THE NEUROBIOLOGY OF 50-KHZ VOCALIZATIONS IN RATS

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ABSTRACT

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A wide variety of myomorph rodents (e.g., rats and mice) emit ultrasonic vocalization (USVs) in response to a wide variety of social interactions across their lifespan. Many rodents emit ultrasonic vocalizations during infant maternal separation, rough-and-tumble play, aggression, or mating. It has been proposed that rodents utilize ultrasonic vocalizations as communicative signals, given that it is both difficult for predators to hear these calls due to their rapid attenuation across short distance, and the lack of hearing sensitivity for ultrasonic tones of many rodent predators (Nyby & Whitney, 1978). Ultrasonic vocalization production has been most extensively studied in laboratory rats. Recent experiments suggest that rat USVs vary across two independent dimensions – peak frequency that may index locomotor arousal, and frequency modulation which may index emotional valence. In this paper, we hypothesize that high frequency vocalizations (50-70 kHz) are related to high levels of locomotor arousal, whereas lower tones (20-40 kHz) are related to low levels of locomotor arousal often accompanying freezing. We also hypothesize that frequency modulated (FM) vocalizations, which include trill and step calls, reflect a positive affective rewarding state in the animal, whereas non-FM calls (i.e. constant frequency calls) are unrelated to reward and perhaps may reflect aversion. The apparent arousal and valence encoding of ultrasonic vocalizations would presumably allow for rapid, high fidelity decoding by receivers of the motivational and emotional states of senders. Ultrasonic vocalizations may function by evoking the same emotional state of senders in receivers and thereby coordinate social behavior. The relationship between 50-kHz USVs and reward will further be tested by examining the relationship between
50-kHz calls during rough-and-tumble play and the rewarding value of play (Chapter 1A), and the relationship between electrical and chemical brain stimulation induced USVs and the rewarding value of the stimulation (Chapter 2).
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PREFERATORY NOTE

The overall aim of this dissertation is to examine the affective properties of rat ultrasonic vocalizations in order to further elucidate the neural mechanisms of emotional production in mammals using the tools of neuroscience. This dissertation is divided into 3 principal sections, Introduction & Review, Behavioral Concomitants of Ultrasonic Vocalizations, and the Neural Mechanisms of Ultrasonic Vocalization Production. The first section provides a review and theoretical synthesis of literature linking rat ultrasonic vocalizations to emotional states. This section consists of the initial introductory chapter, and an extensive review of the relationship between 50-kHz ultrasonic calls (which is the primary vocalization studied in this dissertation), reward, and human positive affect that is more extensively summarized in Appendix A in this dissertation. This appendix will appear in the journal *Neuroscience & Biobehavioral Review* early next year (Burgdorf & Panksepp, 2006). The second section of this dissertation (Chapter 1A & 1B) provides experimental evidence that 50-kHz USVs are related to the positive appetitive behavioral aspects of rough-and-tumble play behavior and mating, and that 50-kHz calls are related to the reward value of vocalization-eliciting stimuli. The final section (Chapters 2 & 3) are devoted to the neurobiology of 50-kHz vocalizations using both central and peripheral pharmacology as well as electrical brain stimulation to show that the mesolimbic dopamine system plays an important role in generations of 50-kHz vocalizations. Taken together, the studies of this dissertation provide evidence that 50-kHz ultrasonic vocalizations reflect a positive affective state in rats that may share some similarities to human positive affective states, in that both 50-kHz calls in rats and positive affective states in humans are modulated by the mesolimbic dopamine system.
INTRODUCTION

The Vocal Patterning of Laboratory Rats

Many species of myomorph rodents emit ultrasonic vocalizations (USVs) in the context of social encounters (Nyby & Whitney, 1978; Sales, 1972; Sales & Pye, 1974). USVs serve as an excellent short-distance communication device for rodents, given that they attenuate rapidly in space, especially in the burrow environments in which these rodents typically live. Also, many predators have limited hearing capacity for ultrasonic tones (Nyby & Whitney, 1978; Schwartkopff, 1955). This allows such rodents to communicate to “intended” receivers, with low risk of predation (Nyby & Whitney, 1978).

In rats, ultrasonic calls appear to be produced by a whistle like mechanism (Roberts, 1972). Helium-oxygen mixtures increase the fundamental frequency of resonant cavity utterances but do not alter the fundamental frequency of voiced laryngeal-generated calls. Both rat and mice USVs exhibit an increase in peak frequency in the presence of helium – therefore suggesting that ultrasonic calls are generated by a whistle-like mechanism given that whistle induced sounds increase their peak frequency in the presence of helium whereas voiced sounds do not (Roberts, 1975a). It is important to note that rodent whistle type USVs are probably generated in the larynx by the vocal cords that are held taut with a certain fixed apertures, given that denervation of the larynx has been shown to abolish USVs, and that USVs can still be generated in animals vocalizing through their nose which rules out a whistle like mechanism generated by the mouth (Roberts, 1975a; Roberts, 1975b). However, it is unlikely that the frequency modulated (FM) components of USVs are generated via pure tone whistles given the rapid nature of the frequency modulation (i.e. ~10 ms trill components; Figure 1B). It is probable that FM components of ultrasonic calls are generated either through voicing in the
larynx or articulation in the mouth. Therefore, it appears that rat and mice USVs are generated by two separate components, a whistle component consisting of taut vocal cords held at an aperture, and a FM component, which may consist of voiced laryngeal vibrations and/or articulation in the mouth.

**Behavioral concomitants of ultrasonic vocalization**

USVs first emerge early in infancy in many rodent species, and in the case of mice and rats USVs emerge within the first few days of life (Noirot, 1968; Sales & Pye, 1974). Pup distress vocalizations are triggered exclusively by aversive events such as social isolation and hypothermia (Blumberg & Sokoloff, 2001; Hofer, 1996). These infant vocalizations readily elicit maternal retrieval behavior in many strains of rats and mice (Brunelli, Shair & Hofer, 1994; Cohen-Salmon et al., 1985; Hahn & Lavooy, 2005; Smith, 1976; White et al., 1992). The most parsimonious interpretation of these data is that pups ultrasonic calls communicate an aversive emotional state to their mothers, whereby the moms respond by retrieving the pups. Rat pups stop showing separation-induced distress calls around the time when their ear canals open at approximately 14 days of age (Hofer et al., 2001), suggesting that the vocalization are not intended for non-maternal conspecifics (Noirot, 1966; Noirot, 1968). Alternative explanations of pup distress calls involve a variety of thermoregulatory functions of these calls (Blumberg & Sokoloff, 2001) although not all research groups find these effects (Hofer & Shair, 1993; Shair & Jasper, 2003). It is possible that there are multiple subtypes of pup calls, with one subtype reflecting separation distress while others may be associated with animals attempting to thermoregulate (Moles, Kieffer & D'Amato, 2004). Future research utilizing sonographic measured of the variety of infant separation calls is required to resolve this issue.
Pups isolated from their mothers consistently show ultrasonic distress calls, but not all myomorph rodent mothers show ultrasonic calls in response to separating them from their pups (Sales & Pye, 1974). However, in the few species that do show USVs in response to separation from their pups, the USVs do sonographically resemble the infant separation cries of their young (Sales & Pye, 1974). It is important to note that maternal behavior varies widely across rodents, with even genetically deaf strains capable of exhibiting adequate maternal behavior (D’amato & Populin, 1987). Therefore, mothers must be able to use multiple cues including USVs in order to retrieve and care for their pups.

During adolescence, rats begin to exhibit the two adult forms of USVs namely, the 50-kHz and 20-kHz calls, and cease to exhibit infant separation cries (Burgdorf & Panksepp, unpublished observation). Most other species of adult myomorph rodents also cease to make infant separation calls, and emit adult USVs (Sales & Pye, 1974). In many myomorph species, ultrasonic calls that are emitted in adulthood resemble the rat 50-kHz vocalizations, which are emitted primarily during the appetitive phases of sexual solicitation and to a lesser extent aggressive behavior (Floody, Pfaff & Lewis, 1977; Sales & Pye, 1974; White et al., 1998). In adult female/male rat dyads, the highest rates of 50-kHz USVs occur with pairings of dominant hormonally-intact males and estrous females (McGinnis & Vakulenko, 2003). In rats, adolescent play behavior and mating are the natural behaviors in which the greatest rates of 50-kHz calls are evident (Burgdorf et al., in prep; Knutson, Burgdorf & Panksepp, 2002). In various experimental studies, 50-kHz calls have been found to be tightly linked to reward, with increased rate of 50-kHz USVs exhibited in response to rewarding drugs, electrical brain stimulation, and heterospecific play (i.e. “tickling”) stimulation with vocalization rates strongly predicting the rewarding value of the stimuli (Knutson, Burgdorf & Panksepp, 2002).
Rats and ground squirrels appear to emit ultrasonic alarm calls in response to predators (Blanchard et al., 1992; Wilson & Hare, 2004); mice do not. This may reflect the fact that both rats and ground squirrels live in social groups, and would therefore benefit from emitting ultrasonic alarm calls by potentially reducing the predatory threat to both the caller and perhaps their kin (Shelley & Blumstein, 2004). It is important to note that ultrasonic alarm calls are primarily emitted when rats can avoid predators (e.g., hiding in a tunnel; Blanchard et al., 1991).

In addition to showing 20-kHz calls in response to predators, rats also exhibit 20-kHz calls in response to social subordination, during mating after unsuccessful mounts by the male, as well as after ejaculation (Knutson, Burgdorf & Panksepp, 2002). There is some evidence that 20-kHz calls that occur after ejaculation may be different from the 20-kHz calls emitted during social defeat or in response to predators, with the post ejaculatory 20-kHz USVs being more likely to be frequency modulated than the 20-kHz USVs that occur during aggression (van der Poel & Miczek 1991). In non-naturalist studies, many aversive events including foot shock and withdrawal from drugs of abuse promote 20-kHz calls (Antonidas & McDonald, 199; Burgdorf, Knutson & Panksepp, 2000; Vivian & Miczek, 1991).

**Effect of ultrasonic vocalizations in the receiver**

Most of the studies that examine the effects of USVs on the receiver use one of two methodologies. These studies either utilize the playback of ultrasonic calls (compared to tape hiss noise playback) on the behavior of devocalized animals, or they examine the behavioral differences in social behavior in devocalized animals compared to non-devocalized sham operated animals. Playback of 20-kHz USVs or synthetic tones similar to those produced in response to social defeat and foot shock have been shown to produce either freezing or flight behavior in the receiver (Beckett et al., 1996; Brudzynski & Chiu, 1995). Playbacks of 50-kHz
calls during mating behavior of rats elicit more vigorous appetitive mating behavior in animals that have been devocalized (White & Barfield, 1987; White & Barfield, 1989; White, Gonzales & Barfield, 1993). These data suggest that the affective - behavioral states of the sender induces a similar affective - behavioral state in the receiver, with 20-kHz USVs promoting a negatively valenced avoidance state in receivers and 50-kHz USVs eliciting a positively valenced appetitive state in the receiver. However, other devocalization - playback studies do not show these patterns of results. During aggression, the few 50-kHz or the many 20-kHz USVs emitted by the defeated male rat do not apparently attenuate aggressive behavior in the dominant animal that is defending his territory (Thomas, Takahashi & Barfield, 1983), although other studies have shown that 20-kHz calls are inversely related to aggression (Lehman & Adams, 1977; Sales, 1972). Therefore, it appears that USVs can produce an emotional contagion effect when the emotional states of the animals are congruent (i.e. mating) but the results are not as clear in more aversive social contexts (i.e. aggression).

Recent advances in the study of ultrasonic vocalization in rats

The increased utilization of direct recordings of USVs has made it possible to routinely measure the true characteristics of USVs without the distortion in signals imposed by the transformation of USVs into the human audible range with bat detectors (the devices most commonly used to bring USVs into the human audible range). Bat detectors transform ultrasonic signals into the audible frequency range typically by using one of the following two techniques: Frequency division – in which all recorded frequencies are divided by a fixed number (e.g. 1/10 or 1/6), or Heterodyne – in which only a pre-selected frequency (e.g., 50-kHz) is detected by the bat detector. Through the utilization of high frequency recording, a number of different subtypes of ultrasonic vocalization in the rat are now readily observable (White et al., 1992). First, 50-
kHz USVs can either be non-frequency modulated (constant frequency call, Figure 1A), or frequency modulated (trill and step components, Figures 1B). Similarly, 20-kHz calls appear to differ in both frequency modulation and harmonic structure comparing calls evident during social defeat as compared to post-ejaculation (Figure 1C & 1D). These differences are more apparent under direct recording conditions, given that frequency modulation and harmonic structure of calls can be blunted or artificially altered by bat detector recordings.
Figure 1. Sample sonographic images from high frequency recordings of USVs during mating, and aggression. 1A Constant frequency & 1B Frequency modulated 50-kHz calls that occur during mating. 1C Constant frequency & 1D Frequency modulated 20-kHz calls that occur during aggression and mating respectively as originally described by (van der Poel & Miczek, 1991). Frequency modulation of 50-kHz calls occurs by altering peak frequency with step (~30-kHz) and trill (~75 kHz) components, whereas 20-kHz calls are modulated primarily by articulation and harmonic structure.
Behavioral correlates of individual ultrasonic vocalization subtypes

There appear to be three distinct variants of 50-kHz calls: The constant frequency variety, as well as ones modulated with either trills or step components. Calls modulated with either step or trill components are scored as FM, given that often these calls contain both step and trill components, whereas the constant frequency variety of 50-kHz USVs is scored as non-FM calls. During mating, as well as play behavior, FM calls are positively correlated with appetitive soliciting behaviors (i.e., chasings during mating, and dorsal contacts during play, chapter 1A). Unlike FM 50-kHz calls, constant frequency 50-kHz calls are not correlated to these same appetitive behaviors during mating or play. During heterospecific rough-and-tumble-play behavior (i.e., “tickling”), a similar pattern emerges, with FM but not constant frequency 50-kHz calls being elevated in rats induced to be motivated for tickling by prior social isolation as compared to socially housed control animals (Chapter 1A). During tickling, the reward value of the stimulation as measured by running speed that the animal exhibit to self-administer additional tickling stimulation is significantly correlated ($r = +.60$) with the vocalization rate of FM 50-kHz calls but not constant frequency 50-kHz calls (Chapter 1A).

As previously reported (van der Poel & Miczek, 1991), there appear to be two distinct varieties of 20-kHz calls. The non-FM subtype appears to show little frequency modulation and little harmonic structure and occurs primarily in submissive male rats during aggression, whereas the FM subtypes exhibit either variability in the peak frequency (which appears to be generated by articulation; chapter 1A) or increased harmonic structures of the calls and occur primarily after males ejaculate during mating.
Affective correlates of Adult Ultrasonic Vocalizations

50-kHz and 20-kHz USVs have been proposed to reflect a positive affective state akin to human joy and a negative affective state akin to human anxiety respectively, ideas that have been extensively reviewed elsewhere (Appendix A; Knutson, Burgdorf & Panksepp, 2002, Panksepp, Knutson & Burgdorf, 2002). In brief, stimuli that are aversive to rats such as foot shock, bright light or predatory odors have been show to decrease levels of 50-kHz calls, whereas positive affective stimuli such as mating, rough-and-tumble play, drugs of abuse and rewarding brain stimulation increase levels of 50-kHz calls (Burgdorf et al., 2001; Burgdorf, Knutson & Panksepp, 2001; Knutson, Burgdorf & Panksepp, 1998, 1999). The ability of positive affective stimuli to elicit 50-kHz calls is also positively correlated with the rewarding value of the eliciting stimulus as measured by approach latency (Burgdorf & Panksepp, 2001). In addition, the neural mechanisms involved in the production of 50-kHz calls, namely the activation of the mesolimbic dopamine system and electrical stimulation of the accumbens, septum, and ventral tegmental area (VTA), are similarly associated with positive affective states in humans (Burgdorf et al., 2001a, Burgdorf et al., 2001b, Burgdorf, Knutson, Panksepp & Ikemoto, 2001; Heath, 1972, 1960; Okun et al., 2004). Conversely, rates of 20-kHz USVs are increased primarily by aversive events such as foot shock, environments paired with aversive drugs, withdrawal from drugs of abuse, and social defeat (Covington & Miczek, 2003; Burgdorf et al., 2001; Burgdorf, Knutson & Panksepp, 2001; Panksepp et al., 2005). Also, 20-kHz vocalizations are reduced by drugs that have anxiolytic effects in humans (Miczek et al., 1995).

Utilizing Vocalization Playback to Tests the Affect Value of Ultrasonic Vocalizations

We propose that USVs separately encode emotional valence by frequency modulation and locomotor arousal (i.e. forward locomotion) by peak frequency, both of which allows rats to
transmit and receive high fidelity signals as to the emotional state of the sender animal. It is also possible to produce an emotional contagion effect via the same mechanism allowing for coordination of behavior within groups of rats. Thus, during positive appetitive behaviors (i.e. mating, rough-and-tumble play, and non-agonistic social encounters) 50-kHz FM calls may serve to promote high locomotor arousal (high peak frequency -> high locomotor arousal) and continuation and reinforcement of behavior (Frequency modulation -> positive emotional valence). During predatory threat, constant frequency 20-kHz calls may serve to promote low levels of locomotor arousal (low peak frequency), and avoidance (little frequency modulation).

The two-factor model including valence and arousal is a commonly used model of human emotions, and may be pertinent for animal emotions as well (Knutson, Burgdorf & Panksepp, 2002).

**Overview of Experiments outlined in this dissertation**

In the first Experiment (chapter 1A), USVs were measured during the traditional adolescent and adult social interactions (i.e. rough-and-tumble play behavior, mating, and aggression), and the behavioral concomitants of each vocalizations subtype was analyzed. The results of this study showed that FM 50-kHz calls was related to appetitive mating (chasing and mounts) and play behavior (dorsal contacts) as well as the rewarding value of heterospecific play behavior (i.e. “tickling”). Conversely, 20-kHz calls were related to cessation or avoidance of further social behaviors during mating (post-ejaculatory refractory period) and aggression (freezing). In the second part of Experiment (Chapter 1B), the rewarding value of rough-and-tumble play behavior was assessed by the animals preference for the first 15 min of play behavior in which primarily 50-kHz calls are exhibited, compared to the last 15 min of play in which 20-kHz calls predominated, using a conditioned place preference procedure. The results
of this study show that rats prefer environments paired with the first 15 min of play compared to the second 15 min, and that differential rates of 50-kHz calls exhibited during the first 15 min as compared to the second 15 is positively correlated to the preference for the first 15 min.

In the second Experiment (Chapter 2), the neurobiology of 50-kHz calls was explored by utilizing localized electrical stimulation of various brain areas. 50-kHz USVs were elicited by electrical stimulation of ventral tegmental area (VTA) and the main projection areas of the VTA, namely the lateral hypothalamus, nucleus accumbens, and prefrontal cortex. All animals that exhibited electrical brain stimulation elicited 50-kHz calls also self-administered brain stimulation of these electrode placements (i.e. they self-stimulated). In another experiment, the dopamine receptor antagonist flupenthixol reduced levels of 50-kHz calls in animals that showed reliable brain stimulation induced 50-kHz USVs. Finally, focal injections of analogs to drugs of abuse (e.g., heroin and nicotine) previously shown to be rewarding and to increase dopamine release when administered directly into the VTA were rewarding only to animals that showed elevated levels of 50-kHz calls in response to these drugs.

In the third Experiment (Chapter 3), the role of the A10 mesolimbic dopamine system was further evaluated by using our heterospecific rough-and-tumble play paradigm (i.e. “tickling”). Electrolytic lesions of the VTA reduced levels of tickle induced 50-kHz calls but not 20-kHz calls. Neurochemical lesions of the A10 dopamine system with 6-OHDA also reduced levels of 50-kHz calls but not 20-kHz calls. Similar effects were observed with the dopamine receptor antagonist flupenthixol. However, the histamine receptor antagonist diphenhydramine non-specifically reduced both 50-kHz calls and 20-kHz calls.

In the final Experiment (Chapter 4), the rewarding / aversive effect of the playback of 50-kHz and 20-kHz USVs was examined in a receiver animal. Playback of frequency modulated
50-kHz USVs was rewarding to the receiver (i.e. rats nosepoked more in the hole that triggered playback of this vocalization then in a neutral hole), whereas playback of 20-kHz USVs was aversive (i.e. rats nosepoked less in the hole that triggered playback of this vocalization then in a neutral hole). Thus, these results are consistent with our primary hypothesis that 50-kHz USVs are related to positive affect, whereas 20-kHz USVs are related to negative affect (Knutson, Burgdorf & Panksepp, 2002).

**Overall synopsis of the hypotheses of the experiments covered in this dissertation**

The primary affective theoretical issue explored in this dissertation is that at least one of the subtypes of 50-kHz USVs (i.e. the frequency modulated variety) reflects a positive affective state in rats. Four falsifiable predictions were generated from this primary hypothesis, and these predictions were tested across the experiments covered in this dissertation. All the hypotheses were supported by the experiments that were conducted.

The first prediction is that 50-kHz USVs would be elevated in response to stimuli that has previously been shown to be rewarding to rats. In chapter 1 and 2, this hypothesis was tested by examining if rewarding stimuli (i.e. rough-and-tumble play behavior, mating behavior, compounds that mimic drugs of abuse, and brain stimulation) increase levels of 50-kHz USVs. The results of the experiments conducted in chapter 1 demonstrated that the rewarding social interactions (i.e. rough-and-tumble play behavior and mating) were associated with higher proportion of FM 50-kHz USVs, then aggression which has been found to have a mixed rewarding / aversive quality (Tayler, 1976). The second prediction is that 50-kHz USVs would be decreased in response to stimuli that has previously been shown to be aversive to rats. In the experiments conducted in chapter 2, it was demonstrated that animals that show 50-kHz USVs in response to direct chemical or electrical stimulation of the brain find that stimulation to be
rewarding, whereas animals that do not exhibit 50-kHz USVs in response to these manipulations rarely find the stimulation to be rewarding.

The third hypothesis is that the mesolimbic dopamine system, which has been shown to play a positive modulatory role in human positive affect (reviewed in Appendix A), will also positively modulate levels of 50-kHz USVs. The third hypothesis was tested in chapter 2 by examining the ability of pulses of electrical brain stimulation or drug microinjection that have been previously shown to elevate levels of dopamine to increase levels of 50-kHz USVs. In this study it was found that rats that showed 50-kHz USVs in response to either electrical or chemical brain stimulation found that stimulation to be rewarding. The third hypothesis was also tested in chapter 3 by examining the ability of manipulation that have been shown to decrease dopamine activity in the brain (i.e. dopamine receptor antagonist, electrolytic lesion of the ventral tegmental area, and neurochemical lesion of the mesolimbic dopamine system) to decrease rates of 50-kHz USVs. The results of this study demonstrated that in each of the manipulations that decreased dopamine functioning used in the study resulted in a reduction in FM 50-kHz USVs (which is the subtype of 50-kHz USVs that is most strongly related to reward and positive affect (Appendix A, chapter 1A)

The fourth hypothesis is that playback frequency modulated 50-kHz USVs would be rewarding to rats, and was tested in chapter 4. The results of this study showed that rats self-administer playback of frequency modulated 50-kHz USVs, but not flat 50-kHz USVs. These results are consistent with the experimental findings in chapter 1: FM 50-kHz USVs are strongly correlated to the rewarding value of both rough-and-tumble play behavior and “tickling”, whereas flat 50-kHz USVs are not correlate with these same social rewards.
CHAPTER IA: 50-KHZ CALLS DURING MATING AND AGGRESSION

Adolescent and adult *Rattus norvegicus* exhibit USVs in a wide variety of social encounters, including rough-and-tumble play (Knutson, Burgdorf & Panksepp, 1998), mating (White et al., 1990) and aggression (Thomas, Takahashi & Barfield, 1983; Vivian & Miczek, 1993). USVs have been hypothesized to serve as a useful communication signal for rats, given that many avian predators cannot hear ultrasonic calls, and such calls attenuate rapidly in natural environments (Nyby & Whitney, 1978). Rats that are devocalized prior to social encounters demonstrate deficits in social interaction, which can be ameliorated by the playback of USVs (White & Barfield, 1990; White & Barfield, 1989; White & Barfield, 1987). Thus, USVs appear to help facilitate social behavior in laboratory rats.

Adolescent and adult rats emit two classes of ultrasonic vocalization, namely 50-kHz calls which potentially reflect high levels of behavioral arousal and appetitive motivation, and 20-kHz calls which reflect low levels of behavioral arousal and an aversive motivational state (Knutson, Burgdorf & Panksepp, 2002). During rat aggression, mating, and rough-and-tumble play behaviors, USVs show a transition during test sessions from 50-kHz calls to 20-kHz calls, which mirrors the decrease in appetitive behaviors across test sessions (Burgdorf & Panksepp, in prep; White, et al., 1990). Therefore, USVs may help coordinate social behaviors in both the initiation-maintenance phase as well as the termination phase of adolescent and adult social encounters.

In order to further understand the behavioral and motivational concomitants of these vocalizations, in the present study rat USVs were compared across the primary social behaviors exhibited in adolescent and adult rats, namely rough-and-tumble play behavior, aggression, and mating. In the current study USVs were recorded via direct high frequency recordings.
al., 1992), which allow for the detection of ultrasonic call parameters that cannot be reliably quantified from the transformed recordings of bat detectors (i.e. which does not distinguish constant frequency and frequency modulated 50-kHz calls). Constant frequency (CF) 50-kHz calls are also referred to as flat 50-kHz calls. This experiment, for the first time, sought to quantify USVs recorded without transformation from a bat detector during behavioral episodes of aggression, sex, and rough-and-tumble play, and to correlate these USVs (some of which can only be successfully recorded via high frequency recordings) with these social behaviors. We hypothesize that variants of 50-kHz USVs will be correlated with positive appetitive behaviors (e.g., mounts during mating) and that 20-kHz USVs will be correlated with aversive behaviors (e.g., submissive freezing behavior; Knutson, Burgdorf & Panksepp, 2003).

Method

Housing

Long Evans rats born and bred in the Bowling Green State University animal facilities were used in this study. All animals were weaned at 21 days of age and singly housed in 20 x 40 x 20 cm lucite cages with corn cob bedding. Subjects were maintained on a 12:12 light dark cycle (lights on 8 am), and were given ad libitum access to Purina lab chow and tap water throughout the study. The resident males used in the aggression study and in the mating study were pair housed with ovariectomized females (e.g., as in Vivian & Miczek, 1993).

Experiment 1: Mating Behavior

14 adult male and female rats participated in this study. The experimental mating protocol was similar to Kippen and Pfau (2001). In brief, females were made sexually receptive by subcutaneous injections of estradiol (10 µg) followed 48 hrs latter by progesterone (500 µg), and mating sessions occured 6 hrs after progesterone administration. The mating chamber
consisted of a plexiglass chamber (35 x 26 x 18 cm) with a divider (30 x 0.5 x 20 cm) in the middle that permitted both the males and females to escape from each other (i.e. paced mating). Before testing, both males and females were habituated to the test chamber for at least two 30 min sessions. Testing consisted of a single 60 min session that occurred during the animals’ dark cycle. Mounts and ejaculations and post-ejaculatory refractory periods were scored (number of incidences of behavior, and time at which the behavior occurred) by a trained observer who was blind to the vocalization data from video recordings made from the long side of the mating chamber.

**Experiment 2: Aggression**

Eight male pairs were used in this experiment. All testing was conducted in a separate test room, so that the social activities would not be disruptive to the rest of the colony. Approximately 30 min before testing, the female was removed from the “resident” male’s cage. Social aggression testing consisted of placing the intruder animal into the home-cage of the resident animal for 30 min under dim (~2 Lux) light. A plastic lid was placed on top of the resident’s home cage with a small hole (6 X 6.5 cm) to position the ultrasonic sound sensitive microphone. For the 2 min pre- vs. post-bite analysis, only pairs in which intruders received at least 3 bites, with the first bite occurring within the first 5 min, were used for the analysis (n=6). From these pairs, either the vocalization pattern from the first and / or second set of bites was used for the analysis, so that analysis of calls would be minimally confounded by the potential vocalization effects of previous bites.

All video recorded behavioral data were analyzed blindly and independently of knowledge of the ultrasonic vocalization data. All USVs were also scored by an individual who were blind to experimental conditions. Video tape records were hand scored for three behaviors:
1) dorsal contacts, 2) bites (most of which were accompanied by audible squealing by the resident), and 3) the subsequent freezing behavior. A freezing bout was counted after ~5 consecutive seconds without movement (except sniffing). Total duration of freezing was used for subsequent analyses. Dorsal contacts (which in juvenile rats are indicative of social play solicitations) consisted of one animal having both its front paws on the dorsal surface of the other animal. Dorsal contacts and bites were scored as frequency of occurrence for each animal. Other behaviors that commonly occur during the more serious forms of this type of aggression, (i.e. sustained dominance on-top and submissive supine postures) did not occur at a high enough rate to be scored with the comparatively mild aggression observed in the tested animals (i.e. less than 1 event per test session). USVs were hand scored from sonographic displays.

**Experiment 3: Conspecific Play Behavior**

Before the start of testing, animals were weighed and assigned to play pairs matched on litter, sex and weight. All animals (n=27 play pairs) received 5 min play session in the play chamber on days preceding testing, which served as habituation sessions. Play testing consisted of a single 5 min play session in a 31 X 31 X 32 cm box with corn cob bedding under dim red light. Animals were 35 days of age at testing, and play behavior was recorded with a commercially available video camera and DVD recorder, and high frequency ultrasonic vocalizations were recorded in a similar manner as the previous experiments. Dorsal contacts, Pins (frequency of occurrence as well as duration) were scored by a trained experimenter as previously described (Panksepp, Siviy & Normansell, 1984). USVs and play behavior were analyzed separately in a blind manner.
Experiment 4: Heterospecific Play Behavior

Heterospecific play (i.e., tickling) consisted of vigorous whole-body playful simulation that included repeated pinning of the animal. For all animals, the tickling was done with the experimenter’s (dominant) right hand and consisted of scaled-down rapid finger and hand movements commonly used in human tickling. Methods were identical to Burgdorf & Panksepp (2001). In brief, tickling was conducted in a 45 X 35 X 20-cm opaque plastic box without bedding. Even though the stimulation was rapid, brisk, and assertive, care was taken not to threaten the animals. Approach latency data was collected by placing the rat in one corner facing the palm of the Experimenter's hand, which was placed in the corner diagonal to the rat (distance about 50 cm), and recording the latency in seconds with a digital timer (to 1 s) for the rat to touch the experimenter's hand with its head or front paws. The maximum approach latency was set at 30 s. Before testing, all animals received at least one 2 min tickling session on the day preceding testing that served as a habituation session. Adolescent animals were 1 month old, and adult animals were 6 months old during testing.

Results

Rates of 50-kHz calls were significantly different in response to “tickling”, mating, or aggression (all P’s < .001), whereas rates of 20-kHz calls only differed significantly between tickle and aggression (t(36) = 3.63, P = .001) and between mating and aggression (t(11) = 3.52, P = .005; Figure 2). Percentage of all 50-kHz calls that were frequency modulated was greater during 2 minutes of tickling in adult animals (t(34) = 15.9, P < .0001) or 2 minutes before ejaculation in mating pairs (t(12) = 12.6, P < .0001) than during the two minutes preceding a bite during aggression testing but did not differ between tickling and mating (t(36) = 1.5, P = .15; Figure 3).
Sonographic analysis of the step, trill, and constant frequency components of 50-kHz calls as well as 20-kHz calls revealed that all peak frequencies were significantly different from each other (all P’s < .001; Figure 4). During mating testing, frequency counts of constant frequency 50-kHz, FM 50-kHz, and 20-kHz calls were compared during the 2 minutes before and 2 minutes after each of eight ejaculations. While the rate of constant frequency 50-kHz calls remained unchanged 2 min before ejaculation (Mean ± SEM: 10.0 ± 1.6) and 2 min after ejaculation (11.6 ± 4.7) (t(7) = 0.3, P = .77), FM 50-kHz calls decreased (t(7) = 9.74, P < .001) and 20-kHz increased (t(7) = 5.15, P < .005) across the same time intervals (Figure 5).

During aggression testing, constant frequency 50-kHz calls decreased after a bite compared to 2 minutes before a bite Mean ± SEM 45.7 ± 10.8 and 2 minutes after bites 6.5 ± 4.3 (t(5) = 4.7, P = .006), and frequency modulated 50-kHz calls also decreased after bites (t(5) = 4.5, P < .01) whereas 20-kHz calls increased after the bite (t(5) = 13.7, P < .0001; Figure 6).

In an analysis of a subset of post-ejaculation 20-kHz calls, of the calls that occurred while the male rat was grooming or showing mouth movements (i.e. tongue protrusions) 96% were frequency modulated, whereas only 3% of the calls were frequency modulated when the male rat was not grooming or showing mouth movements (Mann-Whitney U, P < .0001; Figure 7).

In adult female rats receiving tickling stimulation, animals previously shown to have high levels of 50-kHz calls in response to tickling (tickle responders) showed significantly higher rates of FM 50-kHz calls (t(16) = 4.15, P < .001), but similar rates of constant frequency 50-kHz calls and 20-kHz USVs compared to the non-responder animals (Figure 8). Tickle responders also showed shorter approach latencies following tickling then non-responder animals (t(16) = 8.13, P < .0001; Figure 9).
In adolescent animals, isolate housed animals showed higher rates of tickle induced FM 50-kHz calls \((t(30) = 2.14, P < .05)\) but not constant frequency 50-kHz or 20-kHz calls compared to socially housed animals (Figure 10). FM 50-kHz USVs were positively correlated with dosal contacts during play behavior \((r = +.59, P < .005)\), and chases \((r = +.63, P < .005)\) and mounts \((r = +.47, P < .005)\) during mating, they were also positively correlated with the rewarding value of “tickling” stimulation \((r = +.60, P < .005)\). FM 50-kHz USVs were negatively correlated with the duration of the post ejaculatory pause during mating \((r = -.57, P < .005)\). 20-kHz USVs during mating were positively correlated to the duration of the post ejaculatory pause \((r = +.53, P < .005)\), and negatively correlated with chases \((r = -.32, P < .05)\) and mounts \((r = -.27, P < .05)\). Flat 50-kHz USVs were not significantly correlated with any play and mating behaviors, or the hand approach latency following “tickling”. All correlations reported were Pearson correlations (2 tailed) with the exception of comparisons involving 20-kHz USVs in which Spearman correlations (2 tailed) were employed given the non-parametric nature of 20-kHz USVs. All correlations between various USVs and play behavior, mating behavior, and the rewarding value of “tickling” are summarized in table 1.

**Discussion**

These results demonstrate for the first time that it is the frequency modulated subtype of 50-kHz calls that is related to positive appetitive behavior during rough-and-tumble play behavior and mating and is strongly positively correlated to the rewarding value of the vocalization eliciting stimulus during heterospecific play \((r = +.60, \text{see table 1})\). These data are consistent with previous studies demonstrating that 50-kHz calls are associated with positively valenced appetitive behaviors and reward (Knutson, Burgdorf & Panksepp, 2002).
These results also demonstrate for the first time that the frequency modulated subcomponents of 50-kHz calls (i.e. step and trill) can readily be discriminated via the fundamental frequency of the call, and that the rats appear to space the peak frequency of their ultrasonic vocalization across their usable ultrasonic hearing range (20-75 kHz; Figure 2).

This study revealed that frequency modulated 20-kHz calls that occur after ejaculation may be primarily due to a grooming or tongue protrusion artifact. Given that frequency modulated 20-kHz calls have been found to occur in greater frequency during mating than aggression (van der Poel & Miczek, 1991), it is possible that they may reflect different affective states in the sender. Post-ejaculatory 20-kHz calls may reflect a low arousal positively valenced state of satiety. It is also possible that the patterns of tongue protrusions and grooming that occur after mating resemble those seen during postprandial grooming (Berridge & Fentress, 1986); however, further studies involving slow motion analysis comparing postprandial to postejaculatory grooming are required to test this hypothesis. 20-kHz calls that occur after ejaculation are thought be associated with the sexual refractory period of the male rate and to perhaps scare off any other males from mating with the target female such periods of sexual inactivity (Barfield & Thomas, 1986). Studies involving the playback of these USVs are currently underway in order to examine the role of the subtypes of 50-kHz calls in the social and reward behaviors of rats. Overall, it is our hope that the study of the neural mechanisms of 50-kHz calls will shed light into the neural bases of positive affective states in mammalian brains.
Figure 2. (Top) Mean ± SEM 50-kHz & 20-kHz USVs in adult rats during heterospecific play (i.e. “tickling”), mating, or aggression. 50-kHz USVs rates were significantly different between all comparisons (p’s < .01), whereas rates of 20-kHz USVs for “tickle” and mating were significantly different from aggression (p’s < .01) but not from each other (p = .15).
Figure 3. Mean ± SEM percentage of all 50-kHz calls omitted during 2 min of tickling in adult animals or 2 min preceding a bite or ejaculation in aggression and mating studies respectively.

*** P < .001, two-tailed t-test comparing tickling to aggression or mating to aggression.
Figure 4. Mean ± SEM peak frequency of constant frequency 50-kHz calls, 20-kHz calls as well as the trill and step components of frequency modulated 50-kHz calls. All groups are significantly different from each other (p’s < .001).
Figure 5. Mean ± SEM Frequency modulated 50-kHz calls and 20-kHz calls 2 min before and the 2 min after an ejaculation during mating.
Figure 6. Mean ± SEM frequency modulated 50-kHz calls and 20-kHz calls 2 min before and the
2 min after a bite during inter-male aggression.
Figure 7. Percentage of post-ejaculatory calls that were frequency modulated while the animal was engaging in grooming or tongue protrusions during vocalizing (Movement group). *** p < .0001, Chi square test 2 tailed comparing movement vs. no-movement groups.
Figure 8. Mean ± SEM Ultrasonic vocalization rates in adult rats in response to 2 min of heterospecific play (i.e. “tickling”) stimulation in animals that exhibited differential “tickling” induced vocalization rates during an initial screening for vocalization responsivity (high vs. low responders). *** p < .001, between subjects t-test, 2 tailed.
Figure 9. Mean ± SEM Approach Latency for animals to self-administer additional tickling stimulation following the 2 min tickling test. *** p < .001, between subjects t-test, 2 tailed.
Figure 10. Mean ± SEM USVs in adolescent rats in response to 2 min of heterospecific play (i.e. “tickling”) stimulation in animals housed alone or in groups. * p < .05, between subjects t-test, 2 tailed.
Table 1. Pearson correlations between 20-kHz calls, and constant frequency and frequency modulated 50-kHz calls and various subcomponents of play and mating behavior as well as the rewarding value of heterospecific play (i.e. “tickling”) stimulation.

<table>
<thead>
<tr>
<th></th>
<th>“tickling”</th>
<th>Play</th>
<th>Play</th>
<th>Mating</th>
<th>Mating</th>
<th>Mating</th>
</tr>
</thead>
<tbody>
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<td></td>
<td>Reward</td>
<td>DC</td>
<td>Pins</td>
<td>Chases</td>
<td>Mounts</td>
<td>Post-ejaculation pause</td>
</tr>
<tr>
<td>20-kHz</td>
<td>+.12</td>
<td>-.12</td>
<td>+.03</td>
<td>-.32</td>
<td>-.27</td>
<td>+.53</td>
</tr>
<tr>
<td>Flat 50-kHz</td>
<td>+.23</td>
<td>-.14</td>
<td>+.05</td>
<td>-.22</td>
<td>-.15</td>
<td>-.19</td>
</tr>
<tr>
<td>FM 50-kHz</td>
<td>+.60 **</td>
<td>+.59 **</td>
<td>+.35</td>
<td>+.63 **</td>
<td>+.47 **</td>
<td>- .57 **</td>
</tr>
</tbody>
</table>

* P < .05
** P < .005

50-kHz Calls, Pearson Correlation (2-tailed)
20-kHz Calls, Spearman Correlation (2-tailed)
CHAPTER IB. 50-KHZ CALL DURING ROUGH-AND-TUMBLE PLAY

Rough and tumble play behavior in rats is exhibited at its highest levels from 32 to 40 days of age in socially isolated rats (Panksepp, 1981). Within a single test session, the highest rates of rough- and-tumble play behavior occur during the first 5 min of the session and gradually decrease across time (Panksepp, Siviy & Normansell, 1984). This gradual decrease in play behavior has been traditionally attributed to a growing satiety for play (Panksepp et al., 1984).

Recently, we have reported high levels of 50-kHz USVs occur during the first 5 min of rough and tumble play behavior, but which gradually decrease across the five minute session session (Knutson, Burgdorf & Panksepp, 1998). 50-kHz USVs have been used to index positive emotionality, given that rewarding stimuli increase the rates of these calls (i.e. mating, rough-and-tumble play, food, and drugs of abuse), whereas aversive events (i.e. predatory odor, bright light, foot shock, frustrative non-reward, and lithium chloride) decrease rates of 50-kHz calls (reviewed in: Knutson, Burgdof & Panksepp, 2002). This gradual decrease in 50-kHz calls suggests a gradual decrease in positive emotionality across test session, and raises the possibly that negative emotionality may increase across a play session.

The following study examined for the first time the pattern of USVs across a 30 min rough-and-tumble play session while the animals approach “satiation” (Panksepp et al., 1984), whereas our previous study of USVs during rough-and-tumble play only examined the first 5 min of play (Knutson, Burgdorf & Panksepp, 1998) in which the highest levels of play behavior are evident (Panksepp et al., 1984). In the second experiment, we tested the hypothesis that a shift from positive to negative emotionality occurs during longer sessions (30 min) of play by measuring both 50-kHz as well as 20-kHz USVs, which may index positive and negative
emotionality respectively (reviewed in: Knutson et al., 2002) during a 30 min play session. To further evaluate this proposition, we also directly tested whether rats prefer the first 15 min of play compared to the second 15 min of play using a conditioned place preference procedure.

Method

Subjects

46 Long Evans rats born and bred in the Bowling Green State University animal facility were used in this study. All animals were weaned at 21 days of age and singly housed in 20 x 40 x 20 cm lucite cages with corn cob bedding. Subjects were maintained on a 12:12 light dark cycle (lights on 8 am), and were given ad libitum access to Purina lab chow and tap water throughout the study.

Experiment 1A.

Before the start of testing, animals were weighed and assigned to either the resident, intruder, or the control group. All subjects were paired with a littermate and matched by sex. Animals in the resident group were habituated to the testing room for 2 consecutive days for 30 min each day starting the day after weaning (22 days of age). Habituation consisted of moving each animal in their home cage from the colony room to the testing room and then removing the animal’s home cage lid and placing a translucent plastic lid on top of the cage. Two additional play-habituation days occurred on the days after room-habituation, and consisted of 2 consecutive days of placing the intruder in the resident’s cage under the plastic lid for 30 min each day. Testing occurred 4 days after the end of play-habituation (29 days of age) for a subset of 8 pairs and consisted of placing the intruder animal in the resident animal’s cage for 30 min (resident-intruder play). The remaining 11 pairs received an additional day of 30 min play testing 5 days after the previous test session (34 days of age) in a neutral test cage (20 x 40 x 20 cm lucite cages with corn cob bedding).
cm with corn cob bedding) that was not the homecage of either animal in the play pair (non-resident play). These animals were also tested with new play partners, so original resident – intruder pairs were not tested together. For analysis, data from resident-intruder pairs (n = 12) and non-resident play (n = 11) were combined, since no significant differences were found between groups. During testing, USVs were also recorded using a Pettersen D980 ultrasonic detector (Uppsala, Sweden; with a 1:10 frequency division channel and second heterodyne channel tuned to 55-kHz), and the animals were videotaped using a commercially available camera and VCR or DVD recorder. USVs were analyzed via sonogram, and were scored in a blind manner.

**Experiment 1B.**

Following the end of Experiment 1A, the 11 pairs that received non-resident play in experiment 1A began conditioned place preference testing (CPP) for play. Place conditioning was conducted using an unbiased procedure as described elsewhere (Shippenberg and Herz, 1987). The place conditioning apparatus consisted of shuttle boxes made of Plexiglas (30 x 50 x 30 cm high), each equipped with a loose-fitting wire mesh lid. One side of the shuttle box was white with a textured floor (i.e. small ridges and valleys approximately 0.5 mm tall and wide), while the other side was black with a smooth floor. During conditioning sessions, a slide partition divided the two sides, creating two different conditioning “environments.” During the testing sessions of Experiment 1, the partition was replaced with a 20 x 15 cm door that allowed the animal unrestricted access to either environment. The light level (~10 lux) level on each side was matched with a portable lamp suspended over the test chamber. All animals were habituated to the two chamber conditioned place preference apparatus with the divider (20 X 15 cm) removed to allow access to both chambers for 15 min on the day prior to the start of testing.
Habituation occurred 2 days (36 days of age) after non-resident play testing. Place conditioning consisted of 3 consecutive days of allowing each pair to play for the first 15 min on the white side, and then being moved by the experimenter to the black side for the second 15 min of testing. Therefore, the first 15 min of play was conditioned to the white chamber and the second 15 min of play was conditioned to the black chamber of the conditioned place preference apparatus. One of the animals became sick during testing, so only 21 of the total 22 animals were used for place preference analysis. An additional 8 animals that did not participate in Experiment 1A were used in this study and served as control animals. Control animals were placed individually into the white chamber for the first 15 min followed by the black chamber for the second 15 min during all 3 days of conditioning. Place preference testing occurred on the day after the end of conditioning and consisted of allowing the animals to explore both the black and white sides of the place preference chamber (divider removed) for 15 min. USVs and play behavior were recorded and scored in a similar manner as Experiment 1A.

Results

Experiment 1A.

50-kHz USVs decreased during the 30 min test period whereas 20-kHz calls increased across this same period as indicated by a significant Vocalization Type X Trial Block interaction using a 2 x 6 repeated measures ANOVA (F(1,5) = 12.29, P < .0001; Figure 11). Many of 50-kHz USVs scored using the frequency division output of the bat detector were obscured by noise produced by animals moving around in the corn cob bedding substrate. Therefore, in subset of play pairs (n=8), we compared 50-kHz vocalizations sonographically scored utilizing frequency division compared to 50-kHz calls scored through the heterodyne channel tuned to 55-kHz. Frequency division 50-kHz calls were significantly positively correlated with heterodyne scored
50-kHz calls (Spearman’s r = .80, P < .0001). A linear regression revealed a slope of 6.334. Therefore, we multiplied all frequency divided calls in Experiment 1A by the correction factor of 6.334. To further test the validity of this transformation, corrected 50-kHz vocalization scores during the first 15 min of play (mean ±SEM, 249.5 ±61.5) were not significantly different (t(27) = 0.94, P > .05) then the first non corrected 50-kHz calls during the first 15 min of play during the third play place preference conditioning session (mean ±SEM, 346.4 ±89.1) in which no corn cob bedding was present in the cage to produce sound artifacts.

**Experiment 1B.**

Play animals exhibited significantly more time in an environment paired with the first 15 min of play on the place preference test day compared to the habituation day (t(20) = 2.86, P < .01; Figure 12), whereas control animals failed to show this effect (P > .05, data not shown). During the 3rd (final) conditioning day, animals exhibited more 50-kHz calls during the first 15 min (mean ±SEM, 346.4 ±89.1) as compared to the second 15 min (mean ±SEM, 194.5 ±67.9) (t(9) = 3.94, P < .005). 20-kHz calls were not significantly different during the first 15 min (mean ±SEM, 247.1 ±80.2) compared to the second 15 min (mean ±SEM, 276.9 ±95.6) (t(9) = 0.37, P > .05). Exemplar sonograms of 50-kHz & 20-kHz calls from the frequency division channel of the bat detector appear in Figure 13. Place preference scores for the first 15 min were significantly correlated with the degree to which pairs produced more 50-kHz calls during the first 15 min compared to the second 15 min (Pearson’s r = + .77, P < .05).

**Discussion**

This study revealed for the first time that the pattern of USVs shifts from 50-kHz USVs to 20-kHz USVs across a 30 min session, and that rats prefer an environment paired with the first 15 min of play compared to the second 15 min of play. Most importantly this study revealed that
differential rates of 50-kHz USVs during the first and second 15 min of play is positively correlated with the preference for the environment paired with the first 15 min of play. These results suggest that prolonged (> 5 min) rough and tumble play bouts are associated with progressively elevated levels of 20-kHz USVs, and that rats show a preference for environments that are paired with the beginning segments of play over the later segments of play. Our previous report of USVs during rough and tumble play behavior (Knutson, Burgdorf & Panksepp, 1998), which failed to detect 20-kHz USVs was probably due to the relatively short 5 min play bouts the animals received. The high levels of 50-kHz and low levels of 20-kHz USVs in our previous report using 5 min play bouts (Knutson, Burgdorf & Panksepp, 1998) as well as during the first 5 min of testing in this present study suggest that the first 5 min of play is accompanied primarily by positive affect (Knutson et al., 2002).

These results also suggest that well-established decreases in rough and tumble play behavior across a play session (Panksepp, Siviy & Normansell, 1984) could reflect an increase in negative emotionality across the test session given that 20-kHz USVs increase across the play session which has been shown to index negative emotional states (Knutson et al., 2002). During rough and tumble play bouts, pinning durations (Panksepp et al., 1984) as well as freezing durations increase with time (Burgdorf & Panksepp, unpublished observation). Elevated levels of 20-kHz calls which occur primarily during the last 15 min of a play bout also index negative emotionality. Increases in 20-kHz USVs rates have been associated with a wide variety of aversive events including social defeat (Thomas et al. 1983; Tornatzky and Miczek 1995), foot shock (Cuomo et al. 1988; Tonoue et al. 1986), lithium chloride administration (Burgdorf, Knutson, Panksepp & Shippenberg, 2001) and drug withdrawal (Vivian and Miczek, 1991; Barros & Miczek, 1996; Mutschler & Miczek 1998).
Future studies of rough and tumble play behavior need to become increasingly sensitive to the apparent shift from positive to negative emotionality during prolonged test sessions. Studies utilizing longer play bouts (i.e. Gordon et al., 2002) may reflect the presence of mixed emotionality in rats.
Figure 11. Mean ± SEM 50-kHz and 20-kHz USVs during 30 min play sessions.
Figure 12. Mean ± SEM percentage of time spent in the place preference chamber paired with the first 15 min of play behavior both before and after conditioning. ** P < .01, within subject’s t-test (2-tailed).
Figure 13. Exemplars of 50-kHz and 20-kHz USVs that occur during rough and tumble play behavior.
CHAPTER 2: MAPPING OF 50-KHZ CALLS IN THE BRAIN

We have previously shown that rat 50-kHz USVs may index a positive affective state associated with reward (Knutson, Burgdorf, & Panksepp, 2002). Specifically, we have shown that 50-kHz USVs are elevated by food, sex, rough-and-tumble play, drugs of abuse, and anticipation of rewarding electrical brain stimulation (chapter 2; Burgdorf, Knutson & Panksepp, 2000; Burgdorf, Knutson, Panksepp & Ikemoto, 2001; Burgdorf, Knutson, Panksepp & Shippenberg, 2001; Knutson, Burgdorf & Panksepp, 1998). Conversely, aversive stimuli including bright light, predatory odors, foot shock, and aversive drugs decrease levels of 50-kHz calls (Burgdorf, Knutson & Panksepp, 2000; Burgdorf, Knutson, Panksepp & Shippenberg, 2001). During heterospecific play behavior (i.e. “tickling”), rates of 50-kHz vocalization rates are positively correlated with the rewarding value of the stimulation as measured by instrumental approach behavior (at about +.60 to +.80 Pearson correlations; Chapter 1A; Burgdorf & Panksepp, 2001; Panksepp & Burgdorf, 2003).

The neurobiology of reward has classically been studied by examining brain areas that support self administration of electrical brain stimulation (self-stimulation), or by examining the rewarding effects of drugs injected directly into the brain by examining their ability to support self administration, facilitate electrical self-stimulation, or produce a conditioned place preference. While a number of brain areas support electrical self-stimulation behavior (e.g., hippocampus, habenula, and medial dorsal thalamus), the circuits from the prefrontal cortex through the medial forebrain bundle to the raphe are the best studied and support the most robust self-stimulation behavior (Gallistel, Shizgal & Yeomans, 1981; Miguelez & Bielajew, 2004). Within this circuit, the ventral tegmental area (VTA) has been found to be the brain area most
consistently found to support that rewarding effects of opiates, nicotine, barbiturates, and neurotensin when given focally (Ikemoto & Wise, 2004).

In this study, the relationship between the 50-kHz USVs and reward will be further examined by testing the relationship between the ability of electrical brain stimulation (Experiment 1) or injections of a rewarding drug (i.e., DAMGO, see McBride, Murphy & Ikemoto, 1999) into the VTA (Experiment 2) to elicit 50-kHz calls and reward. Given that we have previously shown that 50-kHz USVs reflect a positive affective state in rats and are positively related to reward (reviewed in: Knutson, Burgdorf & Panksepp, 2003), we hypothesize that animals that receive either electrical stimulation or chemical stimulation to brain regions that increases 50-kHz USVs will find the brain stimulation to be rewarding as measured by self-stimulation behaviors and the above neurochemistries in conditioned place preference studies.

Method

65 Long Evans female rats born and bred in the Bowling Green State University animal facilities were used in this study. All animals were weaned at 21 days of age and singly housed in 20 x 40 x 20 cm high lucite cages with corn cob bedding. Subjects were maintained on a 12:12 light dark cycle (lights on 8 am) tested during the light phase, and were given ad libitum access to Purina Lab Chow and tap water throught the study.

Surgery

Subjects were anesthetized with Ketamine (80 mg/kg, i.p.) & Xylazine (10 mg/kg, i.p.) and mounted in a Kopf stereotaxic instrument (Kopf Instruments, Tujunga, CA). After exposing the skull, the skull landmarks bregma and lambda were visualized and aligned on the same horizontal plane. In Experiment #1, Bipolar stainless steel electrodes (Plastic Products, USA) were lowered into the brain aimed at the following brain areas: prefrontal cortex, nucleus
accumbens, septum, ventral pallidum, lateral preoptic area, lateral hypothalamus, ventral tegmental area, pedunculopontine tegmentum, and dorsal raphe. Anatomical localizations of these electrode placements are reported in figure 18. All electrodes were angled 16° from the midline. Three additional holes were drilled for insertion of stainless steel self-tapping anchor screws, and electrodes were secured to the skull with dental acrylic. In Experiment #2, Rats were implanted with unilateral 23 gage guide cannulae (Plastic Products, USA) into the VTA(-5.0 AP, 1.0 ML, 8.0 DV, flat brain). Cannulae were angled 16° to avoid ventricles and were secured to the skull with four jeweler’s screws and dental cement.

**Electrical Brain Stimulation Testing**

After a minimum of a week of postsurgical recovery, animals first received experimenter-delivered brain stimulation to test for stimulation induced USVs using a protocol shown to elicit the maximal dopamine release from electrical stimulation of the VTA (three 10 second 120 µA stimulus trains of continuous 60 Hz sine wave stimulation, with a 30 min inter train interval; Montague et al., 2004). Current thresholds for stimulation-induced vocalizations, motor behaviors (e.g., turning) as well as stimulation-induced feeding were conducted in a 45 X 35 X 20 cm opaque box with laboratory rat chow on the floor. Animals were given ascending 10 continuous pulses of ESB starting at 5 µA, and increasing in 5 µA steps until a behavioral effect emerged (i.e. stimulus induced eating, or ultrasonic vocalization). After which, the stimulation was repeated 4 times, and if the stimulation elicited the behavior in at least 3 of the 4 trials, the current level was recorded as the threshold level. Before each of the stimulation trials, at least 30 seconds was allowed to transpire without the animal exhibiting any ultrasonic vocalizations or eating behavior. Ultrasonic vocalizations and eating behavior were rarely exhibited by animals in the absence of brain stimulation. For self-stimulation tests, subjects were placed in a 34 × 23
× 32.5 cm high translucent box with a 10 x 2 x 25 cm bar elevated 8.5 cm from the floor. Subjects were allowed to bar press for 0.5 to 0.2-s pulses of 50-200 µA pulse-trains on a continuous reinforcement schedule (as in: Rossi & Panksepp, 1992). During this training, subjects received priming pulse-trains and were directed toward the bar as needed. Current intensity was initially set at 20 µA, and was raised by 5 µA every 3 min until vigorous self-stimulation occurred (operationally defined as greater than or equal to 3 bar presses per min). Each subject's optimal current levels and stimulation durations for eliciting maximal barpressing rates was recorded and used on subsequent tests to determine self-stimulation rates.

In a subset of 8 animals that showed reliable brain stimulation induced 50-kHz USVs, the effect of the D1/D2 receptor antagonist Flupenthixol on brain stimulation induced 50-kHz USVs were tested given that dopamine has been shown to play a key role in the rewading effects of brain stimulation as well as drugs of abuse (Ikemoto & Panksepp, 1999) Brain stimulation sessions were conducted in a 34 × 23 × 32.5 cm high translucent box with commutator (Plastics One, USA) and consisted of a single 2 min session each day in which animals received 10 seconds of continuous brain stimulation 40 seconds and 80 seconds into the session. 0.8 mg/kg flupenthixol (Sigma, USA) or vehicle was administered i.p. 2.5 hrs before the start of the test session (Laviolette & van der Kooy, 2001). Drug and Vehicle injections were counterbalanced between animals, and all subjects received a single re-habituation session between the two test days.

USVs were recorded from the high frequency output of a Pettersson D980 bat detector (Sweden) and recorded digitally with a Fostex Fr-2 field recorder (USA). Sonographic analysis of calls was done in a blind manner with SAS lab Pro (Avisoft Bioacoustics, Germany)
Place Conditioning Procedure

Place conditioning was conducted using an unbiased procedure as described elsewhere (Shippenberg and Herz, 1987). The place conditioning apparatus consisted of shuttle boxes made of Plexiglas (30 x 50 x 30 cm), each equipped with a loose-fitting wire mesh lid. One side of the shuttle box was white with a textured floor (i.e. small ridges and valleys approximately 0.5 mm tall and wide), while the other side was black with a smooth floor. During conditioning sessions, a slide partition divided the two sides, creating two different conditioning “environments.” During the testing sessions of Experiment 1, the partition was replaced with a 20 x 15 cm door that allowed the animal unrestricted access to either environment. The light level (~10 lux) on each side was matched with a portable lamp suspended over the test chamber.

Before the start of conditioning, all rats were habituated to the place preference chamber for 15 min in order to determine the compartment preferences for each animal. Two conditioning session was conducted, with the vehicle pairing (0.9% saline) being with the black side of the chamber and the drug injection (100 ng DAMGO; Sigma, USA) pairing with the white side. Each conditioning session lasted 30 min. USVs were recorded during both the drug and vehicle conditioning trials. Each conditioning session had at least 2-3 days separating the sessions which were counterbalanced for drug and vehicle injection order. USVs were recorded during the conditioning sessions. All injections were 500 nl in volume and were injected over 1 min and the injection cannulae was left in place for an additional 30 seconds before removal. After injections, rats were immediately confined to the white compartment following drug injections or the black compartment following vehicle injections. After place preference conditioning, rats were again allowed free access to both chambers for 15 min to test for post-drug conditioning preference. After histological analysis, only animals that had cannulae injection tips that were at
least partially contained within the VTA were used for analysis (n = 18). Histological analysis of placements was done blind manner in respect to the vocalization eliciting or rewarding effects of the injection site.

**Histology**

At the conclusion of behavioral testing, animals were sacrificed with carbon dioxide and their brain was rapidly removed. Brains were placed into a 30% sucrose-10% formaldehyde-0.9% saline solution (w/v) for at least 1 month before slicing. Brains were then frozen and sliced into 50-micron coronal sections with a freezing microtome. Sections through the tips of the electrodes were mounted on microscope slides, and electrode as well as cannulae tips were localized by projecting the slides with magnification onto a table using the atlas of Paxinos & Watson (1998). Histological reconstruction was done in a blind manner in respect to the behavioral effects of the electrical stimulation or microinjection.

**Results**

**Experiment 1**

The minimum self-stimulation rate was set at 3 bar presses / min, which is significantly greater than the free operant bar pressing rate (mean ± SEM) of 0.4 ± 0.2 bar presses / min (t(9) = 13.96, P < .0001). The Mean ± SEM bar pressing rate for the animals that exhibited self-stimulation (n=32) was 22.1 ± 4.1, and for the non-self stimulation animals (n=15) it was 0.3 ± 0.1. The mean bar pressing rates for the self-stimulating animals was significantly greater than the threshold for self stimulation (3 barpresses / min; t(31) = 4.6, P < .001) and significantly less than the threshold for self stimulation for the non self-stimulating animals (t(14) = 18.6, P < .001). The self stimulation animals were further subdivided into two group – a group that showed marginal self stimulation rates (3-9 bar presses / min, n=11), and a group that showed
more robust self-stimulation rates (10 or more bar presses/min, n=20). In the marginal self-stimulation group, 91% of the animals showed repeatable ESB induced 50-kHz USVs, and 50% showed reliable ESB induced 50-kHz USVs. In the group showing more robust self stimulation, 75% showed repeatable ESB induced 50-kHz USVs, and 60% showed reliable ESB induced 50-kHz USVs. Neither group differed significantly in the percentage of animals showing repeatable or reliable ESB induced 50-kHz USVs, rates of ESB induced 50-kHz USVs, current thresholds for self-stimulation, or brain stimulation induced 50-kHz USVs (all P’s > .05). Therefore the data for all self-stimulation animals was combined for subsequent analyses.

In animals that demonstrated self-stimulation with electrode sites in the forebrain (i.e. frontal cortex and basal forebrain, n=13), 77% showed marginal self-stimulation rates compared to mid and hindbrain self-stimulation animals (i.e. hypothalamus, ventral tegmental area, dorsal raphe) in which only 5% showed marginal self-stimulation rates (Chi Square, P < .001). However, no significant differences were found in between forebrain and hindbrain self-stimulation animals in current threshold for reliable ESB induced 50-kHz USVs, optimal self stimulation current, or rates ESB induced 50-kHz USVs. Therefore, the data forebrain and midbrain/hindbrain electrode sites was combined for subsequent analyses.

81% of all self stimulating animals showed repeatable ESB induced 50-kHz calls, whereas none of the non self-stimulators showed repeatable ESB induced 50-kHz calls (Mann-Whitney U, P < .0001; Figure 14). All animals tested that showed repeatable ESB induced 50-kHz USVs also self-stimulated (n = 26). Self stimulating animals also showed a significant increase in 50-kHz call rates to experimenter-delivered ESB compared to baseline (t(31) = 3.10, P < .005), whereas non self-stimulating animals did not show this effect (t(14) = 0.33, P > .05; Figure 15). Also, the increase in 50-kHz call rates in response to experimenter delivered
stimulation was greater for self-stimulating rats compared to non-self stimulating rats (Mann-Whitney U, P = .001). The histological localization of electrode tips are reported in figure 18.

In a subset of 8 animals that showed reliable, stimulation-induced USVs, injections of the D1 and D2 dopamine receptor antagonist flupenthixol reduced frequency modulated (FM) 50-kHz calls (t(7) = 2.31, P < .05), but not constant frequency 50-kHz calls or 20-kHz calls (all p’s > .05; Figure 16).

**Experiment 2**

Animals were classified as DAMGO Vocalizers if they exhibited twice as many 50-kHz calls to DAMGO in the first 5 minutes compared to vehicle injections, or exhibited more 50-kHz USVs calls during both the first and second 5 minute blocks for the DAMGO compared to vehicle injection. The first 5 min after DAMGO injection was the peak drug effect with 71% of all DAMGO elicited vocalizations occurring during the first 10 min of the 25 min that each condition session was recorded for. Animals that did not make this criterion were referred to as DAMGO Non-Vocalizers. DAMGO Vocalizers animals showed a greater increase in 50-kHz USVs in response to DAMGO compared to vehicle injection during the first 10 min of testing compared to DAMGO Non-Vocalizers (Mann-Whitney U, P < .05). Using these grouping, DAMGO Vocalizers (n=6) showed significant place preference for the environment paired with drug injection (t(5) = 3.0, P < .05) whereas DAMGO Non-Vocalizers (n=12) did not show significant place preference for the environment paired with drug injection (t(10) = 0.2, P > .05; figure 17). The histological localization of cannulae tips are reported in figure 19.

**Discussion**

These results demonstrate for the first time that electrical or chemical stimulation of the brain that elicits 50-kHz USVs is rewarding to the animal, providing further support that 50-kHz
USVs are positively related to reward (Knutson, Burgdorf & Panksepp, 2002). The results of experiment 1 provides a preliminary functional neuroanatomy of 50-kHz calls as well as more evidence that 50-kHz USVs are related to reward given that all electrode sites that elicited repeatable 50-kHz calls supported self-stimulation. These results are consistent with the results of Jürgens (1976) who showed the brain-stimulation elicited trill calls are positively related to self-stimulation in many of the same brain regions as tested in this study. A common variant of 50-kHz calls involves a FM trill component, and both the rodent 50-kHz call with trill components and the squirrel monkey trill call are both related to positive appetitive behavior (Jürgens, 1979; White, Cagiano, Moises & Barfield, 1990).

The results of Experiment 2 demonstrate that rewarding drugs that are focally injected into the VTA are primarily rewarding in animals that exhibited elevated levels of 50-kHz calls in association with these injections. Given that these drugs of abuse as well as electrical brain stimulation are thought to at least partially depend on the mesolimbic dopamine system, we also showed that electrical brain stimulation induced 50-kHz calls could be attenuated by dopamine receptor antagonist flupenthixol. In particular, flupenthixol only decreased rates of FM 50-kHz calls (which includes trill calls). Previously, it has been shown that injections of amphetamine into the accumbens shell produces a dramatic 35-fold elevation in rates of 50-kHz calls (Burgdorf et al., 2001).

Although all animals used in these studies were adult female Long Evans rats, it is unlikely that variability in ultrasonic vocalization rates and operant behavior due to estrus cycle explains the results in this study. While levels of USVs do vary during the estrus cycle, this effect is dependent on the presence of a hormonally intact male rat in an experience dependent manner (McGinnis & Vakulenko, 2003). In this experiment, all females were virgins and there
was no contamination with male odors since the equipment used in these studies were only used by female rats. While operant responding for electrical brain stimulation at some sites has been shown to vary modestly across estrus cycle (Stratmann & Craft, 1997), in the current study presence or absence of self-stimulation behavior remained quite stable across test days. Also in both of the present studies, the experimenter was blind to estrous condition of the animal. Therefore, it is probable that any variability induced by estrous state of the animal would be evenly distributed across conditions, and therefore contribute primarily to error variance.

Taken together, these data provide further support that 50-kHz calls are related to reward processes and the mesolimbic dopamine system. Future study will examine the in vivo release of dopamine during the production of 50-kHz calls. Preliminary data already shows that DA is released during rough and tumble play (Panksepp, 1993; Burgdorf, Unpublished data). A comprehensive analysis of the putative neural circuit of 50-kHz USVs is discussed in the general discussion section.
Figure 14: Percentage of animals showing repeatable electrical brain stimulation induced 50-kHz USVs that also show self-stimulation behavior. *** P < .0001, Mann-Whitney U, 2-tailed.
Figure 15: Mean ± SEM rates of 50-kHz calls in response to electrical brain stimulation (120 μA, 10 seconds, bipolar stimulation) associated with maximal dopamine release in animals that exhibit self-stimulation behavior (Montague et al., 2004) in self-stimulation and non self-stimulation animals. *** P < .005, 2-tailed within subject t-test.
Figure 16: Mean ± SEM rates of 50-kHz calls elicited by electrical brain stimulation after pre-treatment with the dopamine D1/D2 receptor antagonist flupenthixol or vehicle. * P < .05, within subjects t-test, 2-tailed.
Figure 17: Mean ± SEM increase in time (sec) spent on drug paired side after drug conditioning compared to pre-conditioning in animals that showed more USVs in response to drug injection during the peak drug effect (twice as may 50-kHz USVs to drug vs. vehicle in peak 5 min block, or more calls in both the peak and next block) referred to as DAMGO Vocalizers whereas animals that did not meet this criterion were referred to as DAMGO Non-Vocalizers. * P < .05 within subject t-test, 2 tailed comparing pre vs. post conditioning.
Figure 18.
Figure 18. Anatomical location of electrode tips in Experiment 1. Anatomical plates adapted from Paxinos & Watson (1998).
Figure 19. Anatomical location of cannulae tips in Experiment 2. Anatomical plates adapted from Paxinos & Watson (1998). VTA – Ventral Tegmental Area. No differences in anatomical placements were found between the responder and non-responder animals.
CHAPTER 3: PHARMACOLOGY OF 50-KHZ CALLS

Rat 50-kHz USVs have been shown to be strongly linked to reward, positive affective states, as well as mesolimbic dopamine functioning (Appendix A; Knutson, Burgdorf & Panksepp, 2002). A wide variety of rewards including rough-and-tumble play behavior, mating, food, drugs abuse, and rewarding electrical brain stimulation increase rates of 50-kHz calls, whereas aversive stimuli such as foot shock, bright light, aversive drugs, and the smell of predators suppress 50-kHz USVs (Knutson, Burgdorf & Panksepp, 2002).

The mesolimbic dopamine system, consisting of primarily the projections from the dopaminergic neurons from the ventral tegmental area (VTA) to the nucleus accumbens, has also been implicated in reward and positive affective states (Appendex I; Ikemoto & Panksepp, 1999; Swanson & Volkow, 2003; Wise, 2004). Recently, we have shown that both systemic and intra-accumbens injections of amphetamine increase rates of 50-kHz calls (Burgdorf, Knutson, Panksepp & Ikemoto, 2001; Knutson, Burgdorf & Panksepp, 1999). We have also shown that rewarding electrical brain stimulation of the VTA using stimulation parameters shown to elicit dopamine release can increase rates of 50-kHz calls (chapter 2; Burgdorf, Knutson & Panksepp, 2000; Montague et al., 2004). All electrode sites thus far tested that show repeatable electrical stimulation induced 50-kHz calls support self-stimulation (chapter 2), which is consistent with the finding that all electrode sites that show electrical stimulation induced dopamine release support self-stimulation (Garris et al., 1999).

In the present set of studies we further tested the role of the mesolimbic dopamine system in the generation of 50-kHz USVs by testing the effect of electrolytic lesions of the VTA and dopamine receptor antagonists on heterospecific rough-and-tumble play (i.e. “tickling”) induced USVs, given that this paradigm by far produces the largest and most stable increases in 50-kHz
ultrasonic calls (chapter 1). Based on our previous findings that manipulations that increase mesolimbic dopamine levels (i.e. amphetamine microinjection and rewarding electrical brain stimulation; Burgdorf et al., 2001; chapter 2) we predicted that lesions or pharmacological blockade of the mesolimbic dopamine system would reduce levels of 50-kHz USVs.

Method

Housing

Long Evans male and female rats born and breed in the Bowling Green State University animal facilities were used in this study. All animals were weaned at 21 days of age and individually housed in 20 x 40 x 20 cm high Lucite cages with corncob bedding. Subjects were maintained on a 12:12 light dark cycle (lights on 8 am), and were given ad lib. access to Purina lab chow and tap water thought the study.

Surgery

0-size insulated insect pin (with a 1 mm active electrode exposed) was lowered bilaterally into the VTA. Lesions consisted of passing 0.5-miliamp anodal DC current for 30 seconds bilaterally into the VTA (-6.0 mm AP, ± 0.5 mm ML, -8.6 mm DV to bregma with bregma and lambda aligned in the DV plane; n=8). Sham lesion animal (n=7) had electrodes lowered into the brain, but no current was passed. Animal were allowed to recover for one-week post surgery before the start of testing, and were tube fed with a 10% glucose solution if they lost more then 5% of presurgery body weight (ie. 5 ml, twice per day) until the animals no longer showed sustained weight loss.

Tickling Procedure

Tickling consisted of vigorous whole-body playful simulation that included repeated pinning of the animal. For all animals, the tickling was done with one hand and consisted of
scaled-down rapid finger and hand movements commonly used in human tickling (see Panksepp & Burgdorf, 2003). Tickling was conducted in a 45 X 35 X 20 cm high opaque plastic box without bedding. Although the stimulation was rapid, brisk, and assertive, care was taken not to threaten the animals. The test chamber was divided into 4 equal size quadrants, and line cross was counted when the animal crossed both forepaws over a line in order to measure locomotor activity.

**Behavioral Testing Experiment 1 & 2**

After 3 days of habituation to tickling, animals that showed high levels of 50-kHz USVs calls in response to tickling (greater then 50 calls / 2 min) were used for these studies. In experiment 1, animals were assigned to either lesion or sham group matched for levels of 50-kHz USVs exhibited on the third habituation day. Lesion and sham animals received 3 consecutive days of tickling 1 week, and 1 month after surgery. In experiment 2, Either the dopamine D1/D2 receptor antagonist flupenthixol (0 and 0.8 mg/kg, i.p., n=20; Sigma, St. Louis MO) in 0.9% saline vehicle or the histamine H1 receptor antagonist diphenhydramine (0 and 40 mg/kg i.p., n=20; Sigma, St. Louis MO) in DMSO vehicle were administered 2.5 hrs (flupenthixol; Laviolette & van der Kooy, 2001) or 15 min (diphenhydramine) before testing. Drug and vehicle test days were separated by a single re-habituation day during which rats received 2 min of tickling without injection. Drug and vehicle administration was counterbalanced between subjects.

**Ultrasonic Vocalization Recording**

USVs were recorded from the high frequency output of a Pettersson D980 bat detector (Sweden) and recorded digitally with a Fostex Fr-2 field recorder (USA). Sonographic analysis of calls was done in a blind manner with SAS lab Pro (Avisoft Bioacoustics, Germany).
Ultrasonic calls were divided into 3 separate categories for analysis 1) frequency modulated (FM) 50-kHz calls, 2) non-frequency modulated 50-kHz calls, and 3) 20-kHz calls. USVs were scored off line in a blind manner from sonogram by a trained observer.

Histology, Brain Samples, and Dopamine Assay

At the conclusion of behavioral testing, animals were sacrificed with carbon dioxide and their brain was rapidly removed with the forbrain dissected into an accumbens and a prefrontal cortex sample for dopamine assay by gas chromatography mass spectrometry. The rest of the brain was placed into a 30% sucrose-10% formaldehyde-0.9% saline solution (w/v) for at least 1 month before slicing. Brains were then frozen and sliced into 50-micron coronal sections with a freezing microtome. Sections through the tips of the electrodes were mounted on microscope slides, and electrode tips were localized by projecting the slides with magnification onto a table using the atlas of Paxinos & Watson (1998).

Results

Experiment 1

Animals receiving electrolytic lesion of the VTA showed a significant reduction in 50-kHz USVs compared to pre-lesion testing both at one week (t(8) = 4.09, P < .005) and 4 weeks (t(8) = 3.95, P < .005), while 20-kHz USVs were unaffected by sham lesions (all p’s > .05; Figure 20A). VTA lesion induced decreases in 50-kHz calls were primarily due to reductions in FM calls and not constant frequency calls that are also referred to as flat calls (Data not shown). VTA lesioned animals exhibited a marginal trend to increased rates of 20-kHz calls compared to pre-lesion testing both at 1 week (t(8) = 2.30, P = .05) and at week 4 (t(8) = 2.17, P = .06), while 20-kHz USVs were unaffected by sham lesions (all p’s > .05; Figure 20B). Dopamine levels (Mean ± SEM as percent of sham lesion levels) in VTA lesioned animals were reduced in the
basal forebrain sample that included the nucleus accumbens (69.3% ± 10.9; t(14) = 2.16, P < .05), but not in the dorsal striatum (i.e. caudate nucleus (96.5% ± 9.0) or the olfactory bulb (80.7% ± 6.6; all p’s > .05). No significant changes in locomotor behavior or body weight were evident during testing in either VTA lesion or sham lesion groups (P > .05).

**Experiment 2**

Compared to vehicle treatment, flupenthixol reduced FM 50-kHz calls (t(17) = 2.34, P < .05), but not flat 50-kHz or calls 20-kHz calls (all p’s > .05; Figure 21). Locomotion as measured by line crosses was also reduced by flupenthixol (Mean ± SE; 1.6 ± 0.4) compared to vehicle (6.8 ± 0.6) treated animals (t(17) = 6.47, P < .0001). In contrast to vehicle treatment, diphenhydramine reduced FM 50-kHz calls (t(19) = 3.19, P < .005), as well as 20-kHz calls (t(19) = 3.11, P < .01), but not flat 50-kHz calls (p > .05; Figure 22). Locomotion as measured by line crosses was not significantly altered by diphenhydramine (Mean ± SE; 5.6 ± 1.5) compared to vehicle (8.0 ± 1.1) treated animals.

**Discussion**

These results demonstrate that disruptions of the mesolimbic dopamine system either by lesions or pharmacological blockade specifically reduce levels of FM 50-kHz calls that are commonly emitted during reward and positive affect, but such dopamine depletions do not effect flat 50-kHz calls or 20-kHz calls which appear not to be related to reward or strong positive emotionality (Chapter 2). Histamine has been shown to increase rates of positive affective vocalizations when injected directly into the squirrel monkey brain (Lu & Jürgens, 1993). Here, peripheral pharmacological blockade of histamine H1 receptor by diphenhydramine reduced both reward related FM 50-kHz calls, and even more robustly decreased aversive 20-kHz calls. This
latter effect is consistent with the anxiolytic actions of diphenhydramine (Halpert, Olmstead & Beninger, 2002).

Additional studies are required to examine the relationship between the mesolimbic dopamine system and 50-kHz USVs by employing selective neurochemical lesions (i.e. 6-OHDA), as well as examine the relationship between 50-kHz USVs and the in-vivo release of dopamine. Pilot work on these questions is currently proceeding, and we predict that these studies will further corroborate the role of dopamine in the neurobiology of positive affect and addictive rewards (Panksepp, Burgdorf & Knutson, 2002).
Figure 20: Mean (± SEM) 50-kHz (top panel) and 20-kHz (bottom panel) USVs in response to 2 min of heterospecific play (i.e. “tickling”) 0, 1 and 4 weeks after receiving either electrolytic lesions of the ventral tegmental area or sham lesions. ** P < .01, within subject t-test, 2 tailed.
Figure 21: Mean (± SEM) USVs in response to 2 min of heterospecific play (i.e. “tickling”) in response to pretreatment with the D1/D2 dopamine antagonist flupenthixol (0.8 mg/kg, i.p.) or vehicle. * P < .05, within subject t-test, 2 tailed.
Figure 22: Mean (± SEM) USVs in response to 2 min of heterospecific play (i.e. “tickling”) in response to pretreatment with the H1 Histamine antagonist diphenhydramine (40 mg/kg, i.p.) or vehicle. ** P < .01, within subject t-test, 2 tailed.
Figure 23. Anatomical description of electrolytic lesions of the ventral tegmental area performed in Experiment 1. Anatomic plates adapted from Paxinos & Watson (1998). Dark: smallest area of damage; light shading depicts the largest spread of damage.
CHAPTER 4. REINFORCING EFFECT OF PLAYBACK OF ULTRASONIC CALLS

We are currently entertaining the possibility that USVs separately encode emotional valence by frequency modulation and locomotor arousal by fundamental frequency, both of which may allow rats to transmit and receive high fidelity signals reflecting the emotional state of interacting animals. It is also possible to produce an emotional contagion effect via the same mechanism, allowing for coordination of behavior within groups of rats. Thus, during positive appetitive behaviors (i.e. mating, rough-and-tumble play, and non-agonistic social encounters) 50-kHz FM calls may serve to promote high locomotor arousal (high peak frequency accompanying high locomotor arousal) and continuation and reinforcement of behavior (frequency modulation accompanying positive emotional valence). During predatory threat, constant frequency 20-kHz calls may serve to promote low levels of locomotor arousal (low peak frequency), and avoidance (little frequency modulation). The two-factor model including valence and arousal is a commonly used model of human emotions, and may be usefull in animal emotion studies as well (Knutson, Burgdorf & Panksepp, 2002).

Since our working hypothesis is that the frequency modulated component of USVs are related to positive affect, we tested two hypotheses that are put forward in this paper, namely that rats will perform an instrumental response to self administer playback of frequency modulated USVs of both the 50-kHz and 20-kHz varieties, while playback of constant frequency 50-kHz and 20-kHz USVs will be avoided by rats.
Method

Housing

27 adult female Long-Evans rats were used in this study. All animals had been bred and born at the BGSU animal facility, pups were weaned at 21 days of age, and isolate housed in 20 x 40 x 20 cm high plastic cages until testing. Animals were maintained on a 12:12 light: dark cycle, with lights on at 7 am. Animals were given *ad libitum* access to food and water throughout the study and were tested during the animals light cycle.

Apparatus

An unbiased operant self administration chamber was used in this study. The apparatus consists of a 30 X 30 X 50 cm Lucite box with two holes (each 5 cm from the floor, 3.1 cm in diameter) on opposing walls. Nosepoking by rats broke a photo beam, and with the aid of a personal computer, the frequency and duration of all nosepokes were tabulated. Nosepokes in the active hole elicited playback of a tape loop of the various types of USVs via a fostex FR2 field recorder (USA) into a pre-amplifier and speaker (Avisoft Bioacoustics, Germany) that was placed on top of the operant box ceiling-(a 10.5 cm hole accommodated this speaker). USV playback lasted as long as the animal continued to nosepoke in the active hole, whereas nosepokes in the inactive hole triggered no USVs playback. USVs used for these studies had previously been recorded during rat mating, aggression and conspecific or heterospecific rough-and-tumble play. Ultrasonic playback of the different call types were matched for intensity of background noise of the recordings (with 20-kHz USVs at approximately 85db) as well as percentage of time vocalizing.
Testing

Before the start of testing, animals were habituated to the apparatus for 15 min, during which levels of baseline free operant nosepoke were recorded. On three successive days of testing animals received either playback of 1) Constant frequency 50-kHz calls, 2) FM 50-kHz calls, 3) Constant frequency 20-kHz calls, 4) FM 20-kHz calls, or 5) tape hiss in response to active hole nosepokes. Following training, animals received 3 successive test days of 15 min extinction (active hole nosepoke no longer elicits ultrasonic playback).

Results

During the 15 min habituation session, animals showed no preference for the active hole (Mean ± SEM nosepoke duration in seconds) 64.9 ± 6.5 compared to the inactive hole 70.3 ± 8.4 (P > .05). As depicted in Figure 24, active hole nosepoking was greater than inactive hole nosepoking for playback of frequency modulated 50-kHz USVs (t(5) = 2.58, P < .05), did not differ in constant frequency 50-kHz USVs (i.e. flat 50-kHz calls) or tape hiss groups (all P’s > .05), and active nosepoking was decreased compared to inactive nosepoking for playback of either constant frequency or frequency modulated 20-kHz calls (t(9) = 2.76, P < .05). The results from playback of constant frequency 20-kHz and frequency modulated 20-kHz USVs were combined given that the exhibited a similar level of aversion for active nosepoking, both showing a profound suppression of active hole nosepokes (~3% of total nosepoke duration in active hole).

Discussion

This study is the first to demonstrate that rats will actively avoid playback of 20-kHz calls of both the constant frequency and frequency modulated variety. Previous studies utilizing the playback of 20-kHz USVs or 20-kHz tones have elicited freezing and escape behaviors
(Beckett et al., 1996; Brudzynski & Chiu, 1995). However, the results of this study were contrary to our initial hypothesis that frequency modulated calls, regardless of fundamental frequency, would be rewarding to rats given that frequency modulated 20-kHz USVs were as strongly avoided in this study as the unmodulated variety.

On the other hand, the data from this study suggest that playback of frequency modulated 50-kHz USVs appears to be rewarding, whereas constant frequency 50-kHz USVs appear to be neutral. It is important to note that although the data on the playback of 50-kHz USVs is promising, further work is required to thoroughly evaluate such rewarding effects. While the frequency modulated components of 50-kHz USVs (i.e. trills) appear to be generated in the larynx, the frequency modulated components of 20-kHz USVs may be artifacts of grooming and locomotion (as described in chapter 1A). Therefore, it remains possible that only the frequency modulation of laryngeally generated calls may be rewarding to receivers.
Figure 24: Mean ± SEM nosepoke duration in that active hole compared to the inactive hole in groups of rats in which nosepoking in the active hole elicited playback of FM 50-kHz, CF 50-kHz, FM 20-kHz & CF 20-kHz USVs in a combined group, or tape hiss. * P < .05, within subject’s t-test (two tailed) comparing active vs. inactive hole.
DISCUSSION: THE NEUROBIOLOGY OF 50-KHZ ULTRASONIC VOCALIZATIONS AND THEIR RELATIONSHIP TO REWARD

Prefatory Note

The supplementary, extended discussion of these results as well as the theoretical discussion of findings is elaborated in greater detail in Appendix A, which will appear in the journal *Neuroscience & Biobehavioral Reviews* (Burgdorf & Panksepp, 2006).

Summary of Results

There are three principal finding in the experiments included in this dissertation. Rat 50-kHz USVs are increased in response to both rewarding social (i.e. play behavior and mating) and non-social reward (i.e. electrical and chemical brain stimulation) in such a manner that the animals' 50-kHz vocalization response to these stimuli predict their rewarding value. Frequency modulated 50-kHz USVs but not flat 50-kHz USVs are related to positive affective behaviors that occur during mating and play behaviors, are positively correlated with the rewarding value of heterospecific play behavior (i.e. “tickling”), and playback of frequency modulated 50-kHz USVs but not flat 50-kHz USVs is rewarding to conspecifics (chapter 1A; chapter 4). Frequency modulated 50-kHz USVs are modulated by the mesolimbic dopamine system (chapter 2), as is human positive affective states (Appendix A). Taken as a whole, the results of this dissertation suggest that 50-kHz USVs index a positive affective state in rats, and have a similar neural substrate as human positive affective states.

The Primacy of Affect Over Cognition

The standard theory of emotions put forth by most textbooks in psychology is the James Lange theory of emotion (James, 1894), although their actual theoretical stance on emotion may have been misrepresented as solely consisting of the sensation of physiological arousal (see
Ellsworth, 1994). This alternative view suggests that emotions may serve as labels (e.g. Fear) for sensations of physiological arousal and behavioral activation (i.e. flight behavior with high autonomic arousal) in response to eliciting stimuli (i.e., seeing a bear in the woods). A modern recasting of which theory states that emotional responses serve as situation specific attributions to a general and nonspecific autonomic arousal. Thus, in the classic study of Schachter & Singer (1962) human participants given injections of epinephrine attributed their high levels of arousal as anger in the presence of an unruly confederate, or positive affect in the presence of a playful confederate, but only if the participants had not been informed that they were given a sympathetic nervous system stimulant.

Although it cannot be denied that cognitions play an important role in the regulation of affect, and that high physiological arousal can lead to increases in emotional surgency which may lead to misinterpretations of arousal, it may be unreasonable to conclude that the essence of emotion is simply a cognitive attribute or side-effect of peripheral physiological arousal. For instance, Zajonc (1980) has suggested that affect occurs before cognition. Also, ontogenetically, emotional expression such as laughter and crying develop much earlier than language and complex cognitions (Sroufe & Waters, 1976; Panksepp, 1998). Humans with impaired cognitive capacity due to severe cortical damage or neurological degeneration appear to show normal emotional behaviors and experiences (Damasio, 1999). In fact, it appears that in some neurological conditions that diminished cognitive capacity is related to greater emotional expressivity (Panksepp, 1998). In relation to the causal role of physiological arousal in emotional experience, suggestive evidence from “locked in” patients who have minimal to no voluntary movement (they communicate with a specialized EEG apparatus) or physiological
responses to external stimuli show that they have essentially normal basic emotional experiences (Birbaumer, 2005; Kotchoubey et al., 2003).

**Do Emotional Feelings have Causal Efficacy?**

We believe that emotional feeling states do indeed play a causal role in influencing the behavior of an organism, by both serving to both shine an attentional spotlight onto the relevant stimuli in their environment and to guide the organism towards what stimuli to approach / avoid (Panksepp, Knutson & Burgdorf, 2002). Autistic individuals who show a deficit in affiliative feelings and drives show a profound deficit in forming and maintaining strong social attachments, whereas sociopaths show deficits in feelings of guilt and shame, and thereby deficits in obeying the laws of society (Damasio, 1994). In normal individuals, social emotions may help children adhere to social norms (Eisenberg, 2000; Panksepp, 1998). Therefore, humans who have blunted affective responses have been shown to have deficits in certain cognitive activities. Conversely, individuals that exhibit excessive emotional states (i.e. anxiety, depression, mania, and phobia) also show profound deficits in normal functioning (DSM-IV, 1994; Jager et al., 2005).

**Do Non-Human Animals Have Emotional Feelings?**

Currently the weight of evidence supports the conclusions that all mammals share a set of homologous basic emotional behaviors and affective states with humans (Panksepp, 1998, 2005). For the purpose of this dissertation, I will limit my discussion to the emotional states that appear very early in human development, namely separation distress, anger, and joy – all appearing well within the first year of life (Sroufe, & Waters, 1976; Panksepp, 1998). Other more complex affective abilities that develop later in human childhood development such as shame, guilt and
pride may have counterparts with our closer mammalian ancestors (i.e. other great apes) and not with more divergent mammalian species (i.e. rodents).

Thus based on the observation that affective states emerge in human development before language and higher order cognitions, it is reasonable to expect that affects that develop early in human are more likely to be shared with related species (with the exception of sexual desire, which develops during puberty but is probably shared by widely with other mammalian species). Although this argument has in the past been criticized as anthropomorphic, we believe that in the modern age of molecular genetics in which rats and humans have been found to share ~99% of their genes (Gregory et al., 2002), anthropocentricism (namely, that humans are psychologically unique) may be a far greater error.

While the early emergence in childhood of these core affective states makes it plausible that other mammals do have feeling states, it does not demonstrate affect in non-human animals scientifically. In order to empirically validate the existence of affective states in animals, falsifiable scientific propositions need to be addressed. To highlight this line of thinking, I will use the relationship between rat 50-kHz USVs and positive affect in humans.

The first step in this enquiry was to identify a behavior that appeared to share homology with human hedonic laughter, showing a similar behavioral response (i.e. vocalizing) to similar environmental stimuli (i.e. “tickling” and rough-and-tumble play), with a similar developmental time course (occurring around weaning). Based on this, we then tested the falsifiable hypothesis that positive affective stimuli should increase levels of 50-kHz USVs, whereas negative affective stimuli should decrease levels of 50-kHz USVs; both predictions have been affirmed (chapter 1A; chapter 1B; chapter 2; Knutson et al., 2002).
A second falsifiable hypothesis is that rates of 50-kHz USVs should be positively related to the rewarding value of the eliciting stimulus. This has also been observed (chapter 1A; chapter 2; Knutson et al., 2002).

The third falsifiable hypothesis is that the neurobiology of 50-kHz USVs will be related to neurobiology of human positive affect, which has also been supported (Appendix A). Dopamine activity in the accumbens is associated with positive affective states in humans and 50-kHz USVs in rats (Burgdorf et al., 2001; Drevets et al., 2001).

The final prediction we call the Neuroscience Prediction – namely that the neural mechanisms for affective state in a laboratory animal will yield new predictions about how affect may change in humans (Panksepp, 1998). In the case of 50-kHz USVs, we can predict on the basis of the findings presented in Chapter 2 that electrical stimulation of the human dorsal raphe nucleus will elicit laughter and/or positive affective states in humans. Positive affective states have already been reported from brain stimulation of the accumbens or a region near the ventral tegmental area in humans (see: Appendix A). Electrical stimulation of both the accumbens and ventral tegmental area elicit 50-kHz USVs only in animals that find the stimulation to be rewarding (chapter 2). Also, the neurochemical data suggest that analogs to many of the drugs of abuse that elicit positive affect in humans increase rates of 50-kHz USVs in rats (Appendix A). As in any scientific discipline, such work will only provide evidence for or against animals’ affective experiences, and cannot definitively demonstrate that animals do or do not have affective experiences. Science only has “weight of evidence” rather than “proof.” On that dimension, it presently seems likely that both other humans and other mammals do have affective experiences that guide their behavior.
Are 50-kHz USVs in rats similar to human positive affect akin to human joy?

Positive affect in humans is a complex and multifaceted phenomenon. In this comparison between 50-kHz USVs in rats and human positive affect, the analysis follows logically on empirical studies of human and animal positive affect. Perhaps the most robust behavioral correlate of human positive affect is the Duchenne or felt smile that involves activation of the zygomatic and orbicularis oculi muscles (Ekman, Davidson & Friesen, 1990). Duchenne smiles have consistently been shown to be positively correlated with self reports of positive affect as well as left hemisphere alpha EEG dominance, that has been shown to be a marker of both state and trait positive affectivity (Ekman, Davidson & Friesen, 1990). Similarly, Duchenne laughter represents a Duchenne smile in combination with laughter, which may index a positive affective state in humans.

Positive affective states are most robustly elicited by social interactions in humans. Both smiling and laughter occur almost exclusively during positive social interactions (Provine, 2001), and the strongest positive correlate of trait positive affectivity (i.e. subjective well being) is the personality trait of extroversion (Seligman & Csikszentmihalyi, 2000). Although little empirical evidence exists, it is reasonable to assume that rewarding social interactions (as indexed by approach behavior) as compared to aversive interactions (as indexed by avoidance behaviors) elicit positive affect in humans (as indexed by Duchenne smiling / laughter and affective self reports).

In addition, drugs of abuse (Schlaepfer et al., 1998) as well as anticipation of reward (Knutson et al., 2001) have been shown to increase positive affect in humans. In comparison, rat 50-kHz USVs are elicited most robustly by rewarding social interactions such as rough-and-tumble play behavior and mating (chapter 1A), and levels of 50-kHz USVs are positively related
to the rewarding value of these social interactions (Chapter 1A & 1B). Administration of drugs of abuse to rats also elicit 50-kHz USVs (Knutson, Burgdorf & Panksepp, 2002), and the rats that show increased rates of 50-kHz USVs in response to drugs of abuse are the animals that demonstrate place preference for these drugs (Chapter 2). We have also previously shown that anticipation of a wide variety of rewards elevate levels of 50-kHz USVs (Knutson, Burgdorf & Panksepp, 2002).

Comparison of the Neurobiology of human positive affect and 50-kHz USVs

Neurobiological studies of positive affect in humans primarily come from two independent methodologies, direct electrical stimulation of the brain as well as functional brain imaging studies utilizing either Positron Emissions Topography (PET) or Magnetic Resonance Imaging (MRI). Studies utilizing direct brain stimulation in humans have found that stimulation of the nucleus accumbens and the ventral tegmental area elicit positive affect and support self-stimulation in humans. One patient has reported that the stimulation of this brain area was the most pleasurable experience of his life (Heath, 1972). Another patient with a tegmental stimulation electrode referred to the button with which he could self administer stimulation to that brain area as his “happy button” (Heath, 1960). Duchenne laughter has been reported from accumbens stimulation along with increased Likert scale self reports of positive affect (Okun et al., 2004).

However, it is important to note that not all human patients who readily self administer electrical brain stimulation exhibit positive affect during these episodes. Self administration of brain stimulation has also been associated with the tip of the tongue phenomenon, in which the patient repeatedly self administered brain stimulation allegedly in the effort to remember a name.
or event, reporting that the subjective effect of the stimulation was anger and frustration (Heath, 1960). Still, these are a minority of the observations that have been made.

Brain imaging studies have also shown that nucleus accumbens activation is positively correlated with positive affective states in humans in response to a wide variety of positive affective eliciting stimuli (see Appendix A for a comprehensive discussion). PET imaging studies have found that increases in dopamine binding specifically in the accumbens is positively correlated to the euphoric effects of drugs of abuse (e.g., Drevets et al., 2001).

In laboratory rats, 50-kHz USVs are elicited by electrical brain stimulation of the accumbens and ventral tegmental area that have been show to elicit positive affect in humans (Chapter 2). Notably, all animals that showed repeatable ESB induced 50-kHz USVs also exhibited self-stimulation (i.e. self-administered electrical brain stimulation to these brain areas). Similar to the relationship between ESB induced self-stimulation and positive affect in humans, although repeatable ESB induced 50-kHz calls was sufficient for self-stimulation, it was not necessary. Approximately 20% of animals that exhibited self-stimulation did not exhibit repeatable ESB induced 50-kHz USVs. It is possible that these sites have mixed emotional effects with substantial accompanying negative affect.

Similar to human studies that demonstrate a relationship between the ventral striatal dopamine system and positive affect, 50-kHz USVs have also been shown to be strongly modulated by the mesolimbic dopamine system. Injections of amphetamine (which facilitates dopamine activity) directly into the accumbens produce a dramatic elevation in 50-kHz USVs (Burgdorf et al., 2001), which can be antagonized by either D1 or D2 dopamine receptor antagonist (Brudzynski, in press). Additionally, dopamine receptor antagonist have been shown
to attenuate 50-kHz USVs elicited by tickling (Chapter 3), brain stimulation reward (chapter 2), and glutamate injection into the preoptic area (Wintink & Brudzynski, 2001).

Effect of rewarding and aversive stimuli on 50-kHz USVs

A straight-forward prediction from the hypothesis that 50-kHz USVs reflect a positive affective state is that rewarding stimuli would increase rates of these calls, whereas aversive stimuli would decrease these calls. We have shown that anticipation of a wide variety of rewards increase levels of 50-kHz USVs, including food, mating, play, drugs of abuse, and rewarding brain stimulation (Knutson, Burgdorf & Panksepp, 2002). Additionally, we have also found across studies that rates of 50-kHz USVs elicited by a rewarding stimulus are positively correlated to the approach latency for the stimuli between $r = .60$ to $r = .80$ range (Chapter 1A, Knutson, Burgdorf & Panksepp, 2002). Conversely, a wide variety of aversive stimuli have been shown to decrease levels of 50-kHz USVs, including bright light (that is aversive to rats), predatory odor, social defeat, foot shock, aversive drugs (Knutson, Burgdorf & Panksepp, 2002).

A proposed neuronanatomy of 50-kHz USVs

The results of the mapping study of electrical brain stimulation induced 50-kHz USVs (chapter 2) revealed that the circuit for 50-kHz USVs bears a striking similarity to the primary circuit that supports the most vigorous electrical self-stimulation in the brain (Shizgal, 1989, 1997). All electrode sites that exhibited repeatable brain stimulation induced 50-kHz USVs also exhibited self-stimulation behavior (chapter 2). The primary substrate for self-stimulation is believed to be circuit consists of cell bodies in the nucleus accumbens, ventral pallidum, lateral hypothalamus, and lateral preoptic area which send descending myelinated fibers through the medial forebrain bundle and ventral tegmental area and terminate at least in part in the raphe.
nuclei (Shizgal, 1989, 1997). Each of these brain areas that are part of the presumed primary self-stimulation circuit also show brain stimulation induced 50-kHz USVs (Chapter 2).

It has also been show that glutamate stimulation of the lateral preoptic area can increase rates of 50-kHz USVs which are attenuated by pretreatment with a dopamine receptor antagonists (Wintink & Brudzynski, 2001). Given that lateral preoptic area neurons have been shown to contain neurotensin containing neurons that send a direct excitatory projection ventral tegmental area dopamine neurons (Zahm et al., 2001), this circuit possibly explains why both lateral preoptic area glutamate induced 50-kHz USVs are attenuated by dopamine receptor antagonists (Wintink & Brudzynski, 2001) and why neurotensin elevates levels of 50-kHz USVs when injected into the ventral tegmental area (chapter 2). Indeed, direct or indirect activation of dopamine neurons by microinjections of neurotensin, nicotine, muscimol, and DAMGO may explain their ability to increase rates of 50-kHz USVs (chapter 2; Burgdorf, unpublished observation; McBride, Murphy & Ikemoto, 1999; figure 25)

The mesolimbic dopamine system has long been implicated in electrical self-stimulation, with the dopamine projection from the ventral tegmental area to the nucleus accumbens having been shown to be a key projection (Wise & Bozarth, 1987). This dopamine projection, from the ventral tegmental area to the nucleus accumbens, also appears to be an important in the generation of 50-kHz USVs. Electrical brain stimulation parameters that have been shown to elicit maximal dopamine release, do so at electrode sites that support self-stimulation but not at sites that do not support self-stimulation (Garris et al., 1999; Montague et al., 2004). The results of chapter 2 demonstrate that electrical brain stimulation utilizing the above parameters increased levels of 50-kHz USVs only at electrode sites that support self-stimulation (chapter 2). Additionally, amphetamine microinjections into the nucleus accumbens dramatically elevate
rates of 50-kHz USVs (Burgdorf et al., 2001), and lesions that depleted mesolimbic dopamine levels as well as pharmacological blockade of dopamine consistently attenuates rates of 50-kHz USVs (chapter 3).

The final common integrative pathway for the production of vocalizations is the caudal periaqueductal grey, which sends a direct projection to the vocalization central pattern generator in the nucleus ambiguus (Jürgens, 2002). More recently, it is becoming evident that the final common pathway for the generation of positive affective vocalizations may be the dorsal raphe, which could also be considered the caudal ventro medial periaqueductal grey (Jürgens, personal communication). Indeed in the present mapping study, the dorsal raphe produced the most consistent and reliable brain stimulation induced 50-kHz USVs as well as the most vigorous and consistent self-stimulation of any brain area tested. It is possible that the direct projections from the prefrontal cortex, nucleus accumbens, and ventral pallidum to the dorsal raphe contribute to the ability of these areas to support brain stimulation induced 50-kHz USVs (Peyron et al., 1998). As mentioned above, many of the basal forebrain areas that have been shown to both support self-stimulation and brain stimulation induced 50-kHz USVs are thought to produce their self-stimulation effects, at least in part, by their projection to the dorsal raphe (Shizgal, 1989, 1997).

**Alternative non-affective interpretations of 50-kHz USVs**

A variety of alternative hypotheses concerning the nature of rodent USVs have been proposed across the years –

1) 50-kHz USVs are an artifact of locomotion:

The hypothesis that 50-kHz USVs are an artifact of locomotion akin to grunting due to forepaw compression during locomotion was based on an unpublished observation of mating
behavior (Blumberg, 1992). We have demonstrated that only 9% of 50-kHz USVs occur 0 to 0.5 sec after forepaw contact with the ground, in other words 91% of the 50-kHz USVs were not related to forepaw compression movements (Panksepp & Burgdorf, 2003). We have also shown across a series of studies that we could observe increases in 50-kHz USVs with decreases, no change, or increases in locomotor behavior (Knutson, Burgdorf & Panksepp, 2002).

2) 50-kHz USVs reflect a non valenced arousal state:

This hypothesis states that 50-kHz USVs reflect a non-valenced state of arousal, such that both high valenced positive affect (i.e. “joy”) and high valenced negative affect (i.e. “anxiety”) elicit 50-kHz USVs. However, we suggest that frequency modulated 50-kHz USVs reflect a state of positively valenced high arousal given that highly arousing positive valenced stimuli increase levels of 50-kHz USVs (e.g. mating, play, drug of abuse), whereas highly arousing aversive stimuli decrease levels of 50-kHz USVs (i.e. foot shock, predatory odor, bright light, aversive drugs; Knutson, Burgdorf & Panksepp, 2002). However, it remains possible that flat or constant frequency 50-kHz USVs may reflect a non-valenced state of high arousal since they are increased during the more active appetitive aspects of aggressive behavior (chapter 1A) and are neither rewarding or aversive in vocalization self administration testing (chapter 4).

3) 50-kHz USVs reflect a non-positively valenced “wanting” state:

This hypothesis is based on the work of Berbrige & Robinson (2003) which suggests that the appetitive component of reward seeking (i.e. wanting) is strongly modulated by the mesolimbic dopamine system and is not related to positive affective states (i.e. “liking”). However, more recent studies have shown that anticipation of certain rewards do increase levels of the subjective self report of positive affective states in humans (Knutson et al., 2001). In our work with 50-kHz USVs, only rewards that are delivered in a completely predictable manner
elicit 50-kHz USVs, whereas when these rewards (i.e. food and brain stimulation reward) cease to be delivered during extinction, levels of appetitive behavior such as food seeking behavior and bar pressing (both of which index “wanting”) remain high, whereas levels of 50-kHz USVs decrease and levels of 20-kHz calls, which index a negative affective state, increase (Burgdorf, Knutson & Panksepp, 2000). Therefore, it appears likely that 50-kHz USVs index a positive affective appetitive (or “wanting”) state. Although Berridge and Robinson have convincingly demonstrated that sensory pleasure (i.e., “liking”) associated with rats ingesting sapid sucrose solutions is not related to appetitive aspects of seeking sucrose, or mesolimbic dopamine activity, it appears that an appetitive positive affective state, indexed in rat by 50-kHz USVs, is related to positively valenced seeking states promoted by the mesolimbic dopamine arousal.

**Future Directions**

In work summarized in this dissertation as well as our previous work, we believe that we have provided convincing evidence that 50-kHz USVs reflect a positive affective state which 20-kHz USVs reflect a negative affective state in rats. Across these studies we have also developed optimal experimental protocols to elicit high rates of these vocalizations, namely the tickling procedure for eliciting 50-kHz USVs, and the male resident-intruder aggression paradigm for eliciting 20-kHz USVs (Panksepp et al., 2005). Therefore, in future studies we plan to use these measures as indices of emotional states to further elucidate the genetics, neurochemistry, and neuroanatomy of affective states using selective breeding, microarray, and biochemical techniques – experiments which are already underway (Burgdorf et al., 2005; Panksepp et al., 2004).
Figure 25. Proposed neural circuit of rat 50-kHz USVs plotted on a sagittal section (0.4 mm lateral to midline) of the atlas of Paxinos & Watson (1998). Ascending dopamine projections from the ventral tegmental area project to the accumbens and prefrontal cortex, where dopamine activates prefrontal cortex neurons or accumbens neurons. The dorsal raphe is then activated directly from the prefrontal cortex, and indirectly from the ventral tegmental area. The dorsal raphe then activates central pattern generator neurons in the ambiguous. PFC – Prefrontal cortex, Acc – Accumbens, VP – Ventral pallidum LPOA – Lateral preoptic area, VTA – Ventral tegmental area, DR – Dorsal raphe, NA – Nucleus Ambiguus.
REFERENCES


APPENDIX A: THE NEUROBIOLOGY OF POSITIVE EMOTION

Prefatory Note

Appendix A is presented as it was submitted in the journal Neuroscience & Biobehavioral Review early next year (Burgdorf & Panksepp, 2006).
The Neurobiology of Positive Emotions.

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Abstract

Compared to the study of negative emotions such as fear, the neurobiology of positive emotional processes and the associated positive affect (PA) states has only recently received scientific attention. Biological theories conceptualize PA as being related to i) signals indicating that bodies are returning to equilibrium among those studying homeostasis, ii) utility estimation among those favoring neuroeconomic views, and iii) approach and other instinctual behaviors among those cultivating neuroethological perspectives. Indeed, there are probably several distinct forms of positive affect, but all are closely related to ancient sub-neocortical limbic brain regions we share with other mammals. There is now a convergence of evidence to suggest that various regions of the limbic system, including especially ventral striatal dopamine system is implemented in an anticipatory (appetitive) positive affective state. Dopamine independent mechanisms utilizing opiate and GABA receptors in the ventral striatum, amygdala and orbital frontal cortex are important in elaborating consummatory PA (i.e. sensory pleasure) states, and various neuropeptides that mediate homeostatic satisfactions.

Keywords: Affect, Emotions, Pleasure, Play, Seeking, Foraging, Vocalizations, Dopamine
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1. Introduction

Recently, scientific study of positive emotions has been receiving increased experimental attention. Extraversion and gregariousness are among the best predictors of subjective well-being and positive affectivity; along with being happier, people who experience high subjective well-being typically have better health outcomes and longevity (Fredrickson, 2004; Seligman & Csikszentmihalyi, 2000). In this paper, we will review the recent scientific evidence on the neurobiology of positive emotions that has emerged from human and animal research.

An abundance of neuroscience evidence indicates that whereas the cognitive aspects of emotions, such as the recognition of happy and sad faces, require neocortical processing, the experiential states of happiness and sadness, as well as the other basic affective states are strongly dependent on sub-neocortical limbic circuitries that we share with the other mammals (Damasio, et al., 2000; Liotti & Panksepp, 2004; Panksepp, 1998). Although there are no unambiguous objective indicators of subjectively experienced affective states that commonly accompany emotional and motivational arousals in humans, because of abundant corroborative evidence (Panksepp, 2005), it is becoming more acceptable to entertain that such affective states of consciousness may arise from sub-neocortical brain dynamics that we share with other animals as well.

Throughout the 20th century, there was resistance to entertaining brain/mind entities such as affective states in animals, but modern neuroscience, with its many well demonstrated neuroanatomical and neurochemical homologies across all mammalian species, now provides ways to evaluate such possibilities in more scientifically rigorous ways than was possible during earlier eras (Panksepp, 1998, 2005). For instance, it is now clear that the neurochemistries of
opiate and psychostimulant addictions are organized similarly in the brains of all mammals, and it has been argued that we cannot make sense of such behavioral changes unless we begin to take affective experiences more seriously in the lives of other animals (Knutson, Burgdorf, & Panksepp, 2002; Panksepp, Knutson, & Burgdorf, 2002; Panksepp, Nocjar, Burgdorf, Panksepp & Huber, 2004). Although there is still a strong and re-vitalized neo-behaviorist tendency to see all behavioral change in animals as arising from unexperienced, pre-conscious brain processes, our goal for this essay is to discuss why it may be wiser to provisionally conclude that ancient forms of consciousness such as affective experiences do exist in the neurodynamics of other animals, and why such brain functions may be all important for behavioral neuroscientists to consider more openly (Panksepp, 2003a, 2005). Animal behaviorists (e.g., Marc Bekoff, Marian Dawkins, and Don Griffin) are increasingly accepting the likelihood that animals experience their lives, and that such issues are essential for discussing animal welfare issues (see McMillan, 2005 for a recent summary of such work). In our estimation, such views are more consistent with the mass of available evidence, and they provide a straight-forward strategy for shedding empirical light on very important neuro-mental functions, critical for advancing understanding that can inform psychiatric practice (Panksepp, 2004). Such issues are almost impossible to study with any neuroscientific precision in human beings. In this paper, we will largely restrict our coverage to anticipatory eagerness and gustatory pleasure, since those are among the best studied and least controversial affective responses that may remain conserved across most mammalian species.

Although not covered in detail, we would also note that the primitive emotional concepts of positive and negative affect (PA and NA, respectively), which are increasingly central to modern psychological analyses of emotional experiences (Lambie & Marcel, 2002; Russell,
are rather general and non-specific ways to conceptualize emotional feelings. It could be argued that PA and NA are merely semantic-conceptual ways to parse the many kinds of “good” and “bad” feelings that the nervous system can construct, and that a neuroscientific analysis must seek endophenotypes that are neurobiologically ingrained affective processes. For instance, a recent personality test designed to evaluate various basic emotional tendencies in humans provides evidence that NA states can be constituted of specific negative feelings such as fear, anger and sadness, while PA can be constituted of specific positive feelings such as those related to playfulness, nurturance and exploratory-seeking urges (Davis, Panksepp, & Normansell, 2003). Whether the more general categories of PA and NA simply reflect convenient ways to talk about such conceptual groupings of desirable and undesirable feelings, or whether they have neurobiological realities above and beyond their class identifier status, is an issue that remains to be resolved. Since so much effort in psychology has been devoted to development of general PA and NA concepts (Davidson, et al., 2002 *Handbook of Affect Science*), we will here utilize that scheme as a guide to talking about more specific affective feelings that require a more resolved taxonomy. Although all investigators of such neuro-mental processes surely recognize that they can only be indirectly monitored through the use of various psychological and behavioral measures, it remains to be widely recognized that affective states cannot be scientifically understood without neural analyses.

### 1.1. Measurement of Positive affective states

Positive affective (PA) states can either be scientifically measured via self-report Likert-type rating scales or by examining unconditioned behavioral responses. Thus, the positive
emotion of happiness is typically measured by asking subjects to rate their current level of happiness using either a verbally anchored scale or a pictorial one such as the self-assessment manikin in which subjects identify their emotional state by choosing cartoon characters that match their mood states along valence (bad to good mood), arousal, and power/surgency dimensions (Lang, 1995). For example, emotional states elicited by viewing pictures of human babies are rated high on positive valence and moderately on arousal (Lang, 1995). Alternatively, happiness could be measured behaviorally, for instance by the presence of Duchenne smiling, which has been found to be positively correlated with human subjective self-report of positive emotion (Ekman, Davidson & Friesen, 1990).

In animals, the utilization of conceptual-psychological scales is not possible, and hence all measures have to rely on behavioral analyses. In general, investigators have the option of focusing on the study of unconditioned-instinctual behavioral tendencies or conditioned-learned behavioral changes. Both are useful, but the former may be more useful for a brain systems analysis of the critical neural components, if one makes the simplifying dual-aspect monism assumption that affective feelings may directly reflect the neurodynamics of brain systems that generate instinctual tendencies (Panksepp, 2005). However, the study of learned behaviors is also critical, for that level of analysis provides the opportunity to validate the likely presence of experiential components as measured by various approach and avoidance behaviors, especially conditioned place preferences and aversions to locations where organisms experienced experimental imposed variation of their internal states (studies that are very hard to conduct ethically and logistically in humans).

A great deal of animal work has been conducted on various negative affective processes (Panksepp, 1998), especially fearful behaviors, even though many leading investigators still do
not accept the concept of affect as being of any relevance (or even reality) within their neuro-
behavioristic ontologies. They commonly ignore abundant work done on other negative 
emotions, such as separation-distress, which has relied heavily on vocal measures of emotions 
such as the analysis of separation calls (Panksepp, 2003b), not to mention the variety of positive 
emotions such as playfulness and other social emotions that exist in animal brains (Panksepp, 
1998, 2005). However, it is becoming increasingly clear that the mapping of the separation 
distress system in animal brains has striking anatomical correspondences to human sadness 
systems highlighted by PET imaging (Damasio, et al., 2000; Panksepp, 2003b), even to the 
extent that human sadness is accompanied by low opioid tone in the relevant limbic circuits 
(Zubieta, et al., 2003), a principle first revealed through animal brain research (Panksepp, 1981, 
2003b). However, to keep this contribution manageable, our coverage here will be restricted to 
several key exemplars of positive affect.

1.2. Biological approaches to understanding positive affect

Many psychometric models of positive emotions conceptualize positive affective states as 
originating from approach and various consummatory behaviors. From this perspective, all 
emotions can be categorized on a two or three dimensional Cartesian planes, with an approach-
avoidance valence dimension and an arousal dimension (Cabanac, 1979; Knutson, Burgdorf & 
Panksepp, 2002; Lang, 1995; Russell, 2003; see Figure 1), and often a third, and less well 
conceptualized dimension of surgency or power of a feeling. For example, joy would be 
categorized as approach + high arousal whereas sensory postprandial pleasure would be 
approach + low arousal. Approach based psychometric models of positive emotion have also 
been used to conceptualize stable PA traits states such as subjective well-being (Davidson,
Jackson & Kalin 2002; Panksepp, Burgdorf & Knutson, 2002) as well as the personality trait of extroversion which is associated with high levels of PA (Diener, 1998). However, as already noted, both positive and negative affect could be broken down into a variety of specific emotions, affording a more refined view of the many distinct species of affect that may exist in the brain (Davis, et. al., 2003; Panksepp, 1998).

From this perspective, what is needed in the study of affect is a more resolved taxonomy that hopefully will eventually map onto distinct brain systems. For instance, it could be argued that positive and negative affect are simply conceptual class identifiers rather than “natural kinds” and it is easy to envision many distinct categories of positive affects—for instance, i) those that emerge from homeostatic bodily need states which are alleviated by specific sensory-motor consummatory activities, ii) those that reflect emotional action processes (e.g., play and investigation), and iii) those that emerge as general background feelings related to various forms of satisfaction, distress, and relief (Ostow, 2004; Panksepp, 2004; Panksepp & Pincus, 2004). So far neuroscience has had relatively little to say about such issues, except for the recognition that many of these feelings are substantially due to activities of the limbic system (MacLean, 1990) and specific sub-systems coursing through those sub-neocortical regions of the brain (Panksepp, 1998). Since most of that work comes from the study of laboratory animals, where discussion of internal subjective states continues to be shunned, it is an understatement to say that the nature of affective experience remains a conceptually and empirically underdeveloped territory. Future developments along these lines will require investigators to be willing to take objective behavioral measures, especially of spontaneous behaviors (e.g., emotional vocalizations) and parallel preference and aversion paradigms, as potential indicators of affective experiences.
An emerging human research tradition does, in fact, attempt to infer PA from unconditioned behaviors that are correlated to self-reported PA states. Perhaps the classic example is the Duchenne smile, also known as the felt smile (Ekman, Davidson & Friesen, 1990). Such approaches suggest that from a felt smile one can infer PA due in part to the strong positive correlation between Duchenne smiling and subjective self-report of positive affect in humans (Ekman, Davidson & Friesen, 1990). However, it is increasingly recognized that such measures may lack validity in many adult human studies, because of various cognitive-instrumental ways humans regulate their affects, including cultural display rules that commonly make such indicators fuzzy signals of emotional feelings. However, such instinctual displays may be more veridical readouts of internal affective states in infants who are not yet skilled in cognitive control of their behavioral displays, since they have not fully assimilated cultural expectations.

In fact, human infants exhibit certain patterns of oral-facial behaviors exclusively to sweet solutions that adults find highly palatable (Ganchrow et al., 1983; Rosenstein & Oster, 1998). Adult primates as well as rodents also exhibit these hedonic taste reactivity reactions exclusively to highly palatable solutions (Berridge, 2000, 2004). Positive emotional vocalizations that are exhibited during anticipation of rewards, as well as during pro-social interactions such as conspecific reunion as well as rough and tumble play, have also been used as a behavioral index of positive affective states in primates (Jürgens, 1976, 1979) and rodents (Knutson, Burgdorf & Panksepp, 2002; Panksepp, Knutson & Burgdorf, 2002). Many of the conclusions derived from the study of these unconditional behaviors have been validated by corresponding place preference and place aversion paradigms (Burgdorf & Panksepp, 2000).
With regard to the sensory aspects of positive affects, various pleasures reflect the capacity of certain stimuli to return the body to homeostasis. Although there are many historical antecedents to this idea from Plato onward, the modern discussion goes back to the work of Michel Cabanac (1971). For example, a warm stimulus would be experienced as pleasurable by a cold individual, with the magnitude of the pleasure being proportional to the ability of the stimulus to return the body to homeostatic conditions. This has been referred to as sensory alliesthesia (Cabanac, 1992). Positive emotional responses are also thought to reflect the relative utility of eliciting stimuli -- an old idea (e.g. Bindra, 1978; Young, 1966) that fits modern neuroeconomic principles (Shizgal, 1997; Panksepp, Knutson & Burgdorf, 2002).

1.3. Conditioned place preference studies of limbic chemistries

Many studies have found that the rewarding effects of addictive / euphoric drugs are mediated by sub-neocortical systems as indicated via self-injections studies as well as conditioned place preference studies Opiate agonists that bind preferentially to µ-opiate receptors are euphorgenic, whereas kappa selective opiates generate negative affect in humans (Schlaepfer et al., 1998). When injected directly into the brain, µ-opiate agonists (morphine, endomorphin 1, DAMGO) produce conditioned place preference consistently when injected into the ventral tegmental area, nucleus accumbens, periaqueductal gray and lateral ventricle (Bals-Kubik et al., 1993, Olmstead, Franklin 1997; Terashvili et al., 2004), whereas kappa selective agonists (U50,488H) produce conditioned place aversion when injected into many of these same brain regions (Bals-Kubik et al., 1993). Cocaine produces conditioned place preferences and self-administration when injected directly into the accumbens, prefrontal cortex, and olfactory tubercle (Goeders & Smith, 1993; Gong, Neill & Justice, 1996; Ikemoto, 2003), whereas
amphetamine place preference seems restricted to the accumbens and ventral palladium (Carr & White, 1986; Gong, Neill & Justice, 1996). Alcohol and nicotine produce place preference and self administration when injected into the ventral tegmental area (Laviolette & van der Kooy, 2003, Rodd et al., 2004). In sum, most of the drugs that are euphorogenic in humans are rewarding when injected directly into the ventral striatum or its cortical afferents in rats.

A number of peptide systems have been implicated in positive affective states since the peptides have been rewarding when given peripherally or microinjected directly into the rodent brains. Neurotensin and CART, peptides closely associated with brain dopamine, exhibit place preferences when injected into the ventral striatum (Glimcher, Giovino & Hoebel, 1987; Kimmel et al., 2000). Neuropeptide Y, a peptide involved in food intake and alleviating anxiety, yields place preference when injected into accumbens and perifornical hypothalamic nuclei, with its rewarding effects being partially dissociable from its hyperphagic effects (Brown, Coscina & Fletcher, 2000). Oxytocin, which is involved in creating social bonds and the pleasures of social contacts shows place preference when given peripherally (Liberzon et al., 1997), but it has been difficult to see this effect centrally, even though in unpublished work we find socially-induced place preference to be facilitated by oxytocin (Panksepp, 1998). These studies implicate a number of sub-neocortical circuits in the generation of affective states.

1.4. Affect is largely a sub-neocortical process

Before we proceed, let us briefly emphasize why investigators should entertain the likelihood that affect largely has a sub-neocortical locus of control. At present, many investigators and theoreticians remain skeptical about the fundamental role of sub-neocortical
systems in the elaboration of affect, partly because they feel consciousness is only a characteristic of the higher heteromodal cortical functions in humans and perhaps several other highly cerebrated species. Modern brain imaging studies which demonstrate results contrary to the evidence long provided by animal brain research, tend to highlight the arousal of many higher cortical regions in the emotional-cognitive processes aroused by exteroceptive stimuli (for summaries, see Lane & Nadel, 2000).

Modern brain imaging, especially with fMRI may be yielding deceptive findings, at least for understanding the nature of affect: Investigators, by using perceptually driven methodologies, are typically visualizing the cognitive components of emotional processing rather than core affective states. fMRI does not appear to have the proper characteristics to pick up true affective changes (which have a time-course which is not well suited for fMRI parameters). PET is much better; indeed Damasio et al.'s (2000) study picked up patterns that are rarely evident in fMRI studies. The ever increasing imaging of unconscious emotional processes (starting with Morris, et al, 1998) is pertinent only for understanding the sensory-perceptual aspects of emotional stimuli, not affective states. Indeed, most investigators that use such methodologies fail to evaluate potential affective changes empirically, which may compromise the generality of their conclusions concerning the affective dimensions of experience, which may be most relevant for clarifying psychiatrically relevant emotional issues. Thus, although fMRI may be a fine tool for identifying brain cognitive correlates of emotional experiences (generated very rapidly to specific sensory or cognitive contingencies), as currently used, it appears to be a blunt tool for analyzing how affect is generated in the brain. Since emotional affects emerge relatively slowly (e.g., sadness), they may not be as readily linked temporally to precipitating stimulus events as are emotional perceptions.
Fortunately, PET imaging is better suited for visualizing affective responses of the brain, and an increasing number of Experiments using PET during the past few years have been more concordant with the animal data than fMRI studies. Perhaps the most compelling evidence comes from Damasio, et al. (2000), who asked individuals to achieve deep, existentially experienced feeling states of anger, fear, sadness and happiness via personal reminiscences. When subjects truly experienced those feelings, radioactive water was infused and PET images were constructed. The results affirmed abundant arousals in sub-neocortical brain regions, accompanied by substantial reductions of blood flow in many higher brain areas, suggesting a narrowing of information processing in neocortical systems during intense emotional states (Liotti & Panksepp, 2004).

Various studies have also highlighted the importance of sub-neocortical regions in human affective experiences such as air hunger (Liotti, et al., 2001), the taste of chocolate (Small, et al., 2001), the appetite for various rewards including winning money (Knutson, et al., 2001), the sex-specific appeal of pretty faces (Aharon, et al., 2001), the pleasure of musical peak experiences (Blood, et al., 2001), male sexual arousal (Redoute, et al., 2000) and orgasmic pleasure (Holstege, et al., 2003), as well as the sexually differentiated experience of rectal distention (Kern, et al., 2001). All of these studies report arousals of various subcortical brain areas implicated in the generation of affect by animal research, as well as those mesocortical zones, especially orbitofrontal, anterior cingulate and insular cortices that MacLean (1990) originally highlighted in his Limbic System concept (which has been increasingly attacked by a growing number of cognitive neuroscientists more accustomed to working on the higher informational functions of the brain. It is claimed that the Limbic System is not a coherent anatomical or functional entity (which can be said for any region of the brain), without realizing that the
concept was originally used to designate visceral regions of the brain that are critical for elaborating emotionality, a general conclusion that continues to be supported by modern research.

The extended viscerally-focused brain regions known as the limbic system, descending deep into the medial diencephalon and upper brainstem, do appear to comprise the fundamental neuro-geography of spontaneous emotional behavior and affective experience in both humans and other mammals (and probably birds and reptiles also). These systems are regulated and further parsed by higher cortical activities, but aside from the role of mesocortical areas like the insula in the pleasures and displeasures (e.g., disgust and pain) of certain sensations, there is little evidence that higher neocortical regions are essential for generating affective experiences that accompany emotional arousal, even though they may be essential for the cognitive memories associated with those states. Accordingly, we should be devoting more effort to studying the details of basic emotional systems in appropriate animal models (e.g., as summarized in Panksepp, 1998). These systems, which appear to be homologously organized in all mammals, are largely inaccessible for causal human research.

In this vein, we should also recall that emotional feelings have typically been much easier to activate in humans through stimulation of sub-neocortical circuits that mediate the instinctual emotional behaviors in our fellow animals, than through higher brain stimulation (for reviews see Heath, 1996; Panksepp, 1985). A most recent striking example was Bejjani et al.’s (1999) observation of sudden onset of depression by stimulating midline diencephalic structures near the subthalamic nuclei, and mirth by stimulating the nucleus accumbens (Okin et al., 2004). In sum, the evidence is substantial for a sub-neocortical locus of control for the generation of experienced affects that accompany various emotional states and consummatory responses.
2. Neurobiological findings in positive affect

Causal analysis of the brain substrates of affective change have typically been achieved by direct electrical and chemical stimulation of human and animal brains, findings which have often been corroborated with PET imaging of brain activity changes in humans. The weight of evidence suggests that brain systems which support affective change are concentrated in similar subcortical regions of the brain.

2.1. Electrical stimulation of the brain (ESB) studies

In the late 1940’s Robert Heath discovered that human psychiatric patients would press a button to self-administer electrical brain stimulation to a variety of brain areas. During these self-stimulation sessions some subjects would occasionally report that the stimulation produced a PA state, with one subject describing the switch that electrically stimulated his mesencephalic tegmentum as his “happy button” (Heath, 1960); another of Robert Heath’s patients described electrical stimulation of the septum as the most pleasurable experience of his life (Heath, 1972). However, neither of these cases described by heath included reports of brain stimulation induced laughter. It is important to note that Heath’s definition of the septum included the Nucleus Accumbens (Heath, 1954; Heath, 1972). Electrical Stimulation of the nucleus accumbens has been shown to elicit smiling laughter and euphoria, with laughter response being highly correlated with euphoric response (Okin et al., 2004). Patients treated for Parkinson’s disease with deep brain stimulation electrodes in the subthalamic nucleus show hedonically experienced
laughter in response to ESB (Krack et al., 2001). Hedonic felt laughter has also been elicited from electrical brain stimulation of the supplementary motor cortex in one epileptic patient (Fried et al., 1998), but the affect may have been coincident to the recruitment of sub-neocortical systems.

In non-human primates, deep brain electrical stimulation of the striatum as well as the midbrain regions can elicit vocalizations that are normally exhibited when the animals unexpectedly find palatable food or are reunited with conspecifics, and in most cases ESB that elicits these vocalizations can serve as a positive reinforcer for that animal (Jürgens, 1976). Similarly, ESB of many of these same regions also produces PA vocalizations in other mammals (Kyuhou & Gemba, 1998; Burgdorf & Panksepp, unpublished observation).

2.2. Ventral Striatum systems

Psychostimulants such as cocaine or amphetamine elicit positive affective states in humans, which have been found to depend in part by the action of these drugs on Dopamine (Volkow & Swanson 2003). Additionally, global dopamine function in humans as measured by a metabolite of DA has been found to be implemented in subjective well being as well as extroversion (Depue & Collins 1999). Psychostimulant induced PA has been found to be positively correlated with increases in DA levels (inferred from a decrease in raclopride binding) in the ventral but not the dorsal striatum in humans (Drevets et al., 2001; Volkow & Swanson 2003; Martinez et al., 2003) Similarly, microinjections of psychostimulants into the ventral but not the dorsal striatum elicit PA vocalizations in rats (Burgdorf et al., 2001; Brudzynski, personal
communication). A synopsis of the evidence for dopamine arousal in PA is summarized in Table 1.

The ventral striatum has been found to be recruited in multiple forms of positive affective states. Increases in brain metabolic activity as measured by brain imaging in humans have been found in response to PA induced by anticipation of reward (Knutson et al., 2001, as well as to PA induced by music (Blood & Zatorre, 2001). In addition, whereas increases in metabolic activity are seen in anticipation of reward in the ventral striatum, the actual receipt of a monetary reward is related to a decrease in ventral striatal activity (Knutson et al., 2001). Human male orgasm/ejaculation is associated with increases in Ventral Tegmental Area (VTA) activity (Holstege et al., 2003). In non-human animals the ventral striatum has also been implicated in various PA states, perhaps ones that can be psychologically distinguished. Whereas injections of dopamine agonists into the ventral striatum elicit PA vocalizations in rats (Burgdorf et al., 2001), injections of morphine into the ventral striatum facilitates hedonic taste reactivity responses (Peciña & Berridge, 2000).

2.3. Frontal cortex

The orbital frontal cortex has been found to be activated in fMRI brain imaging of positive emotional states related to taste induced PA (Kringelbach et al., 2003) olfactory induced PA (Rolls, Kringelbach & de Araujo, 2003) as well as somatosensory induced PA (Rolls et al., 2003). PA states induced by music (Blood & Zatorre, 2001) as well as mothers viewing pictures of their newborn babies (Nitschke et al., 2004) have also been shown to increase orbital frontal
activity. In non-human primates, a subset of orbital frontal cortex neurons are activated specifically be taste stimuli that are palatable to the monkey (Thorpe, Rolls & Maddison, 1983).

Lesion studies of the frontal cortex show definitively that the right frontal cortex is important in the neurobiology of positive affect, strongly suggesting laterality in positive affect in humans. Patients with right frontal lesions are more likely to present with symptoms of mania, whereas left frontal lesions patient are more likely to present with depression (Robinson et al., 1988). Additional evidence for the laterality of PA comes from EEG studies, with generalized PA states associated with increased left cortical power in the alpha frequency compared to the right hemisphere, and generalized negative affective states associated with decreased left cortical activation (Davidson, 2004; Tomarken et al., 1992).

2.4. Amygdala

The classic description of the Klüver- Bucy syndrome is that after bilateral temporal lobe lesions (including the amygdala) monkeys exhibit flat affect (Bucy & Klüver, 1955). Recently, it has been shown that monkeys with specific lesions of the cell bodies in the amygdala show deficits in the expression of fear (Kalin et al., 2001). Similar to animal studies, patients with amygdala damage have more deficits in processing static negative emotional than positive emotional stimuli (Adolphs et al., 1999), even though they are more responsive to dynamic-moving stimuli (Adolphs, et al., 2003). Also, there is considerable evidence that selective bilateral amygdala damage, as in Urbach-Wiethe disease, does not seriously compromise the ability of patients to have many affective experiences (Damasio, 1999). Brain imaging studies reveal that PA inducing stimuli such as music (Blood & Zatorre, 2001) odor (reviewed in Zald,
decrease amygdala activation. In general negative emotional stimuli are more effective in increasing amygdala activity than positive ones in fMRI studies (Zald, 2003). Taken together, these studies suggest that positive emotions tend to reduce amygdala activation, and that the principal role of the amygdala in emotion is in the information processing related to negative valenced emotions.

3. A recent analysis of social-joy in the rat brain

Our work on the play systems of the brain was initiated over a quarter of a century ago (first summarized in Panksepp, Siviy & Normansell, 1984). Eventually this work led to the discovery of play vocalizations (Knutson, Burgdorf & Panksepp, 1998) and soon thereafter, the remarkable finding that tickling could also evoke these 50 kHz chirpy laughter-like vocalizations (Panksepp & Burgdorf, 1999, 2000). After extensive behavioral analysis, it seemed evident that it is justified to provisionally consider this substrate as one that may mediate ancient forms of social joy and laughter (Panksepp & Burgdorf, 2003). The aim of this short section is to highlight very recent Experiments that bring us closer to understanding the underlying circuitry.

3.1. Rat 50-kHz ultrasonic vocalizations as a measure of positive affect

Just as in humans, we believe emotion-related vocalizations are one of the best ways to map out positive affect circuits of the mammalian brain. Abundant ultrasonic chirps of the 50-kHz variety are evident during the anticipatory phase of rat sexual behavior (Barfield & Thomas,
1986), anticipation of rewarding brain stimulation or drugs of abuse during rough-and-tumble play behaviors (Knutson, Panksepp & Burgdorf, 2002), as well playful, Experimenter administered manual somatosensory stimulation (i.e. tickling) (Panksepp & Burgdorf, 1999, 2000, 2003) that can reinforce arbitrary operant responses (Burgdorf & Panksepp, 2000). In humans, the anticipation of an imminent and highly predictable reward elicits PA. Similarly, anticipation of eminent reward in rats has been shown to elicit 50-kHz vocalizations. However, when the reward is omitted during an extinction trial (e.g. empty food cup place into the animals cage for their daily 1 hr feeding session, instead of the full food cup they have received each day for a week), 50-kHz calls decrease and negative affective 20-kHz calls increase although the motivation for the reward presumably remains high. Therefore, 50-kHz calls seem to reflect a positively valenced incentive motivational state. Modest amounts of 50-kHz calls are also evident during aggressive behavior (Thomas, Takahashi, & Barfield, 1983; Takahashi, Thomas & Barfield, 1983). However, 50-kHz calls during aggressive encounters occur primarily during the brief social investigation that occurs before biting occurs, after which negative affective 20-kHz ultrasonic calls dominate (Panksepp and Burgdorf, 2003; see Table 2). 20-kHz ultrasonic calls have been associated with negative affective states including morphine and cocaine withdrawal (Covington & Miczek, 2003), footshock, aversive drugs, and social defeat (Knutson, Burgdorf & Panksepp, 2002).

Of all manipulations that elicit 50-kHz ultrasonic calls in rats, Experimenter administered tickling behavior in individually-housed adolescent rats elicits the highest rates of calling, which are strongly and positively correlated with the rewarding effects of the stimulation (Burgdorf & Panksepp, 2000; Panksepp & Burgdorf, 2000). This relationship also holds true for 50-kHz vocalizations that occur during rough-and-tumble play behaviors among adolescent rats
(Burgdorf & Panksepp, submitted), electrical brain stimulation (Burgdorf & Panksepp, Submitted), and administration of drugs of abuse (Knutson & Burgdorf & Panksepp, 2002; Burgdorf & Panksepp, submitted). From this evidence, we have hypothesized that 50-kHz calls reflects an appetitive positive affective state akin to primitive human joy and laughter (Panksepp & Burgdorf, 2003; Knutson, Panksepp & Burgdorf, 2002).

Tickling responsivity generally declines in adulthood, especially in animals that have not be offered such experiences during adolescence (males generally become less responsive than females). Approximately half of such socially-housed adult female rats, placed into isolation housing several days before tickle testing, do show reasonably high levels of tickle induced 50-kHz ultrasonic vocalizations but the remaining half remain very unresponsive. The adult female rats that show high levels of tickle induced 50-kHz calls find the tickling stimulation to be more rewarding than the low responder animals (Figure 2), a similar relationship between the 50-kHz vocalizations and reward has been found using place preference, instrumental choice, and bar-pressing paradigms (Knutson, Burgdorf & Panksepp, 2002). With the aide of digital sound acquisition equipment (Fostex, USA) and a computer based sonographic analysis program (Avisoft Bioaccustics, Germany), which do not modify the ultrasonic signal to be heard in the human audible range with a bat detector, we are able to detect a variety of different types of 50-kHz calls first described in White et al. (1990). Of these various 50-kHz subtypes, it is the frequency modulated (primarily with trill components) variety in which the high tickle adult females exhibit more then the low responders (Figure 3).

3.2. Neurochemical control of 50-kHz ultrasonic vocalizations
Given that dopamine receptor antagonists have been found to reduce positive affective states in humans (e.g. those induced by psychostimulants), we tested the D1/D2 receptor antagonist alpha-flupenthixol in our tickling paradigm at a dose shown to block the rewarding effects of psychostimulants, but that does not produce conditioned place aversion (Mackey & van der Kooy, 1985). We found that alpha-flupenthixol specifically reduced the frequency modulated 50-kHz calls, without affecting non frequency modulated 50-kHz calls or aversive 20-kHz calls (Figure 4).

Conversely, psychostimulant induced positive affect has been found to be positively correlated with increased dopamine levels in the nucleus accumbens (NAcc) as inferred by decreased raclopride binding (e.g. Drevets et al., 2001). In rats, injecting amphetamine directly into the NAcc robustly elevates local dopamine levels at doses that are rewarding to the animal. We found that amphetamine given peripherally or directly into the NAcc increases levels of 50-kHz ultrasonic vocalizations (Burgdorf et al., 2001; Knutson, Burgdorf & Panksepp, 1999). The greatest elevations in 50-kHz calls were seen in animals injected with amphetamine directly into the medial shell subregion of the NAcc. In this subregion, only rewarding stimuli have been found to elevate dopamine levels, with aversive stimuli decreasing dopamine levels (Di Chiara, 2002).

In general, the drugs that are addictive to humans (e.g. opiates and psychostimulants) also elevate dopamine levels in the NAcc (Di Chiara & Imperato, 1988). In addition, to their addictive and dopamine facilitating qualities, these drugs have also been shown increase positive affect when given to humans. While drug craving and withdrawal effects may better account for the long-term addictive effects of these drugs (Berridge & Robinson, 1993, Koob & Le Moal, 2001), they do not contravene the acute euphorogenic effects. In addition to amphetamine, we
have tested a subset of these positive affect inducing drugs injected directly into the brain areas in which they are most rewarding. So far, we have found elevations in 50-kHz calls in response to nicotine, opiates, and barbiturates when injected directly into the VTA, which is the brain area most closely tied to their rewarding effects (Burgdorf & Panksepp, submitted).

In the case of opiates, only the animals which show elevated 50-kHz ultrasonic vocalizations in response to opiates administered into the VTA find these same microinjections to be rewarding (Figure 5). We have shown that re-exposure to an environment previously paired with a rewarding dose of morphine elevates levels of 50-kHz calls, whereas aversive drug paired environments decrease 50-kHz calls compared to vehicle (Burgdorf, Knutson, Panksepp & Shippenberg, 2001). When injected into the VTA, both the GABA-A receptor agonist muscimol and antagonist bicuculline are rewarding, while only the rewarding effect of muscimol is blocked by dopamine receptor antagonists (Laviolette & van der Kooy, 2001). We have found that VTA injections of muscimol but not bicuculline elevate levels of 50-kHz calls (Figure 6), again suggesting that the rewarding effects of dopamine are linked to 50-kHz calls.

The final link to the human neuroscience literature on positive emotion is intracranial self-stimulation. In their research programs both Robert Heath and Sem-Jacobsen and their colleagues reported some patients in which electrical brain stimulation produced positive affective states. When self-stimulation was evaluated, electrode placements yielding 50-kHz calls were repeatedly self-activated. While positive affect may have been sufficient for self-stimulation, it does not appear to be necessary, with some patients self administering stimulation which lead to frustration and not to positive affect. In rats, we have shown that stimulation of electrode sites that supported self-stimulation provoke more 50-kHz calls than sites which do not support self-stimulation (Figure 7). In the subset of animals in which electrical stimulation
triggered 50-kHz calls in a reproducible manner, all of these animals showed self-stimulation. Similar to tickle induced USVs, D1/D2 antagonist alpha-flupenthixol decreased frequency modulated 50-kHz in animals that showed reliable ESB induced 50-kHz calls (Figure 8). However, some animals did show self-stimulation without showing ESB induced 50-kHz calls. Therefore, similar to the human studies, positive affect seems to be sufficient but not necessary for self-stimulation. In other words, self-stimulation may reflect several distinct affective processes—an issue in need of further attention through sophisticated neuro-behavioral analyses.

4. The locus of control for affective processes

Although Experimental manipulations of sub-neocortical limbic areas of the brain tend to produce the strongest affective experiential changes in humans, and emotional behaviors in animals, there is still considerable controversy about whether other animals can have affective experiences. The traditional solution has been to suggest that all conscious experiences require neo-cortical participation. However, we would argue that the more parsimonious, data-based view is that ancient pre-propositional forms of consciousness, such as raw affective experiences, can be elaborated completely within sub-neocortical limbic regions of the brain, and that a host of affective processes are elaborated there (Panksepp, 1998, 2003, 2005). There are strategic benefits to be had if we accept, as a provisional working hypothesis, that all other mammals have basic forms of affective consciousness, not that dissimilar from our own. Such reasonable views offer many new and robust research strategy for working out important experiential aspects of the human mind from thirst and hunger to lust and loneliness (Panksepp, 1998, 2003). This, of course, is not the same as to argue that the other animals have much propositional cognitive
consciousness that would allow them to think about their affective states in ways we humans are
prone to do, even though the analysis of cognitive-emotional interactions is a challenge that
needs to be addressed (Paul, Harding & Mendl, 2005).

If one considers all the available evidence, the following conclusion seems inescapable:
A variety affective networks were present in all our mammalian ancestors, and still exist in all
living mammals. These internal value codes allow the nervous system to reference many other
behaviors with respect to the survival value (utility) of environmental objects and behavioral
actions. The importance of such brain mechanisms for survival may have “discouraged” the
weeding or dramatic genetic modifications of the infrastructures. Even though there are surely
abundant species-specific elaborations upon these foundations (e.g., rats intrinsically fear the
smell of cats; humans and most other mammals do not), the general neural principles may be
conserved (e.g., executive neurochemistires). As higher brain functions emerged, some of the
lower functions may have actually become less affectively conscious because those higher
functions operated more effectively by inhibiting lower functions (Liotti & Panksepp, 2004).
Thus, it remains possible that other animals are, in fact, more intensely affective than humans, at
least with respect to the core affects which do not depend heavily on cognitions (e.g., sensory
alliestesia, Cabanac, 1979, 2005). To find some support for such a view, we have to go no
further than young children who are typically much more emotional than their parents. In other
words, some of our lower affective functions may have been experiencing more intense prior to
the emergence of the higher cognitive functions — higher mental functions that many cognitive
scientists still deem essential for having any form of internally experienced states at all.

A cortical "read-out" explanation of affective experience is unparsimonious, and
proponents of such a perspective have yet to effectively deal with many apparent paradoxes with
such a view, the main one being the strong evidence that we always get much stronger affective responses by manipulating the sub-neocortical limbic loci of control for emotions, than by manipulating higher neocortical functions of the brain.

What is "gained" by a sub-neocortical limbic focus? We could capitalize on simple and straightforward empirical strategies for pursuing many of the important human issues, such as psychiatrically relevant feeling-disorders through animal research (Panksepp, 2004). Why do so many still find it more important to marginalize the affective consciousness of animals, when the acceptance of such processes opens up robust mechanistic strategies to tackle some of the greatest problems that neuroscience has yet to solve? It is all too easy to simply assert that these ancient limbic mechanisms only generate unconscious emotional outputs, but that is an opinion that currently flies against a rather large body of evidence (Panksepp, 1998, 2005). Although many of our cognitive capacities may be deeply unconscious, that may not be the case for affective states that help to conditionally and unconditionally valuate the world.

In making such arguments, it is important to re-emphasize that most modern fMRI brain-imaging studies of “unconscious emotions” are dealing with unconsciousness at the cognitive (perceptual information-processing) level, and practically none of those studies has monitored affect (by taking measures of changes in valence, arousal and surgency levels). Until they do that, they should only claim that they are dealing with cognitively unconscious processes, while saying nothing about affective states. In other words, too many investigators have simply failed to even consider the possibility that affective consciousness has distinct neural principles (Panksepp, 2003). Indeed, it has recently been demonstrated that emotional information presented tachistoscopically under the absolute detection threshold (1 msec) can yield reliable
changes in emotional feelings, specifically on the measure of surgency, using Lang’s Mannakins (Panksepp, Shevrin, Snodgrass & Brakel, 2004; for summary see Panksepp, 2004).

Our own work is based on the assumption that the animal work can tell us more about affective consciousness than any type of ethically conceivable human work. Conversely, the animal work may tell us much less about how the human cognitive apparatus (most people's meaning for the term "consciousness") operates. Although it may be strategically wise for the time being to simply focus on positive and negative affect measures (as can be done by various preference and aversion studies), in the future we may need a more resolved taxonomy to make sense of how the mammalian brain is functionally organized (Panksepp, 1998).

5. Conclusions

There appear to be at least 2 distinct classes of PA states represented in the brain, with separate but overlapping neuroanatomical substrates. An appetitive PA system, devoted to foraging and reward-seeking, associated in part with the effects of psychostimulants such as cocaine and amphetamine is dependent in part on the ventral striatal dopamine system. A nearby PA system involved in processing sensory pleasure such as pleasurable touch and hedonic tastes involves the opiate and GABA system in the ventral striatum and orbital frontal cortex. These classical distinctions between appetitive and consummatory processes, have been encapsulated in motivational theories which distinguish the brain substrates of expectancy type processes, such as seeking and wanting, from consummatory reward processes (Berridge & Robinson, 2003; Ikemoto & Panksepp, 1999; Panksepp, 1981, 1982, 1986, 1998).
This distinction between appetitive and consummatory PA systems is well illustrated by the work of Jürgens (1976), in which electrical brain stimulation revealed two distinct rewarding brain circuits that elicited two separate call types. The more appetitive PA call is normally exhibited when monkeys unexpectedly find palatable food or are reunited with a conspecific after a long separation, whereas the second call is exhibited during nursing as well as conspecific grooming.

Although there is still a vigorous movement to relate activity in the appetitive part of this system under the concept of reward consummation pleasures (Wise, 2004), we believe that a disciplined distinction between the positive feelings from sensory pleasures and the appetitive energization (encapsulated well in human exclamations such as “I was so excited, It was such fun!”) needs to be made in order to understand how emotional behaviors and subjective affective experiences are generated by specific types of brain activities. Some are finally beginning to make such distinctions, while others continue find such “spooky” neurodynamic concepts troublesome in our aspirations to have a mechanistic understanding of brain and mind.
References


Figure Legends

Figure 1: Mapping of fitness concerns to an affective circumplex. Potential increases in fitness create a vector moving up and to the right, which generates positive feelings (i.e. PA: positive affect) involving high arousal, while removal of potential decrements in fitness creates a vector moving down and to the right, which generates positive feelings involving low arousal. Potential decreases in fitness create a vector moving up and to the left, which generates negative feelings (i.e. NA: negative affect) involving high arousal, while removal of potential increments in fitness creates a corresponding vector moving down and to the right, which may generate different negative feelings involving low arousal. Adapted from: Knutson, Burgdorf & Panksepp (2003).

Figure 2: Mean (±SEM) latency to approach the Experimenters hand to self administer tickling stimulation in adult female long Evans rats which have previously been found to exhibit either low or high levels of 50-kHz ultrasonic vocalizations in response to tickling. Testing protocol was similar to Panksepp & Burgdorf (2000). *** P < .001, between subjects t test, 2-tailed

Figure 3: Mean (±SEM) ultrasonic vocalizations in animals which have previously been shown to exhibit either high or low levels of 50-kHz ultrasonic vocalizations. Testing protocol was similar to Panksepp & Burgdorf (2000). Ultrasonic vocalizations were recorded from the high frequency (untransformed) output of a Pettersson D980 bat detector onto a fostex fr-2
field recorder with a 196-kHz, 24 bit sampling rate. *** P < .001, between subjects ttest, 2-tailed

Figure 4: Mean (±SEM) ultrasonic vocalization in adult female long Evans rats during tickling following pretreatment with vehicle or alpha-flupenthixol (0.8 mg/kg, i.p). Testing protocol was similar to Panksepp & Burgdorf (2000). Ultrasonic vocalizations were recorded from the high frequency (untransformed) output of a Pettersson D980 bat detector onto a Fostex fr-2 field recorder with a 196-kHz, 24 bit sampling rate. * P < .05, within subjects t-test, 2-tailed

Figure 5. Mean (±SEM) place preference score (time on drug side testing minus habituation) in rats conditioned with a single 30 min pairing of 100 ng DAMGO in 500 nl over 1 min microinjected unilaterally into the VTA on the drug paired side, and a single 30 min pairing of vehicle microinjection in the vehicle paired compartment using an unbiased place preference procedure. The responder group consisted of animals which exhibited at least twice as many 50-kHz calls during the first 5 min proceeding, or more calls during first and second five min in response to DAMGO injection as compared to vehicle injection. * P < .05, within subjects t test, 2-tailed.

Figure 6: Mean (±SEM) Ultrasonic vocalizations following unilateral microinjections of 50 ng muscimol (Top) or 50 ng bicuculline (Bottom) into the ventral tegmental area in 500 nl over 1 min. * P < .05 within subject t-test (2 tailed) comparing total 50-kHz calls (flat + frequency modulated) in muscimol vs. vehicle conditions.

Figure 7: Mean (±SEM) 50-kHz ultrasonic vocalizations in response to non contingent electrical stimulation (120 µA, 60 Hz for 10 sec) in adult female long Evans rats implanted with bipolar electrodes in the ventral tegmental area, accumbens, cingulate, bed nucleus stria
terminalis, and tegmental pedunculopontine nucleus. The self stimulator group consisted of animals which subsequently showed reliable self-stimulation behavior ($3 \geq$ barpresses min). * P < .05 within subjects ttest, 2 tailed.

Figure 8: Mean (±SEM) 50-kHz ultrasonic vocalizations in response to non contingent electrical stimulation (120 µA, 60 Hz for 10 sec) in the subset of animals that reliably showed ESB elicited 50-kHz vocalizations following pretreatment with vehicle or alpha-flupenthixol (0.8 mg/kg, i.p). * P < .05 within subjects ttest, 2 tailed.
Figure 1.
Figure 2.
Figure 3.
Figure 4.

Mean Ultrasonic Calls / 2 min

- **Vehicle**
- **Flupenthixol (0.8 mg/kg)**

- **20-kHz**
- **Flat 50-kHz**
- **FM 50-kHz**

* Indicates significant difference.
Figure 5.

![Graph showing mean place preference score (sec) with two groups: DAMGO Non-Vocalizers (n=12) and DAMGO Vocalizers (n=6). The graph indicates a significant difference between the two groups with a star (*) notation.]
Figure 6.

![Graph showing Mean Ultrasonic Calls / 2 min for different conditions: Vehicle, Muscimol (50 ng). Conditions include 20-kHz, Flat 50-kHz, and FM 50-kHz. The graph indicates a significant difference marked by an asterisk (*).]
Figure 7.

*Mean ESB induced USVs / 30 sec*

- **No Self Stimulation n=10**
- **Self Stimulation n=22**
Figure 8.

- Mean ESB induced USVs / 2 min

- Vehicle

- Flupentixol (0.8 mg/kg)

20-kHz Fat 50-kHz FM 50-kHz
Table 1: Evidence that Dopamine modulates an appetitive type of positive affective states.
Dopamine does not seem to be involved in consummatory positive affective states (Berridge & Robinson, 2003). Although the positive case for the role of Dopamine in PA is given, in whole we agree that brain dopamine is only “loosely correlated with subjective pleasure” (Wise, 2004).

Psychostimulant induced PA is associated with Dopamine activity in the ventral striatum (Drevets et al., 2001; Martinez et al., 2003; Volkow & Swanson, 2003).

Psychostimulant induce PA is attenuated be Dopamine receptor antagonists (Jönsson et al., 1971; Newton et al., 2001; Romach et al., 1999).

People with 9/9 Dopamine transporter polymorphisms show diminished subjective and physiological effects of amphetamine including the euphoric effects (Lott et al., 2005).

Personality trait of extroversion (which is highly correlated with positive affect) is associated with Dopamine Functioning (Depue & Collins 1999).

The disporic effects of Dopamine receptor antagonists is associated with striatal dopamine binding (Voruganti et al., 2001).

Drugs of Abuse (but not aversive stimuli) increase Dopamine levels in the N. accumbens shell region of rat brain (Di Chiara, 2002).

50-kHz ultrasonic vocalizations, which is a rat model of PA, is modulated by DA (Knutson, Burgdorf & Panksepp, 2002).
Table #2: Alternative non-affective interpretations of 50-kHz ultrasonic vocalizations in rats, with rebuttal

**Hypothesis 1:** 50-kHz cannot reflect a positive affective state because they occur during aggression.

**Response:** 50-kHz calls occur primarily *before* the onset of aggression, after which aversive 20-kHz calls predominate (Miczek & de Boer 2005; Burgdorf & Panksepp, Unpublished Observation). Male resident intruder interactions that result in aggressive behavior exhibit 50% less 50-kHz vocalizations than non-aggressive encounters. Resident winner animals exhibited only 50-kHz positive emotional calls and no 20-kHz negative emotional calls and find aggressive interactions rewarding, whereas intruder looser animals exhibit both 50-kHz & 20-kHz calls, and find aggressive interactions ambivalent (Takahashi, Thomas & Barfield, 1983; Thomas, Takahashi & Barfield, 1983; Taylor, 1976).

**Hypothesis 2:** 50-kHz calls are an artifact of locomotion.

**Response:** In a recent study (Panksepp & Burgdorf, 2003) we found that only 9% of 50-kHz calls occur within 0.5 second of locomotor activity. Also, we have found increases in 50-kHz calls with manipulations that increased, did not change, or decreased locomotion (Knutson, Burgdorf & Panksepp, 2002).

**Hypothesis 3:** 50-kHz calls reflect states of high arousal without necessarily positive affect.

**Response:** High arousing negative affective stimuli such as foot shock, predatory odor, bright light, flustrative non-reward, and lithium chloride *decrease* levels of 50-kHz calls (Knutson, Burgdorf & Panksepp, 2002).