EVALUATING CONSTANT FREQUENCY 50 kHz ULTRASONIC VOCALIZATIONS
AS AN EXPRESSION OF ANXIETY IN RATS

by

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Chapter 1. Introduction

Neuropsychiatric disorders are a prevalent and significant health concern in the United States. Mood and anxiety disorders are especially widespread, with 18% of the adult population in the United States meeting diagnostic criteria for anxiety disorders in a 12-month period alone, and over 40% doing so over a lifetime (Kessler, Chiu, Demler, & Walters, 2005). Mental health services, including pharmaceutical treatments, are expensive. In the United States, the cost for mental health services totaled $57.5 billion for a one year period; an average of $1,931 per person seeking care (National Institute of Mental Health, 2006). It is therefore vitally important to improve our understanding of the etiology of mental illness and to develop novel interventions to alleviate the psychological and financial burdens experienced by a significant portion of our population.

Basic research provides a critical contribution to meeting the challenge of improving mental health through the development of animal models of human disorders which enable us to better understand the physiological mechanisms involved in these disorders, and to test potential therapeutic interventions. Due to important ethical constraints and purely practical reasons, animal models are indispensable for research that requires invasive methods and testing. However, affective disorders can be particularly difficult to model in non-human animals because they cannot be assessed or diagnosed based on objective physiological measures alone. A strong animal model should include a variety of measures that assess multiple symptoms of the disorder upon which the model is based (Koob, 2012). Creating a strong rodent model for affective disorders has therefore proven difficult because of the
inability to directly assess emotional state and the fact that diagnoses in humans depends heavily upon self-report (Nestler & Hyman, 2010)

Although lacking language, rich emotional content can nonetheless be extracted from the vocalizations of many animals (Brudzynski, 2010). It has long been recognized that an animal’s vocalizations can reliably signal positive and negative emotional state (Darwin, 1872), but technology and careful analysis of vocal expressions have only recently allowed us to use vocalizations as a quantitative measure of emotional intensity among some animals. Animal models of various human disorders can therefore be enriched or developed with increased understanding of emotionally-relevant vocal signals. The vocalizations of rats are can provide window into the animal’s affective state (Burgdorf, et al., 2008). Some of these vocalizations are produced as part of a suite of behaviors associated with emotional response. Because the emotionally-relevant vocalizations of rats can be non-invasively recorded and assessed across a wide variety of experimental paradigms, they can be an important tool in modeling typical and pathological emotion in rodents (Burgdorf & Moskal, 2010). Further study and understanding of rat vocalizations is therefore highly valuable for basic and pre-clinical research.

This dissertation is concerned with testing the novel hypothesis that a certain type of rat vocalization heretofore not described as being a motivational vocalization is in fact produced when the rat is experiencing anxiety. If the data support the hypothesis, including the quantification of this type of vocalization would improve existing animal models of anxiety disorders. Anxiety disorders affect approximately 40 million adults in the United States in a given year (Kessler, et al., 2005). A number of disorders with different symptom
clusters are included in broad label of anxiety disorders, but they similarly describe people for whom fear and uncertainty occupy an exaggerated part of their lives. Not only is quality of life impacted by pathological anxiety, but these disorders are often associated with physical illness such as cardiac disorders, hypertension, and gastrointestinal problems (Härter, Conway, & Merikangas, 2003). Because of its prevalence and associated mental and physical health risks, it is crucial that we utilize every practical tool when modeling normal and pathological anxiety, including careful analysis of animal vocalizations.

**Fear and Anxiety**

Fear and anxiety are emotional states associated with patterns of behaviors and autonomic arousal that prepare or facilitate an animal’s response to a threat. While these two states share many similarities, they are generally dissociated by clinicians and pre-clinical researchers alike. Although there is no universally accepted theory regarding the relationship of these states, it is generally accepted that they are distinguished according to the nature and/or certainty of a threat; with some distinction in the patterns of behavioral and autonomic response (for review, Lang, Davis, & Öhman, 2000; Grillion, 2008). In addition, evidence from studies of both human and non-human animals has shown that separate, though highly inter-related, neural systems mediate anxiety and fear. The following sections will focus on defining anxiety and fear, and how they are modeled in rodents.

Fear is elicited by an imminent threat, and drives defensive behaviors and autonomic changes that facilitate the defense against the threat (Blanchard et al., 1993; Perusini & Fanselow, 2015; Perusini & Fanselow, 2008). These defensive behaviors change as a function of the threat’s proximity or certainty, as well as environmental factors (e.g., opportunity for...
escape). Blanchard et al. (1993) described that as proximity between a rat and a predator (cat) decreases, the rat will change from freezing (a non-action defense wherein the rat is hyper-vigilant), to attempting to flee, and finally resulting to defensive attack if other options of avoidance have been exhausted. In accordance with this distinction, pain threshold is increased by fear of an inescapable threat, and decreased by anxiety (Rhudy & Meagher, 2000; Rhudy & Williams, 2005).

Anxiety is related to uncertainty of threat. The anxious state is marked by an increase in risk assessment, and the suppression of non-defensive behaviors such as eating, play, or mating (Blanchard, et al., 1993, Davis, 2006). Unlike the clear freeze/flight/fight responses that are the indicators of fear, the behavior of an anxious animal is often less overt and can be difficult to accurately assess within different situations or contexts (Lang, et al., 2000; Ramos, 2008; Perusini & Fanselow, 2015). In general, this is an emotional state wherein the animal is experiencing a state of conflict, often between avoidance of a stressor, and the drive to investigate or approach the source of the potential threat (Lister, 1990; McNaughton & Corr, 2004). Diffuse or uncertain cues that indicate a potential or uncertain threat will elicit anxiety.

**Rodent models of anxiety.** Many behavioral tests for anxiety have been developed for use with rodent subjects; the most common of which assesses the animal’s behavior in a context that generates conflict via generalized or uncertain threat, including the open field, elevated plus maze, and the light-dark chamber. The validity of any one of these tests as an overall indicator of anxiety has long been called into question (Ramos, 2008), especially when used as an animal model for human anxiety. It has been suggested that each behavioral
assay measures different, but overlapping, aspects of anxiety. Therefore, multiple, sequential tests are frequently used to assess anxiety. The open field, first introduced by Hall (1934) as an assessment of “emotionality,” is one such tool for assessing anxiety in which the rodent is placed into an open, well-illuminated arena and observed over a period of time. The subject then engages in cautious exploration of the apparatus, tending to avoid the center of the field, yet occasionally doing so thereby exhibiting a conflict between avoidance of the potentially threatening area and the drive to investigate. Many variables have been suggested to quantify anxiety in the open field, the most robust of which are measurements of locomotor activity, and the duration in (or number of times crossing) the central region (Walsh & Cummins, 1976, Royce, 1977, Stanford, 2007). The elevated plus maze (EPM) is similar to the open field both conceptually and in terms of its popularity (Pellow, Chopin, File, & Briley, 1985; Litvin, Pentkowski, Bobbe, Blanchard, & Blanchard, 2008). The EPM apparatus consists of four arms radiating from a central platform raised from the ground; two opposing arms are enclosed by high walls and the remaining walls are open to the room. The theoretical basis of this test is that it balances a rat’s natural preference for enclosed spaces and fear of heights, with its natural drive to explore its environment (Pellow et al., 1985). A rat with high anxiety will spend more time in the enclosed arms, enter open arms less frequent than closed arms, and will spend more time assessing risk by leaning out over open arms from a protected location. Merali and colleagues (2003, 2004) have demonstrated that novel environments in general are anxiogenic for rodents as evidenced by suppressed intake of palatable foods.
Acoustic startle has been a valuable tool for distinguishing between anxiety and fear. A loud, unexpected sound will elicit a rapid, stereotyped startle response in rats. The magnitude of the startle response is potentiated over baseline levels while an animal is experiencing both a fearful or anxious state. To demonstrate fear-potentiated startle, the startle-inducing acoustic stimulus is produced during the presentation of another stimulus (e.g., a light) that the animal has previously been trained to predict imminent shock (Brown, Kalish, & Farber, 1951). This fear-potentiated startle is not observed in the period between conditioned stimulus presentations, or to stimuli not paired with shock (Davis and Astrachan, 1978). Anxiety also potentiates the startle response. Two well documented procedures for demonstrating an anxiety-potentiated startle are extended exposure bright light and previous training with repeated footshock (for review, see Walker, Toufexis, and Davis, 2003). Both of these situations increase startle responses compared to control animals. As discussed in following sections, fear-potentiated, and anxiety-potentiated startle are not only dissociated by the types of stimuli that elicit these emotional states, but also by the neural systems and pharmacological agents that control them.

**Neural correlates of fear and anxiety.** Fear and anxiety are largely mediated by structures in the extended amygdala (Davis & Shi, 2006). The basolateral region of the amygdala receives processed input from sensory systems via thalamic and cortical efferent connections and is crucial for acquisition of conditioned fear and anxiety (LeDoux, 2000; Sigurdsson, et al., 2007), as well as unconditioned expressions of fear and anxiety (Koo, Han, & Kim, 2004; Kim et al., 2013). The central nucleus of the amygdala receives direct input from the basolateral amygdala, and is critical for initiating the behavioral and autonomic
responses associated with fear, such as conditioned freezing, analgesia, and bradycardia via projections to multiple targets in the brainstem and hypothalamus (Ledoux, 2000; Helmstetter, 1992; Kapp et al., 1979). Another direct target of the basolateral amygdala is the bed nucleus of the stria terminalis (BNST). The BNST and central amygdala are highly similar structures that in turn project to many of the same downstream targets (Alheid, 2003). While the central nucleus of the amygdala is critical for the expression of fear, a preponderance of data supports the view that the BNST mediates anxiety. Gewirtz, McNish, and Davis (1998) demonstrated that lesions of the BNST blocked the sensitization of the startle response that occurs following repeated footshock while having no effect on conditioned fear-potentiated startle. Walker and Davis (1997b) similarly showed that lesions of the BNST block light-enhanced startle, but not fear-potentiated startle. Duvarci, Bauer, and Paré (2009) investigated the effect BNST lesions on inter-individual variability in anxiety. In this study, animals with BNST lesions exhibited reduced variation in anxiety-related behavior, with consistently low anxiety on the EPM, and were less likely to generalize fear from a conditioned stimulus, to a stimulus that was never paired with shock. Recently, Kim and colleagues (2013) used optogenetic techniques to show that variably increasing or decreasing activity of the BNST could be used to accordingly increase or decrease anxiety as evidenced by shifts in behavioral measures on the open field and EPM, as well as respiratory rate, a physiological indicator of anxiety. In addition, using both multiunit recording, and optogentic inhibition during exploration of the EPM, it was demonstrated that input from the basolateral amygdala to the BNST was important for the expression of anxiety-related behaviors (Kim, et al., 2013).
**Pharmacology.** The validation of a novel model of anxiety requires not only a comparison with existing models, but also rigorous testing with pharmacological agents known to act as anxiolytic and anxiogenic drugs (Lister, 1990; Pellow et al., 1985, Ramos, 2008). Anxiety can be modulated by drugs affecting many neurotransmitter systems. Some of the most commonly used to selectively modulate anxiety in rodents those acting on the GABA-ergic system such as benzodiazepines, barbiturates, and ethanol. Additionally the central administration of corticotrophin releasing hormone has been used in recent years to dissociate the central control of anxiety or fear.

Benzodiazepines have long been identified as potent modulators of the anxious state. This class of drugs binds allosterically to the benzodiazepine site located between the $\alpha$ and $\gamma$ subunit of a GABA$_A$ receptor. The presence of diazepam, an archetypical anxiolytic benzodiazepine, increases the binding affinity of GABA and the probability of ion channel opening in response to its presence (Morton, Morton, Hall, & Hall, 1999, p. 48). Like other benzodiazepines, diazepam has overall sedative effects, but its anxiolytic properties can be observed at dosages lower than those needed to demonstrate observable sedation in the EPM (Pellow et al., 1985). Furthermore, diazepam selectively attenuates anxiety and not fear or depression, making it an ideal drug for demonstrating if a behavior is associated with anxiety (Blanchard, Blanchard, & Rogers, 1990). GABA$_A$ receptor antagonists, such as pentylenetetrazole (PTZ) can be used in low doses as anxiogenic drugs.

Another preparation used to increase anxiety in rodents is the infusion of corticotrophin releasing hormone (CRH) into the lateral ventricles. In addition to binding to receptors in the hypothalamus as part of the well established, full body stress response via the
hypothalamic-pituitary-adrenal (HPA) axis, CRH is implicated in generating anxiety via binding to extra-hypothalamic sites, particularly within the extended amygdala (Makino, Gold, & Schulkin, 1994a,b). Intracerebroventricular (i.c.v.) infusions of CRH result in a potentiated startle response, an effect that can be attenuated by treatment with the benzodiazepine chloradiazepoxide (Swerdlow, et al., 1986), and delayed administration of the CRH receptor antagonist α-helical CRH. Because this enhanced startle response is not blocked by lesions of the paraventricular nucleus of the hypothalamus, and intrathecal CRH infusions do not result in a similar elevation in startle, it is suggested that the effect is independent of HPA activity (Liang, et al., 1992a). Lee and Davis (1997a,b) demonstrated that the primary binding site CRH causing this enhanced startle was most likely the BNST as the effect was blocked by lesions of this site, as well as by local application of CRH receptor antagonists, and the effect was observed following local-only application of CRH. When these techniques were used with other potential neural candidates, such as the dorsal hippocampus and central nucleus of the amygdala, there was not a similar modulation of the startle response. Other evidence of the anxiogenic properties of i.c.v infusion of CRH comes from Merali, et al. (2004) who demonstrated increased neophobia, as assessed by suppressed consumption of palatable food in a novel context, is a behavioral effect that is not blocked by inactivation of the central nucleus of the amygdala. Anxiety can lead to increased cocaine cravings in humans (Sinha, Catapano, & O’Malley, 1999), and the reinstatement of cocaine self-administration in rats (Ahmed & Koob, 1997). Erb and Steward (1999), demonstrated that this anxiety-driven reinstatement is dependent on the activation of CRH receptors in the BNST, as reinstatement following repeated footshock was blocked following the
administration of the CRH antagonist D-Phe₁₂₋₄₁ to the BNST, and similar reinstatement was observed following local administration of CRH. Neither infusion of D-Phe₁₂₋₄₁ or CRH to the amygdala resulted in any effect. Therefore, i.c.v. administration of CRH, and the use of CRH receptor antagonists can also be used as a means to modulate anxiety. This method has the advantage over the use of GABA-ergic drugs in validating a novel animal model in that the effects are more specifically related to anxiety, and motoric confounds can be avoided with this technique which are not avoidable with systemic administration of GABA-ergic drugs.

**The Ultrasonic Vocalizations of Rats**

Like many rodents, rats are capable of vocalizing in the sonic range (e.g., an audible squeak) as well as in the ultrasonic frequency range. Sonic vocalizations are typically produced by rats facing imminent, inescapable threat (Litvin, Blanchard, & Blanchard, 2007). The majority of adult rat vocalizations are produced in the ultrasonic range. Rats begin producing ultrasonic vocalizations (USVs) shortly after birth and continue to make USVs throughout adulthood. A large body of research supports the general categorization of rat USVs into three types based on the age of the rat and the mean peak frequency of the calls. Newborn rat pups produce USVs with a mean peak frequency of 40 kHz. Around three weeks after birth, rats no longer produce the pup vocalizations and begin to produce the vocalizations typical of adults. Adult rat USVs are categorized as either 22 kHz or 50 kHz USVs, a categorical distinction based not only on mean peak frequency, but also on dissociable behavioral and neural correlates (for review, Taylor, 2013; Knutson,
Adult rat USVs. Adult rat vocalizations distinctly cluster into two broad categories referred to by their typical peak frequency: 22 kHz (Fig 1a), and 50 kHz (Fig 1b).

Vocalizations in the 22 kHz range (~17-35 kHz) have a typical call duration of 300 to 4000

Figure 1. Spectrograms of USVs produced by adult Long Evans rats. a) 22 kHz calls have a constant frequency longer duration than 50 kHz calls. b) 50 kHz USVs have short durations and can be produced in a variety of frequency patterns.
ms. These vocalizations are usually produced with a constant frequency, or slight linear frequency modulation. USVs in the 50 kHz range (~35-70 kHz) are produced with a shorter duration than 22 kHz calls, typically ranging from 30 to 50 ms (Kuntson et al., 2002; Portfors, 2007). These two main categories of calls are typically easy to dissociate based on spectral features because the temporal and frequency characteristics rarely overlap. Estimates of overlap in these domains are less than 1% in naturally produced calls, showing no intermediate call type in juvenile and adult rats (Brudzynski, 2007).

**Dissociating USV Call Types**

**22 kHz Ultrasonic Vocalizations.** A large body of evidence suggests that the two broad categories of rat USVs are associated with different emotional states, with 22 kHz calls reflecting negative affective state. This general claim has been supported the assessment of USVs in conjunction with various behavioral and pharmacological manipulations of emotional state. These calls are promoted by fear, social defeat, frustration, and withdrawal from addictive drugs (for review, Portfors, 2007).

Early work with distinguishing the role of 22 kHz calls often used the intruder paradigm (Sales, 1972). The intruder paradigm, in which an unfamiliar male rat was placed into the housing cage of a larger, dominant male, is used to elicit aggressive encounters between rats. USVs were recorded throughout the procedure in conjunction with other behavioral measures. By selectively devocalizing either the intruder or the resident rat (Thomas, Takahashi, & Barfield, 1983) it was shown that 22 kHz USVs are emitted almost exclusively by the intruder, and usually starting just prior to the point of physical aggression and continuing thereafter.
Jourdan, Ardid, & Eschalier (2002), in a study of chronic and acute pain in rats, reported that 22 kHz USVs are not directly produced in response to physical pain. Rather, 22 kHz calls are produced in association with the elevated negative emotional response resulting from the anticipation of pain. Furthermore, the rate of vocalization increased when the anticipated shock-induced pain was greater in intensity. 22 kHz calls are not reliably produced while actively experiencing the shock-induced pain itself (Jourdan, et al., 2002).

22 kHz USVs can also be elicited by the surprising loss of a reward. Coffey et al. (2013) trained rats for two weeks on a continuous reinforcement schedule to condition an auditory CS to the availability of a 20% sucrose solution, and then reported that subjects produced a “transient bout of short 22 kHz USVs” (p. 261) on the first day that they were downshifted to a 25% reinforcement schedule. Similarly, Burgdorf et al. (2000) reported that rats will make 22 kHz USVs in response to an unexpected termination of appetitive electrical brain stimulation in ventral tegmental area.

**50 kHz vocalizations.** Unlike 22 kHz USVs, which exhibit fairly homogenous spectral features, the vocalizations within the 50 kHz category include a wide range of spectral/temporal arrangements. Wright, Gourdon, & Clarke (2010) analyzed the temporal and spectral features of over 20,000 USVs and classified them into 14 subtypes. While reporting the specific 50 kHz subtypes may eventually help in identifying different signal values among the calls, current research suggests at least two 50 kHz call types are associated with different situational promoters. These 50 kHz USV subtypes are frequency modulated (Figure 2a) and constant frequency (Figure 2b) calls. Studies involving the assessment of 50 kHz USVs inconsistently report and define the distinction between frequency modulated
Many studies that investigate the dissociation between the broad categories of 22 kHz and 50 kHz USVs have used paradigms that modulate affective state, and support the interpretation that 22 kHz USVs signal a negative affective state, and 50 kHz USVs indicating positive (for review, see Knutson et al., 2002; Brudzynski, 2009; Portfors, 2007). 50 kHz call production is has been well-explored within the context of conspecific play. Juvenile rats in particular engage in physical play characterized by pouncing and pinning. This behavior is rewarding as evidenced by the finding that subjects will prefer a testing environment in which they have previously played. Anticipation of play is clearly linked with 50 kHz USV production (Knutson, Burgdorf, & Panksepp, 1998). Juvenile rats produce few 50 kHz USVs when placed into a novel environment, but exposure to the context alone promotes 50 kHz USVs if it has been paired with opportunity for play. Adult rats also
produce 50 kHz USVs in anticipation of play and, interestingly, vocalize at a significantly higher rate even though they will spend less time actually interacting with the conspecific (Willey & Spear, 2011; Willey, Varlinskaya, & Spear, 2009). Heterospecific play with a human can also be rewarding to rats. Rats will perform operant tasks for the reward of tickling (i.e., hand play mimicking rat play interactions) from a familiar experimenter (Burgdorf & Panksepp, 2001). Such tickling can be initially aversive and elicit 22 kHz USVs, but when rats are 'tickled' by an experimenter on a daily basis, rats will soon approach the hand of the experimenter and produce a higher number of 50 kHz calls (Knutson, Burgdorf, & Panksepp, 1998; Mällo et al., 2007).

The production of FM 50 kHz USVs appears to be related to activation of the mesolimbic dopaminergic pathway. Microinfusions of amphetamine into the shell of the nucleus accumbens results in the production of 50 kHz USVs (Burgdorf, Knutson, Ikemoto, & Panksepp, 2001). Burgdorf et al. (2007) extended this research and demonstrated that electrical stimulation of the ventral tegmental area (VTA), and nucleus accumbens selectively leads to increased rates of FM, but not CF 50 kHz vocalizations. The production of tickling-related FM 50 kHz vocalizations was blocked by the administration of the non-selective D1/D2 receptor antagonist flupenthixol. Finally, lesions of the VTA led to a selective overall decrease in FM 50 kHz vocalizations when lesioned animals were tickled by experimenters (Burgdorf et al., 2007). Because these regions are associated with intra-cranial self-stimulation and drug reward, this provides strong support for the theory that FM 50 kHz vocalizations are indicators of positive affect.
It has been proposed that because FM 50 kHz calls are associated with positive behavioral expressions and the neural correlates of the mesolimbic dopaminergic reward system these vocalizations can help reveal the affective components of chronic drug use (Ahrens, Ma, Maier, Duvauchelle, & Schallert, 2009). In this study, three intravenous injections of amphetamine were delivered over five days. Two weeks later after the repeated injections, the same dose of amphetamine was once again administered to subjects. FM 50 kHz USVs showed sensitization, and it was therefore argued that FM 50 kHz USVs provide an emotional measure for this effect to compliment the commonly used locomotor measures (Ahrens, et al., 2009).

*CF 50 kHz USVs.* Whereas FM 50 kHz are expressions of positive affect, it has been proposed that CF 50 kHz USVs are not related to affect, but serve a social coordinating function (Wöhr et al., 2008, p 774). One finding that has been citing in support of this hypothesis is that CF 50 kHz calls are produced by subordinate rats prior to intermale aggression in the intruder paradigm, or when the intruder is separated by a screen, but still threatened by the aggressor in proximity. However, there is no change in the behavioral profile of the aggressive interaction in this paradigm even when the intruder rat is devocalized, or the aggressive rat deafened (Thomas, Takahashi, & Barfield, 1983). These findings can be interpreted to suggest that no pro-social signal is being received, or that it is at least not functioning as an effective communicative signal. Indeed, many situations known to reliably elicit higher numbers of CF 50 kHz calls are not in social situations such as during the exploration of a novel context (Wöhr et al., 2008, Shetty & Sadananda, 2017).
Proportionally higher numbers of CF 50 kHz USVs are often produced in non-appetitive situations, and it has been suggested that this 50 kHz USV subtype may be produced as a social contact call not linked with emotion (Burgdorf et al., 2008, Wöhr et al., 2008). Studies have yet to confirm this function empirically and the primary stimulus for eliciting CF 50 kHz calls has not been specifically explored. As reviewed above and elsewhere (Taylor, 2013), the affective motivation hypothesis for USV production in general is strongly supported by the literature. The argument of the hypothesis is that arousal of certain affective states lead the rat to vocalize in predictable patterns as part of a natural suite of physiological and behavioral responses to that state, although in some instances vocalizing may be able to be withheld by the rat (Blanchard et al., 1991). Through auto-conditioning USVs to the unconditioned stimuli that elicit the calls (Kim et al., 2010; Parsana et al., 2012), and possibly other mechanisms, these calls gain functional significance for rats and may be able to serve as communicative signals. CF 50 kHz USVs have not yet been linked to a specific affective state despite their suggested functional role.

**Preliminary Evidence That CF 50 kHz USVs Indicate Anxious State**

We hypothesize that CF 50 kHz USVs are specifically linked to anxiety. This novel hypothesis is derived from the interpretation of research utilizing both social and non-social paradigms, and our initial investigations have yielded support for this idea. This section will highlight some of the evidence drawn from the literature as well as recent endeavors by our own research group as initially described by Taylor (2013).

In the intruder paradigm, rats produce both 22 kHz and 50 kHz USVs depending on whether the subjects are actually engaged in aggressive fighting or one rat is merely being
threatened by the other (Vivian & Miczek, 1992, Vivian & Miczek, 1993). Detailed spectrographic analysis from the work of Vivian and Miczek (1992, 1993) revealed that the majority of 50 kHz calls were constrained to a narrow bandwidth and consisted of either simple linear change, or a constant frequency (i.e., not FM calls). The administration of the anxiolytic drug diazepam resulted in a decreased rate of 50 kHz USVs during threat of attack, but not during the actual aggressive encounter. However, diazepam administration resulted in was no change in 22 kHz vocalizations during either phase of testing (Vivian & Miczek, 1993). Similarly, Burgdorf et al. (2008) demonstrated that the proportion of CF subtype of 50 kHz USVs was significantly greater prior to the onset of aggression in the intruder paradigm than during ostensibly appetitive tasks such as conspecific play and “tickling” by a familiar human researcher.

As reviewed above, various experimental evidence suggests a link between social stimuli and CF 50 kHz call production. However, this association may be primarily motivated by anxiety, and thereby may provide a signal of social anxiety. In support of this view, CF 50 kHz USVs have been observed in a variety of social situations not obviously linked with anxiety, but may nonetheless include situations of uncertain threat within a social context. CF 50 kHz USVs are often produced during conspecific play, albeit in a lower proportion of total 50 kHz USVs compared to FM 50 kHz calls. It is possible that the presence of these calls indicates some form of social anxiety in an agonistic encounter. For example, Hamed et al. (2009) reported that individually housed rats produced a high number of 50 kHz calls when placed into a novel context with another rat for social interaction. In this study, these increased 50 kHz USVs exhibited a mean frequency bandwidth within the
range often defined as CF 50 kHz calls (Hamed, et al., 2009; Burgdorf, et al., 2008). Furthermore, pretreatment with the anxiolytic diazepam before the social encounter increased the overall number of 50 kHz calls produced, and the USVs exhibited a significantly larger mean frequency bandwidth than saline controls (Hamed, et al., 2009). Although not discussed in the original study, the change in mean bandwidth can be interpreted as a selective decrease in the number of constant, or nearly constant frequency 50 kHz USVs. An increase in FM 50 kHz calls would also be explained by the decrease in anxiety as a result of the diazepam injection. An important caveat when assessing USVs in paradigms that utilize social interaction is that it is difficult to separate which rat is producing the calls. Future research could examine whether there is a difference in the distribution of 50 kHz USV subtypes elicited by subordinate and dominate rats within these ostensibly appetitive paradigms.

**Summary**

Rats are one of the most commonly used animals for modeling human disorders, and models reliant upon indices of emotion are enriched when USVs are assessed. Both FM 50 kHz and 22 kHz USVs are now frequently utilized as indicators of affect in a variety of experimental paradigms. Narrow bandwidth and CF 50 kHz USVs, perhaps due to the ambiguity regarding the stimuli that drive their production and potential function, are often either discounted from analysis entirely, or are combined with other 50 kHz subtypes.

A preponderance of data supports FM 50 kHz and 22 kHz calls as innate calls generated by emotional states that can be utilized as communicative signals through experience. Current literature posits a social contact function to CF 50 kHz USVs, but has
not put forth an associated primary motivating state. Establishing the motivational state associated with the production of CF 50kHz USVs will allow for a greater understanding of their significance across a variety of social and non-social paradigms, and potentially further enrich animal models of affective disorders. We have hypothesized that CF 50 kHz USVs signal anxious state (i.e., the emotional response to less certain or imminent threat) similar to the signal of positive affect reflected in FM 50 kHz calls. Because they can be assessed non-invasively and across multiple testing paradigms, a vocal signal of anxiety would serve to help bridge various behavioral indicators of anxiety that are often specific to the testing paradigm, and frequently rely on movement-related measures alone. In the experiments that follow, we test the veracity of this hypothesis via analysis of the vocal and overt behavior of rats during behavioral, pharmacological, and neurological manipulations of emotional state.
Chapter 2. Differential Patterns of Constant Frequency 50 and 22 kHz USV Production Are Related to Intensity of Negative Affective State

Laboratory rats are frequently used as an animal model for the study of human neuropsychiatric disorders. A special challenge exists for the development and interpretation of nonhuman animal models for affective disorders, because we lack objective, biological markers for emotions, and instead rely on constellations of behavioral assessments (Fernando & Robbins, 2011; Koob & Zimmer, 2012; Litvin, Pentkowski, Pobbe, Blanchard, & Blanchard, 2008; Nestler & Hyman, 2010; Ramos, 2008). In the last few decades, researchers have discovered that recording rodent ultrasonic vocalizations (USVs) provides a powerful and noninvasive method for assessing an animal’s emotional state (Burgdorf, et al., 2009; Burgdorf & Moskal, 2010; Knutson, Burgdorf, & Panksepp, 2002; Miczek, Weerts, Vivian, & Barros, 1995).

Adult rat USVs are classified based on the vocalization peak frequency, and can be broadly categorized as either 22 kHz, or 50 kHz calls (Brudzynski, 2007; Portfors, 2007). These two distinct USV categories correspond to very different emotional states. Twenty-two kilohertz USVs signal a negative affective state in the rat and are frequently produced when the rat encounters an aversive or threatening stimulus. This includes exposure to unconditioned stimuli, such as the presence of a predator (Blanchard, Blanchard, Agullana, & Weiss 1991), tail shock (Van der Poel & Miczek, 1991), unexpected reward loss (Coffey, et al., 2013), aversive brain stimulation (Brudzynski, 2007), as well as in response to conditioned stimuli, such as a tone or context paired with footshock (Wöhr, Borta, &
Schwarting, 2005; Kim, Kim, Covey, & Kim, 2010; Jelen, Soltysik, & Zagrodzka, 2003). In addition, 22 kHz vocalizations are closely associated with other behaviors elicited by threatening stimuli, for example freezing and escape (Brudzynski, 2007; Blanchard, et al., 1991). The acoustic features of 50 kHz calls are quite variable. They can be subdivided into different call types, in some cases up to 14, based on the fundamental frequency, degree of frequency modulation, and behavioral states that elicit these calls (Burgdorf, et al., 2008; Wright, Gourdon, & Clarke, 2010). A preponderance of evidence supports frequency modulated 50 kHz USVs (FM 50 kHz USVs) as a signal for a positive affective state in the rat (Burgdorf, et al., 2008; Wiley, Varlinskaya, & Spear, 2009; Costa, Morelli, & Simola, 2015). However, a definitive link between other 50 kHz call types and corresponding emotional states have not been clearly delineated.

Unlike the FM 50 kHz USVs that are associated with positive affect, there is another call category that has been observed in a variety of behavioral paradigms, but has not been viewed as being related to affective state; these calls are in the 50 kHz category and they are often produced with a narrow bandwidth (< 15 kHz) or composed of only a constant frequency. The term “flat call” has been used to describe both of these categories of non-appetitive 50 kHz USVs (Burgdorf, et al., 2008; Mahler, et al., 2013; Wöhr, Houx, Schwarting, & Spruijt, 2008; Wright, et al., 2010). For clarity, we hereafter refer to the larger category of “flat” 50 kHz USVs as narrow bandwidth calls, and the 50 kHz USVs subtype lacking frequency modulation as constant frequency (CF) calls. Narrow bandwidth 50 kHz USVs, and particularly CF 50 kHz USVs, are produced in a variety of social and non-social situations, many of which may be mildly aversive or be associated with uncertain
threat. For example, Wöhr, et al., (2008) reported that narrow bandwidth 50 kHz USVs are produced when a rat is separated from a cagemate, and that calling rate declined after repeated separations. Rats also produce these narrow bandwidth calls while exploring a novel context (Wöhr, et al., 2008). Additionally, the 50 kHz calls recorded during agonistic encounters using the intruder paradigm; interestingly, these calls are produced almost exclusively by the subordinate rat (Thomas, Takahasi, & Barfield, 1983). One interpretation of these results is that the narrow bandwidth calls serve a social communicative function (Wöhr, et al., 2008). Another interpretation that is not mutually exclusive with a social function, is that the narrow bandwidth calls are related to a mildly aversive state, particularly when the animal experiences uncertainty regarding a threat encounter.

We sought to determine whether emotional state modulates the production of CF 50 kHz and narrow bandwidth USVs. In order to assess changes in USV call type production across a continuum of increasingly negative affect, we used a manipulation wherein the certainty and predictability of threat was increased throughout a single session. In Experiment 1, the primary test session consisted of an introduction to a novel environment, followed by a series of six, temporally consistent footshocks. We predicted that upon exposure to the novel environment and during initial footshocks rats would engage in risk assessment behavior, which was measured by quantifying rearing activity (Borta & Schwarting, 2005; cf. Eschorihuela, et al., 1999). During this period of risk assessment, we predicted that rats would also produce CF 50 kHz USVs. However, after repeated exposure to temporally predictable footshocks, we predicted that animals would begin to engage in behavior associated with elevated negative affect due to the certainty of threat, which was assessed by
measuring freezing behavior (Fanselow, 1980; Grossen & Kelley, 1972). Similarly, we predicted that animals would produce 22 kHz vocalizations when the threat was expected based on the experience with the repeated footshocks (Wöhr, et al., 2005; Kim, et al., 2010). Additionally, to manipulate the animal’s emotional state, subjects were given intraperitoneal (IP) injections of the anxiolytic diazepam (1, 2.5, or 5 mg/kg doses) or saline prior to behavioral testing. We predicted that diazepam would attenuate the intensity of the negative affective state of the animal and this change in affective state would reduce CF 50 kHz and 22 kHz calling behavior during the testing session.

Furthermore, because the predictions of our single-session design were partially dependent on the assumption that exposure to the novel environment was itself mildly aversive, additional behavioral tests were conducted in Experiment 2. Animals were pre-exposed to the footshock environment with or without access to familiar palatable food, and then received the same footshock procedure used in Experiment 1 on the following day. Suppression of food consumption, rearing, freezing, and ultrasonic calling behavior were measured as indexes of intensity of negative affect. Conflict due to the presence of palatable food in the novel environment was predicted to elevate CF calling behavior, and pre-exposure was predicted to reduce calling and rearing behavior associated with increasingly negative affective state during footshock sessions.

**Experiment 1 - Methods**

**Subjects**

Forty adult female Long Evans rats (Harlan Laboratories, IN) forty-five to sixty days old served as experimental subjects. Subjects were housed in standard polycarbonate cages
in a temperature- and humidity-controlled room on at 12:12 hr light cycle with lights on at 0800. Throughout testing, food and water were available ad libitum. Animals were handled for 5 min per day for three days preceding the experiment to acclimate the rats and forthcoming injection procedures. All research was conducted with approval from an Institutional Animal Care and Use Committee (#14/02).

**Apparatus and Materials**

Behavioral testing occurred in an test chamber housed within a sound attenuating cubicle with a single viewing window (Med Associates, St. Albans, VT). The ceiling and two side walls (front and rear) of the test chamber were constructed of clear Plexiglas; the remaining side walls were composed of interchangeable stainless-steel plates. The floor of the chamber was composed of stainless-steel rods measuring 0.5 cm in diameter, spaced 1.5 cm center-to-center. Footshocks were generated by a standalone aversive scrambler/shocker (Med Associates, St. Albans, VT). The test chamber was housed within a sound attenuating cubicle equipped with a viewing window (Med Associates, St. Albans, VT). A single UltrasoundGate Condenser Microphone (CM 16; Avisoft Bioacoustics, Berlin, DE) was positioned outside the test chamber, but inside the sound attenuating cubicle, at the center of one side. A matrix of holes allowed for the passage of sound between the test chamber and microphone. Sound was recorded continuously during the testing session and data were digitized and saved for later call classification (250 kHz sample rate, 16 bit resolution; Avisoft Recorder, v. 5.2.07, Avisoft Bioacoustics, Berlin Germany). Video recording was also obtained for all testing sessions by a webcam (Logitech, Fremont, CA) positioned above the test chamber at the ceiling height of the cubicle.
**Drug Administration**

Diazepam (Sigma, St. Louis, MO) concentrations were prepared by dissolution into 0.9% saline with a few drops of Tween 80 and mixed via sonication. Diazepam (1, 2.5, or 5 mg/kg) or saline was administered via a single IP injection to subjects 30 min prior to behavioral testing in a volume of 2 ml/kg.

**Procedure**

The testing procedure was as described in Taylor (2013). All subjects were individually housed for one week prior to testing. A segment of PVC pipe was added to the home cage for enrichment while individually housed. Rats were then randomly assigned to one of the three diazepam groups (1 mg/kg, \(n = 12\); 2.5 mg/kg, \(n = 8\); 5 mg/kg, \(n = 8\)) or saline control group (\(n = 12\)). On the testing day, subjects were placed in the test chamber and allowed to explore for 2 min after which time the first footshock (0.5 mA, 0.5 s) was delivered. A variable 60 s interstimulus interval (ISI, ± 10 s) separated the subsequent footshocks. Six shocks in total were delivered and subjects were returned to their cages 2 min after the final shock. The test chamber was thoroughly cleaned between subjects and allowed to dry. Bedding beneath the steel rod floor was also changed between subjects.

**Data Analysis**

**Rearing analysis.** Rearing activity was defined as a vertical body movement resulting with only the rear paws in contact with the floor of the chamber. Excluded from this count were instances wherein rearing immediately preceded jumping or climbing. A count of rearing activity was made per 20 s bin by two independent observers; both experimenters were blind to the treatment conditions. Inter-observer agreement was 95%.
Counts were collapsed to create a total rearing count per subject during the pre-shock period, and then compared between groups using a one-way ANOVA with subsequent Tukey’s LSD post hoc tests. All statistical analyses were performed using PASW Statistics (Armonk, New York, version 18.0).

**Freezing analysis.** Freezing was defined as a lack of all non-respiratory movement, and was assessed every 8 s during the session by two independent observers blind to experimental condition. Inter-observer agreement was 94%. These observations were converted to percent time freezing (number of freezing observations/number of observations * 100) for the initial 2 min pre-shock period and subsequent inter-shock-intervals. The percent time spent freezing was compared between groups within the session using a mixed-model repeated measures ANOVA, followed by a one-way ANOVA and subsequent Tukey’s LSD post hoc tests when no interaction was found to compare the overall percent time spent freezing between groups.

**USV classification.** USV recording and analysis was conducted as described in Taylor (2013). Spectrograms for each recording session were calculated with SASLab Pro software (Version 5.2.07, Avisoft Bioacoustics, Berlin, DE) using 1024 FFT length, 100% frame size, FlatTop window, and 96.87% window overlap. USVs were then separated from background noise by a user-defined amplitude threshold and individually marked with section labels for automatic analysis of call mean peak frequency, mean bandwidth, and the time it was produced during the session. Mean peak frequency was defined as the frequency with the greatest sound intensity calculated over the average of the entire call. Each call was categorized as 22 kHz (20-35-kHz) or 50 kHz (>35-kHz) based on mean peak frequency.
Figure 3 provides example calls in the 50 kHz category that were classified into one of three subtypes: Constant Frequency (CF, Fig. 3A), Frequency-Modulated Step (FM Step, Fig. 3B), and Frequency-Modulated Other (FM Other, Fig. 3C). CF calls were defined as those calls with a constant frequency and frequency bandwidth less than 10 kHz. FM Step calls were classified based on a bandwidth less than or equal to 15 kHz, and exhibited small, stepwise increases in frequency. The calls classified into the FM Other category included both increasing and decreasing frequency components, and/or had an overall bandwidth greater than 15 kHz.

Effect of repeated footshock on USV production. We hypothesized that if CF calls are related to a mildly aversive state, then subjects would produce primarily CF 50 kHz calls in the first half of the session, whereas if they are related to a more intensely negative
affective state, then 22 kHz calls would be produced in the last half of the testing session. The number of CF 50 kHz and 22 kHz calls was therefore compared in time bins (first or second half of the six shocks) to determine whether the transition between call types would conform to this pattern. Differences in number of call types during these two time bins during the test session was explored using a 2 x 2 chi-square test using call type (CF or 22 kHz) and time bin in the contingency table. Similarly, the number of USVs in the three different 50 kHz subtypes before and after the administration of the first shock was compared using a chi-square test. This assessment explored whether the predominant call type changed after the first footshock was encountered.

**CF 50 kHz USV production.** CF 50 kHz USVs were produced by subjects receiving saline or 1 mg/kg dose of diazepam, but not by animals receiving the higher doses of diazepam. Differences prior to footshock administration were therefore assessed by comparing the number of CF 50 kHz calls produced across the 2 min period using an independent t-test between saline and 1 mg/kg diazepam. Additionally, a likelihood ratio was calculated to quantify the time of initiate CF 50 kHz USV production between groups.

If the rate of CF 50 kHz USV calling can be used to assess a mildly aversive state, then the rate of calling should increase immediately following shocks early in the session when the shocks are unanticipated. To determine whether calls coincided with footshocks, an inter-call interval histogram was calculated for each rat (custom-written software, LabVIEW, v. 7.1, National Instruments, Austin, TX). To generate histograms, the time interval between the first call and each successive call was calculated; and this process was repeated for each successive call in the data set produced between the time of the first and fourth footshock.
This analytical approach is used for capturing neural spike timing to phase-locked stimuli. In this analysis, if the calls are phase-locked to the time of shock administration, then histogram should show peaks of calling time-locked to the onset of each shock. However, if call timing is unrelated to temporally reoccurring events, then the distribution should be flat. To statistically measure differences between drug treatment groups in post-shock call-rate distributions, the number of CF 50 kHz calls occurring in the 10 s period immediately following each of the first three shocks was then compared between groups using a mixed-model repeated measures ANOVA. Only the first three shocks were analyzed because the CF calls were no longer regularly produced after the third shock.

**22 kHz USV production.** The rate of 22 kHz production was calculated for the interval following each of the final three footshocks by dividing the number of 22 kHz USVs by the total time of the interval. These time bins were used because few subjects produced 22 kHz USVs prior to the fourth footshock. The rate of 22 kHz USV production was compared between groups within these bins using a mixed-model repeated measures ANOVA, followed by an independent t-test when no interaction was found to compare the overall rate of 22 kHz production during the session. A t-test, rather than a one-way ANOVA was used for this analysis because USV production occurred almost exclusively in only the saline- and 1 mg/kg diazepam-treated animals.

**Experiment 1 - Results**

**Rearing Activity**

Video data from one rat in the 1 mg/kg diazepam group was lost due to equipment failure and could therefore not be used to score freezing or rearing behavior. Figure 4A
displays the rearing activity for all groups during the 2 min pre-shock period of the session. Rearing activity was significantly affected by drug pre-treatment, $F(3, 34) = 8.795, p < .001$. Subsequent Tukey’s LSD post hoc tests indicated that the 2.5 ($M = 6.25, SE = 0.84$) and 5 mg/kg ($M = 4.38, SE = 1.46$) diazepam doses significantly reduced rearing activity as compared to 1 mg/kg diazepam ($M = 10.40, SE = 1.23$) or saline ($M = 13.50, SE = 1.71$).

While no other significant differences were found between drug or treatment conditions, a general decrease in overall rearing activity was observed between saline treated animals and increasing doses of diazepam. Rearing activity decreased for all groups across the session. This behavior occurred most frequently in the pre-shock period and was virtually absent after the third footshock (Figure 4B).

**Freezing**

![Figure 4. Rearing and freezing behaviors are modulated by drug administration and repeated footshock.](image-url)

(A) Rearing activity prior to footshock was attenuated by increasing doses of diazepam (DZP). (B) Rearing activity was lower following the first footshock in the control group, especially in the second half of the session, whereas the opposite pattern was observed in time spent freezing. (C) Subjects pre-treated with 2.5 mg/kg of diazepam spent significantly less time freezing than all other drug groups following the administration of footshock. Bars represent mean and SE; * indicates $p < .05$. 
The overall percent time spent freezing for all groups is shown by group in Figure 4C. There was a significant effect of drug treatment on freezing behavior, $F(3, 34) = 3.823, p = .019$. Tukey’s LSD post hoc test indicated that 2.5 mg/kg of diazepam ($M = .33, SE = .068$) resulted in significant reduction in time freezing as compared to 1 mg/kg ($M = .57, SE = .047$) and 5 mg/kg doses ($M = .55, SE = .079$), as well as saline control ($M = .57, SE = .039$). Increased time freezing was observed following the third footshock as compared to the first.

**Figure 5. CF 50 kHz call production decreases as 22 kHz call production increases.** Each dot in the scatterplot is a call produced by subjects during the testing session. The calls above the grey shading correspond to CF 50 kHz subtype and below are the 22 kHz USVs produced by animals in the (A) saline, and (B) 1 mg/kg diazepam groups. Dotted lines indicate time of footshock.
half of the session (Figure 2B). During video coding, qualitative differences were observed in the 5 mg/kg dose of diazepam group in comparison with other groups in that many subjects in this group demonstrated poor motor coordination and sedation throughout behavioral testing.

**Ultrasonic Vocalizations**

The majority of subjects in the control and 1 mg/kg diazepam groups produced both 50 kHz \( (n = 10:12 \text{ and } 10:12, \text{ respectively}) \) and 22 kHz USVs \( (n = 11:12 \text{ and } 10:12) \). However, calling behavior was largely absent in the 2.5 mg/kg and 5 mg/kg diazepam

![Figure 6](image.png)

**Figure 6. The distribution of 50 kHz USV subtypes was affected by footshock.** The dominate subtype of 50 kHz ultrasonic vocalization (USV) transitioned from frequency modulated (FM) step to constant frequency (CF) following the first footshock. A similar distribution in subtype of 50 kHz calls out of all 50 kHz calls was seen in both the saline (A) and diazepam 1 mg/kg (B) group across the session and exhibited a similar change following shock.
groups for both 50 kHz ($n = 0:8$ and $0:8$, respectively) and 22 kHz USVs ($n = 0:8$ and $1:8$).

**Effect of Repeated Footshock on USVs.** The CF 50 and 22 kHz USVs produced by all subjects in the saline and 1 mg/kg diazepam groups is illustrated in Figure 5. The temporal variation in the number of CF 50 kHz and 22 kHz USVs produced changed as a function of repeated footshocks. Specifically, the rate of CF 50 kHz calls decreased as the rate of 22 kHz call production increased.

**50 kHz USVs.** One subject from the 1 mg/kg diazepam group was excluded from USV analysis (produced a number of 50 kHz USVs > 2X SD). A similar overall and shock-related change in the distributions of 50 kHz USV subtypes was observed in the 1 mg/kg diazepam-treated and control animals (Figure 6A, B). For both groups, the number of FM Other calls comprised less than 5% of total 50 kHz calls, whereas FM Step calls were produced in high numbers and in a pattern similar to CF 50 kHz calls. A 2 x 2 (Call Type x Time Bin) chi-square test was used to investigate changes in 50 kHz call type between the pre-shock period and the period between the first and fourth shocks (after which time no 50 kHz USVs were observed). The chi-square test indicated a significant association between call type and time bin, $X^2 (1) = 69.971$, $p < .001$, $\phi = -.249$, with CF 50 being the 50 kHz USV subtype more strongly associated with the period following the onset of footshocks.
**CF 50 kHz USVs.** An independent measures t-test indicated that a marginally significant effect of drug treatment on CF 50 kHz calls during the pre-shock period, \( t(17) = 2.011, p = .060 \) (Figure 7), in that fewer calls were produced by 1 mg/kg diazepam-treated compared to saline control subjects.

Figure 8 displays the inter-call interval histograms generated from calls produced between the first and fourth footshock. A tri-modal distribution, indicating that CF 50 kHz call production was phase-locked with the timing of the mild footshocks, was observed in the saline-treated animals, whereas the 1 mg/kg diazepam-treated animals produced did not show elevated calling after the initial footshocks. Analysis of the 10 second period following the first three footshocks yielded significant main effects for group, \( F(1, 17) = 6.330, p < .001 \), and post-shock interval, \( F(2, 34) = 8.457, p = .001 \). A marginally significant interaction effect was also indicated, \( F(2, 34) = 3.054, p = .060 \). Overall, 1 mg/kg of diazepam (\( M = 1.82, SE = 0.61 \)) treatment decreased CF 50 kHz call production compared to saline control (\( M = 3.93, SE = 0.58 \)) in the period immediately following footshocks.

| Table 1 |

<table>
<thead>
<tr>
<th>Subjects Producing CF 50 kHz USVs During Footshock Session</th>
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<tr>
<td><strong>Group</strong></td>
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<tr>
<td></td>
</tr>
<tr>
<td><strong>Experiment 1</strong></td>
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<tr>
<td>Diazepam (1 mg/kg)</td>
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<tr>
<td>Saline</td>
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<tr>
<td><strong>Experiment 2a</strong></td>
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<tr>
<td>-</td>
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<tr>
<td><strong>Experiment 2b</strong></td>
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<td>Stress</td>
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<td>Control</td>
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Onset of CF 50 kHz USV production. Table 1 summarizes the number of subjects in each group that produced CF 50 kHz calls, and whether calls of this type were initially produced prior to or following the first footshock. There was a significant association between treatment and onset of CF 50 kHz call production, $X^2 (1) = 7.192, p = .007$.

22 kHz USVs. No overall difference in the rate of 22 kHz USVs was observed between the saline ($M = 0.16, SE = 0.054$) and 1 mg/kg diazepam ($M = 0.19, SE = 0.069$) groups, $t(19) = 0.321, p > .05$.

Experiment 2- Methods

Apparatus and Materials

The novel environment and footshock sessions for Experiment 2a and 2b occurred in the operant test chamber described in Experiment 1. For Experiment 2a only, the chamber was modified by the attachment of the food dish onto one wall at a height of 4 cm from the floor.
The equipment and procedures used for video and ultrasonic recording within the test
chamber were identical to those described in Experiment 1.

Procedure

**Experiment 2a – Consummatory behavior in the novel environment.** Rats \((n = 8)\) were individually housed for one week prior to testing. Then, on four successive days, animals were given nine minutes of access to two pieces (approximately 35 mg) of Fruit Loops cereal (Kellogg, Battle Creek, MI) in a round dish (3 cm diameter). The latency to the onset of eating, and total amount consumed was recorded each day. Food and water remained available *ad libitum* in the home cage throughout the experiment.

*Days 1 & 2: Home Cage.* For the first two days, the cereal was presented within the animal’s home cage. Each rat consumed all available cereal within the given time, at the conclusion of which the food dish was removed from the home cage.

*Day 3: Novel Environment.* Subjects were placed within the novel environment and allowed to freely explore for nine minutes with open access to the food. Video and ultrasound recordings were collected for the entire duration of the session. Animals were returned to their home cage at the conclusion of the session.

*Day 4: Footshock.* Subjects were returned to the operant box and again provided with open access to the cereal throughout the session. Footshocks were administered, and additional data were collected using the procedures detailed in Experiment 1.

**Experiment 2b – Context pre-exposure and injection stress.** Rats were individually housed for one week prior to testing, and then randomly assigned to either the stress group \((n = 10)\) or control group \((n = 10)\). Behavioral testing occurred over two
subsequent days. Thirty min prior to testing animals in the stress group received a sham i.p.
injection (insertion of needle, no infusion), whereas control animals received no injection.
The first day of behavioral testing consisted of exposure to the novel environment of the
operant chamber for 9 min in the absence of any acute stressors. Approximately 24 hours
later, subjects were returned to the chamber wherein footshocks were administered using the
procedure detailed in Experiment 1. Video and ultrasound recordings were collected
throughout each session as previously described.

**Data Analysis**

**Food Consumption.** In Experiment 2a, the latency to begin eating following the
access to food dish was assessed for each by a trained observer through direct observation
(Days 1 & 2) or through video recordings (Days 3 & 4). The mean latency to begin eating on
Day 2 and Day 3 of testing was compared using a paired sample t-test to determine whether
the novel context mediated suppression of consummatory behavior.

**Rearing analysis.** Rearing activity was assessed throughout novel environment and
footshock sessions as described in Experiment 1. For novel environment sessions the
behavior was collapsed into 100 s time bins for the first 8 min and compared across time
using a repeated measures ANOVA to determine changes in risk assessment within the
session. When significant within-subjects effects were indicated, Tukey’s LSD post hoc tests
were conducted to determine specific differences between the first and final minute of
testing. For Experiment 2b, between group comparisons were also assessed via a mixed-model repeated measures ANOVA.

A comparison of rearing activity during the first two minutes of testing on the novel environment and footshock sessions was conducted to determine whether previous exposure to the context reduced this measure of risk assessment. A paired samples t-test was used in Experiment 2a, and a mixed model repeated measures ANOVA was used for the results of Experiment 2b to determine possible effects of treatment condition.

**Freezing analysis.** Freezing was assessed during the footshock session as described in Experiment 1. The percent time spent freezing was compared within the session using a repeated measures ANOVA in Experiment 2a. For Experiment 2b, between group comparisons were also assessed by mixed-model repeated measures ANOVA.

**USV classification.** The classification of USVs was performed as described in Experiment 1.

**50 kHz USV production.**

**50 kHz USV subtype distribution.** The relative proportion of 50 kHz USV subtypes for Experiment 2a and for each group in Experiment 2b was calculated for the novel environment and footshock testing sessions. To determine whether the proportion of CF 50 kHz USVs increased following footshock administration – as was observed in Experiment 1 – the distribution in 50 kHz USV subtypes during the footshock session was further assessed within the preshock (first 120 s), and postshock (> 120 s) bins.

**CF 50 kHz USVs.** For novel environment sessions, the number of 50 kHz USVs was first binned into 100 s time bins for the first 8 min. For each subject, the number of calls per
bin was divided by the total number of calls produced throughout the entire session as a way to normalize USV production across high and low calling rats. A repeated measures ANOVA was used in Experiment 2a to determine whether the production of CF 50 kHz USVs decreased with prolonged exposure to the environment in the absence of additional stressors. When significant within-subjects effects were indicated, Tukey’s LSD post hoc tests were conducted to determine specific differences between the first and final minute of testing.

A direct within-subjects comparison in calling behavior across days could not be conducted because several subjects in both Experiment 2a and 2b vocalized on one, but not both sessions of testing.

22 kHz USVs. Few rats produced 22 kHz USVs throughout Experiment 2. No 22 kHz USVs were produced during novel environment sessions, and during footshock sessions only one rat in Experiment 2a, and four rats in Experiment 2b (Stress $n = 2:10$, Control $n = 2:10$) produced calls of this type. Due to the infrequent calling behavior, no further analysis was performed on the collected 22 kHz USV data.

**Experiment 2 - Results**

**Food Consumption Latency**

All rats reliably consumed all palatable food within the given time when provided access inside their home cage. A comparison between the latency to consume food on Day 2 ($M = 25.0, SE = 5.35$) and Day 3 ($M = 334.8, SE = 48.87$) could not be conducted because only half of the subjects consumed the palatable food on Day 3. However, for those rats that consumed food on both days, there was a significant increase in latency to eat within the novel environment, $t(3) = 6.338, p = .007$. 

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Rearing

Experiment 2a. Rearing activity generally decreased throughout the novel environment testing session, $F(4, 28) = 7.054, p < .001$, with significant differences in rearing between the first 100 s ($M = 10.250, SE = 0.412$) and the 500 s time bin ($M = 3.500, SE = 1.1282$), as indicated by post-hoc LSD test, $p = .003$.

The amount of rearing activity during the first 2 min of behavioral testing significantly declined between the first ($M = 11.5, SE = 0.823$) and second day ($M = 6.25, SE = 0.773$) of behavioral testing, $t(7) = 12.747, p < .001$.

Experiment 2b. Rearing activity generally decreased throughout the novel environment testing session, $F(4, 72) = 5.941, p < .001$, with significant differences in rearing between the first 100 s ($M = 11.500, SE = 0.568$) and the 500 s time bin ($M = 7.700, SE = 0.897$) indicated by post-hoc LSD test, $p = .001$. No between-subjects effect was found for group, $F(1, 18) = .006, p = .938$.

Rearing activity in the first 2 min of behavioral testing declined significantly between the first ($M = 13, SE = 0.690$) and second day ($M = 11.15, SE = 0.478$) of behavioral testing, $F(1, 18) = 5.809, p = .027$, but was not differently affected by treatment conditions, $F(1, 18) = 0.370, p = .551$.

Freezing

Experiment 2a. Percent time freezing increased throughout the footshock session, $F(5, 35) = 13.103, p < .001$, with significant differences in freezing between the first intershock interval ($M = .146, SE = .097$) and the minute following the final shock ($M = .875, SE = .077$) indicated by post-hoc LSD test, $p < .001$. 

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**Experiment 2b.** Percent time freezing increased throughout the footshock session, \( F(5, 90) = 60.766, p < .001 \), with significant differences in freezing between the first intershock interval (\( M = .008, SE = .008 \)) and the minute following the final shock (\( M = .819, SE = .047 \)), as indicated by post-hoc LSD test, \( p < .001 \). However, no between group differences were indicated, \( F(1, 18) = 0.000, p = .991 \).

**Figure 9.** Effect of previous experience on 50 kHz subtypes. CF 50 kHz calls were the most frequently produced 50 kHz USV subtype in (a) Experiment 2a, as well as in the (b) Control and (c) Stress groups in Experiment 2b. During the footshock session (shaded region) the lowest proportion of CF calls and highest proportion of FM other calls was observed when re-exposed to the test environment prior to the administration of footshock.
50 kHz USVs

50 kHz subtype distribution.

Experiment 2a. The distribution of 50 kHz call types for the novel environment and footshock sessions is shown in Figure 7. The majority of 50 kHz USVs produced throughout the novel environment session were of the CF type, followed by the FM Other, and then FM Step type calls.

Experiment 2b. The distribution of 50 kHz call types for the novel environment and footshock sessions is shown in Figure 7. The distribution of 50 kHz USV subtypes was similar for both groups during novel environment sessions. CF type calls were the most common, then FM Other, and FM Step type calls.

CF 50 kHz USVs.

Experiment 2a. During novel environment sessions, a significant within-subjects effect, $F(4, 20) = 4.208, p = .012$, indicated that the production of CF 50 kHz USVs generally decreased throughout the session. Rats produced lowest proportion of calls during the 500 s bin ($M = .04, SE = .026$). Relative to the 500s bin, call production was significantly higher during the 200 ($M = .41, SE = .081$), 300 ($M = .15, SE = .063$), and 400 ($M = .24, SE = .063$) time bins ($ps < 0.05$). However, the proportion of calls during the 500 s time bin was not significantly different from the 100 s time bin ($M = .16, SE = .055, p = .126$).

The majority of subjects produced CF 50 kHz USVs during both the novel environment ($n = 6:8$) and footshock sessions ($n = 6:8$). However, a direct comparison in calling behavior across days could not be conducted because two rats only called on one of the two days. A general effect of experience was nonetheless observed. While all vocalizing
rats began producing CF 50 kHz USVs within the first two minutes of the novel environment session, only two rats produced calls prior to the administration of the first shock during the footshock session, following which all vocalizing rats produced CF 50 kHz USVs.

Experiment 2b. During novel environment sessions, a significant within-subjects effect, $F(4, 52) = 2.674, p = .042$, indicated that the production of CF 50 kHz USVs generally decreased throughout the session. Rats produced lowest proportion of calls during the 500 s bin ($M = .08, SE = .029$). Relative to the 500 s bin, call production was significantly higher during the 100 ($M = .25, SE = .070$), 200 ($M = .27, SE = .045$), and 300 ($M = .27, SE = .043$) time bins, but not the 400 s time bin ($M = .13, SE = .038, p = .299$).

The majority of subjects in both the Stress and Control groups produced CF 50 kHz USVs during both the novel environment ($ns = 7:10$ and $8:10$) and footshock sessions ($ns = 8:10$ and $10:10$). However, a direct comparison in calling behavior across days could not be conducted because two rats called on only one of the two days. A general effect of experience and group was nonetheless observed. While most vocalizing rats in both the Stress and Control groups began producing CF 50 kHz USVs within the first two minutes of the novel environment session ($ns = 5:7$ and $7:8$), less than half of rats in the Control group that vocalized during the session produced calls prior to the administration of the first shock during the footshock session ($n = 4:10$), whereas the majority of rats in the Stress group ($n = 7:8$) began vocalizing prior to footshock.

Table 1 summarizes the number of subjects in each group that produced CF 50 kHz calls, and whether calls of this type were initially produced prior to or following the first
footshock. There was a significant association between treatment and onset of CF 50 kHz call production, $X^2 (1) = 4.568, p = .033$.

**Discussion**

Previous rat USV research has suggested that the production of narrow bandwidth 50 kHz calls serves as a social signal not obviously associated with emotional state (Burgdorf et al., 2008; Wöhr et al., 2008). Here we explored the relationship between affect and the production of CF 50 kHz USVs by measuring the vocal and overt behavior of rats following pharmacological and contextual manipulations of emotional state. The results showed that CF 50 kHz calls were produced during the exposure to the novel environment, and call production declined with prolonged exposure to the environment in the absence of a stressor. Additionally, when six, temporally consistent footshocks were administered to the animals in the environment, CF 50 kHz USVs were produced during the preshock exposure to the environment and potentiated by the first three footshocks. Animals pre-treated with a low dose of diazepam were less likely to produce 50 kHz USVs during the baseline pre-shock period, and they produced fewer CF 50 kHz calls following the first three footshocks. The type of USV produced by rats changed as the footshocks were repeated. Following 2-4 footshocks, animals switched from producing CF 50 kHz USVs to generating more 22 kHz calls. Interestingly, the overt behavior of the animal also changed systematically with the vocal behavior; during the baseline animals exhibited rearing behavior, and then began to freeze with repeated footshocks. In sum, the data are consistent with the view that female Long Evans rats produce CF 50 kHz calls during mildly aversive situations and 22 kHz calls when experiencing an elevated level of negative affect.
Motoric confounds are a concern for the interpretation of the data from the animals receiving high dose diazepam (5 mg/kg), as they appeared sedated during behavioral testing and USV production was largely abolished. In animals receiving the moderate dose of diazepam, a significant reduction in rearing activity and freezing was observed, and USV production was absent. Only the lowest dose (1 mg/kg) attenuated CF 50 kHz production, but this dose did not affect 22 kHz USV production. Although the selective suppression of 50 kHz, but not 22 kHz calls following a low dose of diazepam has not previously been described using a footshock paradigm, it is consistent with observations of the vocalizations produced under threat of attack from a conspecific (Vivian & Miczek, 1993). In some footshock procedures, a low dose (1 mg/kg) of diazepam is effective in reducing 22 kHz USVs (Naito, Nakamura, Inoue, & Suzuki, 2003; Jelen, et al., 2003). However, the majority of the studies on anxiety- and fear-related calling behavior in rats have focused more on 22 kHz USVs, and a higher drug dose is required to suppress call production, which is frequently at or near levels associated with sedation (for review, Sanchez, 2003). These differences may very well be due to methodological differences as there is substantial variability in the procedures and sample population used across these experiments and in comparison to the current study. Here we observed a selective reduction in CF 50 kHz calling behavior, without a reduction in 22 kHz calls with our lowest drug treatment group in young adult female rats. The differential effects on calling behavior argue against a nonspecific motoric confound, and is more consistent with the view that the call type suppression is associated with the modulation of affective state resulting from diazepam administration.
It should be noted that the observation that 50 kHz USVs are produced during aversive situations is not new (Sales, 1972), but the production of the narrow bandwidth 50 kHz USVs is more frequently observed in social contexts (Burgdorf, et al. 2008). Previous research has shown that USVs in this range are produced by subordinate male and female rats prior to agonistic encounters with conspecifics, and when placed in a context previously paired with such encounters, and novel context exposure (Thomas, et al., 1983; Haney & Miczek, 1993; Wöhr, et al., 2008). The current study extends these findings, by providing support for a link between CF 50 kHz USVs and a negative affective state via pharmacological and environmental manipulations of affective state independent of social context. We observed 50 kHz USV emission from naïve rats when exposed to the chamber, and that the rate of calling decreased over time in the absence of footshock. The interpretation that the novel context induced neophobia in the rats is supported by the increased latency to consume familiar, palatable food. Furthermore, it is unlikely that the presence of a palatable food was the stimulus eliciting 50 kHz USVs, because no differences were observed in the vocal behavior of subjects with or without access to food in the chamber, and we did not observe frequency-modulated USVs that are associated with positive affective state (Burgdorf, et al., 2008; Wiley, et al., 2009; Costa, et al., 2015). Finally, when animals were later returned to the chamber, approximately half of the subjects previously exposed to the context without i.p. injection stress did not produce CF 50 kHz USVs until the first footshock was administered. However, nearly all subjects in the injection stress group, with or without prior exposure to the context, began vocalizing in the two minutes prior to the first footshock. We interpret these findings as evidence of reduced
negative affect induced by the environment until the animals encountered an acutely aversive stimulus. In sum, CF 50 kHz vocalizations, therefore, may signal a low level, negative affective state.

The theory that 22 kHz USVs can be used as a model for anxiety or fear has existed decades (Tonoue, Ashida, Makino, & Hata, 1986; Cuomo, et al., 1988; Sanchez, 2003), and many experiments describe a link between the imminence of threat and changes in vocal behavior. An encounter with a predator will elicit defensive behaviors, such as escape or freezing, along with 22 kHz USVs, but the decreased proximity to the predator will eventually elicit a different set of behaviors, such as audible vocalizations and attack (Blanchard, et al., 1993). Similarly, 22 kHz and audible vocalizations are produced during a bout of conspecific fighting, but that 50 kHz USVs were more common when a previously defeated rat is re-exposed to the dominant rat with the protection of a barrier (Thomas, et al., 1983), or to the context of the aggressive encounter (Tornatzky & Miczek, 1994). Rats also produce 22 kHz USVs following acutely aversive stimuli such as air puff or electric shock, or when exposed to conditioned stimuli that signal the imminence of these stimuli (but see Jelen, et al., 2003). The intensity of these aversive stimuli is related to the rate of 22 kHz calling and time spent engaged in defensive behavior (e.g., freezing). Low predatory imminence and diffuse, anxiogenic cues (e.g., bright light, open spaces) elicit defensive behaviors reflecting risk assessment, and are not associated with 22 kHz USVs. The results of the current study are consistent with this existing literature regarding the transition between different defensive behaviors as certainty of threat increases.
Our data argue that the intensity of negative affective state is an important modulator of call type production. What remains unclear is whether animals are simply increasing their level of anxiety, level of fear, or if animals are transitioning from a state of anxiety to a fearful state. There is variable terminology and definitions for anxiety and fear, and debate continues whether or not anxiety is adaptive (cf. Avery, Clauss, & Blackford, 2016; Blanchard & Blanchard, 2008; Rau & Fanselow, 2007; Sylvers, Lilienfeld & LaPrairie, 2011). In our view, anxiety and fear are adaptive behaviors (but see, Blanchard & Blanchard, 2008), as risk assessment is necessary when animals are exposed to a potentially threatening context and appropriate fear-related behaviors are necessary when threat is certain (Perusini & Fanselow, 2015; Faneslow & Ponnuamy, 2008; Davis, 2006). We suggest that animals exposed to repeated footshocks in this paradigm transition from a state of anxiety to fear because of the pattern of behavioral responses and the pharmacological modulation of the behaviors. 1) During the unsignaled footshock testing, animals initially engage in rearing behavior (risk assessment) and then engage in freezing behavior (learned fear); 2) the temporal consistency of the footshocks may allow the animals to identify a timing cue necessary to generate a fearful response to a certain threat; 3) animals produce two different types of USVs that overlap with rearing and freezing, CF 50 kHz and 22 kHz, respectively; 4) the moderate and lower doses of the anxiolytic drug diazepam selectively attenuated baseline rearing and CF 50 kHz calls, respectively; 5) pre-exposure to the environment without footshock resulted in a reduction in calling behavior when returned to the environment prior to the initial shock administration. In sum, the dissociable behavioral
patterns are consistent with the view that affective state is being modulated, perhaps indicating that animals are transitioning from an anxious to a fearful state.

Rats are one of the most commonly used animals for modeling human emotional disorders, and these models are enriched when USVs are measured because they can be assessed noninvasively, they allow for dynamic assessment of change for correlation with overt behavior, and can be measured in conjunction with a range of validated procedures and techniques. Both FM 50 kHz and 22 kHz USVs are now frequently utilized as indicators of positive and negative affect, respectively, in a variety of experimental paradigms. The USVs produced in the 50 kHz range are quite diverse and can be classified into at least 14 different categories (Wright et al., 2010). Narrow bandwidth and constant frequency 50 kHz USVs are frequently either discounted from analysis entirely, or are combined with other 50 kHz subtypes, perhaps because of the ambiguity regarding their behavioral significance. The current experiments support the hypothesis that CF 50 kHz vocalizations are associated with negative affective state, and may reflect a low intensity negative affective state, for example when animals are engaged in risk assessment or a situation with an uncertain threat. Our results suggest that dissociating the CF 50 kHz call type from other 50 kHz USVs can increase the sensitivity of behavioral measurements for rodent models of emotional disorders. Future work can extend these results to determine whether the low level of negative affective state does indeed correspond to a state of anxiety. Such an additional behavioral correlate of anxiety would improve assays of this complex emotional disorder that is a prevalent concern in our society today.
Chapter 3: Sex-related differences in CF 50 kHz and 22 kHz USVs during negative affective state

Non-human animal models are valuable for the study of human neuropsychiatric disorders and the development of novel therapeutic treatments. The measurement of rat ultrasonic vocalizations (USVs) has become an important component of rodent models that involve affective processes including animal models of affective disorders, because they can be assessed non-invasively and across a variety of behavioral paradigms (Burgdorf, et al., 2009; Burgdorf & Moskal, 2010; Knutson, Burgdorf, & Panksepp, 2002; Miczek, Weerts, Vivian, & Barros, 1995). Sex differences in the prevalence of affective disorders in humans are well documented (Kessler, McGonagle, Swartz, Blazer, & Nelson, 1993; Kessler, Chiu, Demler, Merikangas, & Walters, 2005; Mclean, Asnaani, Litz, & Hofmann, 2011). Sex differences also exist in calling behavior between male and female rats tested in different procedures (Panksepp & Burgdorf, 2003; Blanchard, Agullana, McGee, Wiess, & Blanchard, 1992). Therefore, investigating rodent sex differences in USV calling behavior is an important consideration when developing novel paradigms for assessing emotional information contained within a rat’s vocal repertoire.

Adult rat USVs are broadly categorized based on the peak frequency of the vocalization as either 22 kHz, or 50 kHz calls (Brudzynski, 2007; Portfors, 2007). Twenty-two kilohertz USVs are fairly homogenous in spectral features (Brudzynski, 2007), and because they are reliably produced in response to aversive or threatening stimuli are generally considered to be a signal of negative affective state in the rat (Blanchard,
Blanchard, Agullana, & Weiss, 1991; Brudzynski, 2007; Wöhr, Borta, & Schwarting, 2005; Kim, Kim, Covey, & Kim, 2010). Unlike the 22 kHz category, 50 kHz USVs are heterogeneous and can be subdivided into different call types based on the fundamental frequency, degree of frequency modulation, and behavioral states that elicit these calls (Burgdorf, et al., 2008; Wright, Gourdon, & Clarke, 2010). A substantial body of evidence supports the interpretation that frequency modulated (FM) 50 kHz USVs signal a positive affective state in the rat (Burgdorf, et al., 2008; Wiley, Varlinskaya, & Spear, 2009; Costa, Morelli, & Simola, 2015). Although it has been posited that other types of 50 kHz USVs may serve a pro-social role in rats (Wöhr et al., 2008; Shetty & Sadananda, 2017), a link between other specific subtypes and internal state has only recently been described (Taylor, Urbano, & Cooper, 2017).

Unlike FM 50 kHz USVs, 50 kHz USVs consisting of a constant frequency (CF) or modulated within a narrow frequency bandwidth are not associated with positive affective state (Burgdorf, et al., 2008). CF 50 kHz USVs are observed in a variety of social and non-social contexts including exposure to novel environments (Wöhr, Houx, Schwarting, & Spruijt, 2008; Shetty & Sadananda, 2017), mating (Burgdorf, et al., 2008; Barfield & Thomas, 1986), and agonistic encounters (Thomas, Takahasi, & Barfield, 1983; Haney & Miczek, 1993). We recently tested the hypothesis that CF 50 kHz USVs may serve as a signal of negative affective state, such as might be elicited by mildly aversive situations, or conditions wherein there is greater uncertainty of threat (Taylor, Urbano, & Cooper, 2017). In this study, female Long Evans rats experienced six temporally consistent, mildly aversive footshocks within a single test session with or without being previously exposed to the
testing environment. During footshock sessions, subjects exhibited a pattern of USV production in which a high rate of CF 50 kHz USVs were produced prior to, and especially following the first footshock; the production of CF 50 kHz USVs declined after repeated footshocks concomitant with an increase in the rate of 22 kHz USVs. Furthermore, CF 50 kHz USVs and other anxiety-related behavior were found to be attenuated by previous exposure to the context or by systemic administration of diazepam (Taylor, et al., 2017).

Sex differences in USV production has been reported in various behavioral paradigms. Adult female rats produce more 50 kHz USVs than male rats during heterospecific (i.e., “tickling”) play with human experimenters, although the opposite is true of adolescent rats (Panksepp & Burgdorf, 2003). Female rats also produce 22 kHz USVs at a greater rate than male rats following exposure to a predator (Blanchard, Agullana, McGee, Wiess, & Blanchard, 1992a). During the acquisition phase of tone- or contextual fear conditioning, Graham, Yoon, Lee, & Kim (2009) reported an interaction between sex and strain, with female Long Evans rats spending more time producing 22 kHz USVs than male Long Evans rats, but an opposite trend was observed in Sprague Dawley rats (Graham, Yoon, Lee, & Kim, 2009; Kosten, Lee, & Kim, 2006). However, sex differences in the production of 50 kHz USVs following footshock has not been reported.

We have yet to compare CF 50 kHz call production between male and female subjects. In the current study, we therefore sought to determine whether there were differences in the calling behavior of male and female rats within the exploration of a novel environment, and throughout a session of increasingly certain threat wherein threat certainty is manipulated by the administration of six temporally consistent mild footshocks (0.5 mA,
As in Taylor, et al. (2017), rearing and freezing behaviors were also assessed to provide secondary measures of risk assessment and perceived certainty of threat. Based on the sex differences previously observed in the vocal and defensive behavior of Long Evans rats in response to uncertain and certain threat (Blanchard, Shepard, Carobrez, & Blanchard, 1991; Blanchard, Blanchard, Agullana, & Weiss, 1991; Blanchard, et al., 1992, Graham, et al., 2009), we predicted that in comparison to male subjects, female rats would engage in elevated rearing behavior and CF 50 kHz USV production during novel context exposure and during initial footshocks. Furthermore, we predicted that female rats would produce more 22 kHz USVs earlier in the footshock session and at overall higher levels than male rats, but that differences in freezing behavior would not be observed (cf. Gupta, Sen, Diepenhorst, Rudick, & Maren, 2001).

Methods

Subjects

Twenty-four male and 24 female Long Evans rats that were 60 days old at the time of testing were used as subjects (Harlan Laboratories, IN). Rats were housed as described in Chapter 2. All subjects were individually housed for one week prior to testing, and then randomly assigned to novel environment (n = 12 male, 12 female) or unsignaled footshock condition (n = 12 male, 12 female). While individually housed, a 15 cm segment of PVC pipe was added to each cage for enrichment. Animals were handled for 5 min per day for three days preceding the experiment. All research was conducted with approval from an Institutional Animal Care and Use Committee (#14/02).

Apparatus and Materials
All behavioral testing occurred in the testing context described in Chapter 2.

**Procedure**

**Footshock sessions.** Footshock sessions were conducted exactly as described in Chapter 2.

**Novel environment sessions.** Subjects were placed in the test chamber and allowed to explore the test chamber for 9 min in the absence of any discrete stimulus, and then returned to the home cage. The test chamber was thoroughly cleaned between subjects and allowed to dry, and bedding beneath the steel rod floor was also changed between subjects.

**Data Analysis**

**Rearing analysis.** Rearing activity was assessed for both the novel environment and footshock sessions. Rearing was defined as a vertical body movement resulting with only the rear paws in contact with the floor of the chamber. Excluded from this count were instances wherein rearing immediately preceded jumping or climbing, as well as grooming behavior. A count of rearing activity was made per 20 s bin during the first 2 min of the session by two independent observers; both experimenters were blind to the treatment conditions. Inter-observer agreement was > 93%.

**Freezing analysis.** Freezing was defined as a lack of all non-respiratory movement, and was assessed every 8 s during the session by two independent observers blind to experimental condition. Inter-observer agreement was 94%. No freezing was observed during the novel environment. These observations were converted to percent time freezing (number of freezing observations/number of observations * 100) for the initial 2 min pre-shock period and subsequent inter-shock-intervals.
**USV classification.** USV recording and classification was conducted exactly as described in Chapter 2.

**50 kHz subtype distribution.** The distribution of 50 kHz USV subtypes was calculated by dividing the number of calls categorized within each subtype by the total number of 50 kHz USVs produced in the session.

**CF 50 kHz USV production.** The rate of calling during the two min preshock period and subsequent inter-shock intervals was calculated by dividing the number of calls per time bin by the number of seconds in each bin. For clarity of comparison, the same time bins were used in the novel environment sessions although no discrete stimulus was presented.

**22 kHz USV production.** The rate of 22 kHz USV production was calculated as described for CF 50 kHz calls. No rats in the novel environment condition produced 22 kHz USVs, so the 22 kHz USVs analysis was performed only for the unsignaled footshock session.

**Statistical Analyses.** Acoustic and overt behavioral data were collected during the entire session for both conditions. For clarity of comparison, dependent variables were identically binned by time in both conditions to correspond to events of the footshock condition: initial 2 min, five ~ 60 s ISI time bins, and final 2 min. The rate of rearing activity during the initial 2 min was compared between groups in each condition using independent samples t-test. The rate of CF 50 kHz and 22 kHz USVs, and the percent time freezing were compared between male and female rats throughout the session using mixed-model repeated measures ANOVA. No 22 kHz USVs or freezing were observed prior to the first shock. Therefore, the pre-shock time period was excluded from ANOVA for both
variables. When the data were heteroscedastic, a log(10) transformation was conducted on the data prior to the analysis. All statistical analyses were performed using IBM SPSS Statistics (Armonk, New York, version 22.0).

Results

Novel Environment – Rearing

There was a marginally significant between-sex difference in rearing behavior during the initial 2 min of the novel environment session, t(22) = 1.960, p = .06. Male rats (M = 0.083, SE = 0.007) tended to engage in a higher rate of rearing than female rats (M = 0.065, SE = 0.006).

Novel Environment – Ultrasonic Vocalizations

50 kHz subtype distribution. The majority of male (n = 10:12) and female rats (n = 8:12) produced 50 kHz USVs during the novel environment sessions. Overall, the

Figure 10. CF 50 kHz USVs during novel environment exposure for male and female rats. The rate of CF 50 kHz USV production decreased across the novel environment session for both male and female rats. No significant differences were indicated for group. Numbered time bins represent the preshock time and succeeding post-shock intervals defined in the footshock sessions.
predominate call subtype was the CF type for both male and female rats (67% and 57%, respectively), followed by FM Other (22% and 34%), and then FM Step calls (11% and 9%).

**CF 50 kHz USVs.** The rate of CF 50 kHz USV production during the novel environment session is shown in Figure 10. A significant within-subjects effect, $F(6, 96) = 5.633, p < .001$, indicated that the rate of CF 50 kHz USVs generally decreased throughout the session. There was not a significant between group effect for sex $F(1, 16) = 3.245, p = .090$, although females ($M = 0.193, SE = 0.057$) tended to call at a higher rate than male rats ($M = 0.096, SE = 0.033$). There was no interaction between time and sex $F(6, 96) = 0.633, p = .703$.

**Footshock – Freezing**

Two subjects, one male and one female, were excluded from freezing analysis due to unusually enhanced freezing behavior (> 2 X SD of the mean) during the first inter-shock

![Figure 11. Freezing during footshock sessions for male and female rats.](image)

Percent time freezing increased following each footshock. There were no differences in freezing between male and female rats. Numbered time bins refer to interval following each footshock.
interval. A significant within-subject effect of time, $F(5, 100) = 76.329, p < .001$, indicated that subjects increased freezing as a result of repeated footshocks (Fig 11). No between-group sex differences were found for freezing behavior $F(1, 20) = 0.909, p = .352$. Similarly, no interaction between sex and time was found, $F(5, 100) = 1.813, p = .117$.

**Footshock – Rearing**

There was no significant between-sex difference in rearing behavior during the initial

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**Figure 12. CF 50 kHz and 22 kHz USVs during footshock session for male and female rats.** CF 50 kHz call production decreases as 22 kHz call production increases. Each dot in the scatterplot is a call produced by an individual subjects during the testing session, the top plot combines all of the animals in each group. To show the individual variation in calling behavior, two exemplars showing the calling behavior in male (left) and female (right) rats.
Footshock – Ultrasonic Vocalizations

Effect of Repeated Footshock on USVs. The temporal variation in the number of CF 50 kHz and 22 kHz USVs produced changed as a function of repeated footshocks. Specifically, the rate of CF 50 kHz calls decreased as the rate of 22 kHz call production increased (Fig. 12).

The majority of male (n = 11:12) and female rats (n = 9:12) produced 50 kHz USVs during the novel environment sessions. Overall, the predominate call subtype was the CF type for both male and female rats (53% and 55%, respectively), followed by FM Other...
The rate of CF 50 kHz USV production during the footshock session is shown in Figure 14. During footshock sessions, a significant within-subjects effect of time, $F(6, 108) = 14.361, p < .001$, indicated that the rate of CF 50 kHz USVs decreased throughout the session. A significant main effect of sex, $F(1, 18) = 4.643, p = .045$, indicated (33% and 38%), and then FM Step calls (14% and 7%) (Fig. 13).

**CF 50 kHz USVs.** The rate of CF 50 kHz USV production during the footshock session is shown in Figure 14. During footshock sessions, a significant within-subjects effect of time, $F(6, 108) = 14.361, p < .001$, indicated that the rate of CF 50 kHz USVs decreased throughout the session. A significant main effect of sex, $F(1, 18) = 4.643, p = .045$, indicated.

**Figure 14.** CF 50 kHz USVs during footshock sessions for male and female rats. The rate of CF 50 kHz USV production decreased throughout the footshock session for both male and female rats. Females engaged in an overall higher rate of CF 50 kHz calling than male rats. * indicates $p < .05$ for between-subjects effect.

The rate of 22 kHz USV production increased throughout the footshock session for both male and female rats. Males engaged in an overall higher rate of 22 kHz calling than female rats. * indicates $p < .05$ for between-subjects effect.

**Figure 15.** 22 kHz USVs during footshock session for male and female rats. The rate of 22 kHz USV production increased throughout the footshock session for both male and female rats. Males engaged in an overall higher rate of 22 kHz calling than female rats. * indicates $p < .05$ for between-subjects effect.
that females \((M = 0.125, SE = 0.031)\) called at a higher rate than male rats \((M = 0.069, SE = 0.036)\). There was no interaction between time and sex \(F(6, 108) = 0.385, p = .887\).

22 kHz USVs. Both male \((n = 5:12)\) and female \((n = 9:12)\) rats produced 22 kHz USVs during footshock session. A Chi-square test indicated no difference in the proportion of subjects producing 22 kHz calls by sex \(X^2 (1) = 2.743, p = .098\). Significant within-group differences across time showed that there was an increase in 22 kHz USV production as a result of repeated footshock, \(F(6, 72) = 18.407, p < .001\) (Fig. 15). A significant between-group effect for sex, \(F(1, 12) = 5.243, p = .041\), indicated that male rats produced 22 kHz USVs at a higher overall rate than female rats. There was no significant interaction between sex and time, \(F(6, 72) = 1.607, p = .158\).

Discussion

We evaluated the USV production, rearing, and freezing behavior of male and female Long Evans rats during exploration of a novel environment and a series of unsignaled footshocks. No sex differences were observed in rearing behavior in either the novel environment or footshock sessions. Male and female rats exhibited a gradual decrease in rearing across time in the 9 min novel environment session. During the unsignaled footshock session, rearing rapidly decreased and freezing increased with repeated footshock administration. Consistent with previous work in our lab (Taylor, et al., 2017), we found that the CF subtype was the most frequently produced type of 50 kHz in both the novel environment session and the baseline unsignaled footshock testing. During unsignaled footshock testing, the rate of CF 50 kHz USVs decreased across the session, but CF 50 kHz USVs were still emitted by rats following the initial footshock presentations. Furthermore,
during the footshock session the decline in CF 50 kHz USV production occurred as 22 kHz call production increased. Female rats generally produced more 50 kHz USVs than males regardless of context, whereas male rats produced more 22 kHz USVs during footshock sessions. The similar pattern of results between male and female rats and suggests that the association between type of USV and affective state is similar between the sexes. It is also consistent with the hypothesis that both CF 50 kHz and 22 kHz USVs may signal the intensity of the negative affective state.

Contrary to our expectations, male rats produced more 22 kHz USVs than female rats during the administration of a six unsignaled footshocks. Previous work using similar methods have shown that females engage in higher levels of 22 kHz USV production than male rats when presented with a threatening stimulus (Graham et al., 2009; Blanchard, et al. 1992a). Although the presence of sex-related behavioral differences during the acquisition of conditioned fear varies by strain, female rats tend to express less freezing than male rats (Pryce, Lehmann, & Feldon, 1999; Kosten, Lee, Kim, 2006; Gupta, et al., 2001). In a direct comparison of sex and strain differences during fear conditioning, Graham et al. (2009) found that during the acquisition phase of tone-shock conditioning 22 kHz call production was higher in female than in male Long Evans rats, although both sexes exhibited similar freezing. Whereas we found that only 50 kHz call production was increased in female rats, compared to male rats, and that 22 kHz production was elevated in male rats compared to females. The differences in the presentation of a discrete cue (tone), the number and intensity of footshocks are variables that may account for the differences in calling behavior observed in this study compared to the findings observed by Graham et al. (2009). The
production of 22 kHz USVs is often associated with other behavioral indices of fear, such as escape from predator or freezing (Blanchard, Blanchard, Agullana, & Weiss 1991, Wöhr, Borta, & Schwarting, 2005, Kim, Kim, Covey, & Kim, 2012). Therefore, although our findings are inconsistent with previous reports of sex differences in the 22 kHz USV calling behavior among Long Evans rats, the results are consistent with an interpretation of enhanced expression of negative affective state in male as compared to female rats within the footshock session.

Both male and female rats produced 22 kHz and 50 kHz USVs in the footshock sessions, and sex-related differences were observed in the calling behavior as footshocks were repeated. Because there were no parallel sex-related differences in rearing or freezing it is tempting to speculate whether the combined measurement of CF 50 kHz and 22 kHz USVs provide a more sensitive or nuanced phenotypic marker of negative affective state. Differences between male and female rats in terms of emotional response to aversive stimuli are well documented (Blanchard et al., 1992a; Kosten et al., 2006; Toufexis, 2007; Dalla & Shors, 2008; Graham et al., 2009). This is true of both conditioned and unconditioned responses. Compared to males, female rats often exhibit enhanced anxiety-related behavior, especially within contexts of uncertain or less imminent threat, although in some cases this has been attributed to differences in activity level (Dalla & Shors, 2008; Simpson, Ryan, Curley, Mulcaire, & Kelly, 2012; cf. Toufexis, 2007). Female rats did produce more CF 50 kHz USVs during the footshock session, which we have previously argued serve as an indicator of a low level of negative affective state (e.g., situations with a uncertainty relating to threat). However, male rats produced more 22 kHz USVs, which are well-established as a
response to actual or imminent threat. If defensive behaviors increase along a single dimension as certainty of threat increases we would expect that a measure with more sensitivity than freezing would show elevated levels throughout the footshock session (i.e. that females would engage in more CF 50 kHz and 22 kHz calling than males). As we have argued elsewhere (Taylor, et al., 2017), these two types of USVs may be associated with different behavioral states: CF 50 kHz may be associated with risk assessment behaviors (e.g., rearing) when threat (imminence or identity) is less certain, whereas 22 kHz USVs may be more associated with defensive behaviors relating to greater imminence of threat (e.g., freezing). Even if female rats persisted in CF 50 kHz because of higher level of risk-assessment activity in response to the initial mild footshocks, it is surprising that they would not quickly shift to 22 kHz USV production that equaled or exceeded that of male rats because female rats are generally more sensitive to footshock stimulation. Such dissociation could be explored in the future by varying the number, predictability, and intensity of footshocks.

The imminence of threat can elicit dissociable defensive behavioral responses which are largely supported by divergent neural systems (Davis & Shi, 2006; Davis, Walker, Miles, & Grillon, 2010; Perusini & Fanselow, 2015). Fear-related responses to imminent threat, or cues conditioned as signals of imminent threat rely upon the well-established circuit within the amygdala resolving in projections from the central nucleus of the amygdala (CeA) to targets in the brainstem. In contrast, anxiety-like responses to less certain threat or diffuse cues are modulated by activity in the bed nucleus of the stria terminalis (BNST). The BNST is sexually dimorphic in both humans and rats. The BNST differs between sexes not only
anatomically, but in terms of responsiveness to and modulation by circulating hormones such as testosterone and progesterone (del Abril, Segovia, & Guillamón, 1986; Lebron-Milad & Milad, 2012; Lebow & Chen, 2016). Numerous studies investigating the changes in anxiety- and fear-related behavior across estrous-cycle and during lactation supports the BNST rather than the CeA as driving many of the observed differences in emotional behavior between male and female rats. For example, Toufexis (2007) reported that among overiectomized rats, progesterone reduces the CRF-enhanced acoustic startle response, a procedure known to depend upon BNST activation; but not fear-potentiated startle, which is mediated by the CeA (Lee & Davis, 1997a,b). Light-enhanced acoustic startle, like the enhancement resulting from CRF administration, likewise depends on the BNST and not the CeA (Walker & Davis, 1997). Male rats exhibit greater light-enhanced startle responses than female rats, but this increased response is related to the level of testosterone (Toufexis, 2007). The neural correlates of CF 50 kHz USVs have not previously been explored. Because of the sex-related differences in CF 50 kHz observed in the current study occurred in situations of lower certainty of threat, the BNST may be involved in controlling the production of this type of vocalization.

The assessment of rat USVs can significantly enrich animal models of neuropsychiatric disorders by providing a dynamic and non-invasive measure of emotional state. CF 50 kHz USVs are produced in association with negative affective state, especially when threat certainty or imminence is low (Taylor et al., 2017). In the current study, female rats were found to produce more CF 50 kHz USVs, but fewer 22 kHz USVs than male rats across a procedure of increasing imminence of threat. The difference in calling behavior
observed in this paradigm suggests a difference in intensity or the transition between emotional states in male and female rats, and therefore reinforces the importance of considering the sex of subjects used in animal models of emotion. The analysis of the CF subtype of 50 kHz USVs may therefore serve as a valuable tool for exploring sex-related differences in emotion and modeling affective disorders.
Chapter 4. Corticotropin-releasing factor and α-helical CRF modulate 50 kHz and 22 kHz USVs and anxiety in rats

The measurement of rat ultrasonic vocalizations (USVs) has become an important component of rodent models that involve affective processes including animal models of affective disorders because they can be assessed non-invasively and across a variety of behavioral paradigms (Burgdorf, et al., 2009; Burgdorf & Moskal, 2010; Knutson, Burgdorf, & Panksepp, 2002; Miczek, Weerts, Vivian, & Barros, 1995). The USVs of rodents are quite diverse. Spectrographic analysis of rat USVs suggests that as many as fifteen distinct types of vocalizations can be classified based on various acoustic features (Wright, et al., 2010). While some types of USVs have been well studied, others have received less attention. Further research regarding less understood types of USVs may provide a more nuanced view of affective and motivational states in rodents.

Rodents produce USVs within a wide range of natural and experimental settings, and some of the USVs produced by rats are associated with emotional states. USVs are commonly classified and described based on mean peak frequency and patterns of frequency modulation (Knutson, Burgdorf, & Panksepp, 2002; Wright, et al., 2010). Only two types of USVs are widely accepted as a signal of affective state: 22 kHz USVs and frequency modulated (FM) 50 kHz USVs (Schwarting, Jegen, & Wöhr, 2005; Portfors, 2007; Burgdorf, et al., 2008). Twenty-two kilohertz USVs are produced by rats experiencing negative affective state, and particularly when an acutely aversive stimulus is encountered or imminent including following electric shock (Van der Poel & Miczek, 1991), during
conspecific fighting (Thomas, Takahasi, & Barfield, 1983; Vivian & Miczek, 1992),
presence of a predator (Blanchard, Blanchard, Agullana, & Weiss, 1991), and aversive brain
stimulation (Brudzynski, 2007). These expressions of negative emotional state are also
elicited by conditioned stimuli such as auditory cues or context cues that are associated with
such aversive stimuli (Wöhr, Borta, & Schwarting, 2005; Kim, Kim, Covey, & Kim). The
spectral characteristics of 22 kHz USVs are fairly stereotyped, exhibiting a constant
frequency or linear frequency modulation within a narrow bandwidth (Brudzynski, 2007). In
contrast with 22 kHz calls, the acoustic features of vocalizations within the 50 kHz category
are quite variable, and can be used to describe or identify many different subtypes (Burgdorf,
et al., 2008; Wright, Gourdon, Clarke, 2010). A preponderance of evidence supports FM 50
kHz type – which is classified via wide frequency bandwidth and rapid oscillations in
frequency – as a signal of a positive affective state (Burgdorf, et al., 2008; Costa, Morelli, &
Simola, 2015; Willey, Varlinskaya, & Spear, 2009). Although it has been posited that other
types of 50 kHz USVs may serve a pro-social role in rats (Wöhr et al., 2008; Shetty &
Sadananda, 2017), a link between other specific subtypes and internal state has only recently
been described (Taylor, Urbano, & Cooper, 2017).

Constant frequency (CF) and other narrow bandwidth 50 kHz USVs have been
observed in a variety of behavioral paradigms, and unlike FM 50 kHz USVs are not
associated with positive affect. Many of the situations in which rats produce USVs of this
type can be interpreted as mildly aversive for the rat, or are associated with uncertainty of
threat, such as when exploring a novel environment (Wöhr et al., 2008; Shetty & Sadananda,
2017; Taylor, Urbano, & Cooper, 2017), prior to conspecific attack (Thomas, Takahashi,&
Barfield, 1983), and following unexpected, short duration, mild footshock (Taylor, Urbano, & Cooper, 2017). We recently tested the hypothesis that CF 50 kHz USVs may serve as a signal of negative affective state, such as might be elicited by mildly aversive situations, or conditions wherein there is greater uncertainty of threat (Taylor, Urbano, & Cooper, 2017). In this study, rats experienced six temporally consistent, mildly aversive footshocks within a single test session. Subjects exhibited a pattern of USV production in which a high rate of CF 50 kHz USVs were produced prior to, and especially following the first footshock; the production of CF 50 kHz USVs declined after repeated footshocks during the period of time during which the rate of 22 kHz USVs increased. Furthermore, in an additional experiment, the rate of CF 50 kHz USVs during the baseline exploration period prior to the first footshock was attenuated by pre-exposure to the context on the preceding test day. Last, CF 50 kHz USV production and risk-assessment behavior was reduced when subjects were pretreated with a 1 mg/kg dose of the anxiolytic diazepam (Taylor, et al., 2017). Based on these findings, we have suggested that measurement of this call type may therefore provide greater sensitivity of negative affect along a continuum of increasingly intensity and/or certainty of threat.

Pharmacological agents are an important tool used to determine whether a behavior is related to emotional state (Lister, 1990; Pellow et al., 1985; Ramos, 2008) and have been valuable in understanding the association between FM 50 kHz and 22 kHz USVs. FM 50 kHz USVs are elicited or increased following the administration of drugs of abuse including cocaine and amphetamine (Ahrens, Ma, Maier, Duvauchelle, & Schallert, 2009; Maiher, Ahrens, Ma, Schallert, & Duvauchelle, 2010). In contrast, 22 kHz USVs can be attenuated
following treatment with anxiolytics (Vivian & Miczek, 1992; Jelen, Soltysik, & Zagrodzka, 2003; Naito, Nakamura, Inoue, & Suzuki, 2003), and are enhanced by anxiogenic drugs such as pentylenetetrazole (Jelen, Soltysik, Zagrodzka, 2003) and corticotropin-releasing factor (CRF; Swiergiel, Zhou, & Dunn, 2007). The experimental modulation of the CRF-system via intracerebroventricular (ICV) microinfusion of CRF or CRF receptor antagonists results in bi-directional modulations in defensive behaviors such as freezing, acoustic startle response, and the emission of 22 kHz USVs (Swerdlow, Geyer, Vale, & Koob, 1986; Dunn & Berridge, 1990; Liang, Melia, Miserendion, Falls, Campeau, & Davis, 1992). These drug-related changes are less likely to be confounded by general motoric effects associated with peripherally administered anxiolytics (e.g., benzodiazepines) and anxiogenics. The effect of ICV CRF and CRF antagonists on non-appetitive 50 kHz USVs has not previously been explicitly explored. We therefore used CRF and α-helical CRF, a nonselective CRF receptor antagonist, to further explore our hypothesis regarding the association between CF 50 kHz USVs and negative affective state within our single session, temporally consistent, mild footshock paradigm.

Two experiments were conducted to evaluate the effect of ICV infusion of CRF and α-helical CRF on the production of CF 50 kHz and 22 kHz USVs while the intensity of a rat’s negative affective state was modulated via repeated mild footshock. In Experiment 1, rats received ICV infusions of CRF or vehicle 30 min prior to behavioral testing in a single session of unsignaled, temporally-consistent mild footshocks (Taylor, Urbano, & Cooper, 2017). We predicted that relative to control infusions, CRF would increase the rate of CF 50 kHz USV production early in the footshock session and the rate of 22 kHz USVs later in the
session as footshocks were repeated. We expected that the CRF-facilitated increase in calling behavior would be paralleled by an increase in rearing behavior prior to the first footshock, and enhanced freezing later in the session. In Experiment 2, α-helical CRF infusions were used to oppositely modulate defensive behaviors and further investigate the role of CRF within this paradigm. We predicted that α-helical CRF pretreatment would result in effects opposite to those observed with CRF administration: a decrease in CF 50 kHz and 22 kHz USV production, as well as decreased rearing and freezing behavior. In both experiments, all subjects were subsequently assessed in the same drug condition on the elevated plus maze (EPM) as a secondary verification of the drug treatment as an anxiogenic or anxiolytic.

Methods

Subjects

A total of (57) female Long Evans rats (Harlan Laboratories, IN) that were 65-70 days old at the time of surgery were used as subjects ($N = 27$ Experiment 1, and $N = 30$ Experiment 2). Animals were housed as described in Chapter 2. All subjects were individually housed for one week between surgery and testing. All research was conducted with approval from an Institutional Animal Care and Use Committee (#14/02).

Stereotaxic Surgery

All rats were implanted with unilateral guide cannula for drug microinfusion. Subjects were anesthetized with 4% (2-4 L/min vaporized in medical air) isoflurane during induction, and then maintained under anesthesia with 1.5 – 2.5% isoflurane during surgery. The scalp was shaved and cleaned with betadine and the rat was positioned with skull flat in the stereotaxic instrument (Leica Biosystems, Germany). The skull was exposed via an incision
along the midline of the scalp and then burr holes were drilled for cannula and anchor screws. A custom made guide cannula (23 gauge stainless steel tubing, 14 mm) was inserted 1 mm above the lateral ventricle on the left or right side of the brain (0.8 mm posterior to bregma, 1.4 mm lateral to the midline, and 3 mm ventral to dura). The laterality of cannula implant was counterbalanced within groups. The craniotomy was filled with vacuum grease, and the guide cannula was then fixed to skull and anchor screws with dental acrylic. Guide cannulas were occluded with custom-constructed stainless steel stylets except during microinfusions. After surgery, a single subcutaneous injection of sustained release buprenorphine (1 mg/kg) was administered for pain relief. All rats were allowed to recover from anesthesia beneath a heat lamp and then given access to a clear liquid gel diet following surgery. For seven days following surgery, the occluder was removed and reinserted to habituate the animal to the microinfusion procedure, and twice daily visual checks were performed to inspect healing around the implant and animal health.

**Microinfusion procedure**

Microinfusions were conducted 30 min prior to behavioral testing with the footshock procedure and EPM testing. The footshock testing and EPM test were spaced 24 hours apart. In Experiment 1, subjects received either CRF (1 µg/5 µl; Sigma; n=15), or the vehicle, artificial CSF (5 µl; n=12). In Experiment 2, subjects received either α-helical CRF (25 µg/5 µl; Sigma; n=15), the vehicle, artificial CSF (5 µl; n=15). Infusions were be made using a custom made injection cannula (30 gauge stainless steel tubing) that terminated 1 mm beyond the guide cannula when inserted. The injection cannula was connected to a 100 µl Hamilton syringe mounted on an infusion pump (KD Scientific, Holliston, MA). Infusions
were made at a rate of 2.5 µl/min, and the injection cannula was removed one minute after infusion was complete.

**Apparatus and Materials**

**Footshock Apparatus.** Footshock sessions were conducted in the same testing environment and with same equipment described in Chapter 2.

**Elevated Plus Maze.** The apparatus and setup for the elevated plus maze testing was as described in Taylor (2013). The elevated plus maze was composed of four arms (40 x 10 cm) radiating from a central platform (10 x 10 cm) elevated 50 cm above the ground by a central pedestal. Two opposite arms had 40 cm walls to create the closed arms, and the two open arms had only a 0.5 cm curb along the edge. The maze was dark grey and situated in a small partitioned space (curtains 90 cm from arms) in an isolated testing room. Diffuse, low level illumination was provided by a single 45 W bulb located directly above the center of the apparatus at ceiling height. To record USVs, an UltraSoundGate Condenser Microphone (CM 16; Avisoft Bioacoustics, Berlin, DE) was suspended from the ceiling 42 cm directly above the central platform. Sessions were video recorded by a webcam (Logitech, Fremont, CA) for behavioral analysis.

**Footshock Session**

**Procedure.** Footshock sessions were conducted 30 min after microinfusion. The footshock procedure and data collection was identical in both Experiment 1 and 2, and was conducted as described in Chapter 2.
**Rearing and Freezing Assessment.** Rearing and freezing behavior were coded from video recordings by two independent observers blind to experimental condition. Inter-observer agreement exceeded 93% for both measures. Rearing activity was measured in successive 20 s bins, and was defined as a vertical body movement resulting with only the rear paws were in contact with the floor of the chamber. Excluded from this count were instances wherein rearing immediately preceded jumping or climbing. Freezing was measured in successive 8 s bins and was defined as a lack of all non-respiratory movement for at least 4 continuous s during the bin. These observations were then converted to percent time freezing (Number of Freezing Observations/Number of Total Observations X 100) for the initial 2 min pre-shock period and subsequent inter-shock-intervals. Rearing and freezing data were analyzed as described in “Statistical Analyses” section below.

**USV classification.** The recording and classification of USVs was conducted as described in Chapter 2.

The number of calls within each classification was used to determine calling rate per second (Number of Calls/Seconds in Time Bin) within the pre-shock period and subsequent inter-shock-intervals. The rate of CF 50 kHz and 22 kHz USV production, and distribution of 50 kHz USV subtype overtime was then compared as described in the “Statistical Analyses” section below.

**Statistical Analyses.** Acoustic and overt behavioral data were collected throughout the entire session. Dependent variables were binned by time: initial 2 min, five ~ 60 s ISI time bins, and final 2 min. The rate of rearing activity during the initial 2 min was compared between groups in each condition using independent samples t-test. The rate of CF 50 kHz
and 22 kHz USVs, and the percent time freezing were compared between drug conditions throughout the session using mixed-model repeated measures ANOVA. No 22 kHz USVs or freezing were observed prior to the first shock, and therefore the pre-shock time period was excluded from the ANOVA for both dependent variables. Additionally, the number of calls classified as each subtype within the 50 kHz category was determined preceding and following the first footshock to investigate changes in the call type distribution as a result of the acutely aversive stimulus using 2 X 3 Chi-square tests. All statistical analyses were performed using IBM SPSS Statistics (Armonk, New York, version 22.0).

Elevated Plus Maze

Procedure. The EPM procedure was identical for Experiment 1 and 2. Thirty minutes after drug or control microinfusion, rats were placed on the center of the EPM oriented toward one of the open arms. Subjects were observed for 5 min on the EPM, and then returned to the homecage. The apparatus was thoroughly cleaned and allowed to dry between subjects. Video and ultrasonic audio recordings were collected throughout the entire session.

Movement-Related Behaviors. The procedure for behavioral data collection for Experiment 1 and Experiment 2 varied as a result of available resources. In Experiment 1, the number of entries for each rat into closed or open arms, as well as the time spent within the open arms by two independent observers blind to experimental condition. These observations were quantified as the percent open arm entries (Number of Open Arm Entries/Total Arm Entries X 100), and percent open arm time (Time in the Open Arms/Total Session Time X 100). In Experiment 2, videos files were exported for analysis using Noldus
Ethovision XT 11.5 software (Noldus, Wageningen, NL). This software was used to determine the percent open arm entries, percent open arm time, and mean velocity. The dependent variables on the EPM for both experiments were conducted as described in the “Statistical Analyses” section below.

**USV Production.** Ultrasound recording and analysis were conducted as described in the footshock session. However, USV production was rare: no 22 kHz USVs were observed, and few rats produced 50 kHz USVs. Because of the paucity of vocal data during EPM testing, USVs were not analyzed.

**Statistical Analyses.** For Experiment 1, between group comparisons for percent open arm entries and percent open arm time were conducted using one-tailed independent samples t-tests. For Experiment 2, between group comparisons were likewise conducted using one-tailed independent samples t-tests for percent open arm entries and percent open arm time, but also for mean velocity of movement. All statistical analyses were performed using IBM SPSS Statistics (Armonk, New York, version 22.0).

**Histology**

At the conclusion of the experiment, animals were euthanized via carbon monoxide induction and the placement of the cannula was verified by injecting water-resistant blue ink into the guide cannula. The criteria for correct placement of cannula depended on the presence of the ink in both lateral ventricles and the aqueduct of sylvius (Liang, et al., 1992b).
Results

Experiment 1

Histology. Accurate cannula placement via infusion of dye in the ventricles was verified by two independent observers for all subjects in Experiment 1.

Footshock Sessions.

Rearing Behavior. A video recording error resulted in the inability to collect rearing or freezing data for one subject in the CRF group during the pre-shock period. Figure 16 displays number of times subjects engaged in rearing during the pre-shock period. Contrary to our prediction, CRF administration resulted in significantly less rearing than vehicle, $t(25) = 1.570, p < .05$.

Freezing Behavior. The percent time freezing across the footshock session is shown

Figure 16. Effect of CRF on rearing behavior. Rearing prior to footshock was suppressed by CRF. Bars show mean and SE, * indicates $p < .05$.

Figure 17. Effect of CRF on freezing behavior. CRF did not significantly affect freezing.
in Figure 17. Animals exhibited significantly increased freezing across the session, $F(5, 125) = 109.910, p < .001$. No differences in this trend were observed as a factor of group, nor were there overall differences in freezing between groups.

**USV Production.** Figure 18 displays all USVs produced by subjects in both groups. Fifty kHz USVs are most common early in the session, and a transition to 22 kHz USV production is evident.

**50 kHz USVs.** The majority of subjects in both the CRF ($N = 9:15$) and control ($N = 12:12$) groups produced 50 kHz USVs. The distribution of 50 kHz call types was not affected by CRF administration, so data were collapsed across groups to investigate changes in call subtype following footshock. Overall, the predominate 50 kHz USV subtype were CF 50 kHz USVs. A 2 X 3 Chi-Square test indicated a significant association between pre- and

![Figure 18. Scatterplot showing CF 50 kHz and 22 kHz USVs produced by ACSF and CRF groups. Each dot represents one call produced by the rats during the test session. CF 50 kHz USVs decrease as 22 kHz USVs increase. Dotted vertical lines indicate the times of footshock administration. The grey box designates calls in the 22 kHz category.](image-url)
post-shock bin, and 50 kHz subtype, $X^2 (2) = 7.931, p < .05$ (Fig 19).

**CF 50 kHz USV.** The rate of CF 50 kHz USV production across the session is shown in Figure 20. The CF 50 kHz calling rate significantly changed across the session, $F(6, 114) = 19.759, p < .001$. This change in rate over time significantly varied as a factor of group, $F(6, 114) = 3.299, p < .01$, but no overall effect was indicated for group. Additional pairwise comparison of time bin X group revealed that a marginally significant difference ($p = .06$) in calling rate occurred between the first and second shock.

**Figure 19. Distribution of 50 kHz USV subtypes.** The majority were of the CF 50 kHz subtype. A transient increase in all 50 kHz calls occurred after the first footshock. The distribution of call types was also affected by footshock, with a greater proportion CF 50 kHz USVs.

**Figure 20. Effect of CRF on CF 50 kHz USVs.** CF 50 kHz USVs increased following the first footshock, and this pattern of calling was enhanced by CRF administration. $\# p < .06$
The rate of 22 kHz USVs increased throughout the session and were enhanced by CRF. * indicates $p < .05$.

22 kHz USVs. The rate of 22 kHz USV production across the session is shown in Figure 21. Five subjects in each group produced 22 kHz USVs during the session. The 22 kHz calling rate significantly increased across the session, $F(5, 40) = 7.762, p < .001$, and was enhanced by CRF, $F(1, 8) = 7.430, p < .05$. The within session change in rate over time significantly varied as a factor of group, $F(5, 70) = 2.238, p = .069$, and additional pairwise comparison of time bin X group revealed that the groups differed significantly following shocks three ($p < .05$), five ($p < .01$) and six ($p < .05$).

Elevated Plus Maze.

Open Arm Time and Entries. Figure 22 displays the percent time spent in, and entries into the open arm of the EPM.

CRF significantly decreased time spent in the open arms of the EPM, but not the percent of entries into the open arms. * indicates $p < .05$. 

Figure 21. The Effect of CRF on 22 kHz USVs. The rate of 22 kHz USVs increased throughout the session and were enhanced by CRF. * indicates $p < .05$.

Figure 22. Effect of CRF on EPM behavior. CRF significantly decreased time spent in the open arms of the EPM, but not the percent of entries into the open arms. * indicates $p < .05$. 

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Administration of CRF resulted in significantly less time in the open arms, \( t(24) = 1.893, p < .05 \), but not the percent of total entries into the open arms.

**Experiment 2**

**Histology.** Cannula implants were verified via ink infusion in the ventricular system in all but three subjects (1 vehicle and 2 α-helical CRF). Therefore, these three animals were excluded from all analyses.

**Footshock Sessions.**

**Rearing Behavior.** Figure 23 displays number of times subjects engaged in rearing during the pre-shock period. Administration of α-helical CRF resulted in significantly less...
rearing than vehicle, $t(24) = 1.685, p < .053$.

**Freezing Behavior.** The percent time freezing across the footshock session is shown in Figure 24. Animals exhibited significantly increased freezing across the session, $F(5, 125) = 108.466, p < .001$. The rate of increased time freezing across the session varied as a factor of group, $F(5, 125) = 2.680, p < .05$, but no overall effect was indicated for group. Additional pairwise comparison of time bin X group revealed that significant group differences occurred following shock one ($p < .053$) and shock two ($p < .05$).

**USV Production.** Figure 25 displays all USVs produced by subjects in both groups. Fifty kHz USVs are most common early in the session, and a transition to 22 kHz USV production is evident.

**50 kHz USVs.** The majority of subjects in both the α-helical CRF (12:14) and control

![Figure 25. Scatterplot showing CF 50 kHz and 22 kHz USVs produced by ACSF and ahCRF groups.](image)

Each dot represents one call produced by the rats during the test session. CF 50 kHz USVs decrease as 22 kHz USVs increase. Dotted vertical lines indicate the times of footshock administration. The grey box designates calls in the 22 kHz category.
(12:15) groups produced 50 kHz USVs. The distribution of 50 kHz call types was not affected by α-helical CRF administration, therefore data were collapsed across groups to investigate changes in call subtype following footshock. Overall, the predominate 50 kHz USV subtype were CF 50 kHz USVs. A 2 X 3 Chi-Square test indicated a significant association between pre- and post-shock bin, and 50 kHz subtype, $X^2 (2) = 50.083, p < .001$ (Fig. 26).

**CF 50 kHz USV.** The rate of CF 50 kHz USV production across the session is shown in Figure 27. The CF 50 kHz calling rate significantly changed across the session, $F(6, 132) = 32.285, p < .001$. This change in rate over time

![Figure 26. Distribution of 50 kHz USV subtypes.](image)

![Figure 27. The Effect of CRF on 22 kHz USVs.](image)

The rate of 22 kHz USVs increased throughout the session and were enhanced by CRF. # indicates $p < .07$. 

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significantly varied as a factor of group, $F(6, 132) = 2.597, p < .05$, but no overall effect was indicated for group. Additional pairwise comparison of time bin X group revealed that a marginally significant difference ($p < .071$) in calling rate occurred between the first and second shock.

**22 kHz USVs.** The rate of 22 kHz USV production across the session is shown in Figure 28. The 22 kHz calling rate significantly increased across the session, $F(5, 70) = 11.041, p < .001$.

Figure 28. The Effect of ahCRF on 22 kHz USVs. The rate of 22 kHz USVs increased throughout the session and were enhanced by ahCRF. * indicates $p < .05$.

Figure 29. The Effect of ahCRF on EPM Behavior. a) ahCRF significantly increased time spent in the open arms of the EPM, but not the percent of entries into the open arms. B) ahCRF also increased movement speed. * indicates $p < .05$. 

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attenuated by α-helical CRF, $F(1, 14) = 6.494, p < .05$. The within session change in rate significantly varied as a factor of group, $F(5, 70) = 5.722, p < .001$, and additional pairwise comparison of time bin X group revealed that the groups differed significantly during shock five ($p < .01$) and shock six ($p = .01$).

**Elevated Plus Maze.** A total of three subjects, two from the α-helical CRF group, and one from the vehicle group, fell off of the EPM during exploration and therefore were excluded from analysis. Additionally, placement of the microphone resulted in obscured video recording within the one of the closed arms for two animals in the α-helical CRF group. Therefore, although percent open arm time could be determined for these animals, entry into closed arms and average velocity could not.

**Open Arm Time and Entries.** The percent time spent in open arms and open arm entries is shown in Figure 29A. Although no group differences were found in the percent time spent in the open arms, α-helical CRF administration resulted in increased percent open arm entries out of total arm entries, $t(20) = 2.113, p < .05$.

**Velocity.** Compared to vehicle, α-helical CRF administration resulted in enhanced speed of movement on the EPM, $t(20) = 1.801, p < .05$ (Fig. 29B).

**Discussion**

We have proposed the hypothesis that the production of CF 50 kHz USVs and 22 kHz USVs may serve as signals of different levels of negative affective state along a continuum of increasing intensity or certainty of threat (Taylor, et al., 2017). Here we further explored this hypothesis by observing the effects of ICV administration of CRF and α-helical CRF on USV production within a single session of footshock administration. Subsequent behavioral testing
with the EPM was used to verify the efficacy of drug treatment as an anxiogenic or anxiolytic.

The general pattern of behavior observed during footshock sessions was consistent with that previously reported in Taylor, et al. (2017). Rearing and CF 50 kHz USVs were observed more early in the test session, whereas 22 kHz USVs and freezing behaviors were observed more often as footshocks were repeated during testing. The rate of 50 kHz calling exhibited a transient increase between the first and second shock, and a significant increase in the proportion of the CF subtype of 50 kHz USVs was observed following the first footshock. The central administration of CRF and α-helical CRF oppositely modulated the USV behavior as compared to control infusions in a manner consistent with an increase or decrease, respectively, in intensity of negative affect. The exceptions to this drug effect interpretation were that CRF did not modulate freezing behavior in Experiment 1, and that rearing behavior prior to footshock was attenuated by drug treatment in both experiments. On the EPM, CRF and α-helical CRF oppositely modulated exploratory behavior, although no significant difference was observed in percent time spent in the open arms following α-helical CRF. Finally, no locomotor deficits were observed following α-helical CRF administration.

Contrary to our initial expectations, few subjects in either experiment produced USVs on the EPM. The spontaneous emission of 50 kHz USVS has been previously reported on the EPM (Rao & Sadananda, 2015; Shetty & Sadananda, 2017; c.f. Borta, Wöhr, & Schwarting, 2006). Although previous exposure to behavioral testing can lead to decreased rates of USV production (Wöhr et al., 2008), we were surprised that so few subjects were
observed to produce USVs during the five minute EPM test session. One explanation for these results is that our recording procedure was only able to detect a small sample of vocalizations. Because ultrasound attenuates rapidly with distance, and is a highly directional signal, ultrasonic vocalizations can be difficult to detect in many settings; a fact that has been highlighted as a natural advantage for the vocalizations of a commonly preyed upon animal (Blanchard & Blanchard, 1989; Blanchard, Blanchard, Agullana, & Weiss, 1990; Brudzynski, 2009). While we designed our EPM apparatus such that it was truncated in size as compared with more traditional dimensions, some studies previously reporting USVs within contexts similar to the EPM also utilize more than one microphone positioned horizontally (Wöhr & Schwarting, 2007, 2012), rather than a single microphone positioned vertically as done in the current experiments (but see Wöhr et al., 2008). However, because some of the vocalizations recorded in the current experiments were produced with the subject was at the distal ends of the apparatus, this explanation is unlikely to completely account for the lack of observed USVs.

Intracerebroventricular CRF administration was an effective anxiogenic when assessed on the EPM, but resulted in less consistent results during footshock sessions. This expected increase in anxiety-related behavior on the EPM is consistent with previous studies utilizing the EPM (Dunn & Berridge, 1990; Baldwin, Rassnik, Rivier, Koob, & Britton, 1991) as well as other behavioral paradigms exploring the association between CRF and anxiety (Dunn & File, 1987; Spina, et al., 2001; Swiergel, Zhou, Dunn, 2007). CRF did not enhance freezing behavior during footshock sessions, but did increase the production of both CF 50 kHz USVs and 22 kHz USVs. This apparent inconsistency may be due to the fact that
subjects in both groups were freezing almost constantly following the fourth footshock and a ceiling effect therefore prohibited the detection of a drug effect. Another possible explanation is that USVs may provide a more sensitive measure than freezing (Kosten, Lee, & Kim, 2006; Taylor, et al, 2017). The attenuation of rearing behavior observed following CRF administration as well as α-helical CRF is less easy to interpret. One likely explanation is that the anxiogenic effect of CRF predisposed subjects to respond to the novel environment of the footshock chamber with enhanced defensive behavior, and accordingly less exploratory behavior (Britton, Koob, Rivier, & Vale, 1982; Takahashi, Kalin, Vanden Burgt, & Sherman, 1989). Rearing activity can be motivated by the anxiety-related drive to explore or assess potential threats, but it is also been used as an index of novelty-seeking (Thiel, Müller, Huston, & Schwarting, 1999; Ho, Eichendorff, & Schwarting, 2002; Kabbaj, Devine, Savage, & Akil, 2000). Therefore, because rearing activity was significantly attenuated by both CRF and α-helical CRF, it may not be an appropriate measure of anxiety-related behavior within this paradigm.

The results of this study are consistent with previous research showing that CRF and α-helical CRF oppositely modulate the production of 22 kHz USVs (Swiergiel Zhou, & Dunn, 2007; Kikusui, Takeuchi, & Mori, 2000), although these effects have inconsistently been observed during acquisition of conditioned fear – a procedure analogous to our footshock procedure. Importantly, these drug-related changes in 22 kHz USV production in the current study are preceded by similar changes in the production of CF 50 kHz USV within this footshock procedure. The theory that 22 kHz USVs can be used as an index of anxiety or fear has existed for decades, and is supported by a preponderance of data (Tonoue,
Ashida, Makino, & Hata, 1986; Cuomo et al., 1988; Blanchard et al., 1991; Sanchez, 2003). Rats exhibit changes in constellations of behaviors including vocalizations based on imminence of threat. An encounter with a predator will elicit defensive behaviors, such as escape or freezing, along with 22 kHz USVs, but the decreased proximity to the predator will eventually elicit a different set of behaviors, such as audible vocalizations and attack (Blanchard, Yudko Rogers, & Blanchard, 1993). Narrow bandwidth and especially CF type 50 kHz USVs are observed during lower imminence or certainty of threat, such as prior to conspecific attack (Thomas, Takahashi, & Barfield, 1983), when exploring a novel context (Schwarting, Jegen, & Wöhr, 2007; Shetty & Sadananda, 2017), or following unexpected, mild footshock (Taylor, Utbano, & Cooper, 2017) when rats are engaged in postural and locomotor behaviors associated with a lower level of negative affective state. The attenuation of CF 50 kHz USVs along with other anxiety- or fear-related behaviors following α-helical CRF administration is consistent with our previous finding that a low dose of diazepam attenuates CF 50 kHz USV production (Taylor, Utbano, & Cooper, 2017). Additionally, because subjects treated with α-helical CRF did not exhibit decreased exploration or movement velocity compared to controls, it is unlikely that the attenuation in behavioral measures observed in the footshock session are a result of drug-related motoric impairment. The modulation of CF 50 kHz USVs via anxiogenic and anxiolytic drugs provides further support for the hypothesis that this call type is associated with negative affective state during lower imminence of threat.

Although the modulation of established indices of anxious or fearful state via ICV CRF and CRF antagonists has been widely reported, this study is the first to describe changes
in the 50 kHz USVs of rats with this protocol. The neural correlates of non-appetitive 50 kHz USVs are poorly understood, but the modulation of CF 50 kHz USVs via changes in the CRF system suggests potential targets for investigation. The anxiogenic properties of ICV CRF administration are predominately due to the activity of CRF receptor binding outside of the hypothalamus (Dunn & Berridge, 1990), particularly within the bed nucleus of the stria terminalis (BNST; Lee & Davis, 1997a,b), a region of the brain closely related to the amygdala considered as part of the extended amygdala. It has been argued that the BNST is a critical region for coordinating defensive behavioral responses to diffuse, or less certain/imminent cues such as (Liang, Mella, Miserendino, Falls, Campeau, & Davis, 1992; Xu, Liu, Xu, Zhang, Zhang, & Wu, 2012; Kim et al., 2013). Indeed, the CRF-enhanced acoustic startle response and bright-light enhanced startle, but not by a discrete conditioned stimulus depends on the BNST (Walker & Davis, 2002a). The modulatory effect of CRF and α-helical CRF on CF 50 kHz USVs in the footshock session may therefore be related to function of the BNST, and future research is needed to determine if this region plays a role in eliciting vocalizations of this type.

The inclusion of rodent USVs enrich existing and novel animal models that assess emotion because they can be assessed noninvasively, they allow for dynamic assessment of change in emotional state, and they can be measured in conjunction with a range of validated procedures and techniques. The recording of FM 50 kHz and 22 kHz USVs as indicators of positive and negative affect, respectively, is widely used in a variety of experimental paradigms. The USVs produced in the 50 kHz range are diverse. The significance and degree to which call types within this range should be dissociated is poorly understood.
Narrow bandwidth and CF 50 kHz USVs are quite distinct from the appetitive FM 50 kHz USVs, but perhaps because of the ambiguity regarding their behavioral significance, are often omitted from analysis or not parsed from other 50 kHz USVs. The current experiments support the hypothesis that CF 50 kHz vocalizations are associated with negative affective state, and may reflect a low intensity negative affective state, for example when animals are engaged in risk assessment, a context with an uncertain threat. Combined with previously reported findings (Taylor, Urbano, & Cooper, 2017), the results of these experiments suggest that dissociating the CF 50 kHz call type from other 50 kHz USVs can increase the sensitivity of behavioral measurements of a rodent’s emotional state. Future work can extend these results to determine how well this behavioral index correlates with other defensive behaviors elicited during variable degrees of threat imminence, and to explore the neural correlates of distinct call types in the 50 kHz range. A better understanding of the emotional nuance encoded by CF 50 kHz USVs would improve assays of complex emotional disorders that are a prevalent concern in our society today.
Chapter 5. The BNST mediates CRF-related changes in CF 50 kHz USVs

Rodents produce ultrasonic vocalizations (USVs) within a wide range of natural and experimental settings, and some of the USVs produced by rats are phenotypic markers of an animal’s emotional state (Burgdorf et al., 2009; Burgdorf & Moskal, 2010; Knutson, Burgdorf, & Panksepp, 2002; Miczek, Weerts, Vivian, & Barros, 1995). The inclusion of rodent USVs along with other behavioral measures therefore enriches novel and existing animal models that assess emotion because they can be assessed noninvasively, they allow for dynamic assessment of change in emotional state, and they can be measured in conjunction with a range of validated procedures and techniques. USVs are commonly classified and described based on mean peak frequency and patterns of frequency modulation (Knutson, et al., 2002; Wright, et al., 2010). The recording of frequency modulated (FM) 50 kHz and 22 kHz USVs as indicators of positive and negative affect, respectively, is widely exploited to reveal an animal’s emotional state in a variety of experimental paradigms (Schwarting, Jegen, & Wöhr, 2005; Portfors, 2007; Burgdorf et al., 2008).

The acoustic features of vocalizations within the 50 kHz range (30-70 kHz) are quite variable; some have argued that there are as many as 14 different subtypes (Burgdorf, et al., 2008; Wright, et al., 2010). The FM 50 kHz USV associated with positive affective state are characterized by rapidly oscillating modulations of frequency with a wide frequency bandwidth. (Wöhr, Houx, Schwarting, & Spruijt, 2007; Burgdorf et al., 2008; Wright, Gourdon, Clarke, 2010). The 50 kHz subtype most commonly dissociated from FM 50 kHz USVs are constant frequency (CF) 50 kHz USVs, sometimes referred to as “flat” calls, and
are characterized by a little or no modulation in frequency (Burgdorf & Panksepp, 2006; Wöhr et al., 2007; Wright, et al., 2010). Rats produce constant frequency (CF) 50 kHz USVs in a variety of social and non-social situations. In contrast to the positive state represented by FM 50 kHz USVs, we have demonstrated that CF 50 kHz USVs are associated with a mildly negative affective state. Our lab has explored the dynamic shifts in rat USVs largely using a single session testing procedure of threat certainty is manipulated via the presentation of six temporally consistent, mildly aversive footshocks (0.5 mA, 0.5 s). In this paradigm, subjects exhibit a distinct change in the pattern of USV production across the testing session. Initially, rats produce a high proportion of CF 50 kHz USVs prior to, and especially following the first footshock; the production of CF 50 kHz USVs declines after repeated footshocks, and the proportion of 22 kHz USVs increase (Taylor, et al., 2017; see Chapter 3, 4). The rate of CF 50 kHz USVs within this paradigm is increased following intracerebroventricular (ICV) administration of the stress-related peptide corticotropin-releasing factor (CRF), and attenuated by previous exposure to the context, systemic administration of diazepam, and ICV administration of the non-selective CRF receptor antagonist, $\alpha$-helical CRF (see Chapter 4).

We have hypothesized that CF 50 kHz USVs are an indicator of anxiety. Anxiety and fear are variably defined, and no single consensus exits on how these terms should be used (cf. Avery, Clauss, & Blackford, 2016; Blanchard & Blanchard, 2008; Rau & Fanselow, 2007; Sylvers, Lilienfeld, & LaPrairie, 2011). We view anxiety and fear as distinct processes; anxiety is driven by either lower-imminence or uncertain threat, and fear is expressed when there is an imminent or certain threat (i.e. fear; Perusini & Fanselow, 2015;
Fanselow & Ponnusamy, 2008; Davis, 2006). Rats produced 22 kHz USVs when escaping from imminent threat of predator (Blanchard, et al., 1991), after repeated footshocks (Schwaring, et al., 2005; Choi & Brown, 2003; Burgdorf et al., 2008), and when exposed to a stimulus that the rat has learned precedes footshock (Kim, et al., 2010; Parsana, Moran, & Brown, 2012, but see Jelen, Soltysik, & Zagrodzka, 2002). Accordingly, we suggest that 22 kHz USVs provide an index of fear along this continuum. The hypothesis that CF 50 kHz USVs are associated with anxiety has been supported by the results of the footshock experiments described in the preceding chapters. Although converging behavioral and pharmacological evidence supports a link between CF 50 kHz USVs and anxiety, the neural control of CF 50 kHz USVs has not be previously explored. Therefore, more research is needed to determine whether the neural systems that drive anxiety- and fear-related behaviors are involved in the production of CF 50 kHz USVs.

The expression of anxiety and fear, at least in part, is supported by separable, but interrelated, neural systems. The bed nucleus of the stria terminalis (BNST) is implicated in anxiety-related behaviors on the elevated plus maze and open field (Duvarci, Bauer, & Paré, 2009; Kim, et al., 2013), as well as certain types of potentiated acoustic startle (Walker & Davis, 1997b; Gewirtz, McNish, & Davis, 1998). Importantly, the modulation of anxiety-related behaviors resulting from ICV administration of CRF and CRF receptor antagonists relies heavily upon the BNST (Walker & Davis, 2002a) as evidenced by the fact that lesions of the BNST selectively abolish CRF-potentiated acoustic startle response (Lee & Davis, 1997). The role of the BNST in the production of USVs has so far received minimal attention (cf. Burgdorf et al., 2007). However, due to its involvement in emotional behavior
generally, and necessary role in mediating ICV CRF-related changes in anxiety-related behavior, the BNST is an ideal initial target to investigate the association between CF 50 kHz USVs and anxiety.

The current study was conducted to investigate the role of the BNST in CRF-related changes in the production of CF 50 kHz and 22 kHz USVs within the context of increasingly certain threat via repeated mild footshock. Rats received electrolytic or sham lesions of the BNST, and then received ICV infusions of CRF or vehicle 30 min prior to behavioral testing in a single session of unsignaled, temporally-consistent mild footshocks. All subjects were subsequently assessed in the same condition on the elevated plus maze (EPM) as an additional measure of anxiety state. We predicted that, in footshock sessions, CRF would modulate rearing behavior and the rate of CF 50 kHz USVs and 22 kHz USVs in a manner consistent with previous observations (see Chapter 4) among sham-lesioned animals, and that these drug-related changes would be abolished or attenuated among BNST lesioned animals. Similarly, we predicted that on the EPM, CRF would decrease exploratory behavior among sham-lesioned animals, but that no differences in behavior would be observed among BNST lesioned animals.

Methods

Subjects

A total of 35 female Long Evans rats (Harlan Laboratories, IN) that were 65-70 days old at the time of surgery were used as subjects. Animals were housed as described in Chapter 2. All subjects were individually housed for one week between surgery and testing.
All research was conducted with approval from an Institutional Animal Care and Use Committee (#14/02).

**Stereotaxic Surgery**

Rats were randomly assigned to either the BNST lesion group ($N = 23$) or the sham lesion group ($N = 12$), and all subjects were implanted with unilateral guide cannula for drug microinfusion. Subjects were anesthetized with 4% (2-4 L/min vaporized in medical air) isoflurane during induction, and then maintained under anesthesia with 1.5 – 2.5% vaporized isoflurane during surgery. The scalp was shaved and cleaned with betadine and the rat was positioned with skull flat in the stereotaxic instrument (Leica Biosystems, Germany). The skull was exposed via an incision along the midline of the scalp and then craniotomies were made for electrode, cannula, and anchor screws insertion. After surgery, a single subcutaneous injection of sustained release buprenorphine (1 mg/kg) was administered for pain relief. All rats were allowed to recover from anesthesia within a heated cage and then given access to a clear liquid gel diet following surgery. For seven days following surgery, the occluder was removed and reinserted to habituate the animal to the microinfusion procedure, and twice daily visual checks were performed to inspect healing around the implant, and health and animal health.

**Lesions.** Electrodes were positioned at three locations $\pm 1.5$ mm from the midline along the anterior/posterior axis of the BNST at $0$ mm, -$0.5$ mm, and -$1.0$ mm from bregma at a depth of -$5.75$ mm from dura. The coordinates used were modified from the location of the BNST in the Rat Brain Atlas (Paxinos & Watson, 2008) based on pilot lesions in age-matched female rats. The depth was adjusted because of the smaller brain and body size of
the adult females in this age range compared to the atlas coordinates which are based on adult male rats. Electrolytic lesions were caused by passing current (0.10 mA, 30 s) through tungsten electrodes (150 \( \mu \)m shaft diameter, 4 M\( \Omega \) tested at 1kHz) at each location. A flat gold clip was placed on the skin of the scalp and served as the ground. For sham animals, electrodes were inserted into the brain, but no current was passed through the electrode.

**Intracerebroventricular cannula implants.** A custom made guide cannula (23 gauge stainless steel tubing, 14 mm) was inserted 1 mm above the lateral ventricle (0.8 mm posterior to bregma, 1.4 mm lateral to the midline, and 3 mm ventral to dura). The laterality of cannula implant was counterbalanced within groups. The craniotomy was filled with vacuum grease, and the guide cannula was then fixed to skull and anchor screws with dental acrylic. Guide cannulas were occluded with custom-constructed stainless steel stylets except during microinfusions.

**Microinfusion procedure**

Microinfusions were conducted 30 min prior to behavioral testing with the footshock procedure and elevated plus maze (EPM) testing. The footshock testing and EPM test were spaced 24 hours apart. Subjects received either CRF (1 \( \mu \)g/5 \( \mu l \); Sigma), or the vehicle, artificial cerebrospinal fluid, ACSF (5 \( \mu l \)). Infusions were be made using a custom made injection cannula (30 gauge stainless steel tubing) that terminated 1 mm beyond the guide cannula when inserted. The injection cannula was connected to a 100 \( \mu l \) Hamilton syringe mounted on an infusion pump (KD Scientific, Holliston, MA). Infusions were made at a rate of 2.5 \( \mu l/\text{min} \), and the injection cannula was left in place for one minute after infusion was complete to allow for diffusion.
Apparatus and Materials

Footshock Apparatus. Footshock sessions were conducted in the same testing environment and with the same equipment described in Chapter 2.

Elevated Plus Maze. The apparatus and setup of the elevated plus maze as described in Chapter 4.

Footshock Session

Procedure. Footshock testing was conducted 30 min after microinfusion. The equipment and procedure for footshock sessions was as described in Chapter 2.

Rearing and Freezing Assessment. Rearing and freezing behavior were coded from video recordings by two independent observers blind to experimental condition. Inter-observer agreement exceeded 93% for both measures. Rearing activity was measured in successive 20 s bins and was defined as a vertical body movement resulting with only the rear paws were in contact with the floor of the chamber. Excluded from this count were instances wherein rearing was immediately preceded by jumping or climbing. Freezing was measured in successive 8 s bins and was defined as a lack of all non-respiratory movement for at least 4 continuous s during the bin. These observations were then converted to percent time freezing (Number of Freezing Observations/Number of Total Observations X 100) for the initial 2 min pre-shock period and subsequent inter-shock-intervals. Rearing and freezing data were analyzed as described in “Statistical Analyses” section below.

USV classification. The recording and classification of USVs was conducted as described in Chapter 2.
The number of calls within each classification was used to determine calling rate per second (Number of Calls/Seconds in Time Bin) within the pre-shock period and subsequent inter-shock-intervals. The rate of CF 50 kHz and 22 kHz USV production throughout the session was then compared as described in the “Statistical Analyses” section below.

**Statistical Analyses.** Acoustic and overt movement behavioral data were collected throughout the entire session. Dependent variables were binned into seven time bins: initial 2 min bin, five ~ 60 s ISI bins, and a final 2 min post-shock bin. The mean count of rearing activity during the initial 2 min was compared using a 2 x 2 Factorial ANOVA (lesion condition x drug group). The percent time freezing, rate of CF 50 kHz USVs, and rate of 22 kHz USVs across the session was compared using a 2 x 2 x 7 (lesion condition x drug group x time bin) repeated measures ANOVA. Only rats producing USVs were included for USV analyses. No freezing behavior or 22 kHz USVs were observed prior to the first shock, and therefore the pre-shock time period was excluded from these analyses. Because of the low number of animal producing 22 kHz USVs within the BNST lesion condition, a Fisher’s exact test was conducted to determine differences in the proportion of subjects calling between drug groups within each lesion condition. All statistical analyses were performed using IBM SPSS Statistics (Armonk, New York, version 22.0).

**Elevated Plus Maze**

**Procedure.** EPM testing was conducted 24 h after footshock procedure for all subjects. Thirty minutes after drug or control microinfusion, rats were placed on the center of the EPM oriented toward one of the open arms. Subjects were observed for 5 min on the EPM, and then returned to the homecage. The apparatus was thoroughly cleaned and
allowed to dry between subjects. Video and ultrasonic audio recordings were collected throughout the entire session.

**Movement-Related Behaviors.** Video files were exported for automated analysis using Noldus Ethovision XT 11.5 software (Noldus, Wageningen, NL). This software was used to determine the percent open arm entries, percent open arm time, and mean velocity. Between-group comparisons of dependent variables on the EPM were conducted as described in the “Statistical Analyses” section below.

**USV Production.** Ultrasound recording and analysis were conducted as described in the footshock session. However, USV production was rare: no 22 kHz USVs were observed, and few rats produced 50 kHz USVs. USVs were not analyzed because of the paucity of vocal data during EPM testing.

**Statistical Analyses.** For each lesion condition, comparisons between drug groups were conducted using one-tailed independent samples t-tests for percent open arm entries, percent open arm time, and mean velocity of movement. All statistical analyses were performed using IBM SPSS Statistics (Armonk, New York, version 22.0).

**Histology**

At the conclusion of the experiment, animals were euthanized via carbon monoxide. Brains were then extracted and stored at 2 °C for 48 h in 10% (w/v) formalin solution, and then transferred to a 30% sucrose formalin solution for one week before being flash-frozen and sliced into 50 μm sections. Every second section from the region containing the BNST was mounted on slides and a Nissl stain was used for microscopic verification of the lesion.
Digital images of individual sections were superimposed over images from the Rat Brain Atlas (Paxinos & Watson, 2007) and the position and extent of lesions were traced by hand.

Results

Histology

Figure 30 displays the placement of lesions for five rats. Two subjects were removed from analysis due to lesion location. Minimal tissue damage was observed in sham-lesioned animals as a result of electrotode insertion.

Footshock Sessions

Rearing Behavior. Figure 31 displays number of times subjects engaged in rearing during the pre-shock period. There were no significant main effects for lesion condition, $F(1, 29) = 1.618, p > .05$, or for drug group, $F(1, 29) = 1.816, p > .05$. No significant interaction was indicated between lesion condition and drug group, $F(1, 29) = 0.845, p > .05$.

Freezing Behavior. The percent time freezing across the footshock session is shown in Figure 32. Among BNST lesioned subjects, two from the ACSF and one from the CRF
group were excluded from analysis as outliers (>2X SD of mean). There was a significant within-subjects effect of time, $F(5, 130) = 76.963, p < .01$. There were no significant main effects for lesion condition, $F(1, 26) = 2.995, p > .05$, or for drug group, $F(1, 26) = 0.554, p > .05$. No significant interaction was indicated between lesion condition and drug group, $F(1, 29) = 0.009, p > .05$. There was no interaction between time and lesion condition, $F(1, 130) = 1.523, p > .05$, drug group, $F(1, 130) = 0.132, p > .05$, or lesion condition by drug group, $F(1, 130) = 0.779, p > .05$.

**CF 50 kHz USV.** The rate of CF 50 kHz USV production across the session is shown in Figure 33. There was a significant within-subjects effect of time, $F(6, 144) = 8.225, p < .001$, and an interaction between time and lesion condition, $F(6, 144) = 2.324, p < .05$, as well as time and drug group, $F(6, 144) = 2.336, p < .05$. Simple main effect analysis showed that CRF administration resulted in a
significantly higher rate of CF 50 kHz USVs after the second footshock among sham
lesioned subjects, $p < .01$, but not compared to BNST lesioned subjects, $p < .01$. There were
no significant main effects for lesion condition, $F(1, 19) = 0.54, p > .05$, or for drug group,
$F(1, 19) = 0.128, p > .05$. No significant interaction was indicated between lesion condition
and drug group, $F(1, 19) = 2.113, p > .05$.

22 kHz USVs. The rate of 22 kHz USV production across the session is shown in

Figure 33. Effect of BNST lesion and CRF on CF 50 kHz USVs. CRF enhanced the production of CF 50
kHz USVs among sham lesioned animals only. * indicates $p < .05$.

Figure 34. Effect of CRF and BNST lesion on 22 kHz USVs. CRF enhanced the production of 22 kHz USVs
among BNST lesioned (A) and sham lesioned animals. This was indicated by the number of subjects calling in
the BNST lesion condition, and by the rate of 22 kHz USVs in the sham lesion condition. * = $p < .056$
Figure 34. A repeated measures ANOVA could not be used to compare animals in the BNST lesion condition (Fig. 34A) because of the low number of animals producing 22 kHz USVs in the ACSF group (ACSF $N = 2:9$, CRF $N = 9:12$). A Fisher’s exact test indicated that a significantly higher proportion of subjects in the CRF group produced 22 kHz than in the ACSF group, $\chi^2 = 5.743$, $p < .05$. There was no drug-related difference in the proportion of subjects producing 22 kHz USVs in the sham lesion condition, $\chi^2 = 0$, $p > .05$ (ACSF $N = 4:6$, CRF $N = 4:6$). There was also no lesion-related difference in the proportion of subjects producing 22 kHz between ACSF drug groups, $\chi^2 = 2.963$, $p > .05$.

Among sham-lesion animals (Fig. 34B), the 22 kHz calling rate significantly increased across the session, $F(5, 25) = 5.213$, $p < .01$, and a marginally significant enhancement by CRF, $F(1, 5) = 6.127$, $p < .057$. The within session change in rate over time did not vary as a factor of group.

Elevated Plus Maze

![Graph A](image)

**Figure 35. Effect of CRF on behavior in the EPM among BNST lesioned animals.** CRF increased anxiety among subjects as indicated by significantly less time spent on and entries into the open arms of the EPM (A), as well as by decreased speed of movement (B). $^* = p < .05$
A total of three subjects in the lesion condition were excluded from analysis. One subject from the ACSF group and one from the CRF group were excluded as outliers (> 2X SD from mean) in measures of percent open arm time and percent open arm entries. One additional subject in the CRF group was excluded from analysis after falling from the EPM. Figure 35 displays the results of the EPM test session within the lesion condition. Contrary to our predictions, CRF administration resulted in attenuated percent open arm time, \( t(14) = 2.360, p < .05 \), percent open arm entries, \( t(14) = 2.377, p < .05 \), and velocity of movement, \( t(14) = 1.789, p < .05 \).

A total of four subjects, all from the CRF group, were excluded from analysis in the sham-lesion condition. Two were unable to receive drug infusion because of cannula blockage, and two fell from the EPM. This loss in subjects within the sham-lesion group resulted in too few subjects to appropriately compare with parametric analysis.

**Discussion**

We have proposed the hypothesis that the production of CF 50 kHz USVs and 22 kHz USVs may serve as signals of different levels of negative affective state along a continuum of increasing intensity or certainty of threat (i.e., anxiety and fear; Taylor et al., 2017). In the current study, we conducted an initial investigation into what role the neural correlates of anxiety and fear serve in the production of CF 50 kHz and 22 kHz USVs via testing the effect of BNST lesions and CRF-mediated changes in vocal and overt behavior within a context of increasing certain threat and on the EPM. Rearing and freezing behavior during footshock sessions were unaffected by lesion or drug administration. The results of the footshock sessions showed that CRF infusions did not modulate CF 50 kHz USVs in BNST lesioned
animals, but increased CF 50 kHz USVs in sham lesioned animals following the second footshock. CRF infusion increased the rate or likelihood to produce 22 kHz USVs among both sham and BNST lesioned animals. In the EPM, CRF reduced exploratory behavior and movement velocity among BNST lesioned animals, but between drug comparisons could not be performed among sham lesioned subjects because of the difficulty in acquiring data in this condition.

Rearing behavior was not affected by CRF administration or BNST lesion. Exploratory behavior including rearing in the presence of uncertain threat has been demonstrated to be associated with activation of the BNST (Kim et al., 2013; Walker & Davis, 1997b). Therefore, the observation that CRF did not attenuate rearing among BNST lesioned animals within this footshock paradigm was consistent with previously reported observations and our predictions at the onset of this experiment. However, although CRF tended to reduce rearing in sham lesioned animals in line with previous studies (see Chapter 4; Britton, Koob, Rivier, & Vale, 1982; Takahashi, Kalin, Vanden Burgt, & Sherman, 1989) this result was not significant. The non-significant difference in rearing behavior among sham lesioned animals may be because rearing is not as sensitive a measure as USVs. In support of this interpretation, we found that rearing was dose-dependently modulated by diazepam, although no significant difference in rearing was observed with the lowest does (1 mg/kg; Chapter 2).

Within this paradigm, we previously reported that ICV administration of CRF resulted in no observable modulation in freezing behavior (see Chapter 3), in the current study, a similar result was found among animals with BNST or sham lesions. A lack of
noticeable difference in freezing behavior may be due to a ceiling effect as subjects tended to spend greater than 80% of the time freezing following the third footshock. Another explanation for the similarity in freezing is that the defensive behaviors modulated by manipulation of the CRF system via ICV infusion are those related to anxiety such as exploratory behavior, and responses potentiated by diffuse environmental cues, i.e. behaviors associated with activation of the BNST, rather than fear-related behaviors (Dunn & Berridge, 1990; Makino, Gold, & Schulkin, 1994a,b; Liang, et al., 1992a; Erb & Steward, 1999; Lee & Davis, 1997). Therefore, the absence of CRF-mediated differences in freezing or due to BNST lesion could be because unconditioned freezing in the presence of an imminent or a higher certainty of threat is associated with fear and activity of the amygdala, rather than the BNST (Hitchcock & Davis, 1986, 1991; Ledoux, Iwata, Cicchetti, & Reis, 1988).

The increased rate of CF 50 kHz USVs as a result of CRF administration among sham, but not BNST lesioned animals indicates that the BNST does indeed play a role in the production or modulation of CF 50 kHz USVs. We predicted that the BNST was an important neural correlate supporting the production of CF 50 kHz USVs, and therefore lesions of this nucleus would attenuate or abolish calling of this type. While ablation of the BNST did abolish the effect of CRF seen among intact animals, there was no difference in CF 50 kHz USVs between BNST and sham lesioned animals in the control infusion group. This result was similar to the observations of rearing behavior between control infusion groups in the sham and lesion condition. A likely interpretation of these results is that while lesions were determined to be bounded within the area of the BNST, they did not completely ablate the nucleus. Unfortunately, the potential for differences in behavior due to small
variations in the regions of the BNST that were ablated cannot be clearly dissociated with the
number of subjects used in this study. Additional research is needed to explore the
consequences of total ablation of the BNST on USVs and specific roles of different regions
within the nucleus.

An interesting pattern of CF 50 kHz USVs was observed following CRF administration among sham animals. Control infusions resulted in the pattern of CF 50 kHz USV production typical within this paradigm: a moderate rate of calling prior to shock, a large increase in calling following the first footshock, and then a decline in calling as shocks were repeated. However, CRF administration resulted in a pattern of calling that contrasts with previous work in that the rate of CF 50 kHz USVs prior to footshock remained constant following the first footshock, and was potentiated only after the second footshock. How the effect of CRF administration could have delayed the typical changes in CF 50 kHz within this paradigm is unclear. It is unclear why the effect of CRF within this paradigm was delayed among sham lesioned animals. One possibility is that electrode insertion resulted in tissue damage to brain regions or connecting fibers dorsal to the BNST involved with the central CRF system. However, it is possible that the results of the sham lesion condition represent an aberration that is difficult to detect in such a small sample size.

CRF administration had a general enhancing effect on the production of 22 kHz USVs regardless of lesion condition, albeit in different analyses. In the sham-lesion condition, CRF increased the rate of 22 kHz USVs consistent with previous observations (Swiergiel, Zhou, & Dunn, 2007; see Chapter 4). Among BNST lesioned animals, subjects receiving CRF in were more likely to produce 22 kHz USVs, although the usual comparison
of rate was impossible due to the low number of animals calling in the control condition. Within the context of the acquisition phase of fear conditioning, a procedure analogous to our footshock session, 22 kHz USVs have been shown to be largely under the control of the amygdala (Choi & Brown, 2003; Koo, Han, & Kim, 2004). However, enhancement in 22 kHz USVs following ICV CRF administration suggests a more complicated picture regarding the control of calls of this type. The CRF-facilitated response has been shown to rely upon the BNST as opposed to the central nucleus of the amygdala, or CeA (Lee & Davis, 1997; Walker & Davis, 2002), which mediates many responses to imminent threat (i.e. fear; LeDoux, Iwata, Cicchetti, & Reis, 1988; Kapp, Frisiger, Gallagher, & Haselton, 1979; Hitchcock & Davis, 1986; Lee & Davis, 1997). Another region of the amygdala, the basolateral amygdala (BLA), projects to both the BNST and CeA (Dong, Petrovich, & Swanson, 2001). Because selective ablation of neurons originating in the BLA but not the CeA attenuate or eliminate 22 kHz USVs during the acquisition of fear conditioning (Koo, et al., 2004), it may be that in addition to the amygdala, the BNST contributes to the production of 22 kHz USVs. Indeed, this speculation is supported by the results of the current study in that among BNST lesioned animals significantly fewer subjects produced any 22 kHz in the vehicle infusion group.

The apparent internal inconsistency of some of the current findings within the BNST lesioned animals may be explained by lesion placement. The goal of our procedure was to ablate the BNST in its majority without attempting to target specific sub-regions of the nucleus. Histological analysis indicated that lesions were contained within the BNST. However, some variation existed in the specificity of position within the nucleus. Regions of
the BNST are heterogeneous in function, with specific sub-regions required for driving
certain behaviors such as respiration and exploratory behavior (Schulkin, Gold, & McEwan,
1998; Dong & Swanson, 2006; Kim et al., 2013). Additionally, stimulation of the different
regions can elicit opposite modulation in general anxiety-related behavior (for review, Forray
& Gysling, 2004). Although distinguishing between effects of lesion damage biased toward
specific regions of the BNST would be valuable, such analyses are impractical for the current
study given the small sample size.

The EPM test was included in this study because it has been widely used to study
both the behavioral correlates of BNST activity, and modulation of the CRF system via ICV
infusion (Duvarci, Bauer, & Paré, 2009; Kim et al, 2013; Dunn & Berridge, 1990; Baldwin,
Rassnik, Rivier, Koob, & Britton, 1991). However, because we were unable to determine the
effects of CRF administration among sham lesion animals, the interpretation of the results of
the EPM test in relationship to footshock sessions is largely speculative. When considered as
an independent test, the enhancement in anxiety-related behavior observed on the EPM
following CRF administration in the BNST lesion condition appears to contrast not only with
our initial predictions, but also with previous work (Dunn & Berridge, 1990; Baldwin, et al.,
1991). The increased anxiety may therefore indicate incomplete or misplaced BNST lesions.
However, it is important to note that all subjects experienced the footshock procedure and
ICV infusions of CRF or ACSF one day before being observed on the EPM. Unpublished
data from our lab shows that previous aversive experience with the unsignaled footshock
procedure used in this study enhances anxiety when subsequently tested on the EPM.
Importantly, CRF can enhance aversive memory via binding to the amygdala (Lee & Sung,
1989; Liang & Lee, 1988). Additional testing is therefore required to dissociate the extent to which the apparently CRF-mediated changes observed with the EPM in the current study are a transient product of CRF infusion or the result of prior experience.

The results of the current study encourage further investigation into the relationship between CF 50 kHz and 22 kHz USVs, and the neural correlates of anxiety and fear. The most notable finding is that the BNST mediates the CRF-enhanced production of 50 kHz USVs within the context of unsignaled footshock. However, there were a number of limitations that we would like to address in the future; particularly the need for additional behavioral data using the EPM and to explore the consequences of full or region-specific lesions of the BNST. Additional work will need to be conducted to determine how the amygdala and the regions of the BNST interact to control defensive behavior and especially USVs within aversive contexts.
Chapter 6. General Discussion

CF 50 kHz USVs as a Signal of Anxious Emotional State

The broad aim of my dissertation was to explore the way in which the acoustic features of a rodent’s vocalizations can be used to determine anxious and fearful emotional states, and to develop a paradigm that shifts emotional state to allow for a dynamic assessment of emotion. The measurement of rat USVs is now widely used as a phenotypic marker of emotional state in a variety of basic and translational research (Choi & Brown, 2003; Coffey, et al., 2013; Ahrens, et al., 2009; Mällo, et al., 2009; Wöhr & Schwarting, 2012; Mahler, et al., 2013). Despite the heterogeneity among USVs, only two types are currently used as indices of affect: 22 kHz USVs as a signal of negative affective state, and the FM subtype of 50 kHz USVs as a signal for positive affective state (Schwarting, et al., 2005; Portfors, 2007; Burgdorf, et al., 2008). Additional information may be able to be extracted from the rich diversity of USV subtypes, but a clear understanding of the motivational states driving this diversity is lacking due to a variety of practices such as inconsistent call-type categorization, selective reporting of only one call type, and the use of recording devices tuned to a narrow frequency bandwidth (e.g., bat detectors). Even so-called “flat” 50 kHz USVs, the most commonly dissociated from FM 50 kHz USVs, has, despite the name, been as variably defined as being composed solely of a constant frequency (Wright, et al., 2010), or else any pattern of frequency modulation less than 15 kHz in frequency bandwidth (Burgdorf, et al., 2008). Previous research has posited that 50 kHz USVs produced with a constant frequency (CF) or within a narrow frequency bandwidth function as social contact call among conspecifics. Based upon a review of the existing
literature and observations in our own lab, we generated the hypothesis that CF 50 kHz USVs, signals anxiety. As reviewed in the following sections, several predictions were generated from this novel hypothesis and tested in the preceding experiments. Based upon the behavioral and pharmacological evidence from these experiments, we conclude that CF 50 kHz USVs are associated with an anxious state; i.e. a negative affective state with low certainty or imminence of threat.

Another central theme to this research has been the development of a paradigm to rapidly modulate negative affective state wherein rats are initially anxious and transition to a fearful state. Other paradigms exist to investigate changes in behavior across increasing intensity of negative affective state. The Anxiety/Defense Test Battery and the Fear/Defense Test Battery (Blanchard et al., 1990; Blanchard et al., 1991; Blanchard et al., 1992), for example, have been used to great effect in exploring anxiety- and fear-related behaviors. However, these tests can be difficult or expensive to run because of the unusual housing conditions often used (i.e. visual burrowing system; Blanchard et al., 1989, 1990), and the use of an actual predator (i.e., cat) or its odor\(^1\). Our procedure, which is analogous to the acquisition phase of contextual fear conditioning, exposes the animal to a series of mild, temporally predictable footshocks within a single testing session. The changes within and transitions between behaviors during the footshock sessions highlights the dynamic shifts in emotional state that can occur over a relatively short period of time. Many of the significant

\(^1\) Pilot tests using cat odor from pet cat hair as described in Blanchard et al. (1990) resulted in no significant changes in behavior. The failure of cat odor to serve as an aversive stimulus was quite possibly due to the fact that many graduate and undergraduate researchers own cats, and the odor is therefore pervasive in the vivarium outside of
differences in behaviors observed throughout these experiments, particularly in the production of USVs, would have been obscured if the dependent variables were only analyzed for the session as a whole. This paradigm is easily replicated with minimal equipment, and provides strong experimental controls and features that can be easily manipulated as needed (e.g., the intensity, duration, and temporal spacing between shocks).

**CF 50 kHz USVs will be elicited by exposure a novel, anxiogenic context**

Exposure to the context of the footshock chamber reliably elicited CF 50 kHz USVs (see Chapters 2 – 5), and calling declined with habituation in the absence of an acute stressor (Chapters 2 & 3). The conclusion that the testing chamber was indeed anxiogenic was supported by the suppression of consummatory behavior regarding a familiar, palatable food (Chapter 2). However, in our experiments few USVs were recording during testing using the EPM (Chapters 4 & 5) despite the fact that they have frequently been reported by other researchers (Rao & Sadananda, 2015; Shetty & Sadananda, 2017; c.f. Borta, Wöhr, & Schwarting, 2006). In an earlier section (Chapter 4, pg 95-96), we suggested that this result may be due to differences in recording procedures. Another explanation for the lack of calls recorded during EPM tests is that subjects are experiencing a level of negative affect too intense to drive CF 50 kHz USVs, but below the threshold of driving 22 kHz USVs. In support for this explanation, unpublished data from our lab demonstrates that previous experience with our footshock procedure results in significantly increased anxiety, as assessed by exploratory behavior, when subsequently tested on the EPM. This explanation appears increasingly likely as the evidence from the previous chapters suggests that USVs may be more sensitive to manipulations of emotional state than movement-related measures.
Although unpublished data from our lab demonstrates that rats produced CF 50 kHz USVs within the Light-Dark Box apparatus, additional research utilizing the EPM and other traditional assays of anxiety is required to more thoroughly address this prediction.

**CF 50 kHz USVs will be potentiated when uncertainty of threat is increased via exposure to a single unanticipated, mild footshock**

In the experiments presented in Chapters 2, 4, & 5, the rate of CF 50 kHz USVs increased from the preshock calling rate in the time period between the first and second shock. Indeed, following systemic administration of diazepam (1 mg/kg), significantly fewer rats elicited any USVs prior to the first footshock in this paradigm (Chapter 2). The majority of significant between-group differences in CF 50 kHz USVs observed in the preceding studies occurred during this time period. The exception to this general result was observed within Chapter 3, wherein subjects produced an abnormally high rate of CF 50 kHz USVs prior to the first footshock. Conspecific scent, especially from the opposite sex, can be a powerful stimulus in driving the production of 50 kHz USVs (Knutson, Burgdorf, & Panksepp, 1998; Brudzynski & Pniak, 2002; Wöhr et al., 2007). Although the testing chamber was thoroughly cleaned between subjects, it is possible that olfactory traces in the testing chamber or room in general potentiated calls prior to the acute, mildly aversive stimulus of footshock. It would be worth investigating whether, if tested independently, male rats typically exhibit the same potentiation in CF 50 kHz USVs following footshock otherwise observed in female rats.

**The vocalizations produced by rats will transition from CF 50 kHz to 22 kHz USVs when certainty of threat is increased via temporally predictable footshocks**
This prediction was repeatedly confirmed throughout the preceding experiments utilizing the unsignaled footshock procedure (Chapters 2 – 5). The interpretation that the repeated footshock drove this change in vocal behavior is supported by the observation that 22 kHz USVs were not produced when footshocks were not administered. Data from an ongoing study in our lab has so far indicated that when footshock timing is randomized, female rats continue to produce CF 50 kHz USVs far later into the testing session and transition to 22 kHz USVs only within the last minute of the session. This ongoing experiment also indicates that when animals are subsequently returned to the context in absence of any acute stressor, rats that previously experienced randomly timed footshocks produce CF 50 kHz, but not 22 kHz USVs; whereas we and others have observed 22 kHz USVs during extinction of contextual fear conditioning when previously exposed to temporally consistent footshocks (Antoniadis & McDonald, 1998, Choi & Brown, 2003). However, additional research including extinction trials is needed to determine whether contextual fear conditioning occurred (i.e., that rats learned certainty of threat within the context), whether subjects were able to predict the timing of shocks, or the change in vocal behavior was driven only by the number or intensity of shocks.

**CF 50 kHz USVs will be modulated by pharmacological agents and procedures known to modulate anxiety**

We have demonstrated that the peripheral administration of a low dose of the benzodiazepine diazepam (1 mg/kg) reduces CF 50 kHz USVs when exposed to the novel environment of the footshock chamber (Chapter 2). The larger dose of diazepam (2.5 mg/kg) was required to significantly reduce the overt motoric behaviors of rearing and freezing, but
this dose abolished USVs within the footshock procedure. The interpretation that these effects were caused by general motoric suppression rather than the anxiolytic properties of the drug was not supported by the results of experiments presented in Chapter 4. Consistent with the interpretation that CF 50 kHz USVs are modulated by anxiolytic and anxiogenic drugs, the central administration of CRF and \( \alpha \)-helical CRF respectively increased and decreased CF 50 kHz USVs within the unsignaled footshock procedure. Additionally, when the EPM was used as a secondary assay of the anxiety-related behavior, it was demonstrated that the \( \alpha \)-helical CRF not only reduced anxiety-related behaviors, but did so without sedation or general suppression of movement. As was seen with dose-dependent effects of diazepam, CF 50 kHz USVs were modulated by CRF and \( \alpha \)-helical CRF although not all overt behaviors were changed by drug administration. These results strongly support the hypothesis that CF 50 kHz USVs are associated with anxiety, and additionally support the idea that USVs may provide a more sensitive or supportive measure than overt behaviors such as rearing and freezing (Wöhr, et al., 2005; Kosten et al., 2006).

**CF 50 kHz USVs will be modulated via ablation or pharmacological manipulation of the BNST; an important neural correlate of anxiety-related behavior**

BNST lesions selectively eliminated CRF-potentiated CF 50 kHz USVs within our footshock paradigm. However, the overall relationship between BNST lesions, CRF administration, and overt and vocal behaviors are difficult to interpret with conviction without additional experimentation due to the limitations previously discussed in Chapter 5 (pg 116-121). Briefly, the most important shortcomings in our initial exploration of this experimental prediction were (a) the incomplete data from the EPM, (b) the small variation
within BNST lesion placement, (c) the non-selective modulation of anxiety- and fear-related behaviors, and (d) the unusual timing of CF 50 kHz USVs relative to footshocks that were produced by sham lesion subjects in the CRF group.

The distinction between the anxiety- and fear-related behaviors at the neural level is well supported by a preponderance of experimental evidence using a variety of techniques including reversible inactivation (Xu, et al., 2012), ablation (Lee & Davis, 1997a; Walker & Davis, 2007), pharmacological manipulation (Liang, et al., 1992a; Toufexis, 2007), and optogenetic stimulation (Kim, et al., 2013). Generally, this line of research has identified the BNST and the CeA as critical neural mediators of unconditioned anxiety and fear, respectively. However, both areas receive input from, and are highly interconnected with not only upstream areas of the brain like the BLA, but also with each other (Alheid, Olmos, & Beltranino, 1995; Dong, Petrovich, & Swanson, 2001; Polous, Ponusamy, Dong, & Fanselow, 2010). In addition, the BNST is a heterogenous area with sub-region specific modulation of anxiety-related behavior such as respiration (Kim, et al, 2013). In light of the complex relationship between these neural systems, it is not surprising that our first experiment investigating the role of the BNST in USV production yielded some ambiguous results. Future research is planned to use direct microinfusion of CRF into the BNST and the CeA in order to better dissociate the role that these nuclei serve in the production of CF 50 kHz and 22 kHz USVs within this paradigm. Additional research utilizing lesion or reversible inactivation of these nuclei will also be beneficial in testing this prediction.
Translational Implications and Concluding Thoughts

An inescapable conclusion of these experiments is that more nuanced emotional information can be decoded from the USVs of rats than was previously understood. Therefore, it is important for researchers to carefully assess the acoustic features of vocalizations, and to adopt a consistent classification terminology. One obstacle to this goal is that, especially within the category of 50 kHz USVs, the classification by subtype is time and labor intensive. This is because USV classification is usually conducted on a call-by-call basis by trained observers in many research groups in addition to our own (Wright et al., 2010; Wöhr & Schwarting, 2012; Kisko, et al., 2015; Shetty & Sadananda, 2017; Burgdorf et al., 2007). Attempts have been made to automate this analysis (Reno, Marker, Cormack, & Schallert, 2013; Barker, Herrara, & West, 2014), but the use of these methods has so far not been widely adopted.

The presence of CF 50 kHz USVs not only within aversive contexts, but also in putatively appetitive contexts such as social interaction (Hamed et al., 2009; Willey et al., 2009) and drug self-administration (Wright et al., 2010, Ahrens et al., 2009) suggests that researchers may also be able to model the transition between anxiety and positive affect via USVs, or else explore the role of conflict and ambivalence within these paradigms. Indeed, the interpretation that CF 50 kHz USVs are associated with anxiety is not incompatible with the hypothesis that CF 50 kHz USVs serve a social contact function. In a social species like rats, the production of CF 50 kHz USVs when alone in an uncertain context (Wöhr, et al., 2008; Rao & Sadananda, 2015; Taylor, et al., 2017) or when presented with an unfamiliar conspecific (Vivian & Miczek, 1992, Vivian & Miczek, 1993) may serve as a pro-social
signal to the listener. The ability of 22 kHz USVs to serve as alarm calls requires autoconditioning; i.e, the animal learning to associate 22 kHz with danger only if the individual has previously produced 22 kHz USVs in response to an aversive stimulus (Kim, et al., 2010; Parsana, Moran, & Brown, 2012). Perhaps a similar process is required for animals to increase exploratory behavior to approach CF 50 kHz USVs (Wöhr & Schwarting, 2007). It would be valuable to explore the effects of experience and social interaction on the functional role of USVs. For instance, the audience-specific production of USVs as observed in Blanchard, et al. (1991), which has never been reproduced in a more typical laboratory setting (Wöhr & Schwarting, 2008) may be due to the use of a more naturalistic social housing conditions of rats in their visual burrowing system.

Finally, the analysis of CF 50 kHz and 22 kHz USVs using our unsignaled footshock paradigm and other assays of anxiety and fear may help basic research to enable a dissociation between these emotional states at the behavioral and neural levels, and can serve to enrich animal models of human affective disorders. Perusini and Fanselow (2015) persuasively argue that animal models that distinctly assess anxiety or fear, when most valuable, should accordingly show distinction in the conditions that elicit these emotional states and the resulting behaviors. For example, variations in the acoustic startle response, a behavioral paradigm often cited in this document, have helped considerably in dissociating the neurobiological correlates of anxiety vs. fear (Davis, et al., 2013), but fall short of the standards put forth by Perusini & Fanselow (2015) in that the same behavior is accepted as both an expression of anxiety and fear. The imminence of a threat, in terms of both distance and certainty, provides distinction for anxiety and fear in condition as well as the resulting
constellation of behaviors (Fanselow & Lester, 1988; Fanselow, 1989; Blanchard, et al., 1989, 1990; Perusini & Fanselow, 2015). The results of the preceding experiments combine with previous research (Blanchard, et al., 1991; Wöhr, et al., 2008; cf. Jelen, et al., 2003) to demonstrate that CF 50 kHz and 22 kHz USVs are consistent with behaviors produced in response to low and high certainty of threat, respectively. We believe that the assessment of CF 50 kHz USVs can therefore help to dissociate anxiety from fear across a variety of behavioral testing procedures, and thereby enrich our understanding of the neurobiological substrates of typical and atypical emotional responses, as well as a providing a novel tool toward the development of therapeutic interventions for emotional disorders.
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ABSTRACT

EVALUATING CONSTANT FREQUENCY 50 kHz ULTRASONIC VOCALIZATIONS AS AN EXPRESSION OF ANXIETY IN RATS

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Adult rat ultrasonic vocalizations (USVs) are a valuable tool for noninvasively assessing an animal’s emotional state. The assessment of USVs can therefore enrich animal models of affective disorders. USVs are broadly classified into one of two frequency ranges: 22 kHz or 50 kHz. One subtype of 50 kHz USVs, constant frequency 50 kHz (CF 50 kHz) calls, is not viewed as signaling a particular emotional state. The data described in this dissertation provide support for the novel hypothesis that CF 50 kHz USVs are related to a low level of negative affective state, when threat is less certain or imminent (i.e., anxiety). The general procedure assessed vocalizations and other traditional behavioral indices of emotional state (i.e., rearing and freezing) in a paradigm of increasing certainty of threat through repeated mild footshocks to determine the vocal and overt behaviors associated with anxiety and fear. In a series of experiments, we assessed changes in these vocal and overt behaviors following modulations in emotional state through behavioral manipulations and peripherally- and centrally-administered pharmacological agents. Within the footshock paradigm, subjects transitioned from producing CF 50 to 22 kHz USVs as footshocks were repeated; a pattern paralleled by a shift from rearing behavior to increased time freezing. We additionally
explored sex-related differences in behavior within this paradigm and determined that female rats provide a better model to explore this hypothesis because of the elevated rate of CF 50 kHz USVs compared to males. The results of these experiments were largely consistent with the hypothesis that CF 50 kHz USVs are related to anxiety. CF 50 kHz USVs are attenuated when the context is familiar and when subjects are pre-treated with the anxiolytics diazepam (1 mg/kg) and the corticotropin-releasing factor (CRF) antagonist α-helcial CRF. In contrast, CF 50 kHz USV production is increased when uncertain threat is first perceived, and when pre-treated with the anxiogenic CRF. The results of these experiments suggest that assessment of CF 50 kHz USVs may enrich existing animal models of anxiety and provide a tool for the development of new animal models and therapeutic interventions.