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Neurobehavioral Correlates of Ultrasonic Vocalization in the Rat

A dissertation submitted in partial satisfaction of the requirements for the degree Doctor of Philosophy

in

Psychology

by

Cara Lyn Buck

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2014
The Dissertation of Cara Lyn Buck is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

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2014
DEDICATION

This is dedicated to my family.

To my parents, Fred and Brooke Buck, who have always given me their unconditional support, encouragement, and love.

To my grandparents, Bob and Barbara Farley and Gene and JoAnn Buck, who paved my way and remain my role models.

To my sisters, Allison, Colleen, and Cailey, who always make life more fun.

And to Matt.
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**POSTERS**


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ABSTRACT OF THE DISSERTATION

Neurobehavioral Correlates of Ultrasonic Vocalization in the Rat

by

Cara Lyn Buck

Doctor of Philosophy in Psychology

University of California, San Diego, 2014

Professor George F. Koob, Chair
Professor Stephan Anagnostaras, Co-Chair

Brain emotional systems are crucial for the etiology of addiction and are a substrate for treatment, but animal models of affective states associated with addiction remain a challenge. Rat 50 kHz ultrasonic vocalizations (USVs) are elicited by appetitive and reinforcing stimuli and are thought to be linked to motivational states, whereas 22 kHz USVs are elicited by aversive stimuli and are thought to reflect a negative emotional state. The hypothesis under test in this research was that USVs provide a novel measure
of positive and negative affective states in the context of reward and stress associated with addiction.

Three studies examined 50 kHz USVs emitted in anticipation for food or alcohol and stress-induced 22 kHz USVs in rats with different levels of motivation and stress. These studies demonstrate that anticipatory 50 kHz USVs represent a stable phenotype of increased motivation for food that involves dopamine and opioid systems, and that during alcohol dependence anticipatory 50 kHz USVs also may be an indicator of context-elicited negative reinforcement learning. In addition, these studies show that alcohol withdrawal-potentiated stress-induced 22 kHz USVs reflect a state of anxiety/arousal that is not mediated by corticotropin-releasing factor.

The current research indicates that rat ultrasonic vocalization is a behavior that is sensitive to changes in emotional and motivational states that can be exaggerated in animal models of compulsive alcohol-seeking and withdrawal. Therefore, the study of USV behavior can help the understanding of motivational mechanisms underlying normal and pathological behaviors.
CHAPTER 1. GENERAL INTRODUCTION

Drug addiction is characterized by compulsive drug seeking, a loss of control in limiting drug intake, and the emergence of a negative emotional state (e.g., dysphoria, anxiety, irritability) that reflects a motivational withdrawal syndrome when access to the drug is prevented (Koob and Le Moal 1997; Koob and Le Moal 2008b). The transition from drug use to drug addiction can be defined by a shift from impulsive to compulsive drug taking (Koob and Le Moal 2008a; Koob et al. 2014). This model describes a process of hedonic dysregulation in which drug-taking transitions from positive to negative reinforcement (Koob and Le Moal 1997). This transition is driven by a cycle of three stages: 1) preoccupation/anticipation, 2) binge/intoxication, and 3) withdrawal/negative affect (Fig. 1.1). The preoccupation/anticipation stage consists of persistent craving and drug-seeking, which leads to binge/intoxication. In the binge/intoxication stage, larger and larger amounts of drug are taken as control over intake weakens. Larger and more prolonged bouts of drug-taking lead to more intense withdrawal/negative affect, and it is the emergence of this negative emotional state that is thought to contribute to the compulsivity associated with dependence through negative reinforcement (Koob and Le Moal 2008a).

Alcoholism is a severe problem in the United States and throughout the world (World Health Organization 2014). There is evidence that with long-term alcohol exposure, reward systems become downregulated, while stress systems driving withdrawal/negative affect such as the corticotropin-releasing factor (CRF) are upregulated (Koob 2008). Increased negative affect combined with decreases in reward
activation is believed to mediate compulsive drug intake through negative reinforcement (i.e., drug is taken for stress-relief purposes) during the transition to addiction.

Rats that are exposed to chronic, intermittent alcohol vapor display several physical and motivational symptoms of alcohol dependence compared with air-exposed nondependent rats (for review, see Vendruscolo and Roberts, 2014). In this model, during alcohol abstinence, rats exhibit increased anxiety-like behavior (Zhao et al. 2007), tail stiffness, abnormal gait/posture, tremor (Macey et al. 1996), hyperalgesia (Egli et al. 2012; Edwards et al. 2012), and dysphoria, reflected by elevated brain reward thresholds (Schulteis et al. 1995). Dependent rats also display escalated alcohol self-administration, increased motivation for alcohol, and persistent alcohol drinking despite punishment (Edwards et al. 2013; Vendruscolo et al. 2012). Corticotropin-releasing factor (CRF) signaling is increased in stress-related brain regions during alcohol dependence (Roberto et al. 2010), and CRF$_1$ receptor antagonists reduce compulsive-like alcohol drinking in dependent but not nondependent rats (Funk et al. 2007; Gehlert et al. 2007; Richardson et al. 2008).

Because drug and alcohol dependence is a condition of altered reward and stress functioning, animal models that measure affective and motivational states are essential for the understanding of the biological basis of the this disorder. Rat ultrasonic vocalizations (USVs) are believed to reflect positive and negative affective states. Distinct ultrasonic vocalizations have been linked to appetitive and aversive states and vocalizations are readily elicited by positive- or negative-conditioned acute or contextual stimuli. Therefore, USVs are well-suited for measuring motivational and affective states
in drug addiction. However, USVs in models of alcohol dependence have been largely unexplored.

**Fifty kHz rat ultrasonic vocalizations**

Short (30-60 ms) calls with frequencies between 35-100 kHz are categorized as 50 kHz USVs (Blanchard et al. 1993; Brudzynski 2013). There are two main types of 50 kHz USVs: frequency modulated (FM) calls and flat calls (Fig. 1.2A). Frequency modulated calls have been more clearly associated with appetitive behavior, especially trills, which are characterized by rapid frequency modulation of large bandwidth (Fig. 1.2A, “trill” and “step-trill FM”).

*Frequency-modulated 50 kHz ultrasonic vocalizations*

Frequency modulated 50 kHz vocalizations are elicited by reinforcing, appetitive stimuli including play (Burgdorf et al. 2008), tickling (Burgdorf and Panksepp 2001), social contact (Brudzynski and Pniak 2002), rewarding drugs (Ma et al. 2010), reinforcing electrical brain stimulation (Burgdorf et al. 2000), and in anticipation of mating or food (Bialey et al. 2000; Burgdorf et al. 2000). Rats also reliably approach playback of FM USVs (Seffer et al. 2014). Additionally, FM calls are suppressed by aversive stimuli such as bright light, and by cues paired with nausea (Burgdorf et al 2001b; Knutson et al. 1998).

Among FM calls, trill calls have been hypothesized to be most strongly associated with a positive affective state. Trills are increased by reinforcing psychostimulants
(Ahrens et al. 2009; Mahler et al. 2013). Also, rats have been shown to “self-administer” playback of trill calls (Burgdorf et al. 2008).

Although FM calls are hypothesized to indicate a positive affective state (Burgdorf et al. 2011), this hypothesis has been challenged by the view that FM calls more generally represent increased motivation or incentive salience that might not necessarily be linked to positive affect (Ahrens et al. 2013; Meyer et al. 2012). Indeed, not all rewarding stimuli elicit FM USVs (Simola et al. 2012; Wright et al. 2012), and FM calls are also observed in aversive conditions (Vivian and Miczek 1993; Wöhr et al. 2008). Additionally, FM USVs are closely associated with mesolimbic dopamine activity, which is involved in both positive and negative affect (Berridge and Robinson 1998).

*Flat 50 kHz ultrasonic vocalizations*

Flat 50 kHz USVs occur in a variety of contexts. They are prominent during non-agonistic conspecific contact (Blanchard et al. 1993) and conspecific contact-seeking (Schwarting et al. 2007; Wöhr et al. 2008). They also occur during agonistic encounters (Sewell 1967), and - along with FM 50 kHz USVs - during play (Knutson et al. 1998), tickling (Burgdorf et al. 2008), copulation (Burgdorf et al. 2008), and in response to reinforcing drugs (Ahrens et al. 2009; Maier et al. 2010). It has been hypothesized that they serve a social coordination function, since they are emitted when seeking contact with conspecifics (Wöhr et al. 2008). Unlike FM 50 kHz calls, there is little evidence that flat 50 kHz calls reflect a positive affective state. For example, when given the opportunity to “self-administer” playback of flat USVs, rats chose flat calls as often as
they chose background tape hiss (Burgdorf et al. 2008), suggesting that flat calls are neither appetitive nor aversive.

Neural mechanisms

A number of studies have shown that mesolimbic dopamine activity is closely related to 50 kHz USV production. Increased dopamine in the mesolimbic pathway increases 50 kHz USVs. Amphetamine injected into the nucleus accumbens, especially the shell, dose-dependently increased 50 kHz USVs, and this increase was reversible with dopamine antagonists (Burgdorf et al. 2001a; Thompson et al. 2006). An intrahypothalamic-preoptic N-methyl-D-aspartate glutamate receptor antagonist decreased 50 kHz USVs (Brudzynski and Pniak 2002). In the same area, glutamate increased 50 kHz calls and a dopamine antagonist reversed this effect (Wintink and Brudzynski 2001).

Both D<sub>1</sub> and D<sub>2</sub> receptor agonists facilitate 50 kHz USVs, and either D<sub>1</sub> or D<sub>2</sub> receptor antagonists can block cocaine-induced USVs (Williams and Undieh 2010). Both a D<sub>1/D</sub> receptor antagonist and lesions of dopaminergic reward areas (i.e., the ventral tegmental area [VTA] and the medial forebrain bundle) decreased tickle-induced FM, but not flat 50 kHz USVs (Burgdorf et al. 2007). Additionally, rats with disrupted dopamine neurons in the medial forebrain bundle emitted fewer trills and more 50 kHz flat calls compared with controls (Ciucci et al. 2009).

Opioids also modulate 50 kHz USV production. Naloxone decreased tickle-induced USVs in single-housed rats and increased them in group-housed rats (Burgdorf and Panksepp 2001). In conditioned place preference test, only rats that had increased 50
kHz calls to a µ-opioid agonist microinjection in the VTA displayed later place preference for the opioid-paired chamber (Burgdorf et al. 2007).

**Fifty kHz ultrasonic vocalizations and drugs of abuse**

Most studies that have investigated the relationship between 50 kHz USVs and drugs of abuse have focused on psychostimulants. Acute amphetamine, methamphetamine, and cocaine injections elicit 50 kHz USVs (Mahler et al. 2013; Natusch and Schwarting 2010; Williams and Undieh 2010; Wintink and Brudzynski 2001), and trill USVs are dose-dependently increased by amphetamine (Wright et al. 2010). Fifty kHz USVs also sensitize with repeated psychostimulant exposure. Sensitization of 50 kHz USVs has been observed with repeated cocaine administration (Browning et al. 2011; Barker et al. 2013; Mu et al. 2009), and trill USVs sensitize with repeat amphetamine injections (Ahrens et al. 2009). Acute alcohol has been shown to slightly increase 50 kHz USVs at a low dose (Willey and Spear 2014).

Cues associated with amphetamine or morphine also elicit 50 kHz USVs (Ahrens et al. 2013; Hamed et al. 2012; Knutson et al. 1999; Simola et al. 2014), and amphetamine-anticipatory 50 kHz USVs sensitize with repeated exposure (Ma et al. 2010).

Finally, individual differences in drug intake and drug-conditioned preference behavior have been linked to differences in 50 kHz USVs (Ahrens et al. 2013; Taracha et al. 2012; Taracha et al. 2014). High- and low-50 kHz USV groups were separated based on rats’ initial USV response to amphetamine. Compared with low-USV rats, high-USV rats showed increased amphetamine-induced and anticipatory 50 kHz USVs along with
increased conditioned place-preference for amphetamine (Ahrens et al. 2013; Taracha et al. 2014). Also, increased 50 kHz USVs to a first-exposure of cocaine predicted faster acquisition of cocaine self-administration (Browning et al. 2011).

**Twenty-two kHz ultrasonic vocalizations**

Calls with peak frequency between 18-32 kHz are categorized as 22 kHz USVs. They are generally long (300-3000 ms) with little or no frequency modulation (Fig. 1.2B). Long 22 kHz USVs have been termed “alarm calls” because they are emitted when rats are threatened, but are not in immediate danger (Blanchard et al. 1991). Rats also emit long 22 kHz calls when attacked and defeated by an aggressive conspecific. Additionally, long 22 kHz USVs are produced by male rats during the refractory period following ejaculation, and are associated with behavioral inhibition (Anisko et al., 1978; Barfield and Geyer, 1972). Rats also emit short (< 300 ms) 22 kHz USVs (Barker et al. 2010; Brudzynski et al. 1993; Coffey et al. 2013; Mahler et al. 2013), though little is known about these calls.

Long 22 kHz USVs are elicited by a potential danger and are thought to indicate a state of intense stress, anxiety and/or fear (Wright and Panksepp 2011). These USVs are induced by predator threat (Blanchard et al. 1991; Blanchard et al. 1992), agonistic encounters (Takahashi et al. 1983; Vivian and Miczek 1993), shocks (Burgdorf et al. 2000; Kaltwasser 1991; Portavella et al. 1993; van der Poel and Miczek 1991), acoustic startle (Kalinichev and Holtzman 2003; Vivian et al. 1994), tactile startle (Barros and Miczek 1996; Pohorecky 2008; Vivian and Miczek 1999), fear-conditioned stimuli (Burgdorf et al. 2000; Molewijk et al. 1995), and intracerebral carbachol injections (Fu
Drug withdrawal and unexpected reward cessation increase distress calls, while acute opioids and anxiolytic drugs decrease them (Barros and Miczek 1996; Burgdorf et al. 2000; Kassai and Gyertyan 2012; Mutschler and Miczek 1998; Vivian and Miczek 1991; Vivian et al. 1994; Williams et al. 2012). Rats also exhibit increased fear/anxiety behaviors (crouched posture, decreased locomotor activity, and increased freezing) along with 22 kHz USVs (Fu and Brudzynski 1994).

Twenty-two kHz vocalizations are increased by distal threats but decreased by proximal threats (Blanchard et al. 1991). During a proximal threat (e.g., direct exposure to a predator; a series of shocks) rats do not emit USVs. Vocalizations begin only when the threat is no longer immediate (e.g., from the safety of the burrow; after shocks have stopped), and stop if the threat returns (Blanchard et al. 1991; Cuomo et al. 1992; Portavella et al. 1993). In sessions of intermittent shocks or startle, rats only call during interstimulus intervals, and the calls often do not begin until after the first 2-4 stimuli (Portavella et al. 1993; Schwarting et al. 2007; van der Poel and Miczek 1991). Additionally, in a conditioned fear paradigm, USVs stopped at the onset of a shock cue, but resumed at the onset of a no-shock cue (Jelen et al. 2003). Therefore, 22 kHz USVs are increased by fear/anxiety, but are suppressed at very high levels of fear/anxiety.

**Neural mechanisms**

Activation of laterodorsal tegmental nucleus (LDT) neurons induces 22 kHz USVs and an intense defensive response (Bihari et al. 2003). The LDT medial projection regions from the anterior hypothalamus to the septum have been referred to as the "medial cholinceptive vocalization strip" (Brudzynski 2001). It has been hypothesized
that production of 22 kHz USVs is caused by activation of the ascending mediobasal forebrain cholinergic pathway (Brudzynski 2001). Carbachol injections into the basal forebrain, septum, diencephalic areas, and anterior hypothalamic-preoptic area of the brain induce 22 kHz USVs and defensive behavior, which match naturally-produced USVs and behavior (Brudzynski 2001; Fu and Brudzynski 1994). Twenty-two kHz USVs are also induced by electrical stimulation of the dorsal periaqueductal gray (dPAG), ventral periaqueductal gray (vPAG), and basolateral amygdala (Kim et al. 2013; Wright and Panksepp 2011).

Stress-induced 22 kHz USVs are reduced by the systemic administration of a number of anxiolytic drugs including benzodiazepines, serotonin agonists, serotonin reuptake inhibitors, opioids, and antipsychotic drugs (De Vry et al. 1993; Jelen et al. 2003; Kassai and Gyertyan 2012; Kikusui et al. 2001; Sun et al. 2010; Tonoue et al. 1986). Of these, benzodiazepines and serotonin agonists reliably and dose-dependently reduce 22 kHz USVs (Kassai and Gyertyan 2012; Vivian et al. 1994).

Stress-induced 22 kHz USVs are increased by CRF infusion into the central nucleus of the amygdala (Ji et al. 2013), however, stress-induced USVs are not affected by CRF$_1$ receptor antagonists (Chen et al. 2014; Kikusui et al. 2000). Interestingly, CRF$_1$ receptor antagonists do reduce context-conditioned USVs (Chen et al. 2014; Kikusui et al. 2000), and this result has led some to hypothesize that CRF mediates the retrieval of fear-conditioned memory (Kikusui et al. 2000).

Twenty-two kHz ultrasonic vocalizations and drug withdrawal
Stress-induced 22 kHz USVs are increased during withdrawal from drugs of abuse including alcohol (Berger et al. 2013; Moy et al. 2000; Williams et al. 2012), benzodiazepines (Vivian et al. 1994), heroin (Williams et al. 2012), and morphine (Vivian and Miczek 1991). Williams et al. (2012) observed a positive correlation between somatic withdrawal scores and stress-induced USVs during spontaneous heroin withdrawal, and showed increased USVs during withdrawal from higher doses of heroin. These results support the hypothesis that 22 kHz USVs reflect an aversive state that is increased during withdrawal from drugs of abuse (Covington and Miczek 2003; Vivian et al. 1994).

Anxiolytic drugs also reduce withdrawal-potentiated stress-induced 22 kHz USVs. During withdrawal from diazepam, both diazepam and a serotonin partial agonist, gepirone, reduced startle-induced 22 kHz (Vivian et al. 1994). In rats made dependent on alcohol by chronic intermittent alcohol vapor, airpuff-stress-induced 22 kHz were reduced by intracerebroventricular (i.c.v.) infusion of a κ-opioid receptor antagonist (Berger et al. 2013). Because κ-opioid receptor activity is thought to contribute to negative affective states associated with alcohol withdrawal (Berger et al 2013; Koob et al. 2014), this further suggests that withdrawal-potentiated 22 kHz USVs reflect an increased negative affective state.

**Current aims**

Rat 50 kHz USVs are elicited by appetitive, reinforcing stimuli and are thought to be linked to motivational states, while 22 kHz USVs are elicited by aversive stimuli and are thought to reflect a state of anxiety or fear. In Chapter 2, food-cue-induced
anticipatory 50 kHz USVs were measured in food-restricted rats over the course of repeated sessions. Additionally, we tested the effects of D₁ and μ-opioid receptor antagonists on these calls. In Chapter 3, alcohol-cue-induced anticipatory USVs were measured in alcohol-dependent and –nondependent rats over the course of alcohol vapor (or air) exposure. In Chapter 4, we explored the relationship between stress-induced 22 kHz USVs and other measures of anxiety and depression. We also tested the effects of a benzodiazepine and of two CRF₁ receptor antagonists on stress-induced 22 kHz USVs in alcohol-dependent and –nondependent rats during alcohol withdrawal.

The current studies aim to address the following questions:

1. Do conditioned, anticipatory 50 kHz USVs reflect motivational state?
2. Do opioids and/or dopamine modulate anticipatory 50 kHz USVs?
3. Do alcohol self-administration anticipatory 50 kHz USVs differ between dependent and nondependent rats, given their differences in compulsive-like alcohol intake? What is the relationship between anticipatory 50 kHz USVs and alcohol self-administration?
4. How do stress-induced 22 kHz USVs relate to other measures of anxiety and/or depression?
5. Are stress-induced 22 kHz mediated by corticotropin-releasing factor in alcohol-dependent and/or –nondependent rats?

Specific predictions were the following:
1. For Chapter 2, it was hypothesized that anticipatory 50 kHz USVs for palatable food would sensitize over repeated sessions. Because other studies have observed changes in motivation behavior with dopamine but not with opioid antagonism (Barbano et al. 2009), it was hypothesized that $D_1$ receptor antagonism would suppress anticipatory 50 kHz USVs while $\mu$-opioid receptor antagonism would not.

2. For Chapter 3, it was hypothesized that anticipatory 50 kHz USVs for alcohol self-administration would initially escalate (compared to nondependent rats) suggesting a initial sensitization of reward systems and then decrease back to baseline or lower over the course of alcohol addiction indicating a disruption of reward systems.

3. For Chapter 4, it was hypothesized that stress-induced 22 kHz USVs would increase in alcohol-dependent rats during withdrawal (compared to nondependent rats). Benzodiazepines and CRF$_1$ receptor antagonism have been previously shown to reduce compulsive-like alcohol drinking in dependent rats (Funk et al. 2007; Lejoyeux et al. 1998). Therefore, we hypothesized that a benzodiazepine and CRF$_1$ receptor antagonism would suppress withdrawal-potiated 22 kHz USVs.
Figure 1.1  The three stages of the addiction cycle: preoccupation-anticipation, binge-intoxication, and withdrawal/negative affect. The figure is from Koob and Le Moal (1997).
Figure 1.2 Classification of 50 and 22 kHz USVs. Representative spectrograms are shown for each call subtype. (A) Frequency-modulated 50 kHz USVs (trill, step-trill, step, and other) and flat 50 kHz USVs. (B) Long and short 22 kHz USVs. The figure is from Buck et al. (2014).
CHAPTER 2. DOPAMINE D₁ AND µ-OPIOID RECEPTOR ANTAGONISM BLOCKS ANTICIPATORY 50 KHZ ULTRASONIC VOCALIZATIONS INDUCED BY PALATABLE FOOD CUES IN WISTAR RATS

INTRODUCTION

Ultrasonic vocalizations (USVs) are thought to reflect positive and negative affective-like states in rats (Brudzynski 2013; Covington and Miczek 2003; Knutson et al. 2002). Frequency-modulated (FM) 50 kHz USVs, particularly calls with rapid-frequency oscillations (“trills”), have been associated with positive emotional states (Burgdorf et al. 2011). Frequency-modulated calls are elicited by reinforcing, appetitive stimuli, such as play (Burgdorf et al. 2008), social contact (Brudzynski and Pniak 2002), tickling (Burgdorf and Panksepp 2001), and psychostimulant drug reward (Ahrens et al. 2009). However, not all rewarding stimuli elicit FM USVs (Simola et al. 2012; Wright et al. 2012), and FM calls have been observed in aversive conditions (Vivian and Miczek 1993; Wöhr et al. 2008). Nevertheless, FM USVs and, more generally, 50 kHz USVs may be an index of motivation during reward anticipation (Ahrens et al. 2009; Ma et al. 2010; Mahler et al. 2013).

Previous reports have also shown that 50 kHz USVs are elicited by cues predictive of rewarding stimuli, such as food (Burgdorf et al. 2000), copulation (Bialy et al. 2000), cocaine (Ma et al. 2010), and rewarding brain stimulation (Burgdorf et al. 2000). However, little is known about the specific call subtypes and the pharmacological mechanisms involved in cue-induced anticipatory USVs.
μ-Opioid receptor antagonism has been suggested to regulate feeding behavior by interfering with the perceived palatability of food, whereas the dopamine system has been suggested to be more involved in the motivation to obtain food (De Tomasi and Juárez 2010; Barbano et al. 2009). Dopamine D₁ receptors, in particular, have been shown to play a role in both anticipatory behavior and food-seeking behavior (Ball et al. 2011; Grimm et al. 2011). We hypothesized that D₁ receptor antagonism but not μ-opioid receptor antagonism attenuates anticipatory USVs. Here, we measured anticipatory 50 kHz USVs in food-restricted rats given intermittent (every 24-48 h) and limited (5 min) access to a highly palatable food for 6 weeks. We then tested the effects of the D₁ receptor antagonist SCH 23390 and μ-opioid receptor antagonist naltrexone on anticipatory USVs and food intake.

MATERIALS AND METHODS

Animals

A total of 16 adult male Wistar rats, weighing 270-380 g at the beginning of the experiment, were used. The rats were housed in groups of two to three in plastic cages in a temperature-controlled (21°C) vivarium on a 12 h/12 h light/dark cycle (lights on at 6:00 AM). During behavioral testing, the animals received 15 g chow (7012 Teklad LM-485, Harlan Laboratories) per day, which was provided 10-60 min after testing. With this food restriction procedure, the rats’ body weights were approximately 80% of their free-feeding body weights. Water was provided ad libitum in the home cages. All of the behavioral tests were conducted during the dark cycle between 6:00 PM and 10:00 PM. All of the procedures adhered to the National Institutes of Health Guide for the Care and
Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee of The Scripps Research Institute.

**Drugs**

Naltrexone and R(+)\(\text{SCH} 23390\) hydrochloride (expressed as salt) were purchased from Sigma (St. Louis, MO, USA). Naltrexone is a preferential nonselective competitive opioid receptor antagonist with higher affinity for \(\mu\)-opioid receptors compared with \(\kappa\)- and \(\delta\)-opioid receptors. \(\text{SCH} 23390\) is a selective dopamine \(D_1\) receptor antagonist. Naltrexone (0.03, 0.06, 0.13, 0.25, 0.5, and 1 mg/kg) and \(\text{SCH} 23390\) (5, 10, and 20 \(\mu\)g/kg) were dissolved in saline and injected subcutaneously in a volume of 1 ml/kg using a within-subjects Latin-square design.

**Experimental procedures**

*Cue-food paired.* Before testing, the rats \((n = 8)\) received 1 week of daily access to the palatable food (Purina TestDiet 1811443 [5 TUL] AIN-76A Rodent tablet chocolate, 45 mg) to prevent neophobia. To minimize novelty-induced USVs, the rats were habituated to the testing environment for several days before beginning the experiment and were always tested in the same cage, and bedding was not changed between sessions. Four rats were tested at a time in separate 22.5 cm × 45 cm × 20 cm plastic cages with wire lids and bedding. Sound-attenuating barriers were placed between adjacent rats. Twenty-four test sessions were conducted 3-7 days per week over the course of 6 weeks. In each session, the rats were placed in the testing cages in a dark room for a variable interval (15-45 min) to prevent cue prediction. A dim light (20 lux) was then presented for 2 min.
Immediately following the light cue, a hopper that contained 20 g of the palatable food pellets was placed in the cage, and the rats were allowed to feed freely for 5 min.

For pharmacological testing sessions, the rats were given saline, SCH 23390 (5-20 µg/kg), or naltrexone (0.03-1 mg/kg) subcutaneously 30 min before recording USVs. The rats were first tested with SCH 23390 (0, 5, 10, and 20 µg/kg) and then tested with naltrexone (0, 0.25, 0.5, and 1 mg/kg). The rats were then allowed chow ad libitum in their home cages for 3 weeks. Because all of the doses of naltrexone decreased the number of USVs, additional lower doses were required to cover the full dose-response. Rats were food restricted for 1 week before the final naltrexone treatments (0, 0.03, 0.06, and 0.13 mg/kg).

Cue only: A separate group of rats (n = 8) was used. All of the procedures were the same as described above, except for the following changes: after the cue light, the rats did not receive palatable food and were returned to their home cages. The rats were tested for 21 sessions, and pharmacological tests were not performed with this group.

Ultrasonic vocalization recording and analysis

Condenser microphones (CM16/CMPA, 10-200 kHz frequency range, Avisoft Bioacoustics, Berlin, Germany) were positioned 5 cm above the wire lids of the testing cages and coupled to an UltraSoundGate 816H data acquisition device (250 kHz sampling rate, 16-bit resolution, Avisoft Bioacoustics). Ultrasonic vocalizations between 10 and 100 kHz were recorded and analyzed using Avisoft SASLab Pro (version 5.1, Avisoft Bioacoustics). Spectrograms were generated with a fast Fourier transform length
of 512 points and overlap of 50% (FlatTop window, 100% frame size), providing a frequency resolution of 419 Hz and time resolution of 1.19 ms. An observer blind to experimental condition classified USVs into seven categories adapted from Brudzynski (2013): trill FM, step-trill FM, step FM, other FM, flat, long 22 kHz, and short 22 kHz.

Ultrasonic vocalization classification

Examples of each USV type are shown in Fig. 2.1. Calls with a mean peak frequency between 30 and 90 kHz were classified into one of five 50 kHz USV categories: trill FM, step-trill FM, step FM, flat, or other FM (Fig. 2.1A). Trill FM and step-trill FM calls were defined as calls with a bandwidth of at least 10 kHz, containing at least one cycle of rapid (about 15 ms) frequency modulation in an inverted-U or sinusoidal pattern. Step-trill FM calls contained an additional low bandwidth (≤ 10 kHz) component. Step FM calls contained at least one frequency jump and monotonic components. Flat calls were monotonic calls with a bandwidth no greater than 5 kHz and duration of at least 10 ms. Other FM calls included 50 kHz USVs that did not meet the requirements for the previous four categories. This category included complex, upward ramp, downward ramp, short, multi-step, and inverted-U subtypes, based on Wright et al. (2010). Calls with a mean peak frequency between 18 and 30 kHz and a flat appearance (bandwidth ≤ 10 kHz) were classified into one of two 22 kHz USV categories: long and short (Fig. 2.1B). Twenty-two kilohertz USVs with a duration greater than 300 ms were considered long calls, whereas 22 kHz USVs with a duration less than 300 ms were considered short calls.
Statistical analyses

The data are expressed as the mean and standard error of the mean (SEM). Prior to any comparisons, all of the dependent variables were subjected to Shapiro-Wilk’s $W$-test of normality. Because the Shapiro-Wilk’s $W$-test of normality was significant for the anticipatory 50 kHz USV counts, the data were square-root-transformed before statistical analysis. Ultrasonic vocalization counts and food intake over 24 sessions were analyzed by one-way repeated-measures analysis of variance (ANOVA), followed by a test for linear trend and the Dunnett post hoc test. Within- and between-subjects coefficients of variation were calculated during the escalation period (sessions 4-24) and compared using Student’s two-tailed $t$-test. Because data transformation did not successfully normalize USV subtype data, nonparametric Wilcoxon tests were used for statistical comparisons. When appropriate, Pearson’s correlations were used. Statistical analyses were performed with Statistica version 10 (StatSoft, Inc.), except for linear trend tests, which were performed with Prism 5 (GraphPad Software, Inc.). For all of the tests, two-tailed values of $p < 0.05$ were considered statistically significant.

RESULTS

Escalation of food intake and 50 kHz ultrasonic vocalizations

Fig. 2.2 shows palatable food intake (in g/kg) across 24 sessions (Fig. 2.2A) and 50 kHz USVs during the anticipation of palatable food per session across 24 sessions (Fig. 2.2B). A one-way repeated-measures ANOVA showed a significant effect of session on palatable food intake ($F_{23,161} = 13.2, p < 0.001$). A linear-trend post hoc test indicated a significant increase in palatable food intake across sessions (slope = 0.085, $R^2$...
= 0.38, p < 0.001). Dunnett’s post hoc comparisons indicated significant increases from session 4 onward (p < 0.001) compared with session 1. The ANOVA revealed a significant effect of session on anticipatory USVs (F_{23,161} = 5.0, p < 0.001). A linear-trend post hoc test indicated a significant increase in USVs across sessions (slope = 0.099, R^2 = 0.14, p < 0.001). Dunnett’s post hoc comparisons indicated significant increases in session 4 onward (p < 0.05), with the exception of sessions 5 and 15 compared with session 1. To confirm that the escalation of USVs was not caused by light exposure alone, the rats were exposed to light only (i.e., no food and light pairings). Although a one-way repeated-measures ANOVA showed a significant effect of session on USVs (F_{21,147} = 2.2, p < 0.01), the linear-trend post hoc test was not statistically significant (slope = 0.009, R^2 = 0.002, p > 0.5). Additionally, Dunnett’s post hoc test did not indicate any sessions that were significantly different from session 1 (Fig. 2.2C).

To test whether the number of anticipatory 50 kHz USVs represents a stable phenotype with reliable interindividual differences, we compared the within- and between-subjects coefficients of variation for anticipatory USVs during escalated sessions (sessions 4-24). Fig. 2.3A shows the number of USVs in each session (session X) vs. the number of USVs in the subsequent session (session X + 1) for each rat. The rats exhibited high interindividual differences, reflected by the higher variability between subjects than within subjects (t_{27} = -3.2, p < 0.01; Fig. 2.3B). Moreover, the number of USVs before escalation (sessions 1-2) was significantly correlated (r = 0.73, p < 0.05) with the number of USVs after escalation (sessions 4-24; Fig. 2.3C).

Fig. 2.4 shows correlations between anticipatory 50 kHz USVs and palatable food intake. Across rats, the averages (sessions 1-24) of 50 kHz USVs were positively
correlated with averages (sessions 1-24) of palatable food intake \( (r = 0.74, p < 0.05, \text{Fig. 2.4A}) \). For five of the eight rats, within-session 50 kHz USV production was positively correlated with subsequent palatable food intake (rat 1: \( r = 0.48, p < 0.05 \); rat 2: \( r = 0.60, p < 0.01 \); rat 4: \( r = 0.57, p < 0.01 \); rat 5: \( r = 0.89, p < 0.001 \); rat 6: \( r = 0.68, p < 0.001 \); Fig. 2.4B). Of the remaining rats, one rat showed a nonsignificant positive trend (rat 3, \( r = 0.40, p = 0.05 \)), and two rats did not show a relationship (rat 7: \( r = 0.10, p > 0.05 \); rat 8: \( r = 0.31, p > 0.05 \)) between within-session 50 kHz USVs and palatable food intake (Fig. 2.4B).

To test whether the escalation of food intake was associated with the escalation of particular subtypes of USVs, anticipatory 50 kHz USVs were classified into five subtypes (trill FM, step-trill FM, step FM, flat, and other FM) for sessions 1, 4, 11, 18, and 24 (Fig. 2.5). Wilcoxon comparisons indicated that the numbers of both other FM and step FM calls were significantly increased in sessions 4, 11, 18, and 24 compared with session 1 (\( p < 0.05 \)). Flat calls were significantly increased in sessions 4, 11, and 24 compared with session 1, and flat calls were significantly reduced in session 18 compared with sessions 4 and 24 (\( p < 0.05 \)). No significant differences were found for trill FM and step-trill FM calls. The 22 kHz USVs were infrequent during the escalation of food intake. Five of the eight animals occasionally emitted short 22 kHz USVs, whereas two rats occasionally emitted long 22 kHz USVs. Wilcoxon comparisons did not show significant differences between sessions 1, 4, 11, 18, and 24 for both short and long 22 kHz USVs, but there was a trend toward an increase in 22 kHz USVs in session 24 (Fig. 2.5A, inset).

A one-way repeated-measures ANOVA revealed a significant effect of SCH 23390 on the number of 50 kHz USVs \( (F_{3,21} = 6.0, p < 0.01, \text{Fig. 2.6A}) \). Dunnett’s post
hoc test indicated that 20 µg/kg SCH 23390 significantly decreased 50 kHz USVs compared with saline ($p < 0.01$), and this decrease was significant within each 50 kHz subtype (Wilcoxon comparisons, $p < 0.05$; Fig. 2.6C). For naltrexone, a repeated-measures ANOVA revealed a significant effect on the number of 50 kHz USVs ($F_{6,42} = 9.1, p < 0.001$; Fig. 2.6B). Dunnett’s post hoc test indicated that naltrexone significantly decreased 50 kHz USVs at all doses tested, with the exception of 0.06 mg/kg, compared with saline ($p < 0.05$). Considering that these data were obtained using two Latin-square designs, we first ruled out any difference in USVs in the two saline groups. A paired $t$-test showed no significant difference between USVs for the two saline tests ($t_{7} = 1.0, p > 0.05$); therefore, these two values were averaged. We then analyzed the same set of data using separate repeated-measures ANOVAs. The ANOVA of low doses (0.03-0.13 mg/kg) revealed an effect of naltrexone on the number of USVs ($F_{3,21} = 8.1, p < 0.001$). Dunnett’s post hoc comparisons indicated that naltrexone significantly decreased 50 kHz USVs at 0.03 and 0.13 mg/kg compared with saline ($p < 0.05$). Similarly, the ANOVA of high doses (0.25-1 mg/kg) revealed a significant effect of naltrexone on the number of USVs ($F_{3,21} = 13.5, p < 0.001$). Dunnett’s post hoc comparisons indicated that naltrexone significantly decreased 50 kHz USVs at all three doses. For 50 kHz USV subtypes, Wilcoxon comparisons between saline and 0.13 mg/kg naltrexone revealed a significant decrease in other FM and flat calls ($p < 0.05$). Palatable food intake during SCH 23390 and naltrexone testing is presented as the percent change from baseline food intake (i.e., the last session before pharmacological testing began) to take into account any change in bodyweight between tests (Fig. 2.6E, F). A repeated-measures ANOVA showed no effect of SCH 23390 on the percent change in palatable food intake ($F_{3,21} = 2.1, p > 0.05$; Fig.
2.6E). For naltrexone, a paired t-test showed no significant difference between changes in food intake for the two saline tests ($t_7 = 0.4, p > 0.05$); therefore, these two values were averaged. A repeated-measures ANOVA of the average saline food intake data and food intake data for all six naltrexone doses did not show an effect of naltrexone on palatable food intake ($F_{6,42} = 0.7, p > 0.05$). The naltrexone data were also analyzed in separate repeated-measures ANOVAs. No significant effect of naltrexone (0.03-0.13 mg/kg) was found on the percent change in palatable food intake ($F_{3,21} = 0.4, p > 0.05$). However, a significant effect of naltrexone (0.25-1 mg/kg) was found on the percent change in food intake ($F_{3,21} = 5.7, p < 0.01$). Dunnett’s post hoc tests indicated that naltrexone significantly decreased food intake at 0.5 and 1 mg/kg compared with saline ($p < 0.01$).

**DISCUSSION**

The present study found that rats emitted anticipatory 50 kHz USVs during the presentation of a cue predictive of palatable food, with robust and stable interindividual differences. The escalation of palatable food intake was associated with the escalation of anticipatory 50 kHz USVs, particularly flat and non-trill FM USVs. Moreover, dopamine D$_1$ and µ-opioid receptor antagonism dose-dependently reduced anticipatory 50 kHz USVs.

The escalation of cue-induced 50 kHz USVs has been observed during the anticipation of various appetitive stimuli, including social interaction (Brudzynski and Pniak 2002; Willey and Spear 2012), copulation (Bialy et al. 2000), cocaine (Ma et al. 2010), and electrical brain stimulation (Burgdorf et al. 2000). However, these effects can be age-dependent, such that adolescent rats do not show escalation of 50 kHz USVs in
anticipation of social interaction (Willey and Spear 2012). The present study showed that adult animals progressively increased the number of 50 kHz USVs emitted during food anticipation over a period of several weeks. Burgdorf et al. (2000) reported similar results, showing escalation of food cue-induced 50 kHz USVs over six sessions. The present study expands these observations by demonstrating that anticipatory 50 kHz USVs have high interindividual differences and low intraindividual variability. Moreover, the initial amount of anticipatory 50 kHz USVs predicted the level of increase in 50 kHz USVs after the escalation of food intake. Furthermore, anticipatory USVs were positively correlated with palatable food intake. Notably, presentation of the cue only (i.e., no food and light pairings) failed to produce escalation of 50 kHz USVs across sessions.

The escalation of food cue-induced 50 kHz USVs was mainly driven by flat, step FM, and other FM calls. All subtypes of 50 kHz USVs tended to increase over time. However, only flat, step FM, and other FM calls reached statistical significance, whereas a nonsignificant trend toward an increase was observed for trill FM and step-trill FM calls. Trill calls have been sometimes linked to appetitive or positive emotional states in rats (Ahrens et al. 2009; Burgdorf et al. 2007, 2008). All of the rats exhibited trill FM and/or step-trill FM calls during the sessions in which USV types were analyzed (sessions 1, 4, 11, 18, and 24). However, we did not observe a significant increase in trill calls over the course of USV escalation. This might have been attributable to a lack of statistical power. Alternatively, the anticipation of food intake may not be linked to positive affect, given that trill calls have been reported not to be exclusively linked to positive affect (Simola et al. 2012; Wright et al. 2012; Vivian and Miczek 1993).
Consistent with this hypothesis, five of the eight animals emitted 22 kHz USVs, and a nonsignificant trend toward an increase in the number of 22 kHz USVs was observed across sessions (more in sessions 11, 18, and 24 than in sessions 1 and 4). Twenty-two kHz USVs have been reliably observed in stressful/aversive situations (Covington and Miczek 2003; Litvin et al. 2007; Miczek et al. 1995). Step FM and other FM calls were also increased during food anticipation. However, the acoustic pattern of these calls is heterogeneous, and their biological significance is unclear (Brudzynski 2013; Haney and Miczek 1994; Mahler et al. 2013; Thomas et al. 1983). Altogether, the present findings suggest that cue-induced anticipatory 50 kHz USVs, at least under the present experimental conditions, may be driven by aspects of positive, neutral, or even negative affect.

Dopamine D_1 and µ-opioid receptor antagonism dose-dependently reduced anticipatory 50 kHz USVs produced by cue presentation at doses that did not affect food intake. Only the highest doses of naltrexone (0.5 and 1 mg/kg) may have slightly reduced food intake. These results are consistent with previous studies that reported that D_1 receptor antagonism decreased both anticipatory behavior and food seeking but not food intake (Barbano and Cador 2006; Barbano et al. 2009; Ball et al. 2011; Grimm et al. 2011), whereas µ-opioid receptor antagonism had mixed results (Barbano and Cador 2006; Barbano et al. 2009). Our results demonstrate that the D_1- and µ-opioid systems modulate anticipatory 50 kHz USVs in situations of heightened motivation for palatable food at doses that minimally affect food intake.

Previous studies reported the effects of µ-opioid and dopamine receptors on 50 kHz USVs (Scardochio and Clarke 2013; Wöhr and Schwarting 2009). Naloxone
suppressed 50 kHz USVs and reduced approach behavior in response to the playback of 50 kHz USVs (Wöhr and Schwarting 2009). High doses of naloxone reduced 50 kHz USVs and increased 22 kHz USVs (Burgdorf et al. 2001b). Other studies reported a role for opioids in the number or characteristics of 50 kHz USVs (Hamed et al. 2012; Simola et al. 2012; Vivian and Miczek 1993). Dopamine administration in the nucleus accumbens (Burgdorf et al. 2001a) and indirect dopamine agonism by psychostimulants (Burgdorf et al. 2001a; Brudzynski et al. 2012; Thompson et al. 2006; Williams and Undieh 2010; Wintink and Brudzynski 2001) have been shown to increase 50 kHz USVs. However, both dopamine agonism and antagonism have been found to reduce 50 kHz USVs (Burgdorf et al. 2007; Scardochio and Clarke 2013; Thompson et al. 2006; Williams and Undieh 2010; Wright et al. 2013). Trill calls and FM calls have been shown to be sensitive to dopaminergic modulation, whereas flat calls have been shown to be somewhat resistant to manipulation of this system (Burgdorf et al. 2007; Wright et al. 2013). In contrast, our results showed that D1 receptor antagonism reduced all 50 kHz subtypes, including flat calls. We also found that anticipatory trill FM, step-trill FM, and step FM calls were more resistant to a µ-opioid receptor antagonist than other FM calls and flat calls, suggesting that the opioid systems may be differentially involved in the generation of different subtypes of 50 KHz USVs during food anticipation. However, increasing the power of the study could reveal a significant effect on these subtypes because we observed a nonsignificant trend toward a decrease in trill FM, step-trill FM, and step FM calls after naltrexone.

In summary, rats emitted 50 KHz USVs during the presentation of a cue predictive of palatable food, with robust and stable interindividual differences, and the
escalation of palatable food intake was related to the escalation of anticipatory 50 kHz USVs, particularly flat, step FM, and other FM 50 kHz USVs but not trill FM or step-trill FM 50 kHz USVs. Anticipatory 50 kHz USVs were dose-dependently reduced by the administration of dopamine D₁ and μ-opioid receptor antagonists, independent of changes in food intake. These results indicate that food cue-induced anticipatory 50 kHz USVs represent a stable phenotype that is mediated by the dopamine and opioid systems.

Acknowledgements

Chapter 2, in full, is a reprint of the material as it appears in Psychopharmacology 2014. Buck, Cara L.; George, Olivier; Vendruscolo, Leandro F.; Koob, George F., Springer-Verlag Berlin Heidelberg 2014. The dissertation author was the primary investigator and author of this paper.
Figure 2.1 Classification of 50 and 22 kHz USVs. Representative spectrograms are shown for each call subtype. (A) Frequency-modulated (FM) 50 kHz USVs (trill, step-trill, step, and other) and flat 50 kHz USVs. (B) Long and short 22 kHz USVs.
Figure 2.2 (A) Escalation of palatable food intake (g/kg/session) and (B) number of 50 kHz USVs during 2 min food-cue presentation in rats that received cue-food pairings. $n = 8$. *$p < 0.05$, different from session 1. (C) Number of 50 kHz USVs during 2 min cue presentation in rats that did not receive cue-food pairings. $n = 8$. 
Figure 2.3 (A) Interindividual differences in cue-induced 50 kHz USVs between sessions after the escalation of food intake (sessions 4-24). Each data point represents one subject. The x-axis shows USVs for each session (X), and the y-axis shows USVs for each subsequent session (X + 1). (B) Coefficients of variation within and between subjects during sessions 4-24. *p < 0.05, significantly different from within-subject (within-subject, n = 8; between-subject, n = 21). (C) 50 kHz USVs emitted during the first 2 sessions versus 50 kHz USVs emitted after the escalation of food intake (sessions 4-24). Each data point represents one subject.
Figure 2.4 (A) Average number of food cue-induced 50 kHz USVs (2 min) vs. average palatable food intake (g/kg) over all sessions (1-24). Each data point represents a single rat, labeled by subject number. (B) Number of cue-induced 50 kHz USVs (2 min) vs. palatable food intake (g/kg) for each session (n = 24), shown for all rats (1-8).
Figure 2.5 Subtypes of USVs in sessions 1, 4, 11, 18, and 24. (A) The number of trill FM (frequency modulated), step-trill FM, other FM, step FM, and flat 50 kHz USVs for each of the five sessions. The inset shows the number of short and long 22 kHz USVs emitted during the 5 sessions. *p < 0.05, different from session 1 within each subtype; †p < 0.05, different from sessions 4 and 24 within flat USVs (n = 8). (B) Distribution of percentage of different subtypes of 50 kHz USVs for each of the 5 sessions.
Figure 2.6 Effects of D₁ and µ-opioid receptor antagonism on cue-induced 50 kHz USVs during the anticipation of palatable food in food-restricted rats. (A) Number of cue-induced 50 kHz USVs (2 min) for each dose (0, 5, 10, and 20 µg/kg, subcutaneous) of the D₁ receptor antagonist SCH 23390. (B) Number of cue-induced 50 kHz USVs (2 min) for each dose (0, 0.03, 0.06, 0.25, 0.5, and 1 mg/kg, subcutaneous) of the µ-opioid receptor antagonist naltrexone. (C) Number of trill FM (frequency modulated), step-trill FM, other FM, step FM, and flat 50 kHz USVs for 0 and 20 µg/kg SCH 23390. (D) Number of trill FM, step-trill FM, other FM, step FM, and flat 50 kHz USVs for 0 and 0.13 mg/kg naltrexone. *p < 0.05, significantly different from the respective subtype after vehicle administration. (E) Percent change in palatable food intake compared with baseline palatable food intake (g/kg) for each dose (0, 5, 10, and 20 µg/kg) of the D₁ receptor antagonist SCH 23390. (F) Percent change in palatable food intake compared with baseline palatable food intake (g/kg) for each dose (0, 0.03, 0.06, 0.25, 0.5, and 1 mg/kg) of the µ-opioid receptor antagonist naltrexone.
CHAPTER 3. ANTICIPATORY 50 KHZ ULTRASONIC VOCALIZATIONS ARE ASSOCIATED WITH ESCALATED ALCOHOL INTAKE IN DEPENDENT RATS

INTRODUCTION

Fifty kilohertz (kHz) ultrasonic vocalizations (USVs) have been linked with positive affective states in rats (Ahrens et al. 2009; Burgdorf et al. 2008; Brudzynski 2013; Brudzynski and Pniak 2002; Burgdorf and Panksepp 2001). However, these calls are also elicited by aversive stimuli (Vivian and Miczek 1993; Wöhr et al. 2008), and the affective state(s) that they represent are not well understood. Previous reports have shown that 50 kHz USVs are elicited by cues that predict rewarding stimuli, such as food (Burgdorf et al. 2000; Buck et al. 2014), copulation (Bialy et al. 2000), cocaine (Ma et al. 2010), and rewarding brain stimulation (Burgdorf et al. 2000). Thus, 50 kHz USVs may be an index of motivation during reward anticipation (Ahrens et al. 2009; Ma et al. 2010; Mahler et al. 2013). Most studies that have investigated the relationship between 50 kHz USVs and drugs of abuse have focused on psychostimulants; few studies have examined 50 kHz USVs in relation to alcohol self-administration.

Rats that are exposed to chronic, intermittent alcohol vapor display several physical and motivational symptoms of alcohol dependence compared with air-exposed nondependent rats (for review, see Vendruscolo and Roberts, 2014). In this model, during alcohol abstinence, rats exhibit tail stiffness, abnormal gait/posture, tremor (Macey et al. 1996), increased anxiety-like behavior (Zhao et al. 2007), hyperalgesia (Egli et al. 2012; Edwards et al. 2012), increased 22 kHz USVs (Berger et al. 2013; Kissler et al. 2013; Williams et al. 2012), and dysphoria, reflected by elevated brain reward thresholds.
Dependent rats also display escalated alcohol self-administration, increased motivation for alcohol, and persistent alcohol drinking despite punishment (Edwards et al. 2013; Vendruscolo et al. 2012). It has been hypothesized that moderate (recreational) alcohol drinking is mediated by its rewarding effects (positive reinforcement). In the transition to dependence, brain stress systems are sensitized, and drinking mainly occurs to alleviate negative affective states during withdrawal (negative reinforcement; Koob et al. 2014).

Therefore, 50 kHz USVs are observed during several situations of increased emotional and motivational valence, and alcohol dependence affects brain reward and stress systems. The hypothesis in the present study was that rats exposed to alcohol vapor to the point of dependence would present a different pattern or amount of 50 kHz USVs compared with nondependent rats. The objective of the present study was to compare 50 kHz USVs emitted during the anticipation of alcohol self-administration in dependent and nondependent rats.

MATERIALS AND METHODS

Animals

A total of 107 adult male Wistar rats, weighing 250-400 g at the beginning of the experiment, were used. The rats were housed in groups of two to three in plastic cages in a temperature-controlled (21°C) vivarium on a 12 h/12 h light/dark cycle (lights on at 8:00 PM). Food and water were provided ad libitum in the home cages except during behavioral testing. All of the behavioral tests were conducted during the dark cycle between 1:00 PM and 6:00 PM. All of the procedures adhered to the National Institutes
of Health Guide for the Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee of The Scripps Research Institute.

**Operant self-administration**

Self-administration sessions were conducted in standard operant conditioning chambers (Med Associates, St. Albans, VT) as previously reported (Vendruscolo et al. 2012). First, the rats were given free-choice access to alcohol (10% w/v) and water for 1 day in their home cages to habituate them to the taste of alcohol. Second, the rats were subjected to an overnight session in the operant chambers with access to one lever (right lever) that delivered water. Food was available *ad libitum* in the operant chambers during this training. Third, after 1 day off, the rats were subjected to one 2 h session and one 1 h session the next day, with one lever that delivered alcohol (right lever). All of the subsequent baseline and test sessions lasted 30 min, and two levers were available (left lever: water; right lever: alcohol). All of the operant sessions were conducted under a fixed-ratio 1 schedule of reinforcement, in which every lever press resulted in fluid delivery (0.1 ml). Once stable levels of baseline intake were reached, the rats were split into two groups: vapor-exposed (dependent) and air-exposed (nondependent).

**Alcohol vapor chambers**

The rats were made dependent by chronic, intermittent exposure to alcohol vapor as previously described (Vendruscolo et al. 2012). They underwent cycles of 14 h on (blood alcohol levels during vapor exposure ranged between 150 and 250 mg%) and 10 h off, during which behavioral testing for acute withdrawal occurred (i.e., 6-8 h after vapor
was turned off, when brain and blood alcohol levels were negligible; Gilpin et al. 2009). Nondependent rats were not exposed to alcohol vapor but were tested for alcohol self-administration together with the dependent rats.

Ultrasonic vocalization recording and analysis

Condenser microphones (CM16/CMPA, 10-200 kHz frequency range, Avisoft Bioacoustics, Berlin, Germany) were coupled to an UltraSoundGate 816H data acquisition device (250 kHz sampling rate, 16-bit resolution, Avisoft Bioacoustics). Ultrasonic vocalizations between 10 and 100 kHz were recorded and analyzed using Avisoft SASLab Pro (version 5.1, Avisoft Bioacoustics). Spectrograms were generated with a fast Fourier transform length of 512 points and overlap of 50% (FlatTop window, 100% frame size), providing a frequency resolution of 419 Hz and time resolution of 1.19 ms. Vocalizations were categorized as trill, other FM, or flat using criteria described in Buck et al. (2014). Here, trill calls included the “trill FM” and “step-trill FM” categories. Other FM calls included “other FM” and “step FM.” Flat calls included the “flat” category from Buck et al. (2014). Microphones were placed inside the sound attenuating cubicle and outside a grate in the wall of the operant chamber. Upon placing the rats in the operant chambers with the levers retracted, anticipatory 50 kHz USVs were recorded for 2 or 3 min. The operant alcohol self-administration session then began, and USV recording stopped. Each rat was always tested in the same operant chamber.

Inclusion criteria
Five different cohorts of rats were used, but no cohort effects were detected. Rats that emitted fewer than 15 USVs in every session were excluded (two dependent rats and three nondependent rats). One rat with high vocalizations at baseline (> 300 calls in 2 min) was excluded. Additionally, rats that had an average of < 10 alcohol reinforcers in sessions 4-6 were excluded (three dependent rats and four nondependent rats). Following these criteria, a total of 13 rats were excluded, and the total number was 94.

**Novelty-induced USVs**

A separate group of rats ($n = 15$) was trained to self-administer alcohol (see section 2.2 above) and made dependent on alcohol (see section 2.3 above). Alcohol anticipatory USVs were recorded as described above (section 2.4). To test novelty-induced USVs, the rats were individually placed in clean housing cages with fresh bedding and wire lids. The microphones were placed 10 cm above the lids, and USVs were recorded for 3 min. The test was performed in a dark room.

**Statistical analyses**

The data are expressed as the mean and standard error of the mean (SEM). Prior to the analysis, the dependent variables were tested for normality using Shapiro-Wilk’s $W$-test. Ultrasonic vocalization counts were square-root transformed to achieve normality. Ultrasonic vocalization counts were analyzed using two-way repeated-measures analysis of variance (ANOVA), followed by the Fisher Least Significant Difference *post hoc* test when appropriate. Student’s $t$-test was used for group comparisons. Pearson’s test was used for correlation analysis. For USV call subtypes, the
proportions of total calls were analyzed using two-way repeated-measures ANOVA. The statistical analyses were performed using Statistica 10 (StatSoft) and Prism 5 (GraphPad) software. For all of the tests, two-tailed values of $p < 0.05$ were considered statistically significant.

RESULTS

Fig. 3.1 shows the results of alcohol self-administration and anticipatory 50 kHz USVs in alcohol-dependent and -nondependent rats. At baseline (i.e., before the induction of alcohol dependence), the two groups of rats displayed similar levels of alcohol intake ($t_{77} = 0.92, p = 0.36$). During alcohol vapor exposure, dependent rats increased their alcohol intake compared with nondependent rats (group effect: $F_{1,77} = 4.16, p < 0.001$; Fig. 3.1A). For alcohol intake in g/kg/30 min (sessions 4, 5, and 6), dependent rats had higher alcohol intake ($1.2 \pm 0.1 \text{ g/kg/30 min}$) compared with nondependent rats ($0.6 \pm 0.04 \text{ g/kg/30 min}$; group effect: $F_{1,77} = 53.42, p < 0.001$; post hoc comparisons: $p < 0.01$). Dependent and nondependent rats’ baseline anticipatory 50 kHz USVs were not significantly different ($t_{77} = 1.15, p = 0.22$). The group x session repeated-measures ANOVA revealed a significant group x session interaction ($F_{5,385} = 2.37, p < 0.05$). Post hoc comparisons indicated that dependent rats displayed increased anticipatory USVs compared with nondependent rats in session 4 only (Fig. 3.1B).

Fig. 3.2A and B show the relationship between anticipatory USVs (averaged over sessions 4-6) and alcohol intake (averaged over sessions 4-6) in dependent and nondependent rats. Anticipatory USVs were positively correlated with alcohol intake in dependent rats ($r = 0.39, p < 0.05$; Fig. 3.2A) but not in nondependent rats ($r = 0.09, p =$
0.59; Fig. 3.2B). To further explore these results, the dependent and nondependent groups were split into high and low alcohol intake subgroups based on the median values of alcohol lever presses (averaged over sessions 4-6). Anticipatory USVs for the high and low alcohol intake subgroups are shown in Fig. 3.2C (dependent rats) and Fig. 3.2D (nondependent rats). In dependent rats, the repeated-measures ANOVA indicated that rats with high alcohol intake emitted significantly more 50 kHz USVs than rats with low alcohol intake (overall subgroup effect: $F_{1,38} = 6.70, p < 0.05$; Fig. 3.2C). For alcohol intake in g/kg/30 min in dependent rats, the high-intake subgroup had higher intake ($1.5 \pm 0.07 \text{ g/kg/30 min}$) than the low-intake subgroup ($0.9 \pm 0.06 \text{ g/kg/30 min}$; group effect: $F_{1,38} = 52.39, p < 0.001$; post hoc comparisons: $p < 0.01$). In nondependent rats, the ANOVA revealed no significant differences in anticipatory USVs between the high- and low-intake subgroups (Fig. 3.2D). For alcohol intake in g/kg/30 min in nondependent rats, the high-intake subgroup had higher intake ($0.8 \pm 0.04 \text{ g/kg/30 min}$) than the low-intake subgroup ($0.4 \pm 0.03 \text{ g/kg/30 min}$; group effect: $F_{1,38} = 50.32 p < 0.001$; post hoc comparisons: $p < 0.01$).

Table 3.1 shows the mean proportions of trill, other FM, and flat calls during baseline and session 6. The repeated-measures ANOVA did not reveal significant differences in trill and other FM call types between dependent and nondependent rats or between baseline and session 6. Flat calls accounted for an average of less than 5% of calls; therefore, this call subtype was not statistically analyzed.

A separate group of dependent rats was split into subgroups of high ($n = 8$) and low ($n = 7$) anticipatory 50 kHz USVs based on the median value of anticipatory 50 kHz USVs for alcohol self administration. The subgroup with high anticipatory USVs had
significantly more anticipatory USVs than the low subgroup ($t_{13} = 5.60, p < 0.001$; Fig. 3.3A), but these subgroups did not significantly differ in the number of 50 kHz USVs emitted in a novel environment ($t_{13} = 1.06, p = 0.31$; Fig. 3.3B). Furthermore, in this group of dependent rats, no significant correlation was found between alcohol anticipatory USVs and novel environment-induced USVs ($r = 0.38, p = 0.16$; Fig. 3.3C).

**DISCUSSION**

In the present study, we report that alcohol-dependent and nondependent rats emitted similar levels of 50 kHz USVs in anticipation of alcohol self-administration, despite showing differences in alcohol intake. However, dependent rats displayed a positive correlation between anticipatory 50 kHz USVs and alcohol intake, whereas no relationship was observed in nondependent rats. Moreover, in dependent rats but not nondependent rats, those that displayed high levels of alcohol intake also displayed an increase in the number of anticipatory 50 kHz USVs compared with rats that displayed low alcohol intake. Baseline alcohol anticipatory 50 kHz USVs (i.e., before dependence induction) did not correlate with subsequent alcohol intake in either dependent or nondependent rats (data not shown). Finally, dependent rats that showed high 50 kHz USVs in anticipation of alcohol showed no difference in 50 kHz USV emission in a novel environment compared with rats that showed low 50 kHz USVs.

Alcohol anticipatory 50 kHz USVs did not substantially differ between dependent and nondependent rats, suggesting that anticipatory 50 kHz USVs are not a general marker of alcohol dependence. However, anticipatory 50 kHz USVs were positively and significantly correlated with alcohol intake in dependent rats but not nondependent rats.
This finding indicates that anticipatory 50 kHz USVs may be qualitatively different for these two groups. Consistent with this hypothesis, in dependent rats but not nondependent rats, we observed that a subset of rats that displayed high alcohol intake also had higher levels of anticipatory 50 kHz USVs compared with low-alcohol-intake rats. Previous studies have reported an increase in 50 kHz USVs in response to cues paired with appetitive stimuli, such as food (Buck et al. 2014; Burgdorf et al. 2000), copulation (Bialy et al. 2000), and cocaine (Ma et al. 2010). Rats that had extinguished methamphetamine self-administration and then given a methamphetamine priming injection or exposed to drug-paired cues robustly emitted 50 kHz USVs and reinstated drug-seeking behavior (Mahler et al. 2013). If these cue-induced USVs are thought to reflect an increased appetitive or motivational state, it may suggest that a subgroup of alcohol-dependent rats (i.e., the high drinkers) in the present study demonstrated an increase in motivational salience (i.e., an increase in alcohol-seeking behavior/craving/incentive salience), reflected by higher anticipatory 50 kHz USVs.

Other studies have also observed individual differences in anticipatory 50 kHz USVs. (Ahrens et al. 2013; Taracha et al. 2012; Taracha et al. 2014). High- and low-50 kHz USV groups were separated based on rats’ initial USV response to amphetamine. Compared with low-USV rats, high-USV rats showed increased amphetamine-anticipatory 50 kHz USVs along with increased conditioned place preference for amphetamine-paired stimuli (Ahrens et al. 2013; Taracha et al. 2014). Unlike amphetamine, acute alcohol does not potently elicit 50 kHz USVs in rats (Blanchard et al. 1993; Willey and Spear 2014). In the current study, however, a dependence-induced
increase in alcohol intake was associated with higher anticipatory USVs, indicating that USVs may be a general marker of interindividual differences in motivational states.

Paulus et al. (2009) suggested that interoception (i.e., the conscious awareness of internal states) under specific conditions (e.g., withdrawal) may be a strong component associated with subsequent drug intake. Thus, the situation of anticipation for alcohol drinking may trigger interoceptive states that serve as cues for both eliciting USVs and favoring a learning process, in which dependent rats associate an environment/situation with subsequent alcohol-induced stress relief (Walker 2012). Interestingly, rats bred for high 50 kHz USVs have shown enhanced associative learning of negative contextual conditioning (Webber et al. 2012).

Most of the anticipatory 50 kHz USVs observed herein were trill or other FM calls. These call types, especially trills, have been hypothesized to indicate a positive affective state (Burgdorf et al. 2011). Others have hypothesized that 50 kHz USVs indicate incentive salience, which may not necessarily be related to a positive affective state (Meyer et al. 2012). Because alcohol vapor-exposed rats display several signs of negative affective-like states during withdrawal (Vendruscolo and Roberts 2014), we speculate that the observed 50 kHz USVs in dependent rats were indicative of negative reinforcement-related incentive salience. However, we cannot exclude the possibility of positive affective states in anticipation of alcohol.

We found that dependent rats that differed in the levels of anticipatory 50 kHz USVs for alcohol self-administration did not differ in 50 kHz USVs emitted in a novel environment. This indicates that the increase in USVs emitted in the self-administration chambers is not attributable to a general increase in the emission of 50 kHz USVs and is
consistent with a previous study (Mu et al. 2009) that reported that rats displayed different patterns of USVs in different environmental settings.

Similar to most neuropsychiatric disorders, alcohol dependence is a heterogeneous condition determined by the interaction between environmental factors and multiple genes. Moss et al. (2007) used nationally representative epidemiological data from the United States and statistically determined that alcohol dependence can be divided into five clinically distinct categories. Earlier, Cloninger (1987) classified alcoholics into Type 1 and Type 2 based on drinking and personality types. Other classification schemes also exist (for review, see Pombo and Lesch, 2009). Despite this knowledge of the heterogeneity of alcohol dependence, few studies have attempted to identify the neurobiological and/or behavioral bases of different subtypes in the context of alcohol dependence. In the present study, the finding that increased anticipatory 50 kHz USVs are associated with increased alcohol intake in alcohol dependence suggests that these two behaviors may share a similar neurobiological basis and that anticipatory USVs may be an potential marker for a specific subgroup of alcohol-dependent rats.

In conclusion, increases in 50 kHz USVs in anticipation of alcohol self-administration are associated with the escalation of alcohol intake during alcohol dependence. More specifically, anticipatory 50 kHz USVs may be an indicator of context-elicited negative reinforcement learning. These findings provide evidence that alcohol dependence comprises a behaviorally heterogeneous group of rats and suggest that anticipatory 50 kHz USVs and high alcohol intake share some biological mechanisms. The study of 50 kHz USVs can help identify a subgroup of alcohol-dependent rats that may better model excessive alcohol drinking in the human condition.
and elucidate the biological mechanisms that are involved in both emotional reactivity and alcoholism subtypes.

**Acknowledgements**

Chapter 3, in full, has been submitted for publication of the material as it may appear in Behavioural Brain Research, 2014. Buck, Cara L.; Malavar, Jordan; George, Olivier; Koob, George F.; Vendruscolo, Leandro F., Elsevier, 2014. The dissertation author was the primary investigator and author of this paper.
Table 3.1

*Proportions of 50 kHz call types*

<table>
<thead>
<tr>
<th>Group</th>
<th>Session</th>
<th>50 kHz call types</th>
</tr>
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<tbody>
<tr>
<td></td>
<td></td>
<td>Trill</td>
</tr>
<tr>
<td>Dependent</td>
<td>Baseline</td>
<td>0.22 ± 0.03</td>
</tr>
<tr>
<td></td>
<td>Session 6</td>
<td>0.22 ± 0.03</td>
</tr>
<tr>
<td>Nondependent</td>
<td>Baseline</td>
<td>0.29 ± 0.03</td>
</tr>
<tr>
<td></td>
<td>Session 6</td>
<td>0.22 ± 0.03</td>
</tr>
</tbody>
</table>

Proportions (mean ± SEM) of anticipatory 50 kHz call types displayed by dependent and nondependent rats during baseline and session 6.
Figure 3.1 Escalation of alcohol self-administration but not anticipatory 50 kHz ultrasonic vocalizations (USVs) in dependent rats compared with nondependent rats. (A) Mean ± SEM number of lever presses for alcohol in dependent and nondependent rats and (B) 50 kHz USVs emitted by rats during 2 min prior to alcohol self-administration. *p < 0.05, difference between dependent and nondependent rats. n = 39-40 per group.
Figure 3.2 Anticipatory 50 kHz ultrasonic vocalizations (USVs) are positively associated with alcohol intake in dependent rats but not nondependent rats. The number of anticipatory 50 kHz USVs (averaged over sessions 4-6) was positively correlated with the number of lever presses for alcohol (averaged over sessions 4-6) in dependent rats (A) but not in nondependent rats (B). $n = 39-40$ per group. (C, D) Mean ± SEM number of 50 kHz USVs emitted during 2 min immediately before the self-administration sessions in the high and low alcohol self-administration subgroups (based on a median split of average alcohol intake during sessions 4-6) in dependent rats (C) and nondependent rats (D). *$p < 0.05$, compared with low alcohol intake rats. $n = 19-20$ per subgroup.
Figure 3.3 Alcohol anticipatory 50 kHz ultrasonic vocalizations (USVs) are not associated novel environment-induced 50 kHz USVs in dependent rats. The mean ± SEM number of anticipatory 50 kHz USVs is shown. Dependent rats were split into high and low alcohol anticipatory USV groups based on a median split. (A) Mean + SEM number of alcohol anticipation USVs emitted during 3 min immediately before the self-administration sessions in the high and low anticipatory 50 kHz USV subgroups (based on a median split of anticipatory USVs). n = 7-8 per subgroup. (B) Mean + SEM number of novel environment-induced USVs emitted during 3 min in the high and low anticipatory 50 kHz USV subgroups. n = 7-8 per subgroup. (C) Correlation between alcohol anticipatory USVs (square-root-transformed) and novel environment-induced USVs (square-root-transformed). *p < 0.05, compared with low anticipatory USV rats. n = 15.
CHAPTER 4. RAT 22 KHZ ULTRASONIC VOCALIZATIONS (USVs) AS A SPECIFIC MEASURE OF ANXIETY AND AROUSAL: POTENTIATION BY ALCOHOL DEPENDENCE AND LACK OF CORTICOTROPIN-RELEASING FACTOR 1 (CRF1) RECEPTOR ANTAGONIST EFFECTS

INTRODUCTION

Rat 22 kHz ultrasonic vocalizations (USVs) are elicited by aversive stimuli and are thought to reflect a negative affective state similar to anxiety (Knutson et al. 2002; Molewijk et al. 1995; Sánchez 2003). Stress-induced 22 kHz USVs are reliably decreased by anxiolytics including benzodiazepines (Kassai & Gyertyan 2012; Millan et al. 2001; Vivian et al. 1994) and serotonin 5-HT1A agonists (De Vry et al. 1993; Millan et al. 2001; Kassai & Gyertyan 2012), and are increased by anxiogenic drugs (Berger et al. 2013; Jelen et al. 2003; Tonoue et al. 1986). Additionally, electrical stimulation of brain nuclei of fear circuits, particularly the dorsolateral periaqueductal gray (dPAG), elicits 22 kHz USVs along with defensive behaviors such as freezing or escape (Brenes et al. 2012; Cabral et al. 2006). Anxiety is a multidimensional construct, and different animal models may measure different aspects of anxiety. The relationship between 22 kHz USVs and other measures of anxiety-like behavior is not entirely clear.

Rats that are exposed to chronic, intermittent alcohol vapor display several physical and motivational symptoms of alcohol dependence compared with air-exposed nondependent rats (for review, see Vendruscolo and Roberts, 2014). In this model, during alcohol abstinence, rats exhibit increased anxiety-like behavior (Zhao et al. 2007), increased 22 kHz USVs (Berger et al. 2013; Kessler et al. 2013; Williams et al. 2012), tail
stiffness, abnormal gait/posture, tremor (Macey et al. 1996), hyperalgesia (Egli et al. 2012; Edwards et al. 2012), and dysphoria, reflected by elevated brain reward thresholds (Schulteis et al. 1995). Dependent rats also display escalated alcohol self-administration, increased motivation for alcohol, and persistent alcohol drinking despite punishment (Edwards et al. 2013; Vendruscolo et al. 2012). Corticotropin-releasing factor (CRF) signaling is increased in stress-related brain regions during alcohol dependence (Roberto et al. 2010), and CRF₁ receptor antagonists reduce compulsive-like alcohol drinking in dependent but not nondependent rats (Funk et al. 2007; Gehlert et al. 2007; Richardson et al. 2008).

We sought to examine the relationship between stress-induced 22 kHz USVs and other measures of anxiety. To better understand the biological significance of 22 kHz USVs, we tested naïve rats in a battery of behavioral models of anxiety-like (elevated plus maze [EPM], defensive burying, and startle reflex) and depression-like (forced swim test [FST]) behavior, and measured stress-induced changes in plasma corticosterone levels and 22 kHz USVs. In separate experiments, we also tested the effects of two classes of anxiolytic drugs, the benzodiazepine chlordiazepoxide and the CRF₁ receptor antagonists R121919 and N,N-bis(2-methoxyethyl)-3-(4-methoxy-2-methylphenyl)-2,5-dimethyl-pyrazolo[1,5-α] pyrimidin-7-amine (MPZP), on stress-induced 22 kHz USVs in nondependent rats and dependent rats during alcohol withdrawal.

MATERIALS AND METHODS

Experiment 1

Animals
A total of 30 adult male Wistar rats, weighing 250-400 g at the beginning of the experiment, were used. The rats were housed in groups of two to three in plastic cages in a temperature-controlled (21°C) vivarium on a 12 h/12 h light/dark cycle (lights on at 8:00 PM). Food and water were provided ad libitum in the home cages except during behavioral testing. All of the behavioral tests were conducted during the dark cycle between 12:00 PM and 6:00 PM. All of the procedures adhered to the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee of The Scripps Research Institute.

Procedure

The sequence of behavioral tests was as follows: an initial airpuff test (airpuff 1) was performed on day 1, followed by EPM testing on day 4 or 5, acoustic startle testing on day 6 or 8, defensive burying testing on day 13 or 14, and FST on days 26-27 or 28-29, concluding with the second airpuff test (airpuff 2) on day 33 or 34. Tail blood samples were collected for corticosterone measurements 40 min after the first airpuff was administered in Airpuff 2. Between tests, rats were left undisturbed in their home cages. Descriptions of each test are provided below. One rat was excluded from the analysis due to data loss in the FST.

Elevated plus maze

The apparatus had four elevated arms (52 cm above the floor), 50 cm long and 10 cm wide, set in a cross-like arrangement, with two opposite arms enclosed by 40 cm high opaque walls and two open arms with a lip (1 mm thick and 5 mm high). A central
platform at their intersection (10 × 13.5 cm) permitted access to any of the four arms. The central platform was under ~10 lux illumination. Each rat was placed on the central platform facing an open arm. The behavior of each animal was recorded for 5 min. The percentage of time spent in the open arms (% open arm time) relative to the time spent in both the open and closed arms was used as an index of anxiety-like behavior.

**Defensive burying test**

The animals were acclimated to the testing apparatus by placing them for 45 min in the testing cage (a polycarbonate rat housing cage with 5 cm of bedding that covered the floor and a small hole centered on the long dimension of the cage, 2.5 cm above the bedding, to accommodate the shock probe on the subsequent test day). For testing, the animals were placed individually in the test cage, and a shock probe connected to a Coulbourn precision shocker (model E13-01, Coulbourn Instruments, Allentown, PA) delivered one 1.5 mA shock (< 1 s) upon contact. As soon as the animal received the shock (verified by a startle response), the probe current was deactivated, and the time the animal spent burying was assessed for 10 min. The test was performed under room lighting conditions. The time the animal spent burying (burying time) was used as a measure of anxiety-like behavior.

**Acoustic startle**

The animals were tested for acoustic startle in SR-LAB startle chambers for rats (San Diego Instruments, USA), which consisted of a Plexiglas cylinder (12.5 diameter and 25 cm long) within a ventilated, sound-attenuating chamber. Four rats were tested in
four chambers simultaneously. Background noise (60 dB) and noise bursts (50-ms duration) were presented by a speaker mounted 24 cm above the cylinder. Piezoelectric accelerometers mounted under the cylinders detected the movements of the animal, which were digitized and recorded by an interface and computer assembly. Startle amplitude was defined as the maximal peak-to-peak voltage that occurred during the first 200 ms after the onset of the startle-eliciting stimulus. Each startle test session consisted of a 5-min stimulus-free acclimation period with 60 dB background noise, followed by presentation of 30 startle-eliciting noise bursts (10 each at 90, 95 and 105 dB in a balanced, random order) with a 30-s fixed inter-stimulus interval. A dynamic calibration system was used to ensure comparable sensitivities across chambers. The house light remained off throughout the test sessions.

**Forced-swim test**

The FST is classically used to screen and validate antidepressants (Lucki 1997, Porsolt et al. 1977). Increased immobility time, decreased latency to show immobility, and escape-oriented behaviors reflect a state of helplessness and despair. The FST was carried out on two days. On day 1, animals were individually placed in a clear Plexiglas cylinder (61 cm height, 25 cm diameter) filled with 24 ± 1°C water at a height of 45 cm. After 15 min, animals were removed from the water and dried with towels before being returned to their home cages. On day 2, animals were placed in the same cylinders for 5 min. Rats’ movements were recorded by video. Behaviors was sampled every 5 s and labeled as “float”, “swim”, or “climb” by an experienced experimenter. The total number of “float” counts per rat was used as an index of immobility.
**Airpuff test**

The airpuff test was based on methods described by Knapp & Pohorecky (1995). Rats were placed in a Plexiglas cylinder (12.5 diameter and 25 cm long) within sound-attenuated chambers. Four rats were tested in four chambers simultaneously. After a 5-min habituation period, airpuffs and USV recording began. A maximum of 15 airpuffs (15-35 psi) were delivered every 15 s, with a 1-min break after every third airpuff. Airpuffs stopped when the rat began vocalizing. Ultrasonic vocalizations were recorded until rats stopped vocalizing for 1 min.

**Corticosterone radioimmunoassay**

Rat whole blood samples were collected on ice from a nick near the tip of the tail and plasma isolated via centrifugation in the presence EDTA (~10% [v/v] 0.5M EDTA) and frozen at -80°C prior to analysis. Plasma corticosterone levels were analyzed using the ImmuChem™ Double Antibody Corticosterone $^{125}$I RIA Kit for Rats and Mice (MP Biomedicals, LLC, Orangeburg, NY, USA) according to manufacturer’s instructions. Standards and plasma samples were run in duplicates, and corticosterone concentrations extrapolated from the four-parameter logistic curve fit to the known concentration standards using SigmaPlot 11.0 (Systat Software, Inc., San Jose, CA, USA).

**Ultrasonic vocalization recording and analysis**

Condenser microphones (CM16/CMPA, 10-200 kHz frequency range, Avisoft Bioacoustics, Berlin, Germany) were coupled to an UltraSoundGate 816H data acquisition device (250 kHz sampling rate, 16-bit resolution, Avisoft Bioacoustics).
Ultrasonic vocalizations between 10 and 100 kHz were recorded and USV durations were measured using Avisoft SASLab Pro (version 5.1, Avisoft Bioacoustics). Spectrograms were generated with a fast Fourier transform length of 512 points and overlap of 50% (FlatTop window, 100% frame size), providing a frequency resolution of 419 Hz and time resolution of 1.19 ms. Microphones were placed inside the sound-attenuating cubicle and outside the Plexiglas cylinder.

**Statistical analyses**

The data are expressed as means and standard errors of the mean (SEM). Prior to the analysis, the dependent variables were tested for normality using Shapiro-Wilk’s W-test. To achieve normality, 22 kHz USV duration, defensive burying time, and corticosterone were square-root transformed, while startle (amplitude) and FST immobility counts were log-transformed. The PCA with an orthogonal normalized Varimax rotation included the following variables: USV 1 duration, USV 2 duration, acoustic startle amplitude, defensive burying time, FST immobility counts, EPM % open arm time, EPM number of closed arm entries, and corticosterone. Factors with eigenvalues above 1 were retained. Student’s t-test was used for comparisons between rats split by high and low factor scores. The statistical analyses were performed using Statistica 10 (StatSoft) software. For all of the tests, two-tailed values of $p < 0.05$ were considered statistically significant.

**Experiment 2**

*Animals*
Animal information was the same as described in experiment 1, except here a total of 68 adult male Wistar rats, weighing 250-400 g at the beginning of the experiment, were used.

Procedure

Three separate groups of rats were used. All rats were trained on alcohol self-administration and then subjected to air or vapor exposure to the point of dependence as previously reported (Vendruscolo et al., 2012). All pharmacological tests utilized a within-subject design, with treatment counterbalanced across two airpuff sessions. Chlordiazepoxide tests were performed on a group of 10 dependent and 10 nondependent rats. Rats were exposed to an airpuff test with no pharmacological treatment during week 7 of vapor exposure (data not reported). The first test session occurred in week 20 of vapor exposure, and the second session was performed two days later. Rats were pretreated with chlordiazepoxide 30 min before the tests began. In this group, 2 nondependent rats never produced USVs, and 1 dependent rat produced a large number of USVs (average duration = 700 s), and so these rats were excluded from the analysis. R121919 tests were performed on a group of 12 dependent and 12 nondependent rats. Rats were exposed to an airpuff test with no pharmacological treatment during week 3 of vapor exposure (data not reported). The first test session occurred in week 6 of vapor exposure, and the second session was performed five days later. In this group, 1 nondependent rat never produced USVs, and 1 nondependent rat produced a large number of USVs (average duration = 596 s), and so these rats were excluded from the analysis. MPZP tests were performed on a group of 12 dependent and 12 nondependent
rats. The first test session occurred in week 8 of vapor exposure, and the second session was performed seven days later. In this group, 3 nondependent rats never produced USVs, and so these rats were excluded from the analysis.

**Drugs**

Chlordiazepoxide (Sigma Aldrich Inc., St. Louis, MO, USA) was dissolved in saline and injected subcutaneously (s.c.; 2 ml/kg) at the dose of 2.5 mg/kg 30 min prior to testing. The CRF$_1$ receptor antagonists R121919 and MPZP were prepared by dissolving in 1M HCl (10% final volume), diluting with 25% (w/v) hydroxypropyl β-cyclodextrin (HBC; Cargill, USA; 80% final volume), and back-titrating with descending concentrations of NaOH (2, 1, and 0.1M; 10% final volume), resulting in a final suspension of 10 mg/mL R121919 or MPZP in 20% HBC (pH 4.5). The animals were administered R121919 (1 ml/kg, s.c.) at the dose of 20 mg/kg 60 min prior to testing or MPZP (2 ml/kg, s.c.) at the dose of 20 mg/kg 30 min prior to testing.

**Airpuff test and ultrasonic vocalization recording and analysis**

The airpuff test and ultrasonic vocalization recording and analysis were the same as described in experiment 1.

**Statistical analyses**

The data are expressed as the mean and standard error of the mean (SEM). Prior to the analysis, the dependent variables were tested for normality using Shapiro-Wilk’s W-test. Ultrasonic vocalization durations were square-root transformed to achieve
normality. Vehicle-treatment USV measurements were pooled across tests and analyzed using a group (dependent vs. nondependent) × session (airpuff 1 vs. airpuff 2) between-subjects ANOVA. Ultrasonic vocalizations were analyzed using two-way repeated-measures ANOVA with drug treatment as a within-subjects factor and group (dependent vs. nondependent) as a between-subjects factor. The statistical analyses were performed using Statistica 10 (StatSoft). For all of the tests, two-tailed values of \( p < 0.05 \) were considered statistically significant.

RESULTS

Experiment 1

A principal component analysis was performed to detect the underlying structure in the relationships between stress-induced 22 kHz USVs, other behavioral measures, and stress-induced corticosterone (Table 4.1 and Fig. 4.1A). This analysis revealed three main factors that together accounted for 58% of the variance. The first factor had high loadings for USVs and startle, with increased USVs associated with increased startle reflex. The second factor had high loadings for EPM behavior and stress-induced corticosterone, with increased open-arm time associated with decreased plasma levels of corticosterone. The third factor only had high loadings for defensive burying time. Immobility in the FST and the number of closed arm entries in the EPM did not load strongly onto any of the three factors.

To investigate these results further, we performed a median split based on the individual scores from the principal component analysis for factors 1 and 2. Rats with high scores on factor 1 showed increased airpuff-induced 22 kHz USVs on airpuff 1 (Fig.
4.1B; $t_{27} = 5.54$, $p < 0.001$) and airpuff 2 (Fig. 4.1C; $t_{27} = 4.01$, $p < 0.001$), and also had increased startle reactivity (Fig. 4.1D; $t_{27} = 4.41$, $p < 0.001$) compared with rats that had low scores on factor 1. Rats with high scores on factor 2 showed an increased proportion of time in the open arm during the EPM test (Fig. 4.1E; $t_{27} = 4.09$, $p < 0.001$), and also had lower plasma levels of airpuff-induced corticosterone (Fig. 4.1F; $t_{27} = 2.91$, $p < 0.01$) compared with rats that had low scores on factor 2.

**Experiment 2**

We pooled the vehicle-treatment vocalizations from rats of all three cohorts (chlordiazepoxide, R121919, and MPZP) and compared the dependent and nondependent groups on airpuff tests 1 and 2 (Fig. 4.2). The ANOVA revealed a significant group effect ($F_{1,56} = 5.2$, $p < 0.05$), with dependent rats showing more USVs than nondependent rats. The ANOVA also revealed a significant session effect ($F_{1,56} = 13.4$, $p < 0.01$), with more USVs on airpuff 1 than on airpuff 2.

Fig. 4.3A shows the effect of chlordiazepoxide on stress-induced 22 kHz USVs in dependent and nondependent rats. The ANOVA revealed a significant treatment effect ($F_{1,15} = 6.0$, $p < 0.05$), with rats that received chlordiazepoxide showing less USVs compared with vehicle-treated rats. Fig. 4.3B shows the stress-induced 22 kHz USVs in dependent and nondependent rats treated with R121919 or vehicle. The ANOVA revealed a significant group effect ($F_{1,20} = 5.2$, $p < 0.05$), with dependent rats showing more USVs compared with nondependent rats. Fig. 4.3C shows the stress-induced 22 kHz USVs in dependent and nondependent rats treated with MPZP or vehicle. The ANOVA did not reveal any significant effects.
DISCUSSION

In the present study, we report that airpuff stress-induced 22 kHz USVs are more strongly associated with startle behavior than with EPM open arm time, defensive burying, FST immobility, or airpuff stress-induced plasma levels of corticosterone. We also report that during alcohol withdrawal, stress-induced 22 kHz USVs are reduced by the benzodiazepine chlordiazepoxide but not by the CRF$_1$ receptor antagonists R121919 and MPZP in both dependent and nondependent rats.

We found a positive association between airpuff stress-induced 22 kHz USVs and startle reflex amplitude. A positive correlation between startle reactivity and startle-induced 22 kHz USVs has been observed previously; however this relationship was seen only in rats bred for high levels of 50 kHz USVs (Webber et al. 2012). Other studies have not found a relationship between startle and USVs. For example, high-anxiety rats – as determined by EPM behavior – displayed increased startle-induced 22 kHz USVs but no difference in startle reactivity compared to low-anxiety rats (Cabral et al. 2009). Additionally, pharmacological manipulations including chlordiazepoxide, gepirone, and olanzapine reduced stress-induced 22 kHz USVs while showing no effect on startle (Sun et al. 2010; Vivian et al. 1994).

The acoustic startle response is a measure of arousal that is reliably elevated by anxiogenic-like stimuli in both humans and laboratory animals and is blocked by anxiolytic-like agents (Davis et al. 1993). Potentiated startle responses (Rassnick et al. 1992; Park et al. 2013) and increased 22 kHz USVs (Berger et al. 2013; Moy et al. 2000; Williams et al. 2012; Vivian & Miczek 1991) have been observed during withdrawal from multiple drugs of abuse, including alcohol and opioids. Moreover, central
administration of CRF has been shown to increase both acoustic startle (Swerdlow et al. 1986, 1989) and 22 kHz USVs (Ji et al. 2013; Swiergiel et al. 2007). CRF$_1$ receptor antagonists suppressed context-conditioned USVs (Chen et al. 2014; Kikusui et al. 2000), while a nonspecific CRF antagonist blocked fear-potentiated, but not basal, startle reactivity (Swerdlow et al. 1989). This lends support to the current hypothesis that some of the neural circuits regulating startle and 22 kHz USVs may overlap.

Studies that have examined the relationship between stress-induced 22 kHz USVs and measures of anxiety and/or depression have found mixed results. Rats that displayed low innate levels of anxiety-like behavior in the EPM displayed more shock-induced 22 kHz USVs and greater freezing behavior than rats with high innate anxiety-like behavior (Borta et al. 2006). However, during alcohol withdrawal, increased anxiety-like behavior as measured in the EPM has been observed along with increased stress-induced 22 kHz USVs (Cabral et al. 2006; Williams et al. 2012). In particular, Williams et al. (2012) observed that an increase in 22 kHz USVs emerged after 2 weeks of alcohol vapor exposure, whereas anxiety-like differences in the EPM did not emerge until 4 weeks into vapor exposure. This temporal dissociation in the emergence of heightened 22 kHz USVs and elevated EPM anxiety-like behavior over the development of alcohol vapor dependence suggests a differential neural regulation of 22 kHz USVs and EPM behavior.

Mixed results have also been reported for the relationship between 22 kHz USVs and depression-like behavior in the FST. Immobility during the FST was increased along with stress-induced 22 kHz USVs in rats during withdrawal from alcohol or heroin (Williams et al. 2012). However, rats exposed to different forms of chronic stress showed diverging profiles of FST and 22 kHz USV behavior. Rats that had been exposed to
shocks had increased FST immobility and decreased shock-induced 22 kHz USVs, whereas rats that had been exposed to chronic restraint stress had decreased FST immobility with increased shock-induced 22 kHz USVs compared to controls (Swiergiel et al. 2007). These findings suggested that increased USVs and immobility may occur concomitantly in some conditions, but are mostly likely dissociated from each other, in agreement with the data presented here. Finally, we did not observe a relationship between 22 kHz USVs and plasma levels of corticosterone, which is consistent with previous studies (Groenink et al. 1996; Naito et al. 2001). However, we did find a relationship between basal anxiety-like behavior and stress-induced corticosterone levels, i.e., more time in the open arms (less anxious-like) associated with lower stress-induced corticosterone release.

CRF<sub>1</sub> receptors, particularly within the central nucleus of the amygdala (Funk et al. 2006), have been shown to mediate compulsive alcohol intake in dependent animals (Heilig and Koob 2007). Infusion of CRF into the central nucleus of the amygdala has also been shown to increase stress-induced 22 kHz USVs in naïve rats (Ji et al. 2013). Therefore, the failure of CRF<sub>1</sub> receptor antagonists to alter stress-induced 22 kHz USVs during alcohol withdrawal in the current report is somewhat surprising. However, CRF<sub>1</sub> receptor antagonists also were unable to reduce acute stress-induced 22 kHz USVs in alcohol-naïve rats (Chen et al. 2014; Kikusui et al. 2000; Millan et al. 2001). A possible explanation for these results is the hypothesis that CRF increases dynorphin activity. In alcohol dependent rats, airpuff stress-induced 22 kHz USVs were reduced by intracerebroventricular (i.c.v.) infusion of a kappa opioid receptor antagonist (Berger et al. 2013). Thus, it may be hypothesized that airpuff stress itself does not cause CRF
release and therefore the effect is not blocked by CRF₁ receptor antagonists. However, airpuff stress in dependent rats and CRF infusion in naïve rats may trigger the release of dynorphin, thereby potentiating USVs.

One limitation of the current study is the lack of a statistically significant group increase in 22 kHz USVs for dependent rats in the chlordiazepoxide and MPZP tests, although dependent rats displayed increased USVs compared with nondependent rats when all groups were analyzed together. A reason for this may be that we observed substantial decreases in airpuff-induced 22 kHz USVs over repeated tests. However, the fact that chlordiazepoxide significantly reduced stress-induced USVs, while two different CRF₁ receptor antagonists had no effect, strongly suggests that our experimental condition was valid and that CRF₁ signaling appears not to play a critical role in airpuff stress-induced 22 kHz USVs.

In summary, these findings suggest that 22 kHz USVs reflect an aspect of the multidimensional state of anxiety that may be related to stress and arousal responses. CRF signaling mediates compulsive-like alcohol drinking, but it does not appear to mediate stress-induced 22 kHz USVs during alcohol withdrawal. Together, the results suggest that stress-induced 22 kHz USVs may be useful to study unique aspects of the complex relationship between emotionality and alcohol dependence.
Acknowledgements

Chapter 4, in full, is currently being prepared for submission for publication of the material. Buck, Cara L.; Logrip, Marian L.; Vendruscolo, Leandro F.; Koob, George F. The dissertation author was the primary investigator and author of this material.
Table 4.1  
Varimax rotated factor loadings

<table>
<thead>
<tr>
<th>Measure</th>
<th>Factor 1</th>
<th>Factor 2</th>
<th>Factor 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Airpuff 1 USV</td>
<td>0.874</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Airpuff 2 USV</td>
<td>0.808</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Startle</td>
<td>0.650</td>
<td></td>
<td></td>
</tr>
<tr>
<td>EPM percent open arm</td>
<td></td>
<td>0.605</td>
<td>-0.563</td>
</tr>
<tr>
<td>EPM closed arm entries</td>
<td></td>
<td></td>
<td>0.515</td>
</tr>
<tr>
<td>Defensive burying time</td>
<td></td>
<td></td>
<td>0.705</td>
</tr>
<tr>
<td>FST immobility</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Corticosterone</td>
<td></td>
<td></td>
<td>-0.768</td>
</tr>
</tbody>
</table>

Only loadings greater than an absolute value of 0.5 are shown. USV = ultrasonic vocalization; EPM = elevated plus maze; FST = forced swim test. n = 29.
Figure 4.1 Principal component analysis on behavioral measures and corticosterone. (A) Factor loadings are plotted for factors 1 and 2. Airpuff 1-induced 22 kHz ultrasonic vocalizations (USV 1), airpuff 2-induced 22 kHz USVs (USV 2), and startle amplitude (startle) loaded positively onto factor 1. Proportion of time spent in the open arm of the elevated plus maze (EPM open arm) loaded positively and stress-induced plasma levels of corticosterone loaded negatively onto factor 2. Measures with loadings less than an absolute value of 0.6 onto both factor 1 and 2 are not shown. (B-D) High and low factor 1 rats were determined using a median split on factor 1 scores. (B) Airpuff 1-induced 22 kHz USVs, (C) airpuff 2-induced USVs, and (D) startle were increased in high factor 1 rats. (E-F) High and low factor 2 rats were determined using a median split on factor 2 scores. (E) EPM open arm was increased and (F) corticosterone was decreased in high factor 2 rats. *p < 0.05, compared with low rats. n = 14-15 per group.
Figure 4.2 Stress-induced 22 kHz ultrasonic vocalizations (USVs) during alcohol withdrawal were greater in dependent rats than in nondependent rats. Vehicle-treatment vocalizations were pooled across clordiazepoxide, R121919, and MPZP tests. Vocalizations are shown by test session (airpuff 1 and 2) for dependent and nondependent rats. *p < 0.05, dependent vs nondependent group effect. n = 60.
Figure 4.3 Stress-induced 22 kHz ultrasonic vocalizations (USVs) during alcohol withdrawal are decreased by chlordiazepoxide but not by the corticotropin-releasing factor 1 (CRF₁) receptor antagonists R121919 and MPZP in dependent and nondependent rats. (A) 22 kHz USVs were decreased by chlordiazepoxide (2.5 mg/kg, s.c.) in both dependent and nondependent rats. n = 8-9 per group. (B) No effects of R121919 (20 mg/kg, s.c.) or (C) MPZP (20 mg/kg, s.c.) were observed. n = 9-12 per group. *p < 0.05, compared with vehicle; + p < 0.05, dependent vs nondependent group effect.
CHAPTER 5. CONCLUSIONS

The present studies demonstrate the following:

1. Anticipatory 50 kHz USVs represent a stable phenotype of increased motivation for food.
2. Anticipatory 50 kHz USV production is mediated by dopamine and opioids.
3. Anticipatory 50 kHz USVs represent a measure of context-elicited negative reinforcement learning in alcohol dependence.
4. Stress-induced 22 kHz USVs reflect an aspect of a multidimensional state of anxiety that may be related to stress and arousal.
5. Alcohol withdrawal potentiated stress-induced 22 kHz USVs are reduced by benzodiazepine anxiolytics but not by CRF₁ receptor antagonism.
6. USVs can be exaggerated by excessive alcohol intake and withdrawal suggesting that they may provide a novel insight into the emotional states hypothesized to contribute to compulsive drug seeking.

These studies indicate that rat ultrasonic vocalization is a behavior sensitive to alterations in emotional and motivational states that can be exaggerated in an animal model of compulsive alcohol seeking. Rat USV is a relatively unexplored field, especially in the context of alcohol dependence and constitutes an interesting behavioral phenotype to study the complex neurobiological mechanisms of reward and stress that underlies incentive salience. The current studies have begun the behavioral and neuropharmacological characterization of rat USVs and further studies will continue to provide new insights into the neurobiological basis of emotional behavior.
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