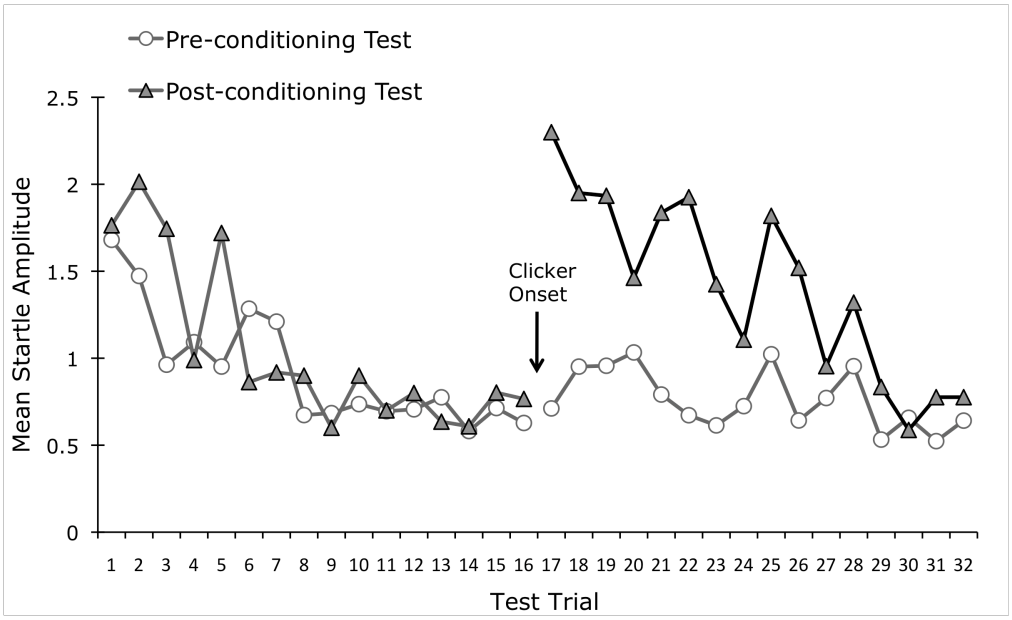

Figure 2. Representative trial-by-trial raw startle data during sustained fear tests (modified sustained fear paradigm with clicker CS).

Conditioning data are plotted for both the pre- (open circles) and post- (filled triangles) conditioning test sessions. Startle responses were evoked every 30 seconds during the 8 minutes prior to CS onset (trials 1-16) and for the 8 minutes during which the CS was presented (trials 17-32). Before conditioning, there appears to be some effect of the 60-Hz clicker stimulus on pre-CS startle, but this habituated to baseline prior to CS onset. After conditioning, potentiation by the clicker CS was clearly evident and especially pronounced on the first test trial after CS onset (i.e., trial 17). Potentiation dropped precipitously from the 1st to the 2nd CS test trial and more gradually thereafter, becoming statistically unreliable approximately halfway through the 8-minute CS.

$\leq$ 0.1 (i.e., what we observe when cage output is sampled in non-startled rats) on baseline

test trials were also excluded.

Inferential Statistics: The primary analyses were between-group comparisons of fear-potentiated scores. Because normality tests indicated significant deviations from normality for a number of datasets, between-group differences were evaluated using distribution-free (non-parametric) Mann-Whitney or Kruskal-Wallis tests and also, to establish statistical robustness, by using *t*-tests and ANOVA on log-transformed scores (Keene, 1995). For all tests, the criterion for significance was 0.05 (two-tailed).

Procedures Specific to Individual Experiments:

Experiment 1: Replicate preliminary sustained fear paradigm

The preliminary sustained fear procedure was originally performed by Dr. David Walker in a different behavioral room, using a similar (but not identical) behavioral apparatus. In the first experiment I tried to replicate Dr. Walker's earlier finding. Using the preliminary sustained fear paradigm (Figure 2.3) 12 rats were fear conditioned for 3 consecutive days. On each conditioning day, animals were exposed to two presentations of an 8-minute continuous 70-db filtered-noise stimulus. Each presentation was paired with 8 randomized 0.4 mA, 500-msec footshocks. The first CS of each session occurred 5 minutes after the rat was placed into the conditioning chamber. On each day, 8-min periods of silence and no shocks occurred after the first and second noise-shock periods.

Figure 2.3

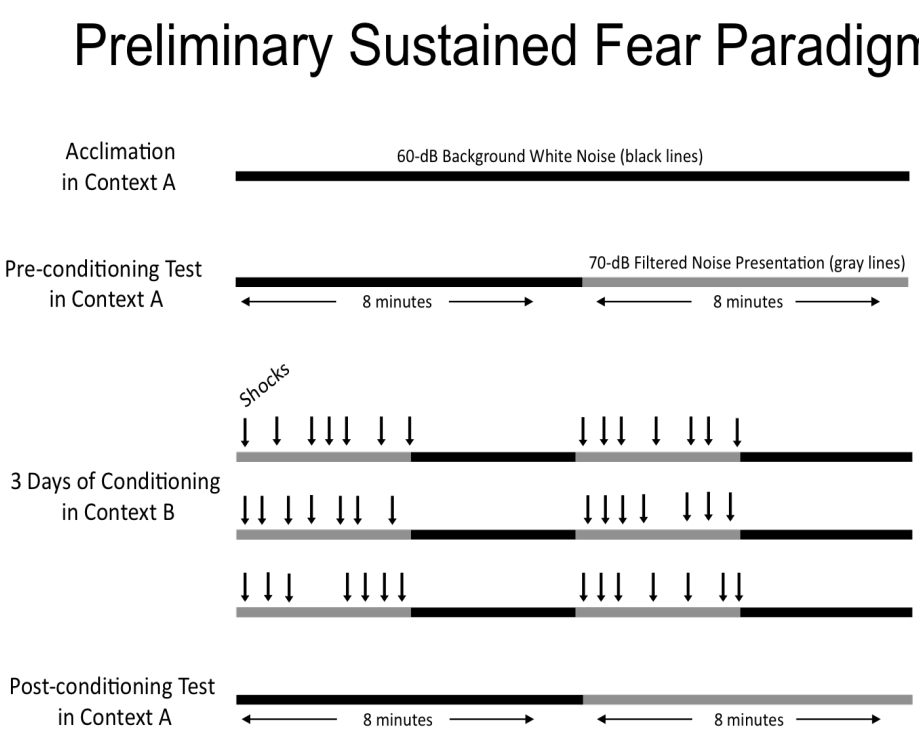

Figure 2.3. Preliminary sustained fear procedures and timeline.

Acclimation, a pre-conditioning test, each of 3 conditioning sessions in which two 8-min presentations of the conditioned stimuli (gray) were paired with randomized footshock (arrows), and a post-conditioning test, took place on separate days. For sustained fear testing, startle was measured before and then during presentation of an 8-minute clicker stimulus. Session and event lengths are not drawn to scale. For a detailed description, see the *Methods* section.

For this and the experiments that follow, testing was performed as described above in the

Common Behavioral Procedures.

Experiment 2: Modify sustained fear paradigm: Varied-CS duration during conditioning

In attempts to enhance the reliability and duration of the sustained fear responses during post-conditioning tests, a modified sustained fear paradigm was tested (Figure 2.4). Twenty-three rats were fear conditioned for 3 consecutive days. On each conditioning day, rats received 8 presentations of variable-duration continuous 70-db filtered-noise stimuli (3 sec, 10 sec, 20 sec, 1 min, 2 min, 4 min, 6 min, and 8 min long), each co-terminating with a 0.4 mA 500-msec footshock. The first CS of each session occurred 5 minutes after the rat was placed into the conditioning chamber. The interval between offset of one CS and onset of the next was 3 minutes. During the first conditioning session, the clicker stimuli were presented in order of increasing duration. During the second and third, they were sequenced randomly.

Experiment 3: Modify sustained fear paradigm: Varied-CS duration during conditioning (using a clicker CS)

In attempts to enhance the strength and duration of the sustained fear responses during post-conditioning tests, we tested the effect of using a more salient stimulus (i.e., a clicker stimulus) within the modified sustained fear paradigm. Sixteen rats were fear conditioned using the modified sustained fear paradigm (described in Experiment 2) replacing the filtered-noise CS with a 70-db 60-Hz clicker stimulus.

Figure 2.4

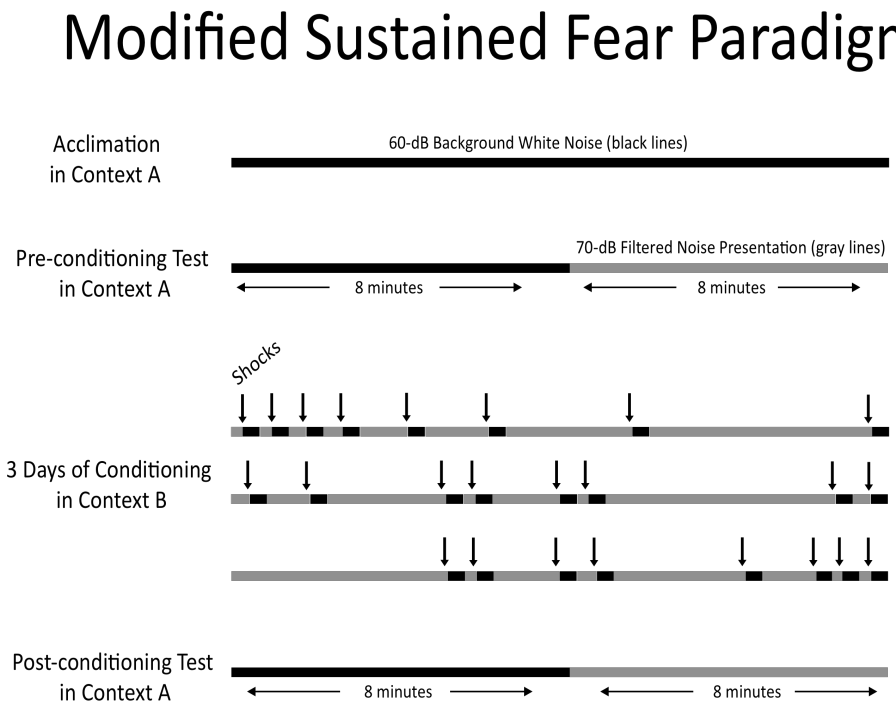

Figure 2.4. Modified sustained fear procedures and timeline.

Acclimation, a pre-conditioning test, each of 3 conditioning sessions in which the conditioned stimuli (gray) of variable duration were paired with co-terminating footshock (arrows), and a post-conditioning test, took place on separate days. For sustained fear testing, startle was measured before and then during presentation of an 8-minute clicker stimulus. Session and event lengths are not drawn to scale. For a detailed description, see the *Methods* section.

Experiment 4: Test modified sustained fear paradigm (using a clicker CS) for sensitization effects

Because the sustained fear procedure is new, it was important to determine if the 60-Hz clicker itself induced any unconditioned startle changes. In three large studies we trained and tested 48 rats in either the presence or absence of the clicker CS.

Experiment 5: Modified sustained fear paradigm (using a clicker CS): Varied number of conditioning days:

Using the more salient conditioned stimulus (i.e., 70-db 60-Hz stimulus), it was important to ensure that we are not unnecessarily overtraining the animals. Using the modified sustained fear paradigm, different groups of 12-16 rats were given either 1, 2, or 3 days of sustained fear training.

Experiment 6: Modified sustained fear paradigm (using a clicker CS): Varied footshock intensity:

To ensure that the shock intensity of 0.4 mA was of an appropriate strength (thereby avoiding less fear-potentiated startle at high shock intensities - (Davis and Astrachan, 1978), we trained different groups of 10-11 rats each in the modified sustained fear paradigm using shock intensities of either a 0.25 mA, 0.3 mA, 0.35 mA, or 0.4 mA shock intensity.

Experiment 7: Replicate preliminary phasic fear paradigm with a clicker CS:

The purpose of this experiment was to determine if the conventional phasic fear paradigm

(Davis, 1986) could reliably produce phasic fear using a 60-Hz clicker conditioned stimulus. Using the preliminary phasic fear paradigm (Figure 2.5), on each of 2 conditioning days, 20 animals were exposed to ten 3.5-sec presentations of a 60-Hz clicker CS, each presentation co-terminating with a 0.35 mA, 500-msec footshock presented at 2, 3, or 4-min randomly ordered intertrial intervals.

Experiment 8: Modified phasic fear paradigm with a clicker CS:

To more directly compare and contrast sustained versus phasic fear responses, we needed to minimize experimental confounds between the two paradigms. We modified the preliminary phasic fear paradigm (Figure 2.6) to ensure that the animals received identical training as those of our sustained fear animals. The only experimental difference between our sustained and phasic fear animals was how are they were tested (i.e., exposed to either a long-duration or short-duration fear stimulus). Sixteen animals were trained with the modified phasic fear paradigm to ensure that we could still produce reliable phasic fear response to a 60-Hz clicker conditioned stimulus.

Results:

Experiment 1: Replicate preliminary sustained fear paradigm

During testing for sustained fear, rats failed to produce any robust sustained fear response (Figure 2.7; left bar).

Experiment 2: Modify sustained fear paradigm: Varied-CS duration during conditioning

By changing the conditioning procedures (i.e., shaping the CS-US exposures), rats

Figure 2.5

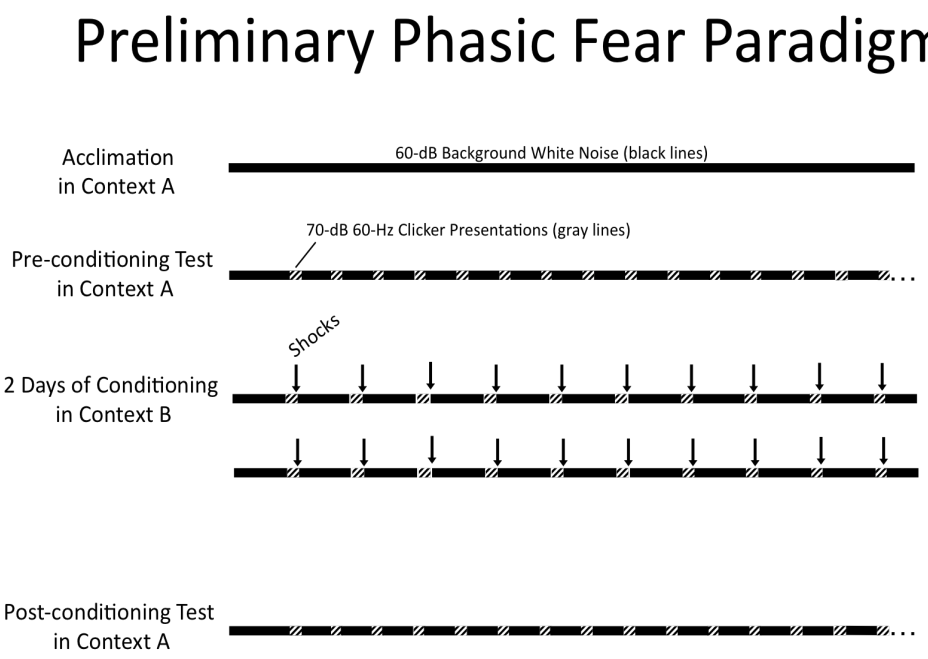

Figure 2.5. Preliminary phasic fear procedures and timeline.

Acclimation, a pre-conditioning test, each of 2 conditioning sessions in which ten 3.5-sec presentations of a 60-Hz clicker (gray) of variable duration were paired with co-terminating footshock (arrows), and a post-conditioning test, took place on separate days. For phasic fear testing, startle was measured in the presence and in the absence, on intermixed test trials, of 3.7-second conditioned stimuli. Session and event lengths are not drawn to scale. For a detailed description, see the *Methods* section.

Figure 2.6

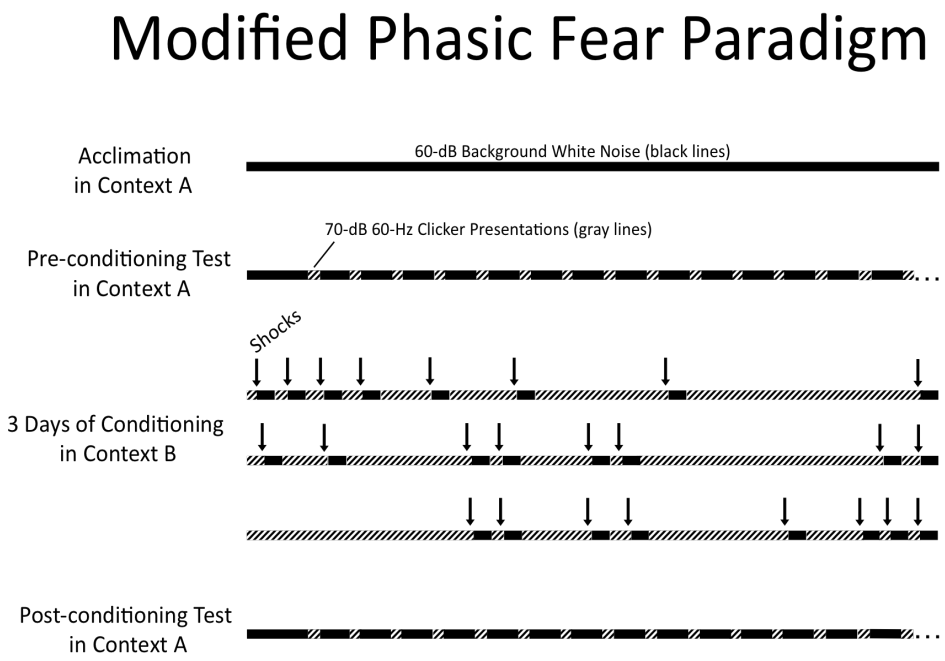

Figure 2.6. Modified phasic fear procedures and timeline.

Acclimation, a pre-conditioning test, each of 3 conditioning sessions in which the conditioned stimuli (gray) of variable duration were paired with co-terminating footshock (arrows), and a post-conditioning test, took place on separate days. With the exception of testing, the sustained and phasic fear procedures were identical. For phasic fear testing, startle was measured in the presence and in the absence, on intermixed test trials, of 3.7-second clicker stimuli. Session and event lengths are not drawn to scale. For a detailed description, see the *Methods* section.

Figure 2.7

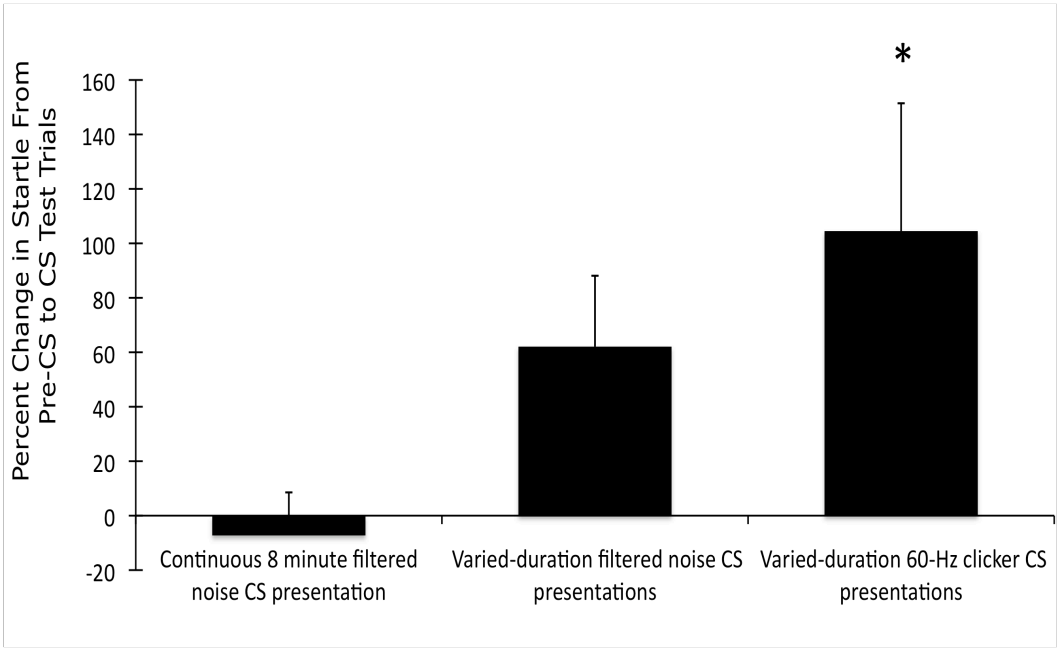

Figure 2.7. Sustained fear responses (preliminary versus modified sustained fear paradigms).

Modification of the preliminary sustained fear paradigm produced greater sustained fear. (* = $p < 0.05$ vs continuous 8 minute filterd noise CS presentation) showed an approximately 60% increase in the percent change in startle from the pre-CS to the CS test trials (Figure 2.7; middle bar). However, the percent change score was not significantly different from Experiment 1's results.

Experiment 3: Modify sustained fear paradigm: Varied-CS duration during conditioning (using a clicker CS)

By using a clicker CS (instead of the preliminary filtered-noise CS), rats showed an approximately 100% increase in the percent change in startle from the pre-CS to the CS test trials (Figure 2.7; right bar). An ANOVA on the log-transformed scores indicated a significant Treatment effect, $F(2, 42) = 4.29, p < 0.05$ which was due to the difference between the preliminary and modified (with a clicker CS) sustained fear paradigm groups ($p < 0.05$ Tukey post-hoc comparisons). Non-parametric analyses yielded similar results.

Experiment 4: Test modified sustained fear paradigm (using a clicker CS) for sensitization effects

Results showed no significant change in startle in the presence of the clicker even after getting footshocks during training, indicating that the prior increase in startle during the clicker could not be attributed to sensitization (Figure 2.8; middle group no-clicker in training, clicker in testing). In addition, animals trained in the presence of the clicker but tested in its absence also did not show any sensitization based on their lack of increased startle during testing. An ANOVA on the log-transformed scores showed a significant

Figure 2.8

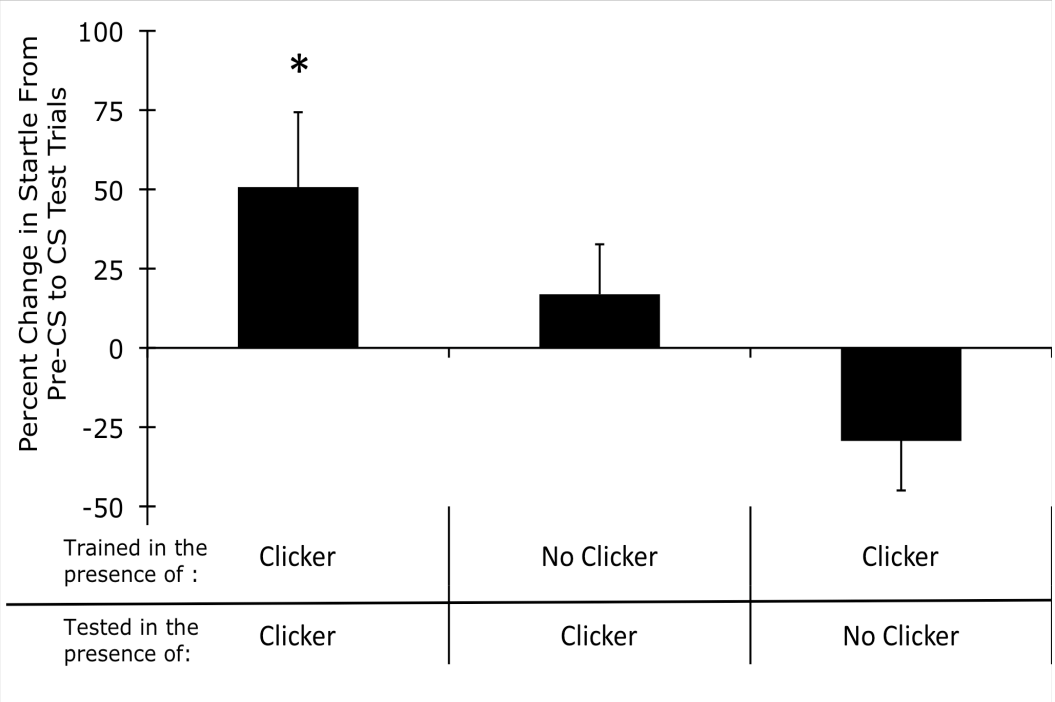

Figure 2.8. Sensitization to the clicker CS.

Results showed no significant effect on startle elicited in the presence of the clicker itself. ANOVA on the log-transformed scores showed a significant Treatment effect between groups, $F(2, 45) = 3.37$. Animals trained in the presence, but tested in the absence of the clicker CS had significantly lower sustained fear than the control group that was trained and tested in the presence of the clicker CS (* = $p < 0.05$, Tukey post-hoc comparisons). A non-parametric Kruskal Wallis analysis on percent change scores yielded non-significant results, H=5.7, 2 d.f., $p= 0.06$.

Treatment effect between groups, $F(2, 45) = 3.37$, $p < 0.05$. A Tukey's posthoc revealed a significant difference between the sustained fear startle response of animals trained in the presence, but tested in the absence of the clicker CS from control animals trained and tested in the presence of the clicker CS, $p < 0.05$. A non-parametric Kruskal Wallis analysis yielded borderline non-significant results, H=5.7, 2 d.f., $p= 0.06$.

Experiment 5: Modified sustained fear paradigm (using a clicker CS): Varied number of conditioning days:

Animals trained with three days of conditioning produced a more robust sustained fear response than those trained for one or two days (although not a significant effect - Figure 2.9). Based on the results we feel that three consecutive days of training is beneficial to the expression of a sustained fear response.

Experiment 6: Modified sustained fear paradigm (using a clicker CS): Varied footshock intensity:

Rats trained with a shock intensity of 0.35 mA showed the most robust sustained fear response as compared to the other shock intensities used (Figure 2.10). ANOVA on the log-transformed scores showed a significant Treatment effect, $F(3, 39) = 4.34$, $p < 0.05$. There was a significant difference between the percent change scores in animals trained with a 0.25 mA shock intensity as compared to those trained with a 0.35 mA ($p < 0.05$ Tukey post-hoc comparisons). Non-parametric analyses yielded similar results.

Experiment 7: Replicate preliminary phasic fear paradigm with a clicker CS:

A clicker CS (in place of the preliminary filtered-white noise CS) was able to

Figure 2.9

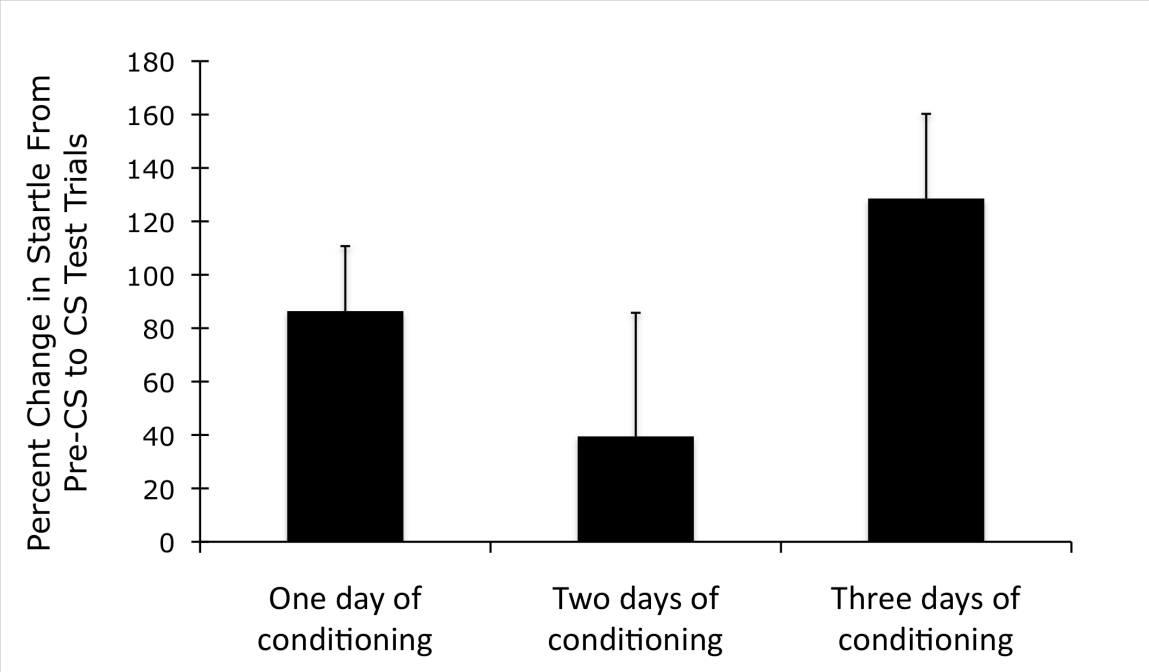

Figure 2.9. Varied number of conditioning days.

Although not a significant effect, data suggest that three days of conditioning (as opposed to one or two) produced higher sustained fear responses. Three days of training did not prove to be detrimental to the expression of a sustained fear response.

Figure 2.10

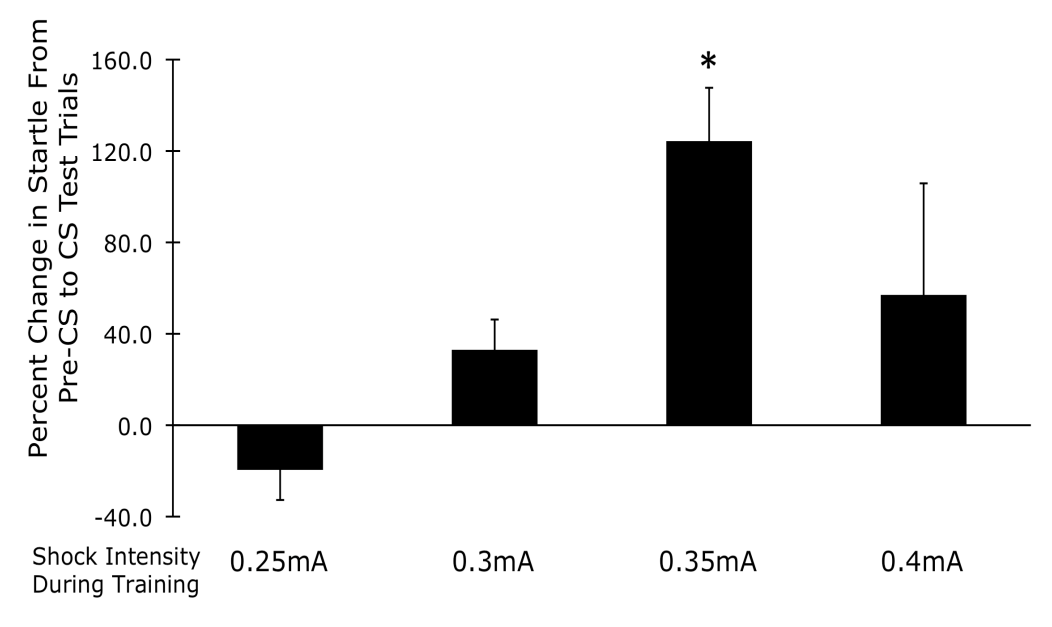

Figure 2.10. Varied shock intensities.

Rats trained with a shock intensity of 0.35 mA showed the most robust sustained fear response as compared to the other shock intensities. ANOVA on the log-transformed scores showed a significant Treatment effect, $F(3, 39) = 4.34$ with a significant difference between the percent change scores in animals trained with a 0.25 mA shock intensity as compared to those trained with a 0.35 mA (* = $p < 0.05$, Tukey post-hoc comparisons).

successfully produce a robust phasic fear response (Figure 2.11; left bar). A paired *t*-test yielded a significant difference between the log-transformed scores of the pre- *vs* post-conditioning tests, $t(39) = -21.9, p < 0.05$. Non-parametric analyses yielded similar results.

Experiment 8: Modified phasic fear paradigm with a clicker CS:

Animals produced robust phasic fear responses, using the varied-duration conditioning regime of the sustained fear paradigm (Figure 2.11; right bar). ANOVA indicated a significant Session effect (pre- vs post-conditioning test) $F(3, 68)=12.9, p < 0.05$ with no significant interaction. Non-parametric analyses yielded similar results.

Discussion:

We have successfully modified two behavioral fear paradigms (i.e., phasic and sustained) and are now able to consistently and reliably produce short- and longer-duration fear responses (respectively) to the same conditioned fear stimulus using the same training procedures and measured with an increase in the startle reflex. Based on the experimental results described above, we feel that our most robust sustained fear responses are reliably produced when we condition animals for 3 consecutive days, using varied duration presentations of a 60-Hz Clicker CS co-terminating with 0.35 mA footshocks. Moreover, the fact that sustained fear was produced using a conditioning procedure indicates that sustained fear is not restricted to only unconditioned stimuli.

Figure 2.11

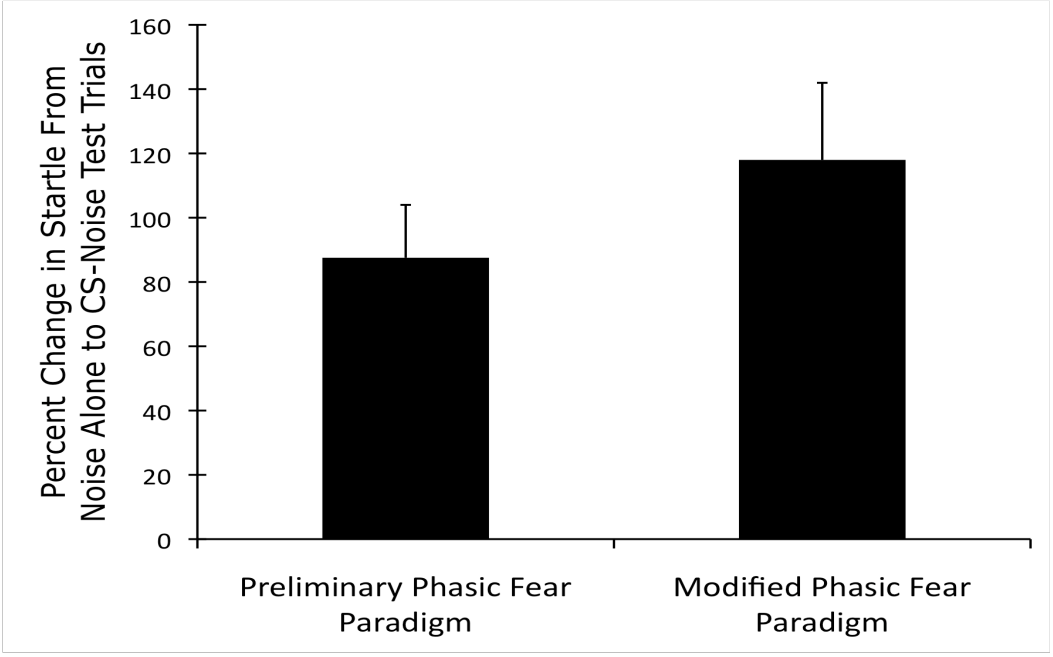

Figure 2.11. Phasic fear responses (preliminary versus modified phasic fear paradigms).

Animals were able to reliably produce robust phasic fear responses, using either the preliminary or the modified conditioning procedures. ANOVA indicated significant a significant Test effect (pre- vs post-conditioning test) $F(3, 68)=12.9, p < .05$ with no significant interaction. Non-parametric analyses yielded essentially the same results.

A proven sustained fear paradigm would serve as an excellent translational research tool for studying anxiety-related behaviors. Studies in humans suggest that sustained fear responses may bear a special relationship to clinical anxiety, more so than that of phasic fear (Davis et al., 2010). For example, researchers observed that startle potentiation to short-duration stimuli that had been paired with shock are no greater in post-traumatic stress (Grillon et al., 2009b) and panic disorder patients (Grillon et al., 2008) than in healthy controls. In contrast, potentiated startle responses *between* CS presentations (a type of sustained fear), *are* greater in these clinical groups (c.f., Davis et al., 2010). Hence, a pre-clinical sustained fear paradigm might provide a more suitable approach with which to study clinically relevant aversive emotional disorders (e.g., post-traumatic stress disorder).

A number of laboratories have used a wide-range of sustained fear paradigms in conjunction with phasic fear paradigms to successfully dissociate the contributions of the CeA versus the BNST (e.g., Hammack et al., 2004; Sullivan et al., 2004; Waddell et al., 2006). Unfortunately, the phasic and sustained fear procedures used in these studies had varied and sometimes complex conditioning and testing components, thus not directly comparing phasic versus sustained fear responses to the *same fear stimulus*. Our modified sustained fear paradigm appears to be behaviorally consistent (as opposed to light-enhanced startle), needs no exogenous hormonal application (e.g., CRF-enhanced startle), uses an innate behavioral reaction (as opposed to operant behavior/conditioned suppression), and is controlled by a discrete, unimodal stimulus (as opposed to the multimodal representation in conventional context conditioning). In addition, testing can be done in both the presence and

absence of the conditioned stimulus (as opposed to conventional context conditioning), and both the training and testing context can be varied (as opposed to conventional context conditioning).

Hence, I believe that our modified fear paradigms provide us with a *unique* opportunity to dissociate the neural processes mediating two very similar, yet different types of fear responses after identical training. These paradigms could potentially play an important role in examining the clinical validity of current and putative anxiolytics.

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Chapter 3

Phasic and Sustained Fear are Pharmacologically Dissociable in Rats

Adaptation from accepted publication:
Miles L, Davis M, Walker DL (2011). Phasic and sustained fear are pharmacologically dissociable in rats. *Psychopharmacology* (in press)

Abstract:

Previous findings suggest differences in the neuroanatomical substrates of short (seconds) versus longer-duration (minutes) fear responses. In this chapter I report that phasic and sustained fear can also be differentiated pharmacologically, based on their response to several treatments that either are or are not clinically effective anxiolytics. For these experiments, short- or long-duration clicker stimuli were paired with footshock. Acoustic startle amplitude was later measured in the absence of the clicker, or within seconds (phasic fear) or minutes (sustained fear) of its onset. Before testing, rats received a single injection of vehicle, the benzodiazepine chlordiazepoxide, the $5HT_{1A}$ agonist and dopamine D2 antagonist buspirone, the selective serotonin reuptake inhibitor (SSRI) fluoxetine, or a 3-week treatment with either vehicle or fluoxetine. Chlordiazepoxide blocked sustained but not phasic startle potentiation. Acute buspirone, which is not anxiolytic in humans, did not affect sustained startle potentiation, but did disrupt phasic increases. Chronic fluoxetine blocked sustained startle potentiation and unreliably reduced phasic increases. Acute fluoxetine affected neither. The results indicate that phasic and sustained fear responses can be pharmacologically dissociated, further validating this distinction, and suggest that sustained startle potentiation may be especially useful as an anxiety model and anxiolytic screen.

Introduction:

A major aim of psychiatric research is to better understand the neural mechanisms of psychiatric disorders, including anxiety. Because the physiological symptoms of healthy fear and clinical anxiety are highly similar, and because it is generally believed that clinical anxiety reflects maladaptive activity within fear circuitry, experimentally-induced fear in healthy controls and in research animals has been used extensively to pursue this goal.

We have used changes in the amplitude of the acoustic startle reflex as a fear measure and anxiety surrogate (Davis, 1986), and have described the neural circuitry that mediates this effect (c.f., Davis, 2006). When evoked by brief presentations of stimuli previously paired with shock, fear-potentiated startle is mediated by direct and indirect projections from the medial central nucleus of the amygdala (CeA) to the primary startle reflex pathway in the pontine reticular formation (Hitchcock and Davis, 1991; Meloni and Davis, 1999; Rosen et al., 1991; Zhao and Davis, 2004). However, when evoked by other treatments, including intra-cerebroventricular infusions of the stress-related peptide corticotropin releasing factor (Lee and Davis, 1997), by startle testing in illuminated versus darkened test chambers (Walker and Davis, 1997b), or by repeated footshock stress (Gewirtz et al., 1998), such increases appear to be mediated not by the medial CeA but instead, by a more rostral extension of the extended amygdala known as the bed nucleus of the stria terminalis (BNST).

In reviewing these and possibly-related findings from other laboratories (e.g., Hammack et al., 2004; Sullivan et al., 2004; Waddell et al., 2006), we have noted that CeA manipulations more consistently disrupt short-duration, rapid-onset/offset fear responses to distinct and imminent threats (phasic fear), whereas BNST manipulations more consistently disrupt longer-duration responses to more sustained stimuli (c.f., Walker et al., 2009b).

To explicitly compare the neurobiological substrates of phasic versus sustained startle potentiation, and to further evaluate the validity of this distinction, I developed a modified conditioned fear-potentiated startle paradigm in which clicker stimuli of variable duration (from 3 sec to 8 min) are paired with co-terminating footshocks. During testing, rats are presented with startle-eliciting noise bursts delivered within seconds (for phasic fear testing) or minutes (for sustained fear testing) of CS onset. In the present study, I used this paradigm to compare the effect on phasic and sustained fear of several pharmacological treatments that are clinically useful for anxiety reduction (i.e., acute chlordiazepoxide, chronic fluoxetine) and others that are not (i.e., acute buspirone, acute fluoxetine). The results are discussed with respect to their implications for the validity of the phasic versus sustained fear distinction, and for the utility of phasic versus sustained fear procedures as anxiety models and anxiolytic screens.

Materials and Methods:

Animals:

Male Sprague-Dawley rats (200–250 g at arrival; Charles River, Raleigh, NC) were housed 4/cage on a 12-hr light-dark cycle in a temperature- and humidity-controlled room with food and water freely available. Behavioral procedures began approximately one week after arrival, and were conducted in accordance with USDA, NIH, and Emory University guidelines.

Apparatus:

Rats were trained and tested in 8 x 15 x 15-cm Plexiglas and wire mesh cages with four 6.0-mm diameter stainless steel floorbars, located within a sound-attenuated behavior chamber. Startle responses were evoked by 50-ms (95 dB) white-noise bursts generated by a computer sound file, amplified by a Radio Shack amplifier (Tandy, Fort Worth, TX, USA), and delivered through Radio Shack Supertweeter speakers located in front of the cage. The same speakers delivered background noise (60-dB, 1-20 kHz) provided by an ACO Pacific, Inc. (Belmont, CA, USA) noise generator. All sound level measurements were made from the center of the cage.

Startle amplitude and shock reactivity were quantified using a PCB Piezotronics (Depew, NY, USA) accelerometer affixed to the bottom of the cage. The accelerometer produces a voltage output proportional to the velocity of cage movement (e.g., produced by the rats' startle response), which is integrated by a PCB Piezotronics signal conditioner and digitized by a GW Instruments (Somerville, MA, USA) InstruNet device. Startle

amplitude was defined as the maximum peak-to-peak voltage during the first 200 ms after each noise burst. Shock responses were similarly quantified, using a 500-ms window concurrent with shock delivery.

The conditioned stimulus (CS) was a 70-dB, 60-Hz clicker stimulus delivered through speakers located behind each chamber. The unconditioned stimulus was a 0.5-sec, 0.35 mA footshock delivered through the floor bars. The sequencing of all stimuli was controlled by a desktop computer using custom-designed software (The Experimenter; Glass Bead Software, New Haven, CT, USA).

Behavioral Procedures:

Experimental Sequence: Rats received two acclimation sessions (days 1 and 2) followed by a pre-conditioning test for sustained or phasic fear (day 3), followed by 3 conditioning sessions (days 4-6), followed 48 hrs later by a post-conditioning test. Thus, some rats were tested for sustained and others for phasic fear, but all rats received the same conditioning procedure. These procedures are described below, and presented graphically in Figure 3.1.

Acclimation: Rats were placed into the test cage and, after 5 minutes, presented with the first of 48 startle-eliciting white-noise bursts (inter-stimulus interval (ISI) = 30 seconds).

Pre-Conditioning Sustained Fear Test: Rats were placed into the test cage and, after 5 minutes, presented with the first of 32 startle-eliciting noise bursts (ISI = 30 sec). The first

Figure 3.1

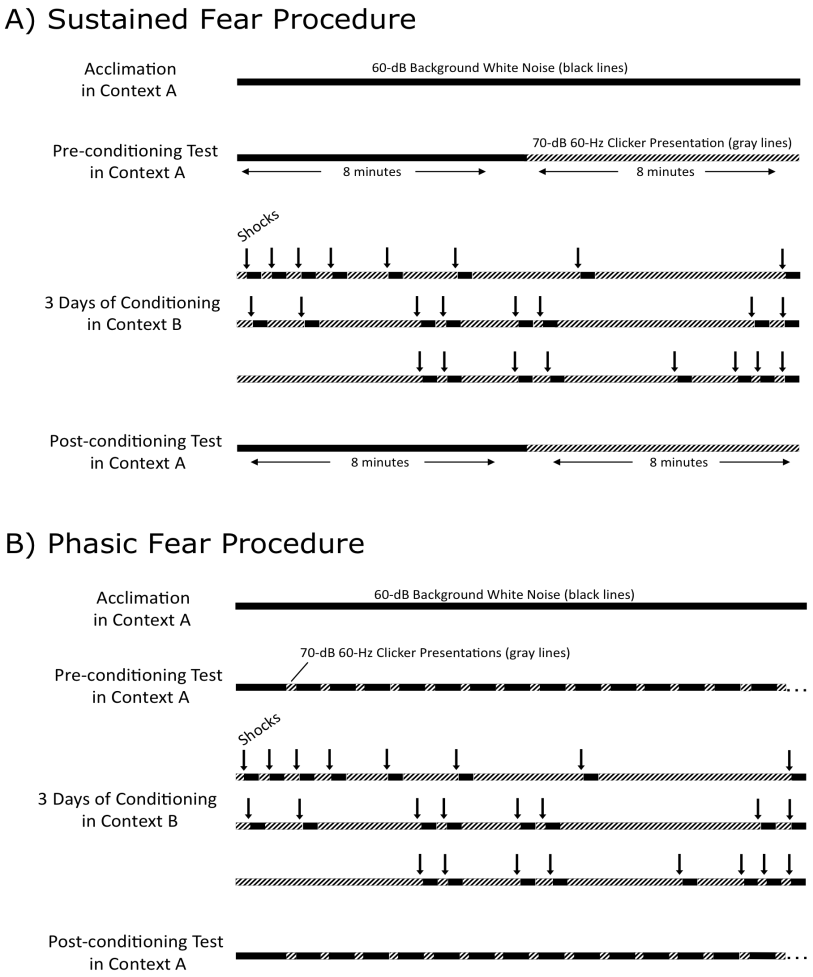

Figure 3.1. Behavioral procedures and timeline

Acclimation, a pre-conditioning test, each of 3 conditioning sessions in which clicker stimuli (gray) of variable duration were paired with co-terminating footshock (arrows), and a post-conditioning test, took place on separate days. With the exception of testing, the sustained and phasic fear procedures were identical. For sustained fear testing, startle was measured before and then during presentation of an 8-minute clicker stimulus. For phasic fear testing, startle was measured in the presence and in the absence, on intermixed test trials, of 3.7-second clicker stimuli. Session and event lengths are not drawn to scale. For a detailed description, see the *Methods* section.

16 were presented in the absence, and the next 16 in the presence of a 60-Hz clicker stimulus.

Pre-Conditioning Phasic Fear Test: Rats were placed into the test cage and, after 5 minutes, presented with the first of 75 startle-eliciting noise bursts (ISI = 30 sec). Thirty of the final 60 were presented 3.2 seconds after onset of a 3.7-sec clicker stimulus and another 30 (intermixed) were presented in its absence.

Fear Conditioning: On each conditioning day, rats received 8 presentations of variable-duration clicker stimuli (3-sec, 10-sec, 20-sec, 1-min, 2-min, 4-min, 6-min, and 8-min), each co-terminating with footshock. The first CS of each session occurred 5 minutes after the rat was placed into the conditioning chamber. The interval between offset of one CS and onset of the next was 3 minutes. During the first conditioning session, the clicker stimuli were presented in order of increasing duration. During the second and third, they were sequenced randomly.

Post-Conditioning Phasic and Sustained Fear Tests: Rats were tested after conditioning, using procedures identical to those described for the pre-conditioning tests.

Context Manipulations: During conditioning, a cotton gauze pad wetted with 0.4 ml of 70% ethanol solution was placed in front of the test cage. A fluorescent light placed behind the cage (150-lux as measured from the middle of the cage) provided constant illumination. During testing, to minimize context-potentiated startle, the cage was dark and no explicit

olfactory stimuli were introduced. In addition, two 5-cm chains hung from the top of the test cage and a sandpaper insert was placed over the floorbars. We have previously found similar changes to be effective in producing discriminable contexts (McNish et al., 1997).

Statistical analyses:

Sustained Fear: Each rat tested for sustained fear received a sustained fear-potentiated startle score. Because our analysis of the control dataset indicated that the first startle response after CS onset was markedly higher than all those that followed, and that sustained fear diminished with time, becoming unreliable after approximately the 4th minute of CS presentation (see Figure 3.2), we calculated a sustained fear-potentiated startle score by dividing mean startle amplitude during the first 4 minutes of the CS (beginning with the 2nd CS test trial) by the mean startle amplitude during the last 4 minutes of the pre-CS period (see Figure 3.2). For presentation purposes, these ratios were converted to percent change scores.

Phasic Fear Measure 1: Each rat tested for phasic fear received a phasic fear-potentiated startle score, defined as the ratio between the mean startle amplitude of all CS test trials and the mean startle amplitude of all intermixed non-CS test trials. For presentation purposes, these ratios were converted to percent change scores.

Phasic Fear Measure 2: An additional measure of phasic fear was computed for rats that received sustained fear testing. This was defined as the ratio between startle amplitude to

Figure 3.2

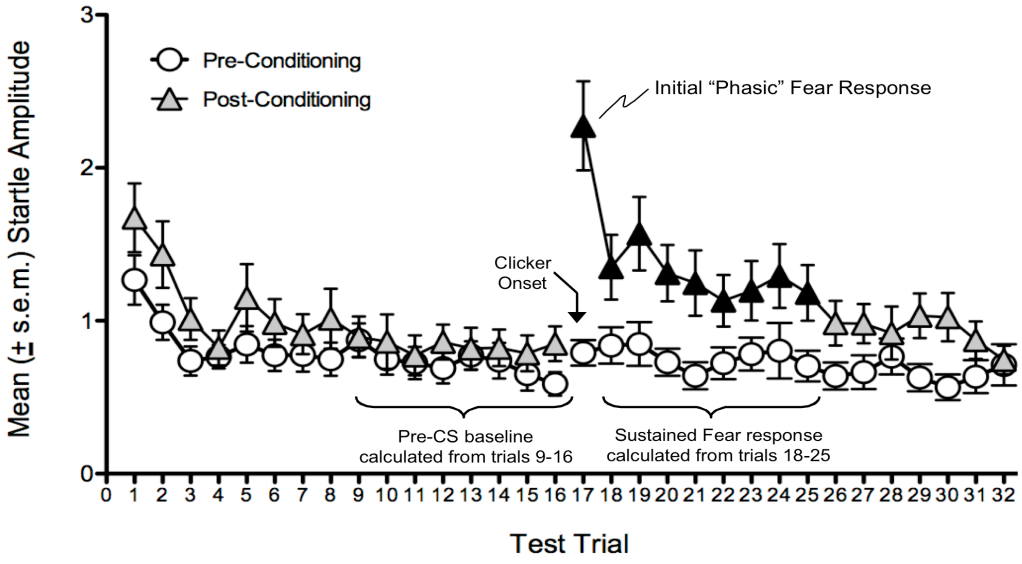

Figure 3.2. Trial-by-trial raw startle data during sustained fear tests. The raw startle data for all control rats used in these studies are plotted for both the pre- (open circles) and post- (filled triangles) conditioning test sessions. Startle responses were evoked every 30 seconds during the 8 minutes prior to CS onset (trials 1-16) and for the 8 minutes during which the CS was presented (trials 17-32). Before conditioning, there was little if any effect of the 60-Hz clicker stimulus on startle. After conditioning, potentiation by the clicker CS was clearly evident and especially pronounced on the first test trial after CS onset (i.e., trial 17), which we consider more akin to phasic than sustained fear. Potentiation dropped precipitously from the 1st to the 2nd CS test trial and more gradually thereafter, becoming statistically unreliable approximately halfway through the 8-minute CS. The percent change scores are based on the ratio (mean startle amplitude across CS test trials 18-25 / mean startle amplitude across pre-CS test trials 9-16) for *sustained fear*, and (mean startle amplitude on trial 17 / mean startle amplitude across pre-CS test trials 9-16) for *phasic fear measure 2*. ▲ = $p < 0.05$ (paired *t*-test)

the very first noise burst after CS onset (i.e., on trial 17 which occurred 19.2 seconds after CS onset) and the pre-CS baseline (i.e., trials 9-16).

For all measures, ratio rather than absolute difference (i.e., startle in presence of clicker – startle in absence of clicker) were used because we have previously found that when fear levels remain constant, ratio, but not difference scores remain stable as well (Walker and Davis, 2002b). We also note that our use of startle responses elicited 19.2 seconds or less as opposed to 49.2 seconds or more, for phasic and sustained fear respectively, is somewhat arbitrary (as would almost certainly be true for any specific time-point), as the transition from one to the other is most likely gradual rather than abrupt. Our aim here was to have one set of measures which was *more* phasic and *less* sustained than the other, but we recognize that each may have elements of both. As will be seen from the results that follow, the data obtained with these scoring methods generally appear to confirm their validity.

Exclusion Criteria: Fear conditioning requires that rats perceive the aversive stimulus. Therefore, we excluded from further analysis the data obtained from rats with footshock reactions of 1.0 or less (the mean of all rats was 3.5) on 12 or more of the 24 conditioning trials. The potentiation data from control rats validated this criterion. Thus, for sustained fear, the mean ($\pm$ s.e.m.) fear-potentiated startle score of rats exceeding this threshold was 83.1 $\pm$ 19.5 % (*N*=55) versus 11.5 $\pm$ 16.4 % for those that did not (*N*=12). For the phasic fear experiments, the mean fear-potentiated startle score of shock-responsive rats was 107.7 $\pm$ 38.9 (*N*=21) versus -26 $\pm$ 23.4 (*N*=3) for excluded rats.

Also, because meaningful ratios cannot be calculated for rats that do not show a baseline startle response, the data from rats with a mean accelerometer output of ≤ 0.1 (i.e., what we observe when cage output is sampled in non-startled rats) on baseline test trials were also excluded. Only two rats failed to meet this criterion. Both had received chlordiazepozide prior to sustained fear testing.

Inferential Statistics: The primary analyses were between-group comparisons of fear-potentiated scores. Because D'Agostino and Pearson omnibus normality tests indicated significant deviations from normality for both the sustained ($K2 = 51.78$) and phasic fear ($K2 = 45.53$) datasets, and because Grubb's test identified several outliers, between-group differences were evaluated using distribution-free (non-parametric) Mann-Whitney or Kruskal-Wallis tests and also, to establish statistical robustness, by using *t*-tests and ANOVA on log-transformed scores (see Keene, 1995). Follow-up comparisons were made using Dunn's (non-parametric) or Dunnett's *t*-test (parametric) for multiple comparisons with a control. Other analyses intended to address specific questions or issues are included where relevant. For all tests, the criterion for significance was 0.05 (two-tailed).

Procedures Specific to Individual Experiments:

Experiment 1: No-Shock Control. Because the sustained fear procedure is new, we wished to determine if the clicker-induced startle changes observed in control rats were

indeed due to conditioning. To this end, 12 rats underwent the sustained fear procedure exactly as described above, but without shock administration during clicker presentations. For this and the experiments that follow, testing was performed as described above in the Common Behavioral Procedures.

Experiment 2: Acute benzodiazepine effect on phasic fear-potentiated startle. 10 minutes prior to the post-conditioning test, rats received an intra-peritoneal (i.p.) injection of either saline (*N*=5), 7.5 mg/kg chlordiazepoxide (*N*=6) or 10 mg/kg chlordiazepozide (*N*=5), doses based on weight of salt. Chlordiazepozide was obtained from Sigma-Aldrich Chemical Co. (St. Louis, MO).

For this and all other acute drug administration experiments, rats received the same drug and dose prior to the pre-conditioning test which allowed us to evaluate drug effects on unconditioned startle amplitude. The sole exception was rats that received 7.5 mg/kg chlordiazepoxide prior to the post-conditioning test but 2.5 mg/kg prior to the pre-conditioning test (we had anticipated using 2.5 mg/kg prior to both, but increased to the higher dose based on the initial potentiation data from rats that received 10 mg/kg chlordiazepoxide).

For all experiments, drugs were administered at 0.1 ml saline/100 g body weight.

Experiment 3: Acute benzodiazepine effects on sustained fear-potentiated startle. 10 minutes prior to the pre- and post-conditioning tests, rats received an intra-peritoneal

(i.p.) injection of saline (*N*=12) or chlordiazepoxide (*N*=11; 10 mg/kg).

Experiment 4: Acute buspirone and fluoxetine effects on phasic fear-potentiated startle. 10 minutes prior to the pre- and post-conditioning tests, rats received a subcutaneous (s.c.) injection of buspirone (*N*=7; 5 mg/kg; Sigma-Aldrich Chemical Co.), an i.p. injection of fluoxetine (*N*=16; 10 mg/kg; Spectrum Chemical, Gardena, CA, USA) or saline (*N*=6, i.p.; *N*=7, s.c.). All doses based on weight of salt.

Experiment 5: Acute buspirone and fluoxetine effects on sustained fear-potentiated startle. 10 minutes prior to the pre- and post-conditioning tests, rats received buspirone (*N*=13; 5 mg/kg s.c.), fluoxetine (*N*=22; 10 mg/kg, i.p.), or saline (*N*=13, i.p.; *N*=12, s.c.). All doses based on weight of salt.

Experiment 6: Chronic fluoxetine effects on phasic fear-potentiated startle. Rats received the first of 21 treatments of either saline (*N*=8) or fluoxetine (*N*=7; 10 mg/kg) by oral gavage approximately 3 hrs after the final conditioning session and for each of the following 20 days. The post-conditioning test was conducted approximately 24 hr after the final injection.

Experiment 7: Chronic fluoxetine effects on sustained fear-potentiated startle. Rats received the first of 21 treatments of saline (*N*=18) or fluoxetine (*N*=20; 10 mg/kg) by oral gavage approximately 3 hrs after the final conditioning session and for each of the following 20 days. The post-conditioning test was conducted approximately 24 hr after

the final injection.

Results:

Sustained fear in control animals. Before presenting results from the individual experiments, I first provide a descriptive account of sustained fear in control rats. Figure 3.2 illustrates the trial-by-trial data pooled from all control rats used in the sustained fear experiments. As shown in this figure, startle amplitude mostly habituated to a stable baseline within the first few trials of the pre-CS conditioning phase and remained relatively stable thereafter. Introduction of the clicker stimulus, between trials 16 and 17, had little if any effect on startle prior to conditioning (open circles), but caused a marked enhancement of startle after conditioning (filled triangles). This enhancement was especially pronounced on the first trial after CS onset (trial 17), which we have scored independently as an ancillary measure of *phasic* fear (i.e., phasic fear measure 2). By the 5th minute after CS onset, potentiation began to wane and was no longer statistically reliable.

Figure 3.2 also shows a modest increase in pre-CS startle amplitude from the pre- to post-conditioning test (i.e., compare open circles to filled triangles on left side of Figure 3.2). Although significant overall (ANOVA on the first 16 trials that preceded CS onset indicated a main effect of Session (i.e., pre- versus post-conditioning), $F(1, 51)=8.54$, $p < 0.05$, the increase was only observed in rats from Experiment 7 – i.e., the group that had a 25-day delay interposed between the two tests (see also Table 3.1). As such, we believe

Table 1. Mean Startle Amplitude (± s.e.m.) in arbitrary units on a linear scale, together with log-transformed fear-potentiated startle (FPS) scores

		Pre-conditioning Test					Post-conditioning Test				
		Startle without clicker	Startle with clicker (phasic)	Startle with clicker (sustained)	Log-transformed Phasic FPS[1]	Log-transformed Sustained FPS	Startle without clicker	Startle with clicker (phasic)	Startle with clicker (sustained)	Log-transformed Phasic FPS[1]	Log-transformed Sustained FPS
Sustained Fear Tests[1]											
Exp 1	No Shock Control	0.64 ± 0.09	0.61± 0.10	0.76 ± 0.11	-0.03 ± 0.08	0.08 ± 0.05	0.56 ± 0.18	0.47 ± 0.08	0.49 ± 0.07	-0.05 ± 0.06	-0.05 ± 0.06
Exp 3	Acute Saline	0.75 ± 0.19	0.69 ± 0.13	0.83 ± 0.24	0.01 ± 0.06	-0.03 ± 0.05	0.58 ± 0.16	1.66 ± 0.72	0.74 ± 0.19	0.25 ± 0.14	0.19 ± 0.09
	Acute Chlordiazepoxide	0.79 ± 0.32	0.52 ± 0.14	1.0 ± 0.33	-0.08 ± 0.15	0.16 ± 0.08	0.80 ± 0.29	1.72 ± 0.86	0.58 ± 0.27	0.22 ± 0.09	-0.13 ± 0.05
Exp 5	Acute Saline	0.73 ± 0.12	0.88 ± 0.15	0.79 ± 0.13	0.05 ± 0.07	0.02 ± 0.04	0.80 ± 0.13	1.74 ± 0.35	1.07 ± 0.20	0.29 ± 0.07	0.10 ± 0.05
	Acute Buspirone	1.39 ± 0.24	1.62 ± 0.30	1.43 ± 0.27	0.06 ± 0.06	-0.01 ± 0.09	1.40 ± 0.31	1.34 ± 0.23	1.73 ± 0.32	0.05 ± 0.10	0.12 ± 0.08
	Acute Fluoxetine	0.80 ± 0.16	0.94 ± 0.17	0.81 ± 0.11	0.06 ± 0.08	0.02 ± 0.05	0.63 ± 0.11	1.25 ± 0.26	0.97 ± 0.18	0.21 ± 0.09	0.18 ± 0.06
Exp 7	Chronic Saline	0.70 ± 0.13	0.78 ± 0.13	0.73 ± 0.10	0.04 ± 0.06	0.01 ± 0.05	1.1 ± 0.21	3.09 ± 0.58	1.70 ± 0.35	0.42 ± 0.06	0.17 ± 0.06
	Chronic Fluoxetine	0.64 ± 0.10	0.67 ± 0.12	0.69 ± 0.12	-0.01 ± 0.08	0.00 ± 0.07	2.20 ± 0.43	2.99 ± 0.58	2.40 ± 0.58	0.13 ± 0.05	0.01 ± 0.04
Phasic Fear Tests											
Exp 2	Acute Saline	0.68 ± 0.12	0.72 ± 0.21	n/a	-0.01 ± 0.11	n/a	0.55 ± 0.11	0.76 ± 0.16	n/a	0.14 ± 0.10	n/a
	Acute Chlordiazepoxide (7.5mg/kg)[2]	0.57 ± 0.13	0.56 ± 0.14	n/a	-0.01 ± 0.05	n/a	0.39 ± 0.13	0.71 ± 0.25	n/a	0.24 ± 0.08	n/a
	Acute Chlordiazepoxide (10 mg/kg)	0.34 ± 0.07	0.40 ± 0.07	n/a	0.08 ± 0.05	n/a	0.20 ± 0.04	0.36 ± 0.04	n/a	0.29 ± 0.11	n/a
Exp 4	Acute Saline	0.62 ± 0.12	0.55 ± 0.11	n/a	-0.03 ± 0.05	n/a	0.50 ± 0.08	1.16 ± 0.36	n/a	0.29 ± 0.07	n/a
	Acute Buspirone	0.81 ± 0.24	0.79 ± 0.29	n/a	-0.04 ± 0.09	n/a	1.12 ± 0.24	1.15 ± 0.24	n/a	0.02 ± 0.07	n/a
	Acute Fluoxetine	0.61 ± 0.07	0.55 ± 0.15	n/a	-0.03 ± 0.07	n/a	0.76 ± 0.12	1.43 ± 0.31	n/a	0.23 ± 0.04	n/a
Exp 6	Chronic Saline	1.26 ± 0.29	1.29 ± 0.47	n/a	-0.04 ± 0.06	n/a	1.42 ± 0.35	2.12 ± 0.45	n/a	0.19 ± 0.06	n/a
	Chronic Fluoxetine	1.09 ± 0.29	1.07 ± 0.28	n/a	-0.03 ± 0.04	n/a	1.82 ± 0.37	2.76 ± 0.69	n/a	0.14 ± 0.07	n/a

[1]For the sustained fear tests, startle potentiation during the 1st test trial after CS onset was used as a supplementary phasic fear measure.

[2]These rats received 2.5 mg/kg prior to the pre-conditioning test, but 7.5 mg/kg prior to the post-conditioning test.

this increase most likely reflects weight gain or something associated with the daily gavage procedure, as opposed, for example, to a generalized context fear response.

The figures that follow show percent change (i.e., from non-CS or pre-CS test trials to CS test trials) scores only. The absolute startle amplitudes from which these change scores are derived are provided in Table 3.1.

Experiment 1: Clicker-induced startle changes require conditioning. During testing, rats that did not receive clicker-shock pairings did not show either phasic or sustained startle potentiation (see Table 3.1).

Experiment 2: Pre-test chlordiazepoxide did not disrupt phasic startle potentiation. Phasic fear-potentiation was not reduced, but was nominally (although not significantly) greater in the chlordiazepozide compared to saline groups (Figure 3.3) based on ANOVA. Baseline startle *was* reduced however, as indicated by a significant Group effect, $F(2, 13) = 4.40$. A Dunnett's *t*-test indicated a significant baseline difference between the vehicle and 10 mg/kg group, $q(9) = 2.95$, but not between the 0 and 7.5 mg/kg group (see also Table 3.1).

Experiment 3: Pre-test chlordiazepoxide blocked the sustained but not initial componentof startle potentiation. As shown in Figure 3.4 (and see also Table 3.1), acute pre-test chlordiazepoxide administration blocked startle potentiation to the long-duration clicker CS. The disruption was statistically robust, being detected by parametric,

Figure 3.3

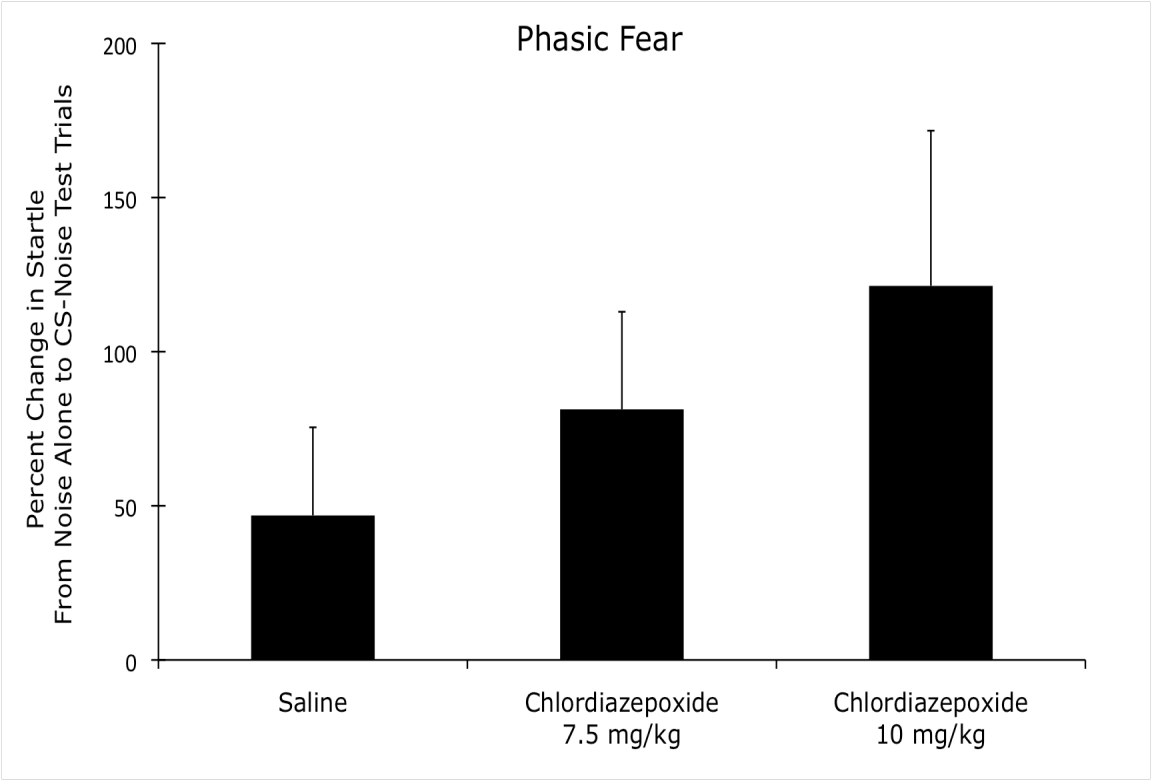

Figure 3.3. Acute chlordiazepoxide effects on phasic startle potentiation

Pre-test chlordiazepoxide did not disrupt phasic startle potentiation to 3.7-sec clicker stimuli.

Figure 3.4

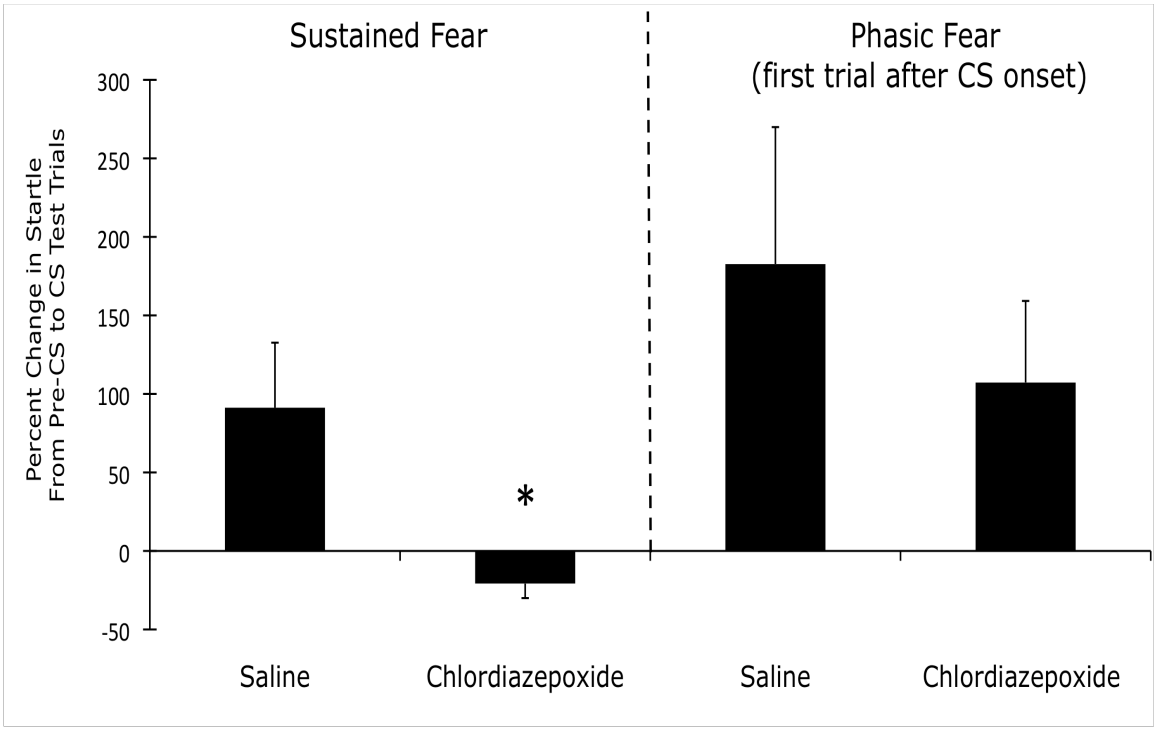

Figure 3.4. Acute chlordiazepoxide effects on sustained startle potentiation

Pre-test chlordiazepoxide (10 mg/kg, i.p.) blocked sustained startle potentiation, but not the initial potentiation to the first startle stimulus after CS onset. * = $p < 0.05$ vs saline

$t(21) = 3.15$, as well as non-parametric, $U=23$, analyses. When limited to the first startle response after CS onset (phasic fear measure 2), a between-group difference was not found using either analysis, consistent with the lack of effect on explicitly trained phasic fear in Experiment 2. There were no between-group differences in baseline startle.

Experiment 4: Pre-test buspirone but not fluoxetine (single injection) blocked phasic startle potentiation. ANOVA on the log-transformed scores indicated a significant Treatment effect, $F(2, 33) = 4.25$, which was due to the difference between the saline and buspirone groups, $q(18) = 2.87$ (Dunnett's *t*-test). Non-parametric analyses yielded essentially the same results. Thus, a Kruskal-Wallis comparison also indicated significant between-group differences, $H=7.74$, with Dunn's multiple comparison test indicating a significant difference between the saline and buspirone groups (rank sum difference = 13.21), but not between the saline and acute fluoxetine groups. These results are shown in Figure 3.5. ANOVA also indicated a significant Group effect on baseline startle, $F(2, 33) = 4.80$. Dunnett's *t*-test indicated a significant difference between the saline and buspirone groups, $q(18) = 3.09$, but not between the saline and fluoxetine groups (see Table 3.1).

Experiment 5: Neither buspirone nor acute fluoxetine blocked sustained startle potentiation. These results are shown in Figure 3.6 (and see also Table 3.1). As in the preceding experiment, phasic potentiation was markedly lower in the buspirone compared to saline groups (i.e., using the first startle response after CS onset as a

Figure 3.5

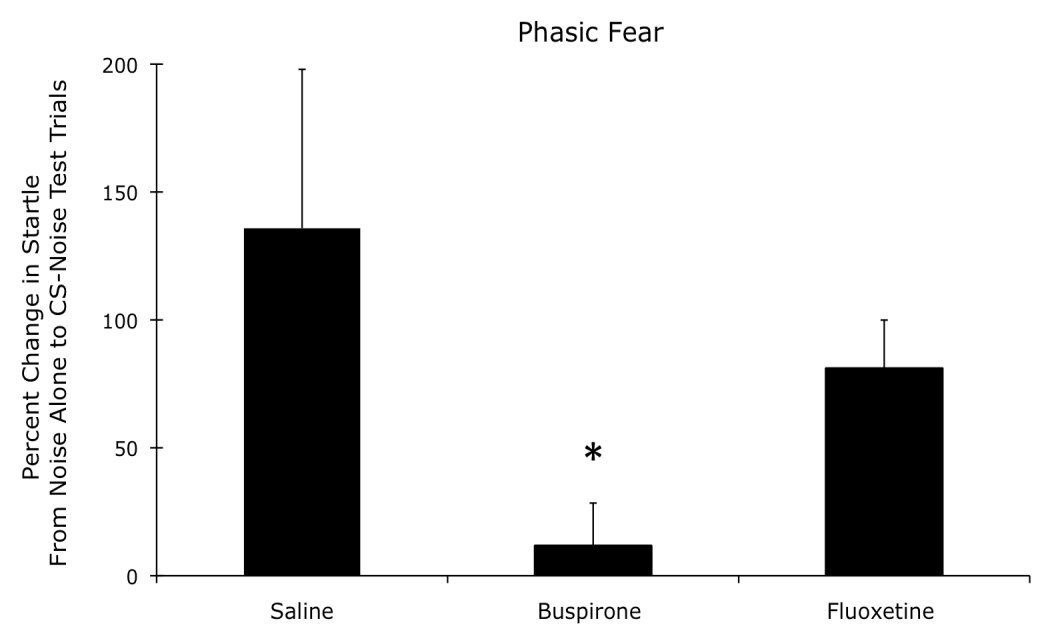

Figure 3.5. Acute buspirone and acute fluoxetine effects on phasic startle potentiation

Pre-test buspirone (5 mg/kg, s.c.), but not fluoxetine (10 mg/kg, i.p.), disrupted startle potentiation to 3.7-second clicker presentations. High variance in the saline group, and the modest difference versus the fluoxetine group, was largely attributable to a single outlier with a fear-potentiated startle score of 826%. Without this rat, the mean (± s.e.m.) for the saline group was 71.8 ± 12.9. * = $p < 0.05$ vs saline (with outlier included).

Figure 3.6

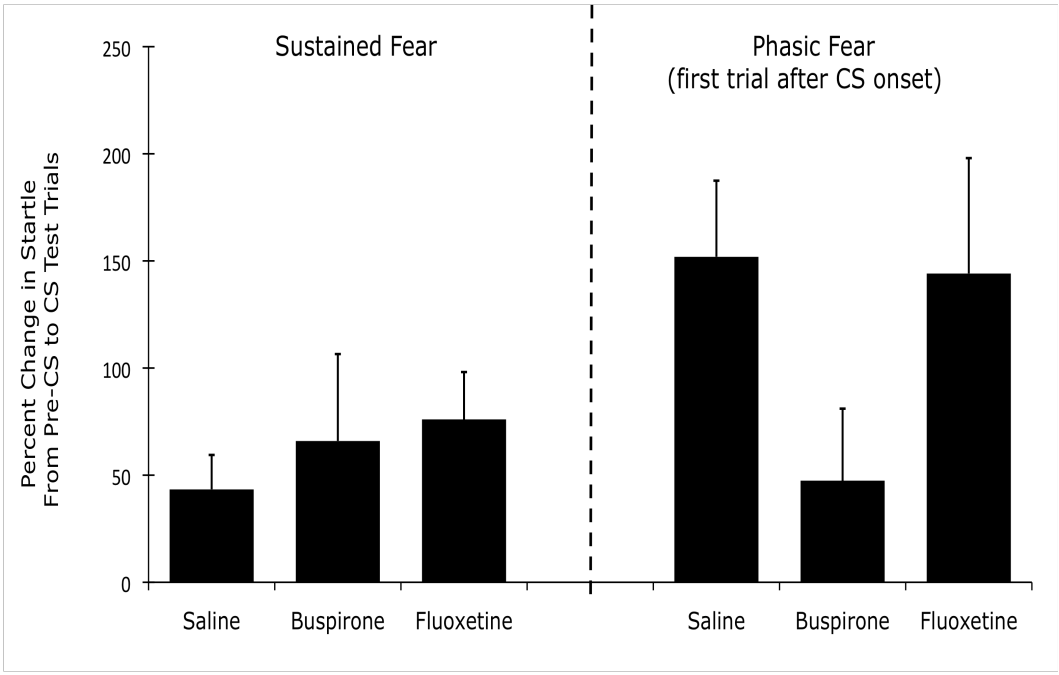

Figure 3.6. Acute buspirone and acute fluoxetine effects on sustained startle potentiation

Neither buspirone nor fluoxetine (single injection) disrupted sustained startle potentiation. The lower level of phasic potentiation in the buspirone compared to saline group was roughly comparable to that seen in Experiment 4, but did not reach significance in this experiment when corrected for multiple comparisons.

supplemental phasic fear measure) – in this case, 47 ± 34% vs. 152 ± 35%, respectively – but this was not statistically significant using either ANOVA or Kruskal-Wallis analyses. The between-group difference with respect to baseline startle was also comparable to the preceding experiment and this *was* significant, $F(2, 57) = 4.91$. As before, this was due to a higher baseline in the buspirone group compared to saline group, $q(18) = 2.47$, Dunnett's *t*-test, which we have found previously (Kehne et al., 1988).

Experiment 6: Chronic fluoxetine does not affect startle potentiation to phasically presented fear stimuli. Neither Mann-Whitney nor *t*-test analyses indicated significant between-group differences ($p > 0.05$ for both). Baseline startle was similarly unaffected. These results are shown in Figure 3.7 (and see also Table 3.1).

Experiment 7: Chronic fluoxetine disrupts startle potentiation to a sustained fear stimulus. As shown in Figure 3.8 and confirmed statistically by an independent-samples *t*-test on the log transformed scores, $t(36)=2.69$, as well as Mann-Whitney analysis of percent change scores ($U=99$), chronic fluoxetine significantly disrupted startle potentiation to the sustained fear stimulus. Fluoxetine also significantly reduced fear-potentiated startle to the first startle stimulus after CS onset (phasic fear measure 2), $t(36)=1.71$ and $U=125$, which was not predicted based on results from Experiment 6. Baseline startle was significantly greater in the fluoxetine group, $t(36) = 2.27$

Figure 3.7

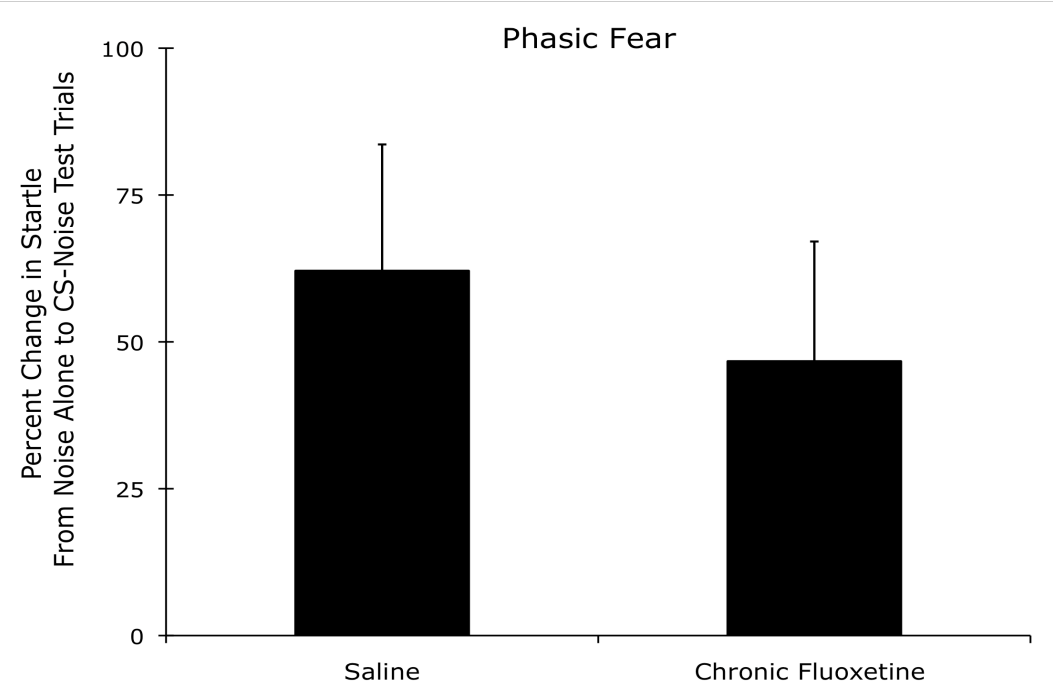

Figure 3.7. Chronic fluoxetine effects on phasic startle potentiation

Chronic fluoxetine (10 mg/kg, p.o., for 21 days beginning approximately 3 hours after the final conditioning session) did not significantly affect startle amplitude increases to phasically-presented fear stimuli

Figure 3.8

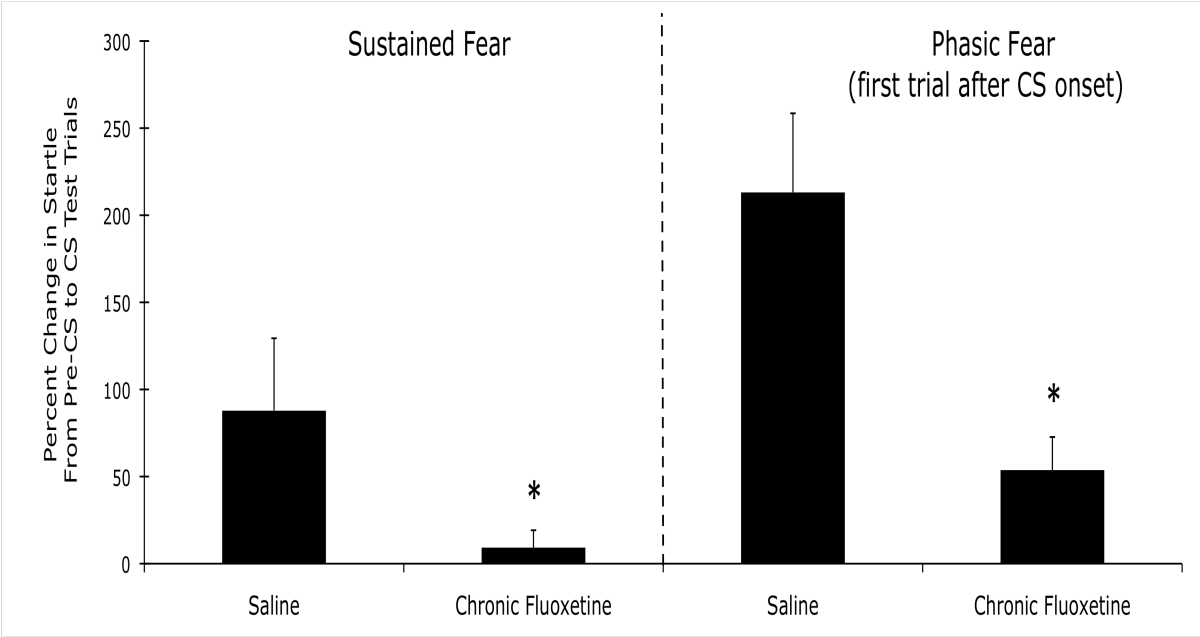

Figure 3.8. Chronic fluoxetine effects on sustained startle potentiation

Chronic fluoxetine (10 mg/kg, p.o., for 21 days beginning approximately 3 hours after the final conditioning session) blocked the sustained increase in startle and significantly attenuated the phasic increase (i.e., the first startle probe after CS onset). * = $p < 0.05$ vs saline

Discussion:

I evaluated the effects on phasic and sustained startle potentiation of several pharmacological treatments that either *are* or are *not* clinically effective for anxiety reduction. Phasic and sustained startle potentiation responded differently, and in opposite directions to several of these treatments, lending support to the validity of the distinction. Moreover, the specific pattern of results suggested that sustained startle potentiation may have greater predictive validity, and might therefore be more useful as an animal model of clinical anxiety, than phasic startle potentiation (see Table 3.2 for a complete summary of findings). The results obtained with each compound are discussed in turn below.

Benzodiazepines allosterically modulate $GABA_A$ receptors to increase the GABA conductance of this inhibitory channel. For many years, benzodiazepines have been the drug of choice for anxiety reduction, being partly supplanted more recently by monoamine reuptake inhibitors because of the greater potential for dependency and abuse with benzodiazepines. It is perhaps surprising then that the effect of benzodiazepines on phasic startle potentiation has been inconsistent, at least in humans, with positive (Bitsios et al., 1999; Graham et al., 2005; Patrick et al., 1996; Riba et al., 2001) as well as negative (Baas et al., 2002; Grillon et al., 2006; Scaife et al., 2005) results being reported, and suggestions that the positive effects that *have* been reported were secondary to sedative effects (Baas et al., 2002) – a problem which

Table 3.2

	Effect on Clinical Anxiety	Effect on Sustained Startle Potentiation	Effect on Phasic Startle Potentiation
Acute Chlordiazepoxide (10mg/kg)	↓	↓	No Effect
Acute buspirone (5mg/kg)	No Effect	No Effect	↓
Acute fluoxetine (10mg/kg)	No Effect	No Effect	No Effect
Chronic fluoxetine (21 days; 10mg/kg)	↓	↓	Variable

Table 3.2. Pharmacological dissociation of phasic vs sustained fear

Sustained fear appears to have greater predictive validity, and might therefore be more useful as an animal model of clinical anxiety than phasic fear.

may be especially pronounced when using absolute difference scores (i.e., from trials without to those with the fear stimulus) rather than percent change scores (Grillon and Baas, 2002; Walker and Davis, 2002b).

Using percent change scores, we found that the benzodiazepine chlordiazepoxide did block sustained startle potentiation, but at the same dose (10 mg/kg) did not significantly influence phasic startle potentiation using either a standard test for phasic startle potentiation (Experiment 2), or the first trial of sustained fear testing as an alternative measure (Experiment 3). It is possible of course that a higher dose *might* have been effective. Doses above 10 mg/kg are generally avoided however due to the emergence of 'non-specific' behavioral effects. In fact, even at 10 mg/kg, baseline startle was reduced by approximately 50% in Experiment 2, which makes the preservation of phasic startle potentiation all the more remarkable. We do not believe that phasic startle potentiation is immune to benzodiazepine administration. Indeed, our lab, using a different protocol for phasic fear training (2 days of training with 10 pairings of a 3.7-sec light and co-terminating footshock per day), has previously observed such effects (Davis, 1979; Walker and Davis, 2002a). However, the current results, in which phasic and sustained startle potentiation data were measured after identical training and, in Experiment 3, in the same rats in the same test session in response to the same CS, seem especially compelling in demonstrating a *differential* sensitivity. Indirect evidence for a greater sensitivity can be found in several other studies. For example, (Guscott et al., (2000) reported a statistically significant disruption of fear-potentiated startle to a 3.7-sec CS by 10 but not 3 mg/kg chlordiazepoxide in rats trained and tested in different contexts, but

significant effects on potentiated startle to the training context itself (sustained fear) at doses as low as 1 mg/kg (i.e., 10-fold difference). In humans, Grillon *et al,* (2006) reported that startle potentiation to an 8-sec CS were not affected by the benzodiazepine alprazolam, whereas startle potentiation to the experimental context during the same test session were significantly reduced.

It is possible that phasic startle potentiation are simply more robust than sustained startle potentiation, and therefore less sensitive to disruption by any means (i.e., a quantitative rather than qualitative difference). However, this explanation would not account for the opposite pattern of results (i.e., a disruption of phasic but not sustained fear) that we observed for buspirone, which we turn to next.

Buspirone, known primarily as a $5HT_{1A}$ partial agonist and dopamine D2 antagonist, potently disrupts phasic startle potentiation in rats when administered shortly before testing (Kehne et al., 1988; Mansbach and Geyer, 1988). In humans however, acute administration is *not* anxiolytic, and in a non-human primate model, does not reduce phasic fear (Winslow et al., 2007). For clinical anxiolysis, chronic administration is required (Goa and Ward, 1986; Goodman, 2004; Jacobson et al., 1985), suggesting that the mechanism of action for effects on phasic startle potentiation in rats (which may not involve serotonin - (Davis et al., 1988)) and for clinical efficacy in humans may be different. In Experiment 4, we replicated the frequently reported effect observed in rats and, in Experiment 5, observed a quantitatively similar difference. In both experiments, an effect on baseline startle was also observed. These baseline effects frequently

accompany (Mansbach and Geyer, 1988; Walker and Davis, 1997a) but are not required (Kehne et al., 1988; Melia and Davis, 1991) for buspirone effects on phasic fear.

In contrast, we saw no evidence for a disruption of sustained fear by buspirone (Experiment 5). As always, it is conceivable that a higher dose might have been effective. However, we previously found that doses as low as 1.25 mg/kg (versus the 5 mg/kg used here) markedly disrupt phasic startle potentiation and that doses half that used here completely abolish the effect (Kehne et al, 1988). Note also that the effect on baseline startle provides a positive control for drug activity. Thus, we believe it unlikely that the failure to disrupt sustained startle potentiation was due to insufficient dosing. Instead, the effect of acute buspirone on sustained startle potentiation appears to reflect more accurately the effect of acute buspirone on clinical anxiety (no effect), than the effect of acute buspirone on phasic startle potentiation.

I should note that buspirone *does* disrupt light-enhanced startle, which is also a sustained increase in startle, albeit to an unconditioned stimulus (Walker and Davis, 1997a; Walker and Davis, 1997b). Assuming that light-enhanced startle reflects anxiety, which we do, the results from that and the present study suggest there may be more than one type of sustained fear (perhaps, for example, conditioned versus unconditioned) with different neural substrates or sensitivity to anxiolytic compounds.

Fluoxetine, a commonly used SSRI, inhibits the reuptake of the monoamine neurotransmitter, 5-HT into the presynpatic cell, thereby increasing the amount of

extracellular 5-HT available to act at pre- and post-synaptic 5-HT receptors. As previously mentioned, chronic monoamine reuptake inhibitors have largely supplanted the use of benzodiazepines for anxiety reduction. Here, we evaluated the effect of acute and also chronic fluoxetine on phasic as well as sustained startle potentiation. Acute fluoxetine, which does not reduce anxiety in humans, had no effect on phasic *or* sustained startle potentiation. In marked contrast, chronic fluoxetine did block sustained increases, but failed to consistently block phasic. As indicated in Table 3.1, chronic fluoxetine also increased baseline startle responses in Experiment 7 and, to a lesser degree, in Experiment 6. These increases on baseline startle amplitude might be a result of actions in the spinal cord. Previous studies indicate that intrathecal administration of serotonin and other serotonin agonists increases startle amplitude, most likely by activating 5-HT_{1A} receptors on spinal motor neurons (Commissaris and Davis, 1982; Davis et al., 1980a; Davis et al., 1980b). It is possible that fluoxetine is increasing startle through a similar mechanism in the present study. Whatever the mechanism, we considered the possibility that these baseline increases could have occluded further fear-induced increases, without affecting fear itself. However, for several reasons, we do not believe this likely. First, the same rats *did* show potentiation to the first startle probe after CS onset (phasic fear) indicating that startle was not at a ceiling and that further increases were indeed possible. Moreover, we have found in other experiments that phasic startle potentiation are unaffected by much larger baseline elevations brought about by i.c.v. CRF infusions or systemic strychnine injections (Walker and Davis, 2002b). Finally, we found no evidence of a correlation between the effect on baseline startle and the effect on startle potentiation. For these analyses, and in keeping with the other analyses reported herein,

we again used parametric (Pearson's) as well as non-parametric (Spearman's) techniques to correlate the baseline increase (expressed as the ratio between the post-conditioning pre-CS baseline and the pre-conditioning pre-CS baseline, or the log transformation of that ratio) with our ratio and log-transformed measures of sustained fear. Neither analysis (conducted on data from fluoxetine-treated rats only) found evidence for a relationship between these variables (Spearman's $r = -0.113$, $p = 0.64$; Pearson's $r = -0.11$, $p = 0.65$). Overall then, we are confident that the abolition of sustained startle potentiation in Experiment 7 was not an artifact of the baseline startle increase.

As previously noted, the effect of chronic fluoxetine on sustained startle potentiation was in contrast to the effect on phasic startle potentiation, which were more variable. Based on the explicit test of phasic fear (Experiment 6), in which startle was elicited 3.2 seconds after CS onset, there was no disruption at all. However, when startle was elicited 19 seconds after CS onset (i.e., to the first probe after onset of the sustained fear stimulus in Experiment 7), startle was significantly reduced but not abolished (as was the more sustained component of potentiation in these same rats).

Disruptions of sustained fear (i.e., to context CSs) by chronic (Li et al., 2001) or sub-chronic (Santos et al., 2006) SSRI administration have previously been reported. In one study (Burghardt et al., 2004), chronic administration of the SSRI citalopram beginning *prior* to training also disrupted freezing to a relatively short 20-second fear stimulus. Because freezing is generally found to persist well beyond CS offset, even when elicited by phasically-presented fear stimuli (Quinn et al., 2002), it is likely that the fear response in that study was more sustained than

phasic. In humans, Grillon and colleagues (2009a) found that chronic citalopram administration had no effect on startle potentiation to an 8-sec fear stimulus, but did reduce the startle potentiation that occurred between stimulus presentations (a more sustained increase which may have reflected context fear). Thus, those results may reflect the same underlying time-dependent dissociation that we have observed in rats.

In interpreting the pattern of results observed across our experiments, it is perhaps relevant that phasic fear potentiation is, in most cases, the stronger response. This could reflect the fact that, during conditioning, a greater number of shocks were experienced by the rats during the early part of the 8-min CS. For example, using these training procedures for sustained fear, half of all footshocks occurred during the first minute of CS presentation (i.e., at 3, 10, 20, and 60 seconds after CS onset). In developing this paradigm, we explored a number of different conditioning protocols, including one in which the ordering of shocks was reversed (i.e., a mirror image of the shock schedule used here in which shock density was greater towards the end of the CS). Invariably however, we find that startle potentiation immediately following the CS onset is greater than potentiation at later times after CS onset.

In any case, and as noted earlier, the greater magnitude of phasic compared to sustained startle potentiation does allow for the possibility that chlordiazepozide and chronic fluoxetine disrupted sustained but not phasic potentiation simply because sustained startle potentiation was the weaker of the two responses. This makes the results obtained with buspirone – which disrupted the stronger phasic response and not the weaker sustained

response – all the more important and highly suggestive of the possibility that phasic and sustained fear are fundamentally, not just quantitatively, different.

To our knowledge, these are the first explicit comparisons of drug effects on short- versus longer duration fear responses. Based on the compounds tested here, the results suggest that sustained fear paradigms may have greater predictive validity, and that the sustained fear itself may be more homologous to at least some types of clinical anxiety than phasic fear. In this regard, other findings by Grillon and colleagues are also relevant. In particular, they observed that startle potentiation to short-duration stimuli that have been paired with shock are no greater in post-traumatic stress (Grillon et al., 2009b) and panic disorder patients (Grillon et al., 2008) than in healthy controls, but that the startle responses that occur *between* stimulus presentations, which as noted earlier may reflect a more sustained type of anxiety to the less-predictive threat context, *are* greater (c.f., Davis et al., 2010). These results lend support to the view that drugs that reduce sustained startle potentiation may be more clinically efficacious than those that preferentially reduce phasic startle potentiation.

The search for such compounds (i.e., drugs that reduce sustained startle potentiation) may be aided by evidence that sustained fear, including fear responses to static contexts, is especially dependent on the BNST (e.g., Hammack et al., 2004; Sullivan et al., 2004; Waddell et al., 2006; Walker and Davis, 1997b) whereas phasic fear responses are more dependent on the medial division of the CeA (c.f., Walker et al., 2009b). Evidence supporting this view is derived primarily from lesion and inactivation studies, but is

consistent with the results of unit recording and imaging studies in rats (Quirk et al., 1995) and humans (Phelps et al., 2001) which have indicated only a transient activation of the amygdala by threat stimuli, but perhaps a more sustained activation of the BNST, the latter which may be exaggerated in subjects with high trait anxiety (Somerville et al., 2010). Perhaps also relevant are recent results from Klumpers et al., (2010) who reported a significant time-dependent correlation between amygdala activation and startle potentiation in healthy humans.

One notable feature of the BNST as well as the *lateral* CeA (which projects to the BNST) is the abundance of so many different neuropeptide-positive cells and terminals (Arluison et al., 1994; Cassell et al., 1986; Gray and Magnuson, 1992; Ju et al., 1989; Roberts et al., 1982; Shimada et al., 1989; Walter et al., 1991; Woodhams et al., 1983) which are not found in the medial CeA. Because peptides often act for long periods of time, as we have found from local BNST infusions of either CRF (Liang et al., 1992) or CGRP (Sink et al., 2011), we believe that accelerated development of small molecule ligands for these receptors, and their evaluation in anxiety models such as the one used here, may be a prudent strategy for the development of new anxiolytic compounds with novel mechanisms of action. We have found, for example, that oral administration of the non-peptide CRF-R1 antagonist GSK876008 disrupts sustained but not phasic startle potentiation to conditioned fear stimuli (Walker et al., 2009a; Walker et al., 2009b), and also startle potentiation evoked directly by calcitonin gene-related peptide (CGRP) infusions into the BNST (Sink et al., 2011) and unpublished observations), where receptors for both peptides are abundant (Chalmers et al., 1995; Christopoulos et al.,

1995; Kruger et al., 1988; Skofitsch and Jacobowitz, 1985). We have also found that intra-BNST CGRP infusions increase anxiety measures in the plus maze, and that intra-BNST infusions of a CGRP antagonist decrease sustained startle potentiation produced by the predator odor 2,5-dihydro-2,4,5-trimethylthiazoline (Sink et al., 2011).

It is also noteworthy that the BNST expresses several types of serotonin receptors, is densely innervated by serotonergic afferents (Freedman and Shi, 2001; Hammack et al., 2009) that in many cases project to CRF-positive BNST neurons (Phelix et al., 1992b), and is especially enriched in serotonin transporters (Smith et al., 1999). These findings suggest that the BNST may be one site of action for SSRI-mediated anxiolysis. The recent observation that anxious temperament in monkeys is correlated with serotonin transporter availability in the BNST is consistent with this view (Oler et al., 2009).

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Chapter 4

The Role of Serotonin (within the Bed Nucleus of the Stria Terminalis) In Sustained Fear

Abstract:

The monoamine neurotransmitter, serotonin (5-HT) has been identified as an active modulator of emotional states. Altered levels and functioning of 5-HT are hallmarks of certain mood disorders (e.g., depression, anxiety). Some of the most commonly used medications to treat anxiety disorders are selective serotonin reuptake inhibitors (SSRI). Chronic SSRI treatment modulates levels of serotonin within the brain and produces an anxiolytic effect, but the literature remains unclear on 5-HT's mechanisms of action within specific anxiety-related circuits.

Previous studies in our laboratory have used a sustained fear behavioral paradigm (with which to elicit anxiety-like responses to a long-duration conditioned stimulus) to show that chronic, but not acute, administration of a SSRI (fluoxetine) reliably blocked the expression of anxiety-like responses, mimicking clinical observations (Miles et al., 2011). The BNST, a forebrain structure found to be necessary in mediating anxiety-like responses, has dense innervation by 5-HT neurons, expresses multiple 5-HT receptor subtypes, and is rich in 5-HT transporters, the site of action of SSRI's. Thus, its physiological properties make the BNST an ideal structural candidate in which to explore serotonin's role in anxiety-like responses.

The goal of this chapter is to determine if systemic fluoxetine mediates its anxiolytic effect through actions within the BNST structure. We trained rats using our sustained fear paradigm, then chronically (21 days) administered either saline or fluoxetine, and

then tested 48hrs after a localized BNST 5,7-Dihydroxytryptamine (5,7-DHT)-induced serotonergic lesion (or vehicle infusion) for the expression of anxiety-like responses. The results suggest that localized serotonergic lesions within the BNST attenuate the anxioytic effect produced by chronic fluoxtine administration. However, localized serotonergic lesions in conjunction with chronic saline administration produced variable behavioral results. Overall the findings suggest that 5-HT within the BNST may be involved in the ability of chronic fluoxetine to reduce sustained fear and, by extension, its ability to reduce anxiety clinically.

Introduction:

Anxiety disorders are a major public health concern, affecting more than 30 million Americans annually, making it one of the nation's most common mental health disorders (DuPont et al., 1996). Anxiety is characterized as a complex emotional state, associated with sustained autonomic, endocrine, and behavioral changes in arousal (Barlow, 2002; Grillon, 2008). Many of the physiological symptoms of anxiety (e.g., impulsivity, changes in cardiovascular/respiratory activity, irritability/mood, sleep dysregulation) are all central nervous system functions modulated by the neurotransmitter, serotonin (5-HT) (Hariri and Holmes, 2006; Ravindran and Stein, 2009). 5-HT is a widely distributed monoamine (Parent et al., 1981) that is synthesized from the amino acid, L-Tryptophan. Presentations of aversive stimuli lead to an immediate change in extracellular 5-HT levels and metabolism (Chaouloff, 1993; Inoue et al., 1993; Shimizu et al., 1992; Tao and Auerbach, 1995), suggesting that 5-HT is a key component in the stress response. Therefore it is not surprising that many effective anxiolytics treatments are those that modulate the body's serotonergic system. There is a large body of literature suggesting a modulatory role of 5-HT in physiological and behavioral systems. However, these studies have shown that while the neurobiological role of 5-HT is important, it is also extremely complex, often producing different neuronal effects depending on target brain area and method of testing. Thus, more investigations are necessary to clarify the role of 5-HT within specific anxiety-related circuits.

Serotonergic neurons originate from within the raphe nuclei of the brainstem. The dorsal region of the raphe nucleus (DRN) is the region that most prominently projects to structures known to be active in aversive emotional states (e.g., the lateral septum, hippocampus, amygdala, BNST) (Commons et al., 2003; Geyer et al., 1976; Jacobs and Azmitia, 1992; Parent et al., 1981; Phelix et al., 1992a). *In vivo* microdialysis studies have shown that modulation of 5-HT levels within the DRN alters the extracellular concentrations of 5-HT in terminal regions (Adell and Artigas, 1991), suggesting that release of 5-HT into these terminal regions are flexible and thus well equipped for mediating the dynamic changes in 5-HT after stress. Investigation of terminal areas rich in serotonin could thus provide insight on the localized mechanisms driving anxiety.

One such terminal region is the bed nucleus of the stria terminalis (BNST), a limbic forebrain structure that has been found to be key in mediating anxiety-like responses in human (Somerville et al., 2010; Straube et al., 2007) and non-human animal subjects (e.g., Commons et al., 2003; Hammack et al., 2004; Kalin et al., 2005; Phelix et al., 1992a; Resstel et al., 2008; Singewald et al., 2003; Somerville et al., 2010; Straube et al., 2007; Sullivan et al., 2004; Waddell et al., 2006; Walker et al., 2009b). The BNST not only receives dense innervation by serotonergic afferents, but also expresses multiple 5-HT receptor subtypes (Commons et al., 2003; Cornea-Hebert et al., 1999; Hammack et al., 2009; Heidmann et al., 1998; Kia et al., 1996; Mengod et al., 1990; Phelix et al., 1992a; Waeber et al., 1994; Wright et al., 1995). Immunohistochemical studies have shown that afferent fibers rich in 5-HTT target the anterolateral BNST and have terminals that surround the soma of corticotropin-releasing factor (CRF) containing neurons within

the BNST (Commons et al., 2003; Hammack et al., 2009; Phelix et al., 1992b). These findings suggest that 5-HT could play an important modulatory role within the BNST.

The question then arises, what is the contribution of 5-HT within the BNST on the expression of anxiety-like responses? Previous studies in our laboratory have used a sustained fear conditioning paradigm to reliably produce sustained (minutes) fear responses to a 60-Hz clicker conditioned stimulus (CS). The sustained fear responses produced by earlier versions of this paradigm have shown to be mediated by the BNST and have proven to have greater predictive clinical validity (and therefore may be a more useful animal model of anxiety) than other types of fear paradigms (Davis et al., 2010; Walker et al., 2009b). As mentioned above, using this sustained fear paradigm we have shown that chronic, but not acute, administration of the SSRI, fluoxetine, reliably blocked sustained fear (Miles et al., 2011). The aim of this study was to determine if the anxiolytic effect of chronic fluoxetine would be altered by depletion of 5-HT in the BNST. Rats were trained using our sustained fear paradigm, chronically (21 days) treated with either saline or fluoxetine, and then tested 48 hrs after a localized bilateral BNST 5,7-DHT-induced serotonergic lesion (or vehicle infusion) for the expression of anxiety-like responses.

Materials and Methods:

Animals:

Male Sprague-Dawley rats (200–250 g at arrival; Charles River, Raleigh, NC) were housed 4/cage on a 12-hr light-dark cycle in a temperature- and humidity-controlled room with food and water freely available. Behavioral procedures began approximately one week after arrival, and were conducted in accordance with USDA, NIH, and Emory University guidelines.

Drugs and Drug Administration:

Fluoxetine (Spectrum Chemical, Gardena, CA, USA) and desipramine hydrochloride (Sigma-Aldrich Chemical Co., St. Louis, MO, USA) were dissolved in physiological saline and delivered in a volume of 0.1 ml/100 g body weight via oral gavage or intra-peritoneal (i.p.) injection (respectively). 5,7-Dihydroxytryptamine creatine sulfate (5,7-DHT) was dissolved in distilled water containing 0.02% ascorbic acid, kept on ice, and was protected from light until BNST infusion. Despiramine hydrochloride is a norepinephrine reuptake inhibitor used to protect noradrenergic neurons from the toxic non-specific effects of 5,7-DHT (Bjorklund et al., 1975).

Apparatus:

Rats were trained and tested in 8 x 15 x 15-cm Plexiglas and wire mesh cages with four 6.0-mm diameter stainless steel floorbars, located within a sound-attenuated behavior chamber. Startle responses were evoked by 50-ms (95 dB) white-noise bursts generated by a computer sound file, amplified by a Radio Shack amplifier (Tandy, Fort Worth, TX, USA), and delivered through Radio Shack Supertweeter speakers located in front of the

cage. The same speakers delivered background noise (60 dB, 1-20 kHz) provided by an ACO Pacific, Inc. (Belmont, CA, USA) noise generator. All sound level measurements were made from the center of the cage.

Startle amplitude and shock reactivity were quantified using a PCB Piezotronics (Depew, NY, USA) accelerometer affixed to the bottom of the cage. The accelerometer produces a voltage output proportional to the velocity of cage movement (e.g., produced by the rats' startle response), which is integrated by a PCB Piezotronics signal conditioner and digitized by a GW Instruments (Somerville, MA, USA) InstruNet device. Startle amplitude was defined as the maximum peak-to-peak voltage during the first 200 ms after each noise burst. Shock responses were similarly quantified, using a 500-ms window concurrent with shock delivery.

The conditioned stimulus (CS) used was either a 60-Hz clicker stimulus delivered through speakers located behind each chamber. The unconditioned stimulus was a 0.35 mA footshock delivered through the floor bars. The sequencing of all stimuli was controlled by a desktop computer using custom-designed software (The Experimenter; Glass Bead Software, New Haven, CT, USA).

Behavioral Procedures:

Experimental Sequence: Rats received two acclimation sessions followed by a pre-conditioning test for sustained fear, followed by conditioning sessions, followed 21 days

later by a post-conditioning test. These procedures are described below, and presented graphically in Figure 4.1.

Acclimation: Rats were placed into the test cage and, after 5 minutes, presented with the first of 48 startle-eliciting white-noise bursts (inter-stimulus interval (ISI) = 30 seconds).

Pre-Conditioning Sustained Fear Test: Rats were placed into the test cage and, after 5 minutes, presented with the first of 32 startle-eliciting noise bursts (ISI = 30 sec). The first 16 were presented in the absence, and the next 16 in the presence of the CS.

Fear Conditioning: On each conditioning day, rats received 8 presentations of variable-duration clicker stimuli (3-sec, 10-sec, 20-sec, 1-min, 2-min, 4-min, 6-min, and 8-min), each co-terminating with footshock. The first CS of each session occurred 5 minutes after the rat was placed into the conditioning chamber. The interval between offset of one CS and onset of the next was 3 minutes. During the first conditioning session, the clicker stimuli were presented in order of increasing duration. During the second and third, they were sequenced randomly.

Rats received the first of 21 treatments of either saline (*N*=33) or fluoxetine (*N*=21; 10 mg/kg) by oral gavage approximately 3 hours after the final conditioning session and for each of the following 20 days.

Figure 4.1

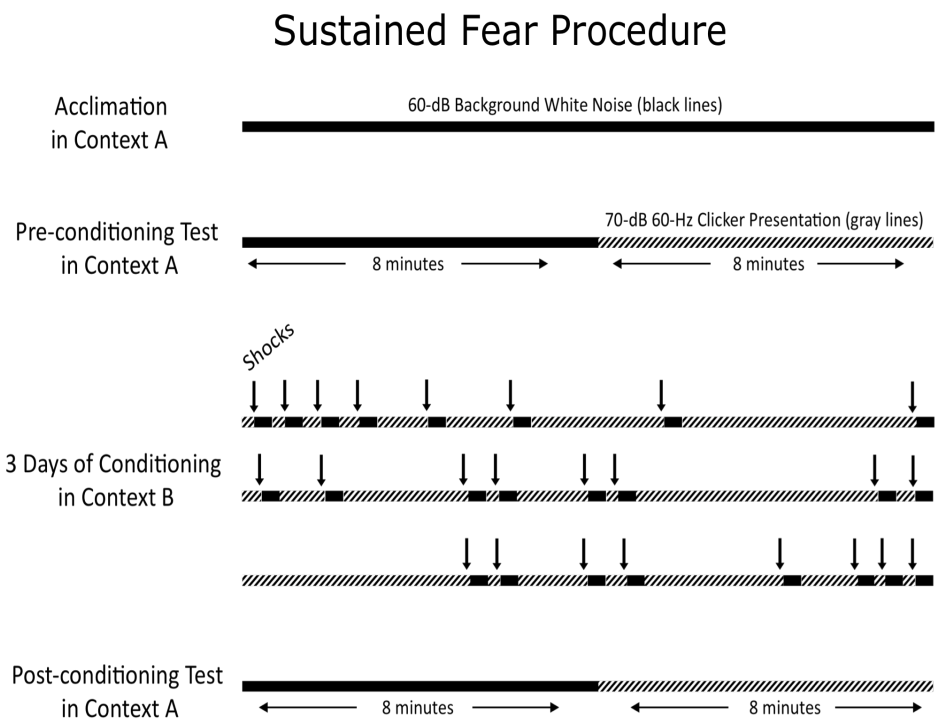

Figure 4.1. Behavioral procedures and timeline.

Acclimation, a pre-conditioning test, each of 3 conditioning sessions in which clicker stimuli (gray) of variable duration were paired with co-terminating footshock (arrows), and a post-conditioning test, took place on separate days. With the exception of testing, the sustained and phasic fear procedures were identical. For sustained fear testing, startle was measured before and then during presentation of an 8-minute clicker stimulus. For a detailed description, see the *Methods* section.

Surgery: On day 19 of chronic saline or fluoxetine treatment, animals were pre-treated with despiramine (25 mg/kg, i.p.) approximately 45 minutes before administration of 5,7-DHT (2mg, calculated as the free base). Rats were anesthetized with 75 mg/kg (i.p.) ketamine (Bioniche Pharma), 0.5 mg/kg (i.p.) Dexdomitor (Orion Pharma), and given an analgesic dose of 1.0 mg/kg (s.c.) meloxicam (Boehringer Ingelheim) to reduce postoperative discomfort rats. Once unresponsive to tail pinch, rats were placed in a Kopf Instruments stereotaxic frame with the nosebar set to –3.8 mm (flat-skull position). Gauge-23 cannulas were inserted bilaterally (20° coronal angle—to avoid the lateral ventricle, 0.3 mm caudal, 5.8 mm ventral, and 3.8 mm lateral to bregma). 5,7-DHT solution (n=27) or ascorbic acid vehicle (n=27) was bilaterally infused in a volume of 1μl at a rate of 1 μl/min. Cannulas remained implanted for 1 minute post-infusion to prevent drug spill out. Head incisions were closed using surgical staples and iodine tincture solution was applied. Post-conditioning tests were given 48 hrs post-surgery.

Post-Conditioning Sustained Fear Test: Rats were tested after conditioning, using procedures identical to those described for the pre-conditioning tests.

Context: During conditioning, a cotton gauze pad wetted with 0.4 ml of 70% ethanol solution was placed in front of the test cage. A fluorescent light placed behind the cage (150-lux as measured from the middle of the cage) provided constant illumination. During testing, to minimize context-potentiated startle, the cage was dark and no explicit olfactory stimuli were introduced. In addition, two 5-cm chains hung from the top of the test cage and a sandpaper insert was placed over the floorbars.

Statistical analyses:

Sustained Fear: Each rat tested for sustained fear received a sustained fear-potentiated startle score. Because our analysis of the control dataset indicated that the first startle response after CS onset was markedly higher than all those that followed, and that sustained fear diminished with time, becoming unreliable after approximately the 4th minute of CS presentation, we calculated a sustained fear-potentiated startle score by dividing mean startle amplitude during the first 4 minutes of the CS (beginning with the 2nd CS test trial) by the mean startle amplitude during the last 4 minutes of the pre-CS period (Figure 4.2). For presentation purposes, these sustained fear-potentiated startle scores were converted to percent change scores.

Exclusion Criteria: Fear conditioning requires that rats perceive the aversive stimulus. Therefore, we excluded from further analysis the data obtained from rats with footshock reactions of 1.0 or less on 12 or more of the 24 conditioning trials (*N*= 15). Because meaningful ratios cannot be calculated for rats that do not show a baseline startle response, the data from rats with a mean accelerometer output of ≤ 0.1 (i.e., what we observe when cage output is sampled in non-startled rats) on baseline test trials were also excluded (*N*=1). Finally, animals within lesion treatment groups whose HPLC analysis revealed a lack of a 5-HT lesion effect (*N*= 4; preseumably due to misplacement of cannulae) were removed from data analysis.

Figure 4.2

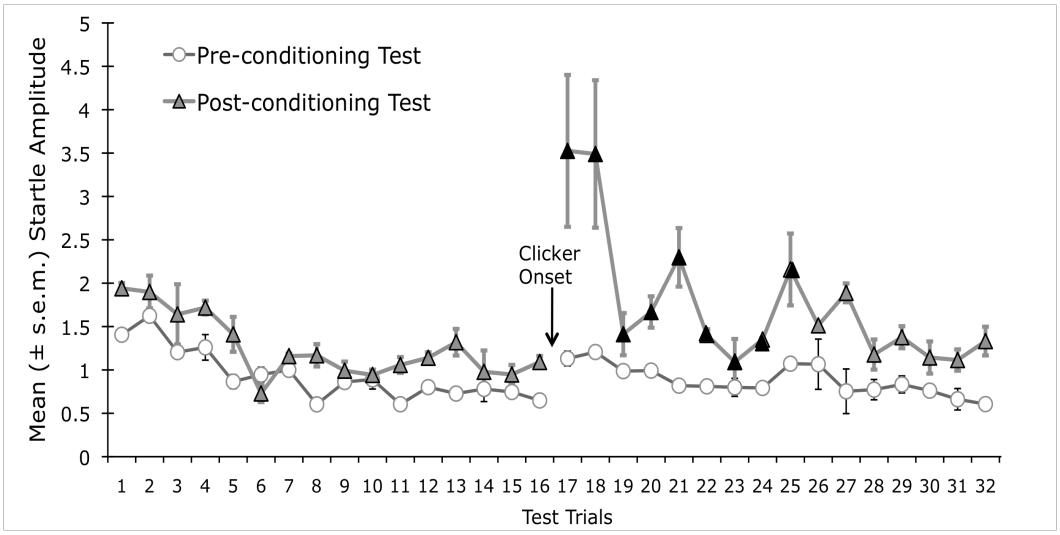

Figure 4.2. Trial-by-trial raw startle data during sustained fear tests.

The trial-by-trial raw startle data for the Saline-Vehicle control group (*N*=16) used in these studies are plotted for both the pre- (open circles) and post- (filled triangles) conditioning test sessions. Startle responses were evoked every 30 seconds during the 8 minutes prior to CS onset (trials 1-16) and for the 8 minutes during which the CS was presented (trials 17-32). Before conditioning, there was little if any effect of the 60-Hz clicker stimulus on startle. After conditioning, potentiation by the clicker CS was clearly evident. The percent change scores illustrated in Figure 3, are based on the ratio (mean startle amplitude across CS test trials 18-25 / mean startle amplitude across pre-CS test trials 9-16) for sustained fear. ▲ = $p < 0.05$ (paired *t*-test)

Inferential Statistics: The primary analyses were between-group comparisons of fear-potentiated scores. Because normality tests indicated significant deviations from normality for a number of datasets, between-group differences were evaluated using distribution-free (non-parametric) Mann-Whitney or Kruskal-Wallis tests and also, to establish statistical robustness, by using *t*-tests and ANOVA on log-transformed scores (Keene, 1995). For all tests, the criterion for significance was 0.05 (two-tailed).

Biochemical analysis:

Animals were anesthetized with isoflurane and decapitated within 30 min of completing their post-conditioning test. Trunk blood was collected from a subset of fluoxetine treated animals (*N*=10). Plasma was separated from arterial blood by centrifugation, supernatant was extracted and stored at -20 ºC until subsequently analyzed by the Dr. James Ritchie Clinical Pathology Translational Research Laboratory at Emory University according to the method described in Synder and Ritchie (2009). Fluoxetine, 10 mg/kg/day produced average serum concentrations of 104 ng/ml fluoxetine and 401 ng/ml of the active metabolite norfluoxetine. These values are consistent with reported clinical serum concentrations (Amsterdam et al., 1997), and are consistent (and relatively low) when compared to other preclinical chronic fluoxetine studies (Czachura and Rasmussen, 2000; Stout et al., 2002).

Brains were rapidly removed, flash frozen on dry ice, and stored in -80ºC until analyzed. Within a week, all brains were sectioned by cryostat and BNST punches were collected from Bregma 0.42 to -0.98mm (Paxinos and Watson Rat Brain Stereotaxic Guide) and

stored in -80ºC until processed by Vanderbilt University's Neurochemistry Core.

Endogenous monoamine levels within BNST punches were determined using High Performance Liquid Chromatography (HPLC). Tissue samples were homogenized using a tissue dismembrator, in 100-750 μl of 0.1M TCA, which contains 10^{-2} M sodium acetate, 10^{-4} M EDTA, 5 ng/ml isoproterenol (as internal standard) and 10.5 % methanol (pH 3.8). Samples were spun in a microcentrifuge at 10000 g for 20 min (Cransac et al., 1996) to isolate monoamines. The pellet was saved for protein analysis. Using an Antec Decade II (oxidation: 0.5) electrochemical detector operated at 33° C, 20 µl samples of the supernatant were injected using a Water 717+ autosampler onto a Phenomenex Nucleosil (5u, 100A) C18 HPLC column (150 x 4.60 mm). Biogenic amines were eluted with a mobile phase consisting of 89.5% 0.1M TCA, 10^{-2} M sodium acetate, 10^{-4} M EDTA and 10.5 % methanol (pH 3.8). Solvent was delivered at 0.6 ml/min using a Waters 515 HPLC pump.

Results:

In Figure 4.2, ANOVA indicated a significant Session effect (pre- vs post-conditioning test) $F(3, 30)=12.9, p < 0.05$. Tukey post-hoc comparisons indicate that post-conditioning startle responses during the CS presentation were significantly higher ($p < 0.05$) than startle responses during the pre-conditioning test (both pre-CS and CS test trials) and the post-conditioning pre-CS test trials.

Figure 4.3 compares the percent change in startle from pre-CS to CS test trials (i.e., sustained fear response) between the four treatment groups. A Kruskal-Wallis test comparing Treatment (Saline vs. Fluoxetine) and Condition (Vehicle vs. 5,7-DHT lesion) yielded no significant interaction, likewise, a two-way ANOVA on log-transformed values showed similar results. As shown in Figure 4.3, the Fluoxetine-Vehicle treatment group had decreased levels of sustained fear as compared to the Saline-Vehicle control group, replicating previous findings (Miles et al., 2011). The Fluoxetine-5,7-DHT treatment group showed higher levels of sustained fear compared to that of the Fluoxetine-Vehicle treatment group, suggesting that chronic fluoxetine might involve 5-HT within the BNST. However, this effect was not significant as shown by a independent samples *t*-test on log values, $t(23) = .49$, $p = 0.62$ and a Mann-Whitney *t*-test on percent scores, $U(25) = 61$, $p = 0.4$.

HPLC analysis was performed to confirm the extent of 5-HT depletion (and NE survival) after vehicle or 5,7-DHT (2mg intra-BNST) administration (Figure 4.4, norepinephrine (white bars) and serotonin (black bars)). ANOVA confirms that in all lesion groups, norepinephrine levels were not significantly different than vehicle controls. However, ANOVA analysis did indicate a Condition effect (vehicle vs. 5,7-DHT lesions), $F(3,57)=32.36$, $p < 0.05$. Tukey post-hoc comparisons of the four treatment groups indicate that the Saline-Vehicle control group had significantly higher levels of 5-HT

Figure 4.3

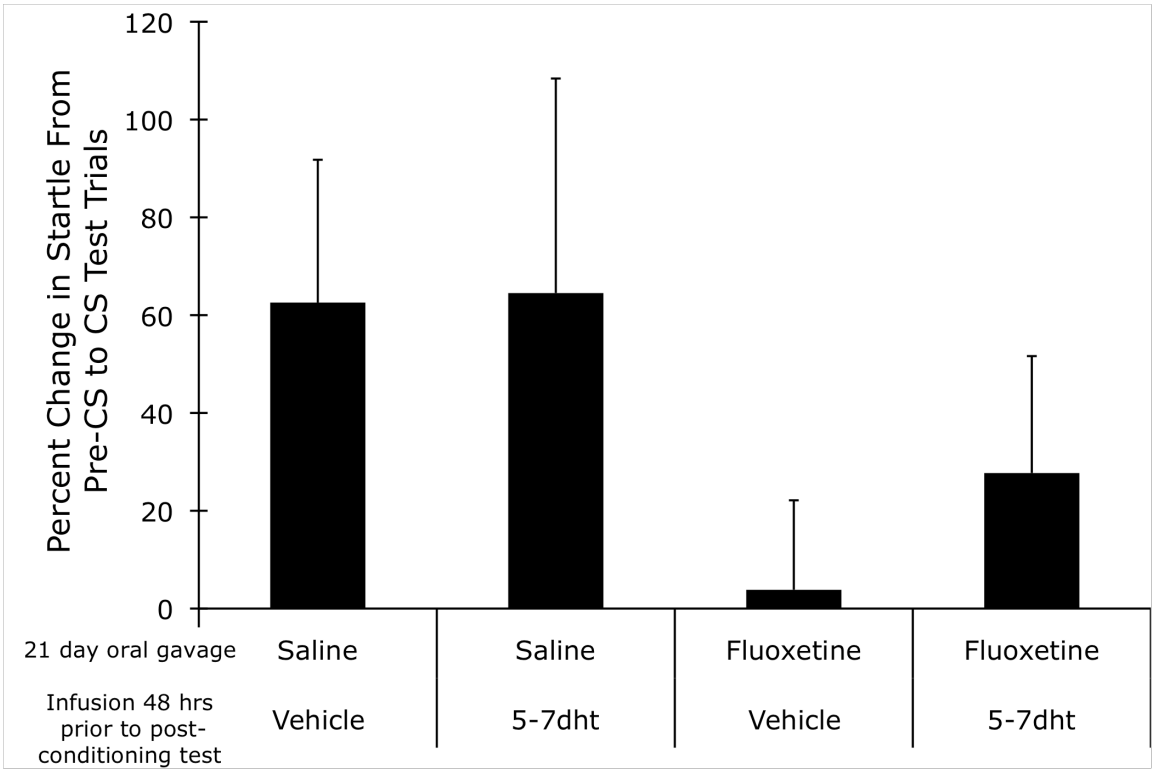

Figure 4.3. The effect of intra-BNST 5,7-DHT lesions on rats treated with 21-days of saline or fluoxetine.

Animals in the Fluoxetine-Vehicle treatment group (*N*=11) produced lower levels of sustained fear (i.e., an anxiolytic effect) as compared to the Saline-Vehicle control group (*N*=16). Animals in the Fluoxetine-5,7-DHT treatment group (*N*=14) produced non-significantly higher levels of sustained fear than Fluoxetine-Vehicle treatment group, but lower levels of sustained fear than Saline-Vehicle controls.

Figure 4.4

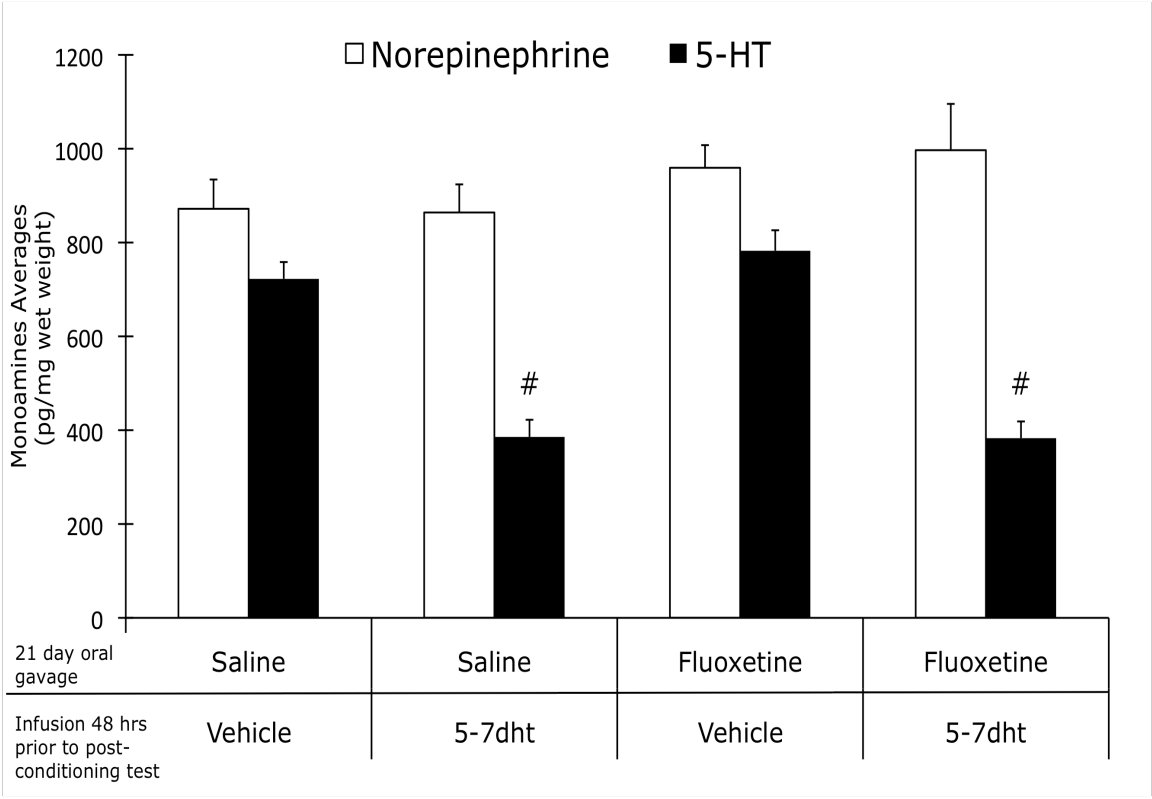

Figure 4.4. The effect of 5,7-DHT on 5-HT and NE Levels in the Bed Nucleus of the Stria Terminalis (BNST)

The effect of lesions produced by 5,7-DHT (2mg intra-BNST) on norepinephrine (white bars) and serotonin (black bars) after 48hrs after neurotoxic treatment. Mean ± S.E.M., N = 11-19 in each group. Significant differences between 5-HT expression in the Saline-Vehicle control group vs 5,7-DHT treatment groups are indicated by #$P<0.01$, Tukey post-hoc comparison.

than the 5,7-DHT lesion groups ($p < 0.05$). Comparisons between the Saline-Vehicle control and the Fluoxetine-Vehicle treatment group were not significantly significant ($p >$ 0.)

Discussion:

We have evaluated the contribution of 5-HT within the BNST on the anxiolytic actions of chronic fluoxetine administration. We were able to replicate our previous findings in that the Fluoxetine-Vehicle treatment group showed decreased levels of sustained fear as compared to the Saline-Vehicle control group (Miles et al., 2011). Although not significant, the Fluoxetine-5,7-DHT treatment group showed higher levels of sustained fear compared to the Fluoxetine-Vehicle treatment group. These findings suggest that 5-HT in the BNST might be involved in the anxiolytic effect of chronic fluoxetine, but clearly more animals would be needed to establish this with statistical significance (power analysis indicates an $N = 32$, a = 0.05, power = 0.50).

The role of 5-HT in the regulation of anxiety-related circuits is complex. Within the literature, the effects of 5-HT have been found to be dependent on a number of variables (e.g., the time course of 5-HT action, extracellular levels of 5-HT, type and subtypes of 5-HT pre-synaptic and post-synaptic receptors (each mediating different G protein signaling pathways), populations of 5-HT receptors (which can change based on extracellular levels of 5-HT), neuronal interactions (post-synaptic signaling to GABA or

glutamatergic neurons)) (c.f., Lowry et al., 2005). In spite of these variables, the vast majority of studies agree that 5-HT plays some sort of role in modulating anxiety-like behaviors (c.f.'s, Griebel, 1995; Handley, 1995).

As stated above, SSRI's are the medication of choice for many types of anxiety disorders (Goldstein and Goodnick, 1998; Goodnick and Goldstein, 1998; Nutt et al., 1999; van der Kolk et al., 1994). Fluoxetine binds to pre-synaptic serotonin transporter on 5-HT neurons and inhibits the reuptake of 5-HT into the pre-synaptic cell, thereby generating an increase in extracellular levels of 5-HT available for pre- and post-synaptic receptor binding (Bel and Artigas, 1993; Bel and Artigas, 1999; Blier and de Montigny, 1987; Fuller, 1994; Goodnick and Goldstein, 1998; Kreiss and Lucki, 1995). Interestingly, acute fluoxetine administration does not reduce anxiety in animals and humans, but only after chronic do anxiolytic effects emerge (Burghardt et al., 2007; Goldstein and Goodnick, 1998; Griebel, 1995; Grillon et al., 2009a; Grillon et al., 2007; van der Kolk et al., 1994). The question then arises, if both acute and chronic fluoxetine treatment block the reuptake of 5-HT and enhance extracellular levels, then why does chronic, but not acute treatment produce an anxiolytic effect?

While there are some conflicting reports, likely due to the various brain regions studied (Beyer and Cremers, 2008; Smith et al., 2000), it is regularly believed that acute fluoxetine administration only *transiently* elevates extracellular concentration levels of 5-HT as compared to the sustained increases seen after chronic administration (Bel and Artigas, 1993; Beyer and Cremers, 2008; Hjorth, 1993; Kreiss and Lucki, 1995; Rutter et

al., 1994; Smith et al., 2000; Tanda et al., 1996). Acute versus chronic increases in extracellular 5-HT can have differential effects on the desensitization of terminal 5-HT_{1B} autoreceptors (Blier et al., 1988), regulation of 5-HT transporter expression (Dewar et al., 1993; Hebert et al., 2001; Hrdina and Vu, 1993; Johnson et al., 2009), and how other local neurotransmitters function (Bymaster et al., 2002; Penttila et al., 2004; Szabo et al., 1999).

However, the leading explanation for the lack of therapeutic effect of acute SSRI treatment and for the delayed therapeutic effect of chronic SRRI treatment is centered on the increase of 5-HT activation at 5-HT_{1A} autoreceptors on 5-HT neurons in the raphe (c.f., Gordon and Hen, 2004; Santarelli et al., 2003). Acute SSRI administration increases availability of 5-HT within the raphe and acts on the somodendritic 5-HT_{1A} Gi-coupled autoreceptor to *decrease* in raphe cell firing (Andrade et al., 1986; Araneda and Andrade, 1991; Artigas, 1993; Blier and de Montigny, 1987; Blier et al., 1990; Chaput et al., 1986; Hjorth and Auerbach, 1994; Sprouse and Aghajanian, 1987; Zifa and Fillion, 1992) which can limit 5-HT levels in forebrain terminal regions (levels vary depending on targeted brain region) (Invernizzi et al., 1992; Kreiss and Lucki, 1995; Rutter et al., 1994). After chronic 5-HT activation (due to chronic fluoxetine administration), these cell body autoreceptors are believed to be desensitized, thereby causing an restoration of cell firing, and hence an *increase* in 5-HT availability in at post-synaptic sites (Artigas, 1993; Blier and de Montigny, 1987; Godbout et al., 1991; Gordon and Hen, 2004; Handley, 1995). Post-synaptic 5-HT_{1A} receptor agonists and partial agonists are found to consistently produce neuronal inhibition and downstream anxiolytic responses (Araneda

and Andrade, 1991; Graeff et al., 1996; Hammack et al., 2009; Levita et al., 2004; Rainnie, 1999). For example, there have been electrophysiological studies suggesting enhanced post-synaptic 5-HT_{1A} receptor function after chronic anti-depressant administration (Blier et al., 1987). Neuroimaging studies reveal that patients with lower levels of ^{11}C 5-HT_{1A} antagonist binding to 5-HT_{1A} receptors had higher scores of anxiety (Tauscher et al., 2001); while binding studies demonstrate that after two weeks of fluoxetine administration there are increased 5-HT_{1A} post-synaptic binding sites (Klimek et al., 1992; Klimek et al., 1994). Additionally, 5-HT_{1A} knock-out mouse studies show a robust increase in anxiety phenotype (not so for knock-outs for 5-$HT_{1B, 2C, 4, 5A, 6}$, or $_{7}$ receptors) (Gingrich et al., 2003). These data suggest that a decrease of BNST 5-HT_{1A} receptor activation will attenuate the BNST's inhibitory tone (Hammack et al., 2009). Please see Griebel (1995) for reviews of relevant studies regarding 5-HT_{1A} receptor's role in mediating anxiolytic effects.

Related to our current study, the BNST receives dense serotonergic innervation from DRN afferents and expresses a number of post-synaptic 5-HT receptor types (i.e., 5-HT_{1A}, 5-HT_{2A}, 5-HT_{2C}, and 5-HT_{7}) (Commons et al., 2003; Cornea-Hebert et al., 1999; Hammack et al., 2009; Heidmann et al., 1998; Kia et al., 1996; Mengod et al., 1990; Phelix et al., 1992a; Waeber et al., 1994; Wright et al., 1995). In electrophysiological studies, Levita *et al* (2004) and Rainnie (1999) have shown that local 5-HT infusions into the BNST preferentially activates 5-HT_{1A} receptors, mediates an inhibitory response in the majority of BNST neurons, and decreases acoustic startle responses. It should be noted, however, that these studies infused very high concentrations of 5-HT (10-50mM),

promoting extracellular levels 1000 times higher than normally seen after chronic fluoxetine administration in representative areas such as the hippocampus (Smith et al., 2000). Likewise, *in vivo* behavioral studies showed that a high concentration of a 5-HT_{1A} agonist (10mM) infused into the BNST can significantly reduce the acoustic startle response, without affecting the general motor activity of the animals, suggesting an anxiolytic profile on this measure (Levita et al., 2004). Studies from Hammack *et al* (2009) show that under a normal state of stress, the net effect of 5-HT release in the BNST is to dampen neural activity via 5-HT_{1A} receptor activation, which in turn could promote an anxiolytic effect. A recent behavioral study corroborates the above findings in that an infusion of a 5-HT_{1A} receptor agonist locally into the BNST produced anxiolytic effects in the elevated plus-maze and Vogel conflict test, and this anxiolytic effect was blocked by pre-treatment of a the 5-HT_{1A} receptor antagonist WAY100635 (Gomes et al., 2011). These studies suggest that administration of chronic fluoxetine may have enhanced 5-HT levels within the BNST, thereby enhancing activation of the post-synaptic 5-HT_{1A} receptors, resulting in an inhibition of neurons in the BNST that led to a decrease in sustained fear responses (Figure 4.3, third bar).

To directly determine the effect of BNST 5-HT on sustained fear responses, we locally infused the neurotoxin 5,7-dihydroxytryptamine (5,7-DHT) to selectively destroy serotonergic neurons. 5,7-DHT is a structural analog of 5-HT. When infused into the brain, the toxin enters the pre-synaptic cells via the 5-HT reuptake receptor, forms hydrogen peroxide, denatures proteins, blocks the formation of ATP, causing destruction of projection neurons and a localized decrease in 5-HT levels (Bjorklund et al., 1975;

Choi et al., 2004; Sinhababu and Borchardt, 1988; Wrona et al., 1986). As shown in Figure 4.4, 5-HT was not fully depleted from the BNST, likely due to our low dose administration. However, even at this low dose our HPLC data revealed siginificant changes in 5-HT and 5-HT's main metabolite, 5-HIAA ratio levels as compared to controls (Figure 4.5). ANOVA indicated a significant Treatment effect $F(3, 50)=8.6$, $p < 0.05$. Tukey post-hoc comparisons indicated significant increases in 5-HIAA /5-HT ratios in the Saline-5,7-DHT treatment group as compared to Saline-Vehicle group, as well as significant increases in the Fluoxetine-5,7-DHT treatment group as compared to Fluoxetine-Vehicle group. Although non-significant, the data also show decreases in 5-HIAA /5-HT ratio levels in animals treated with chornic fluoxetine as compared to those given saline, suggesting elevated levels of tissue 5-HT due to fluoxetine treatment. Our findings suggest that systemic fluoxetine treatment may be affecting local 5-HT levels within the BNST.

Microdialysis studies suggest that despite significant depletions of *tissue* monoamine levels, *extracellular* levels of monoamines can be maintatined at control levels (Abercrombie et al., 1990; Abercrombie and Zigmond, 1989; Castaneda et al., 1990; Hall et al., 1999; Kalen et al., 1988; Kirby et al., 1995; Rex et al., 2003; Robinson and Whishaw, 1988; Romero et al., 1998; Thomas et al., 2000). Findings suggest that until substantial 5-HT depletion (e.g., > 60%, dependent on area) is achieved (Hall et al., 1999), control levels of extracellular 5-HT are likely maintained through multiple neural compensatory mechanisms (Kirby et al., 1995). 5,7-DHT deleptions may produce an increase in 5-HT precursors (Stachowiak et al., 1986), an enhancement in 5-HT synthesis

Figure 4.5

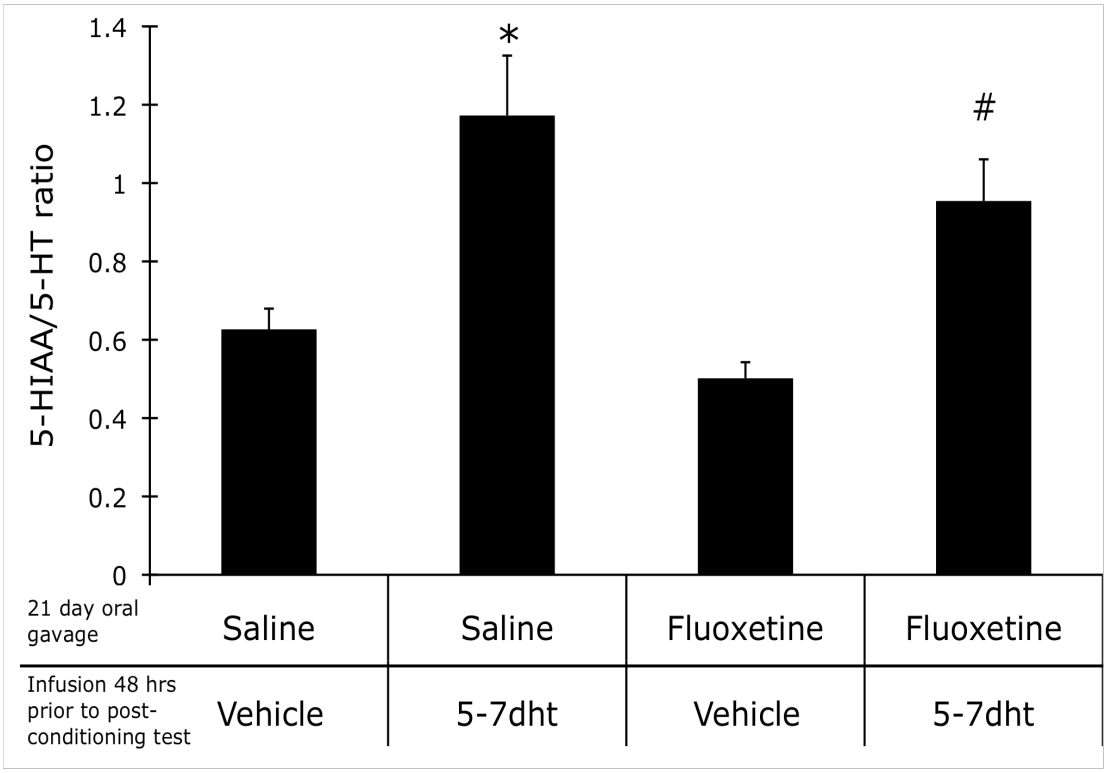

Figure 4.5. 5-HIAA/5-HT ratios within the BNST

HPLC data revealed siginificant changes in 5-HIAA /5-HT ratio levels in groups treated with 5,7-DHT. ANOVA indicated a significant Treatment effect $F(3, 50)=8.6, p < 0.05$. Tukey post-hoc comparisons indicated significant increases in 5-HIAA /5-HT ratios in the Saline-5,7-DHT and Fluoxetine-5,7-DHT treatment groups as compared to their Vehicle controls. These data suggest changes in neural processes due to significant decreases in 5-HT. * < 0.05 (vs Saline-Vehicle), # < 0.05 (vs Fluoxetine-Vehicle).

(Bendotti et al., 1990), and/or changes in serotonergic firing patterns (Hajos and Sharp, 1996). Microdialysis was not performed in this study, and therefore changes in extracellular levels of 5-HT were not determined. However, it is concieveable that due to incomplete lesions, extracellular compensatory 5-HT actions may account for some of the variability in our behavioral data. Future studies might use a higher dose of the toxin to produce more extensive lesions. However, 5,7-DHT also has affinity for NE transporters, and higher doses of 5,7-DHT could potentially decrease NE expression and function (Baumgarten and Bjorklund, 1976). Hence, we would need to do further biochemical studies to develop conditions for a more complete depletion of 5-HT within the BNST.

Our HPLC data confirms that while 5,7-DHT significantly reduced levels of 5-HT within the BNST, this effect was not blocked by fluoxetine treatment. Other studies have found similar results and have suggested this finding may be attributed to the acutely high 5,7-DHT concentration (versus the SSRI) at the binding site (Breese and Mueller, 1978; Fuller, 1978; Fuxe et al., 1978) or that 5,7-DHT may in fact be mediating some of its effects through an alternate site (Choi et al., 2004). Regardless of its mechanism of action, our 5,7-DHT work suggests that decreasing levels of 5-HT within the BNST attenuates chronic fluoxetine's anxiolytic effect (Figure 4.4, fourth bar).

HPLC results revealed a subset of animals in the Fluoxetine-5,7-DHT treatment group that were weakly lesioned (N=5; average 5-HT expression was 5% below control group mean); these animals produced an average 17.4 $\pm$ 19.6 sustained fear response (i.e., weak sustained fear). In comparison, another subset of the Fluoxetine-5,7-DHT treatment

group produced strong lesions (*N*=5; average 5-HT expression was 60% below control group mean) and produced an average 39.7 $\pm$ 19.7 sustained fear response (i.e., average sustained fear). These results suggest that chronic fluoxetine was able to maintain its anxiolytic action due to sufficient 5-HT presence in the weak lesioned animals, but that this anxiolytic response was attenuated in the Fluoxetine-5,7-DHT animals with strong lesions and a low 5-HT presence. In summary, our fluoxetine results suggest that 5-HT within the BNST may play an active role in modulating anxiety-like responses.

Our data show that the Saline-5,7-DHT treatment group's average sustained fear response is non-significantly different than that of the Saline-Vehicle control (Figure 4.3), however, data from our HPLC analysis presents a more complex situation. Weak lesioned rats within the saline-5,7-DHT treatment group (*N*=3; average 5-HT expression was 5% below control group mean) produced an average -6.4 $\pm$ 5.6 sustained fear response (i.e., no sustained fear) (Figure 4.6). Interestingly, a subset of rats within the saline-5,7-DHT treatment group with strong 5-HT lesions (*N*=3; average 5-HT expression was 80% below control group mean) produced an average -1.2 $\pm$ 10.6 (i.e., no sustained fear) (Figure 4.6). It is difficult to determine why Saline-5,7-DHT animals with either weak or strong 5-HT lesions failed to produce appropriate sustained fear responses. Logically speaking, Saline-5,7-DHT animals with weak 5-HT lesions should behave like animals in the Saline-Vehicle control treatment group and produce normal sustained fear responses; however this was not the case. Furthermore, another subset of Saline-5,7-DHT animals with a relatively intermediate level of 5-HT lesion (*N*=3; average 5-HT expression was

Figure 4.6

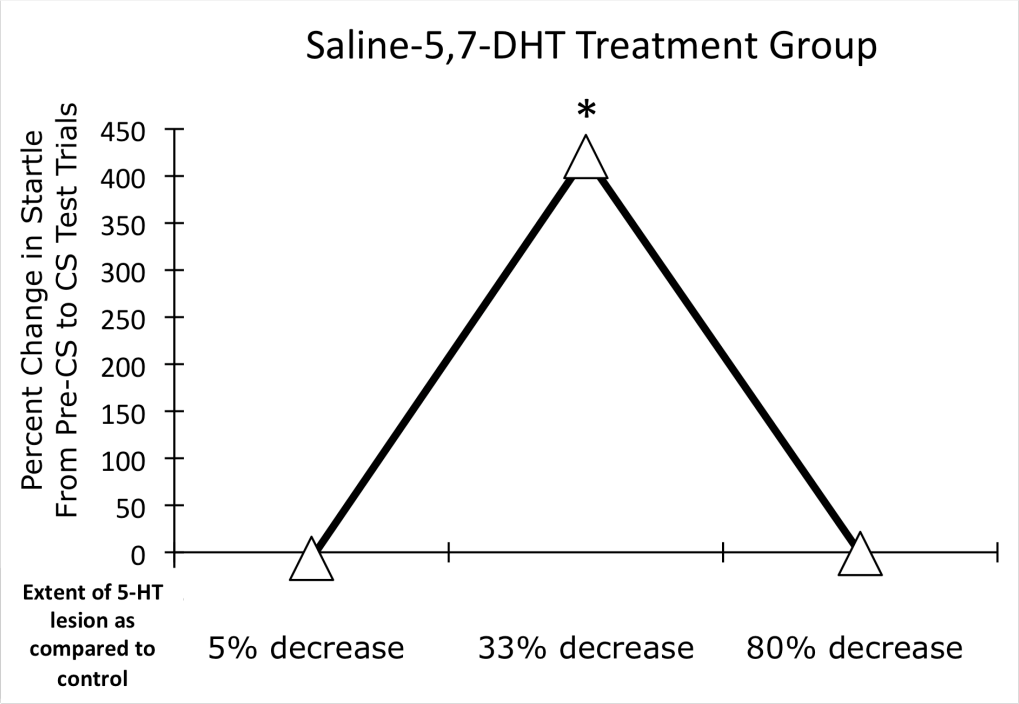

Figure 4.6. The effect of intra-BNST 5-HT depletion on sustained fear

Rats in the Saline-5,7-DHT treatment group with intermediate BNST 5-HT lesions (33% decrease from control) expressed significantly more sustained fear than rats within the same treatment group with either weak or strong BNST 5-HT lesions (5% and 80% decrease from control, respectively), *= $p < 0.05$. The non-monotonic effect suggests that perhaps there is a threshold level of 5-HT needed to produce appropriate sustained fear responses.

33% below control group mean) produced an average 420 ± 195 sustained fear response (i.e., extremely high sustained fear) (Figure 4.6). The non-monotonic effect suggests that perhaps there is a threshold level of 5-HT needed to produce appropriate sustained fear responses, which could account for the high variability in the Saline-5,7-DHT group seen in Figure 4.3.

Supporting our findings of variability, electrophysiological studies show the response of BNST neurons to 5-HT application is not strictly an inhibitory one. Exogenous 5-HT and 5-HT agonists mediate bi-directional modulation on BNST neurons, producing hyperpolarizaition (inhibitory actions via 5-HT_{1A} receptors), depolarization (excitatory actions via 5-HT_2 receptors), and biphasic activity (inhibitory then excitatory actions via 5-HT_{1A}, 5-HT_{2A} and 5-HT_7 receptors) (Hammack et al., 2009; Rainnie, 1999). In addition, chronic stress can shift the balance of BNST 5-HT responses in favor of excitation via increased density of 5-HT_{2C} receptors and a decrease of $5HT_{1A}$ receptors (Ferretti et al., 1995; Lopez et al., 1999; McKittrick et al., 1995; Ossowska et al., 2001; Takao et al., 1997). To further complicate matters, increases and decreases of anxious behavior may be mediated by the expression of multiple receptors in different cell types, and that more than one receptor subtype may mediate actions of 5-HT in a single neuron (Araneda and Andrade, 1991; Davies et al., 1987). It is clear to see why there is such a strong consensus within the literature that sertonin's role in anxiety is a complex one, further complicated by multiple forms of anxiety and potentially different, but overlapping anxiety circuits (c.f., Gordon and Hen, 2004). Nonetheless, we are confident

our study has provided a foundation with which to continue to study the effect of BNST 5-HT on anxiety-like responses.

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Chapter 5

Discussion

This dissertation used a rat model of fear-potentiated startle to investigate the neural processes mediating the expression of phasic and sustained fear startle responses. As discussed in previous chapters, fear-potentiated startle is a potentially powerful translational approach for studying anxiety in healthy human subjects (Davis et al., 2010). Overall findings in this dissertation support the hypothesis that fear responses to short-duration cues (phasic fear) are mediated by different neural mechanisms than fear responses to long-duration cues (sustained fear). The predictive validity of the results suggests that sustained fear paradigms may serve as better models of certain types of human anxiety disorders than phasic fear paradigms.

In chapter 2, I successfully modified two behavioral fear paradigms (i.e., phasic and sustained) to consistently and reliably produce short- and longer-duration fear responses to the *same* conditioned fear stimulus. In Chapter 3, I evaluated the effects of several pharmacological treatments that either *are* or are *not* clinically effective for anxiety reduction on the expression of phasic and sustained fear responses. As predicted, phasic and sustained fear responses (to the same fear stimulus) responded differently, and in opposite directions to several of the drug treatments. The specific pattern of results suggests that sustained fear may have greater predictive validity, serving as a better animal model of clinical anxiety than phasic fear. Cross-species use of sustained fear procedures could provide insights into neurological factors contributing to certain anxiety disorders, and lead to the development of new anxiolytic compounds with novel mechanisms of action. Finally, in Chapter 4, I evaluated the contribution of serotonin (within the BNST) on the anxiolytic actions of chronic fluoxetine. I was able to replicate

findings from Chapter 3 (in that the Chronic Fluoxetine-Vehicle treatment group showed decreased levels of sustained fear as compared to the Saline-Vehicle control group) and provide some suggestive, albeit non-significant, evidence that 5-HT within the BNST may be involved in the anxiolytic effects of chronic fluoxetine.

In Chapter 2, a relevant issue in the design of our sustained fear paradigm is the use of an 8-min CS (as opposed to 2, 4, or even 6-mins). Although the selected time duration in which we measure a sustained fear response is somewhat arbitrary, we suspect there is a *transition* between phasic and sustained fear rather than an abrupt *switch*, so that early times of a long CS primarily reflect phasic fear whereas later time points reflect sustained fear. For example, some laboratories that have dissociated phasic and sustained fear responses have measured phasic fear using durations as short as 10-sec to as long as 60-sec and measured sustained fear with time points as short as 5-min to as long as 10-min (Sullivan et al., 2004; Waddell et al., 2006). Our goal in the design of these paradigms was to compare one fear measure that was more phasic to one more sustained. This was done using an explicit phasic fear paradigm as well as using an internal measure of phasic fear within the explicit sustained fear paradigm (Chapter 3).

Chapter 3 showed a clear pharmacological dissociation of phasic and sustained fear responses. As previously stated, to our knowledge these are the first explicit comparisons of drug effects on short- versus longer duration fear responses. Based on the compounds tested, the findings suggest that sustained fear (versus phasic fear) may be homologous to certain types of clinical anxiety.

In support of our conclusions of predictive validity, Chapter 4's results suggest that 5-HT, as in clinical anxiety, modulates sustained fear responses (Handley, 1995). Although not significant, the data suggest that the normal anxiolytic effect of chronic fluoxetine on sustained fear was reduced by prior depletion of 5-HT in the BNST (produced by local infusion of the 5-HT neurotoxin, 5,7-DHT). To verify this, additional rats would be required and control experiments, depleting 5-HT in a brain area other than the BNST would be necessary. However, depletion of 5-HT in the BNST may not have been the best strategy to test this hypothesis given the opposite effects of BNST 5-HT_{1A} and 5-HT_2 receptor activation, leading to either anxiolytic or anxiogenic effects, respectively. This might explain why both of these results were seen in the Saline-5,7-DHT treatment group.

In fact, it is often the case that behavioral effects in which the BNST has been implicated are often quite variable, as we generally find with sustained fear. A study by Duvarci *et al* (2009) provides clear evidence of inter-individual heterogeneity in BNST-mediated responses. In this study, experimenters trained rats to discriminate between two fear CS's. One group of rats exhibited high discriminative abilities and spent less time in the closed arms of an elevated plus maze (a rodent model of anxiety), however another group of rats showed poor discriminative abilities and portrayed an anxious phenotype in the elevated plus maze. Interestingly, following BNST lesions, the second group's anxious phenotype was abolished and the rats now exhibited high discriminative abilities with little variability. A recent primate study has shown that individual variations in anxious

temperament were predicted by BNST pre-synaptic serotonin reuptake transporter binding (Oler et al., 2009). These observations corroborate clinical findings in anxiety in which a number of variables (e.g., genetic variability, sex differences, and early-life stress) influence individual vulnerability versus resilience in mood and anxiety disorders (Heim and Nemeroff, 2009). I believe our study reflects the individual variations of the BNST-mediated stress response. It would be interesting to perform a sustained fear study with rats grouped as high versus low stress responders to determine if this could potentially lower between subject variability.

Enormous progress has been made in the understanding of the neural mechanisms driving the amygdala and fear (Davis, 1992a; LeDoux, 2000). However, in regards to the BNST and anxiety, many questions remain regarding the mechanisms regulating anxiety-like responses. One notable feature of the BNST is the abundance of different neuropeptide-positive cells and terminals. As mentioned, the BNST is densely innervated by serotonergic afferents (Freedman and Shi, 2001; Hammack et al., 2009) that in many cases project to CRF-positive BNST neurons (Phelix et al., 1992b). There is a proposed negative feedback function in which CRF and 5-HT projections to and from the BNST modulate anxiety-like responses (Hammack et al., 2009). More recently, a pituitary adenylate cyclase-activating polypeptide (PACAP) is being explored in both clinical and preclinical anxiety research studies. Findings suggest that in women, PACAP is differentially associated in those who have post-traumatic stress disorder from those who may be resistant (Ressler et al., 2011). Preclinical studies suggest that PACAP and CRF work together within the BNST to modulate anxiety-like responses (c.f., Hammack et al.,

2010). Therefore, it is possible that among other neurotransmitters and neuropeptides, 5-HT, CRF, and PACAP are working in conjunction within the BNST to modulate anxiety-like responses. Anxiety-related circuits are a complex matter.

Future directions for this research aim to further investigate the complex neural mechanisms mediating sustained fear responses. As discussed above, there are likely a number of overlapping systems working in conjunction to produce anxiety responses, however gaining a better understanding of individual systems will enhance our comprehension of the overall anxiety circuit.

Chapter 4's results provided a sufficient rationale for continuing to investigate the role of 5-HT (within the BNST) in sustained fear. Intra-BNST infusions of 5,7-DHT produced varied levels of 5-HT lesions within subject and treatment groups, thus complicating our interpretation of the behavioral results. In future experiments, I propose to more directly investigate the role of 5-HT (within the BNST) in sustained fear by focusing on 5-HT_{1A} receptor contributions. As mentioned in the previous chapter, this specific receptor subtype has been shown to play a key role in 5-HT's inhibitory action in this area. To determine the role of 5-HT_{1A} receptors (within the BNST) in sustained fear, I propose to give a systemic injection of a specific 5- HT_{1A} receptor agonist (or vehicle) followed by a bilateral, intra-BNST infusion of a 5-HT_{1A} receptor antagonist (or vehicle) prior to the post-conditioning test. The hypothesis is that systemic administration of the 5- HT_{1A} receptor agonist will reduce sustained fear responses (anxiolytic effect) as compared to controls, presumably due to activation of 5-HT_{1A} receptors within the BNST. If so, co-

administration of an intra-BNST 5-HT_{1A} receptor antagonist would be expected to block this anxiolytic effect induced by the agonist and restore sustained fear. In addition, I propose that localized BNST infusion of a 5-HT_{1A} receptor antagonist alone will increase sustained fear responses (anxiogenic effect) as compared to controls, assuming there is a tonic release of 5-HT acting on 5-HT_{1A} receptors in this area to suppress sustained fear. In fact, the degree to which tonic 5-HT release either does or does not occur in different rats could explain the wide levels of variability of sustained fear we typically see in this paradigm. So, a second prediction would be that local infusion of a 5-HT_{1A} receptor antagonist would significantly decrease variability across animals in this test. I believe these proposed experiments will allow for a direct evaluation of role of intra-BNST 5-HT_{1A} receptors in sustained fear responses, more so than a broad 5-HT depletion.

Additional experiments could be proposed to once again investigate whether fluoxetine mediates its effects through 5-HT mechainisms within the BNST. Potential experiments could include chronic fluoxetine administration followed by intra-BNST infusions of a 5-HT_{1A} receptor antagonist prior to the post-conditioning test. Based on previous studies mentioned in the above chapter, we would hypothesize that the antagonist would block the anxiolytic effect mediated by chronic fluoxetine treatment. Findings from these proposed experiments, in either support or disagreement of our hypotheses, would provide exciting results and enhance the field of anxiety research.

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