RAT ULTRASONIC VOCALIZATIONS IN ASSOCIATIVE LEARNING TASKS

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DEDICATION

To my children who put up with me having the dining room table covered with papers and equipment for what seemed to them a long time.

ABSTRACT

This thesis investigated how rat ultrasonic vocalizations (USVs) relate to reinforcement and associative learning under conditions of electrical brain stimulation and natural reward. Adult male Long-Evans rats received either medial forebrain bundle (MFB) stimulation or sucrose pellets while under mild calorie restriction. The goal was to determine whether the number and subtypes of 50-kHz calls reflected dopaminergic activation and learning across repeated sessions.

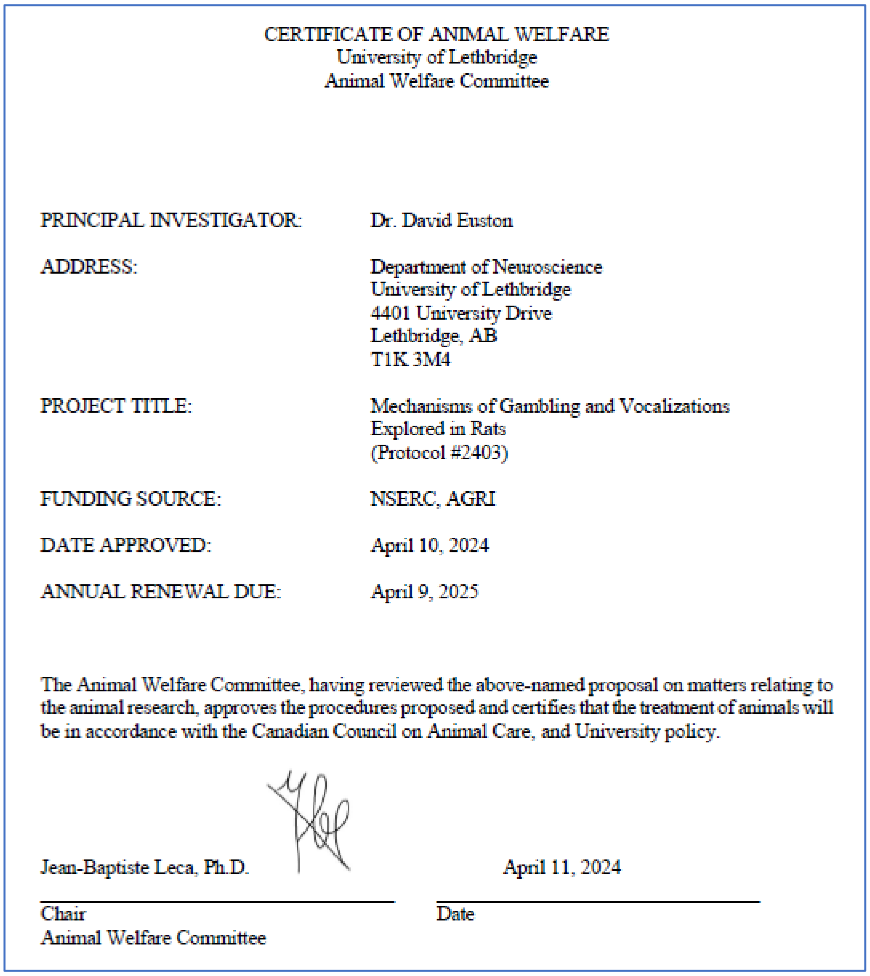
Analyses demonstrated that MFB stimulation produced a clear and consistent suppression of 50-kHz vocalizations immediately following stimulation, indicating that activation of this pathway temporarily inhibited rather than enhanced vocal behaviour. This effect was observed across animals and sessions, suggesting that stimulation interrupts the emission of USVs rather than eliciting calls associated with positive affect. In contrast, food delivery did not significantly alter the number of ultrasonic calls, and there was no evidence that pellet reward alone generated measurable increases in vocal production.

Across days of testing, there was a gradual increase in the overall number of calls made by the rats. This change may reflect increased anticipation of reward as animals became more familiar with the task structure, but it may also represent a non-associative effect of environmental habituation or reduced novelty stress. Together, these findings suggest that while 50-kHz USVs can serve as sensitive indicators of affective and motivational state, their modulation depends critically on experimental context and timing relative to reinforcement.

These results contribute to a more nuanced understanding of ultrasonic vocalizations as behavioural indices of affect. Specifically, they indicate that MFB stimulation, though reinforcing in other paradigms, suppresses ongoing vocalization behaviour, and that food reward alone does not evoke strong appetitive USVs within the parameters tested.

ETHICS STATEMENT

This project was reviewed and approved by the University of Lethbridge's Animal Welfare Committee (AWP #2403).



CERTIFICATE OF ANIMAL WELFARE
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The Animal Welfare Committee, having reviewed the above-named proposal on matters relating to the animal research, approves the procedures proposed and certifies that the treatment of animals will be in accordance with the Canadian Council on Animal Care, and University policy.

Jean-Baptiste Leca, Ph.D.
Chair
Animal Welfare Committee

April 11, 2024
Date

Figure 1. Protocol 2403, Certificate of Animal Welfare

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CR	conditioned response
CS	conditioning stimulus
ESB	electrical stimulation of the brain
FM	frequency modulated
Hz	hertz
IR	infrared
LED	light emitting diode
MFB	medial Forebrain bundle
mg/kg	milligrams per kilogram
mg/ml	milligrams per milliliter
NAc	nucleus accumbens
OFC	orbital frontal cortex
OHDA	hydroxydopamine
PETH / PSTH	peri- event / stimulus time histogram
PFC	prefrontal cortex
RPE	reward prediction error
US / us	unconditioned stimulus
VTA	ventral tegmental area

Chapter 1: Introduction

> "The problem of understanding behaviour is the problem of understanding the total action of the nervous system."
>
> Donald O. Hebb

Happiness is a subjective experience. Some behaviours may reflect happiness. If you want to know if a person is happy, you could ask them, though they may lie to you or they may not know. But how can we know if an animal is happy and what value could it be to know this? Being able to infer positive affect in animals is scientifically valuable because it lets us validate constructs like "liking" vs. "wanting," pinpoint causal pleasure circuits, and develop translational tasks whose drug and stress effects parallel humans. Animal models may help us understand human emotions. If an organism is happy then we assume they are in a positive mental state. In a positive mental state, an organism is less likely to be depressed and is more motivated to action that is conducive to evolutionary survival. While positive emotions help reinforce beneficial behaviours and maintain a state of well-being, negative emotions are the urgent alarm system that ensures an organism survives long enough to experience happiness. They ensure survival in a world full of danger and scarcity. Nature has provided both sets of emotions.

Many events and substances can be viewed as positive in that an organism is more likely to engage in behaviours which support those events. Those events are considered as reinforcing, which means that as a consequence of the event or substance a behaviour is more likely to happen again in the future. Behaviour can be changed using these events and substances as reinforcers in an associative learning paradigm.

Drugs are commonly used as substances that influence reinforcement in real life and the laboratory. In a review of drugs of abuse and electrical stimulation of various brain areas, Wise (1980a) proposed that both drugs of abuse and electrical brain stimulation (ESB) act on the same dopaminergic reward system in the brain, particularly involving dopamine neurons in the ventral tegmental area. Stimulants like amphetamine and cocaine directly enhance dopamine transmission, while opiates and sedatives such as ethanol or benzodiazepines may act indirectly by disinhibiting these dopamine neurons. Overall, all major drugs of abuse facilitate ESB by increasing activity within this shared dopamine-based reward circuitry.

Events that are reinforcing have been found to have an effect on behaviour. For example, Olds (1956) found that electrical stimulation of the brain (ESB) in the ventral tegmental area (VTA) of rats to be extremely rewarding. Electrical stimulation of various brain structures can be thought to be as influential as drugs in terms of behaviour, probably more so. Other events can be reinforcing, such as, sexual behaviours, social and play behaviours, food is if an organism is hungry. These events are often associated with positive mental states, and while we can't know about an animal's mental state except through their behaviour, the USV literature refers to events and substances that are reinforcing and indicative of a "positive" emotional state in the organisms.

Rats make 50-kHz vocalizations in a variety of positive situations. Seidisarouei et al. (2021) looked at the 50-kHz vocalizations of rats and suggests that they are produced in both social and non-social situations. They also saw that there are both quantitative and qualitative properties to the vocalizations. In social situations, such as playing and mating, rats are more vocal. In non-social situations, such as when consuming sugar pellets or sucrose water, drug rewards like cocaine and reward anticipation they still make vocalizations but of a different type. Rats made flat 50 kHz calls mainly during non-social situations. In contrast, they produced more frequency-modulated (FM) calls, especially trills and composites, during social interactions with another rat, indicating higher emotional arousal and social motivation. Thus, flat calls signaled non-social reward, while FM calls marked social engagement.

Some ultrasonic vocalizations (USVs) are strongly associated with social situations and positive affect, yet studies disagree on whether they function as strong reinforcers. Berz et al. (2021) played-back the 50-kHz calls of rats and saw that the sounds caused rats to respond by making more calls and calls of specific subtypes such as the frequency modulated calls. They state that studying the reciprocal nature of rat communication is of value for creating a model for neuropsychiatric disorders. However, another study raises questions about whether 50-kHz calls play a useful role. Kalenscher et al. (2021) tested to see if rat USVs have motivating reinforcing properties on conspecifics. They found that while there is an effect, as produced by audio file playback in a T-maze test, but it was transitory. They suggest that USVs have adaptive survival value in that it helps rats to avoid predation, and in a non-threatening situation, the calls are of value in social relations signaling positive and negative affect. Confusion may arise from conflating the reinforcing consequence of aggression with the aggressor's immediate affective state during the act. Aggression is highly reinforced because its outcome—securing territory or dominance—is advantageous, making the behaviour more likely to be repeated. However, the intense, high-arousal act of fighting itself is associated with a negative affective state (stress or fear-related), which is indexed by the emission of 22-kHz ultrasonic vocalizations, rather than the 50-kHz calls indicative of positive affect. Therefore, while the opportunity to attack is reinforcing, the act is performed under stress, resolving the apparent contradiction.

In terms of the qualitative properties of rat ultrasonic vocalizations, it has been found by Wright et al. (2010), that in response to amphetamine administration, their calls do not simply increase in the number of vocalizations but that there are patterns in the types in the 50-kHz calls referred to as subtypes of calls, associated with specific behaviour. There is a lack of research into what behaviours are associated with specific subtypes of rat ultrasonic vocalizations.

There is evidence that 50-kHz vocalizations are associated with the release of the neurotransmitter dopamine (Scardochio et al., 2015). They found that unexpected electrical stimulation of the medial forebrain bundle (MFB) reliably evoked phasic dopamine release in the nucleus accumbens (NAc), measured via fast-scan cyclic voltammetry. The trill subtype of 50-kHz calls was the most common subtype linked to dopaminergic activity.

Because 50-kHz USVs index positive affect from identifiable neural mechanisms, they offer a tractable signal to investigate emotion reinforcement links in rats, yet they've rarely been integrated into learning tasks. 50-kHz ultrasonic vocalizations produced by rats is associated with positive affect is well established in scientific literature. In researching emotion, Joseph LeDoux points out that emotions can be viewed as biological functions of the nervous system, not simply a psychological state. He points out that emotions can be beneficial to an organism but also detrimental in that to a large extent most mental disorders include emotional disorders. While LeDoux points out that to Darwin an important function of emotional expression is for communication between individuals (LeDoux, 2004), emotions serve other functions as well. From an evolutionary perspective, reinforcement has evolved as a mechanism which insures species-typical responses to appropriate stimuli (Glickman & Schiff, 1967). Research mentioned here will show that rats make 50-kHz calls reflecting their emotional state. There have been few studies looking at the vocalization behaviours of rats in learning paradigms. Reward and punishment systems evolved to help animals naturally learn the right behaviours in the right situations to survive. Learning is guided by biology, not random. Learning occurs where behaviour changes because of its consequences.

As has been suggested (Bernard, 1965), the starting point for research can be based on and observation or a hypothesis or theory. In this research I will experimentally test to see if rats make specific 50-kHz USVs and call subtypes in associative learning paradigms using electrical stimulation of the medial forebrain bundle (MFB) as a reinforcer and sugar pellets for calorie restricted rats. If learning is of adaptive significance, associating learning tasks and behaviours with specific rat ultrasonic vocalizations provides a behavioural model which could be used to test drugs of interest in mental health states.

My hypothesis is that quantity and specific subtypes of rat ultrasonic vocalization in the 50-kHz range will increase in their frequency of occurrence which is caused by an increase of dopamine levels in the ventral tegmental area through the MFB. The increased dopamine is caused by electrical stimulation of the medial forebrain bundle or delivery of sugar pellets to calorie restricted rats (Cone et al., 2014). In associative conditioning tasks, behavioural testing will demonstrate the relationship between reinforcement and both the timing, quality, and the number of 50-kHz vocalizations produced.

The present research is theory-driven and tests explicit predictions derived from contemporary models of dopamine function and associative learning. Rather than treating ultrasonic vocalizations (USVs) as purely descriptive phenomena, this work uses them as a behavioural index of reward processing and anticipation. The central hypothesis is that if dopamine mediates reward prediction and learning, then 50-kHz USVs should progressively shift from occurring after reinforcement to occurring before reinforcement as associations are acquired. Three experiments were designed to evaluate this hypothesis by examining how vocalizations change across time under fixed-interval stimulation, cue-based conditioning with electrical brain stimulation, and cue-based conditioning with food reward in calorie-restricted rats. Together, these studies test whether USVs track the development of expectation and cue–reward learning, and whether these effects generalize across artificial and natural reinforcers. The results are interpreted in the context of dopamine-based models of learning and motivation, while recognizing that mechanistic conclusions about dopamine remain inferential rather than causal.

Rat Ultrasonic Vocalization Behaviour

When do rats emit 50-kHz vocalizations? A wide variety of stimuli cause rats to make 50-kHz vocalizations. Examples are as diverse as tickling a rat’s underside, (Panksepp & Burgdorf, 2000), to social and behavioural situations. Also, a wide variety of pharmacological agents, and electrical stimulation of the VTA of the brain cause rats to produce 50-kHz vocalizations.

Based on a review of rat ultrasonic vocalizations, Burgdorf et al. (2011), found that rats make more 22-kHz vocalizations in situations associated with negative affect and more 50-kHz vocalizations in situations which produce a positive emotional state. Even distress induced by restrictions on behaviour induce 22-kHz vocalizations. For example, depriving rats of spontaneous exploratory behaviour (specifically rearing) induces emotional distress, which is expressed through 22-kHz ultrasonic vocalizations (Faraji et al., 2016). Direct physical pain, such as a bite from a conspecific or an injection of a painful substance, reliably elicits 22-kHz USVs (Borta et al., 2006).

It is worth noting and has been suggested that the USVs produced by rats are a byproduct of movement (Blumberg, 1992). Blumberg found that that vocalizations of infant rats were correlated with their temperature, suggesting infant isolation calls might just be a result of increased breathing that rats do to warm themselves. Brudzynski (2013) points out that irrespective of how the calls are produced the calls have behavioural effects on conspecifics. Whether a devocalized rat responds or not is not really relevant to the issue of whether vocalizations are a by-product of movement. It is interesting that devocalized rats still respond to the vocalizations of non-devocalized rats (Burke et al. (2017). The idea that rat ultrasonic vocalizations are produced simply by-product of movement has largely been refuted and vocalizations are seen as indicative of rat's behavioural states.

Panksepp and Burgdorf (2003) proposed that the 50-kHz ultrasonic vocalizations (USVs) rats emit when tickled are analogous to human laughter and indicate a positive affective state. Because tickling is rewarding and these calls occur in playful, social contexts, the vocalizations likely reflect joy. Consistent with Darwin's view, Ishiyama and Brecht (2016) further argue that ticklish laughter—and, by analogy, rats' 50-kHz USVs—requires a pleasurable mental state. In contrast, Brudzynski suggests that 22-kHz rat vocalizations represent the human equivalent of crying. As with laughter, "crying" may also have a communicative function.

Converging evidence indicates that rats’ 50-kHz ultrasonic vocalizations serve as specific communicative signals that anticipate and shape play—enhancing engagement while helping to prevent escalation into aggression. Converging evidence indicates that rats’ 50-kHz ultrasonic vocalizations serve as specific communicative signals that anticipate and shape play—enhancing engagement while helping to prevent escalation into aggression. For example, Burke et al. (2017) found rats emit more 50-kHz in anticipation of a play partner. They found that USV calls may even serve to reduce the escalation of play to aggression. Knutson et al. (1998) observed that rats emit more 50-kHz USVs during rough-and-tumble play, again consistent with a role for vocalizations in coordinating social interactions. More recent work has shown that the type of calls produced varies with the type of interaction (Himmler et al., 2014).

Evidence suggests that a whole range of positive events such as tickling, sex, food, and play, cause rats to emit 50-kHz calls as a way of broadcasting positive affect. Burgdorf et al. (2008) reported 50-kHz vocalizations occur during mating. Also, male rats emit more 50-kHz calls before ejaculation than after. Brudzynski (2009) reviewed research that looked at play and tickling, both appetitive behaviours, and both were strongly associated with increased 50-kHz vocalizations. Burgdorf et al. (2008) suggests that 50 kHz calls with trills (e.g., trill, composite, complex) are most closely associated with positive affect.

Burke et al. (2017) have stated that it is, “plausible that call subtypes have specific meaning”. Flat calls are the least frequent and, in the frequency modulated (FM) category the trill subtype calls constitute over 75% of calls. Their research showed that specific call categories were emitted during specific behaviours. For example, they found that jumps were associated with trills while just rearing was associated with a lack of calls. This provides evidence that individual call subtypes have specific meaning. As previously mentioned, Burgdorf et al. (2008) pointed out that specific calls (trills and step calls) are positively correlated with appetitive behaviours of mating and play. Knutson et al. (1998) also found that specific subtypes of calls are associated with play and anticipation of play.

Understanding the vocalizations of rats can be used as a model of social behaviour that can be extrapolated to various forms of brain disease as suggested by Simola and Granon (2019). The subtypes of calls can reflect not only individual differences but an individual's emotional state. They suggest that more needs to be known about the way learning affects behaviour and is reflected by USVs.

Conspicuously absent from the current research is the identity of specific categories of 50-kHz vocalizations (subtypes of calls) associated with positive events. The subtypes and timing of calls associated with food reward, drug reward, and with mating was reviewed by Wöhr and Schwarting (2007). They report that 50-kHz call patterns occur during or in anticipation of juvenile play, tickling, mating, food consumption, electrical self-stimulation of the brain, and addictive drugs. Simola (2015) reported that 50-kHz call pattern frequency increases in anticipation of many drugs of abuse. Wöhr and Schwarting (2013) identified two broad types of 50-kHz vocalizations. Rats in isolation produced 50-kHz flat calls and rats searching for a play partner emitted FM 50-kHz call subtypes. These observations were tested via playback recordings to show the FM calls induced social approach behaviour. Only frequency-modulated (FM) 50-kHz calls elicit social approach in rats, reflecting their origin in positive, rewarding contexts such as play or mating. Flat 50-kHz calls, though sometimes produced after isolation, lack the modulation that conveys positive affect and therefore do not invite approach. FM calls activate reward-related regions like the nucleus accumbens, while flat calls do not, indicating that only FM calls serve a clear affiliative, pro-social function.

Wright et al. (2010) identified 14 categories of ultrasonic vocalizations where the subtypes were affected by social context, amphetamine dose, and the time within sessions. The findings revealed that these call subtypes are differentially associated with specific contexts: trill calls were strongly linked to both amphetamine-induced reward and positive social interaction (i.e., with a cage-mate), while flat calls decreased in these appetitive contexts and over time within a session. Furthermore, individual rats exhibited unique and stable "call profiles," suggesting personal vocal repertoires. The authors suggested that their research demonstrated that the vocalizations emitted by rats served both in social inter-rat communication as well as to signal the affective state of the subjects.

To understand the behavioural significance of rat ultrasonic vocalizations, one needs to know the type, quantity and specific timing of calls relative to a target behaviour. Few of the studies done to date have provided a clear indication of which subtypes of 50 kHz calls are associated with specific positive events, such a receipt of food reward or detection of a cue predictive of reward.

Electrical Stimulation of the Brain

As suggested by Claude Bernard, (Bernard, 1965), anatomy is a necessary foundation in the study of physiology. To understand the function, it is necessary to know something about the structures involved. Since the dopaminergic system is implicated in the reinforcing effect of electrical stimulation of various structures in the brain located in the midbrain and forebrain, knowing the location and connections, anatomically, is of value.

There are four major dopamine pathways (Sanescohealth, 2016). The four pathways are:

1. The mesolimbic pathway is involved in the reward system. It begins in the VTA area and sends to the nucleus accumbens.
2. The mesocortical pathways also originates in the VTA but sends to the prefrontal cortex.
3. Nigrostriatal pathway starts in the substantia nigra and goes to the caudate and putamen in the basal ganglia.
4. Tuberoinfundibular dopamine pathway begins in the arcuate and periventricular nuclei of the hypothalamus and projects to the infundibular of the hypothalamus.

Structures involved in dopaminergic system and/or structures known to be responsive to electrical stimulation include the VTA, NAc, amygdala, septum, MFB, locus coeruleus, substantia nigra, basal ganglia, and hypothalamus.

Of some interest is the septum which was cited as a structure of interest for electrical stimulation by Robert Heath and James Olds. The term septum means literally a barrier or partition. The septum has connections with the hippocampus and the hypothalamus and is suggested to serve as a relay station between these structures. It consists of two structures: the septum pellucidum and the verum. Septal nuclei are part of the limbic system. Neurons in the septal nuclei have axons that travel to the medial forebrain bundle.

Electrical stimulation of the basal ganglia has profound effects on behaviour as has been shown by several researchers. For example, ESB was dramatized many years ago by the work of Jose Delgado in the 1960's (Marzullo, 2017). In his research Delgado showed how stimulation, which was delivered by remote control, could stop a charging bull. Also, the work of Robert Heath, who was attempting to help people with many neurological disorders (Kringelbach & Berridge, 2012), also documented the potential effects of electrical brain stimulation (ESB). Brain stimulation can alter mood and motivation, but its effects depend on which circuits are activated. Stimulation of dopamine pathways increases "wanting" or craving, whereas activating opioid and endocannabinoid hedonic hotspots enhances actual "liking" or pleasure. Thus, electrical stimulation may produce strong approach behaviour without true enjoyment. Precisely targeted deep-brain stimulation may relieve depression or anhedonia, but poorly localized stimulation risks inducing compulsive or addictive behaviour. In a recent review of the challenges and future directions of deep brain stimulation in human patients. Lozano (2019) stated that titrating stimulation parameters is necessary to maximize the benefits and reduce the side-effects of the stimulation.

The site for electrical stimulation in this research is the medial forebrain bundle. It has been found that stimulation of the MFB excites the lateral hypothalamic and preoptic regions (Szabó, 1973). Afferent connections through the MFB come from the VTA (Coenen et al., 2018). The MFB also contain efferent connections. It has been found that the absolute refractory period of the MFB neurons in the range of 0.8 to 1.1 milliseconds in rats when stimulated electrically (Rolls, 1971) indicating they recover from stimulation very quickly. The target for the ESB her are the mesocortical and Mesolimbic pathways.

Pioneering work in the study of electrical stimulation of the brain by Olds (1956) showed that when midbrain nuclei and fiber pathways are electrically stimulated rats prefer stimulation over basic survival drives such as eating and mating. Gallistel et al. (1981) showed that electrical stimulation of the medial forebrain bundle in the rat is both positively reinforcing and motivating. They investigated and described the properties of neural tissue in the medial forebrain bundle whose excitation results in reinforcing and motivating effects in the rat. Olds and Milner (1954) found that stimulation of the septal area in rats produced the highest rate of bar presses for electrical stimulation as a reinforcer. More recently, Burgdorf et al. (2007) tested various brain sites to see which brain sites elicited the strongest behavioural responses to electrical stimulation. They found that circuits from the prefrontal cortex to the raphe produce the highest rate of response with the VTA being the most consistent.

Electrical brain stimulation can strongly modulate behaviour and learning—producing reward or aversion and even outcompeting natural reinforcers—yet its effects hinge on which neural elements are activated and on stimulation parameters and can be constrained by habituation. Brocker and Grill (2013) suggests that electrical stimulation of neural tissue is useful--compared to ablation of tissue--in that the stimulation is reversable. When stimulating electrically in the brain it is concerning that one may be stimulating the soma, axons, or dendrites. Exactly what is being stimulated remains an unknown today despite decades of research. The amplitude and time course of the evoked potential are determined by the stimulus (current/charge and pulse width), the tissue's electrical properties and geometry (membrane resistance and capacitance, axial resistance, conductivity/anisotropy), and the electrode–tissue interface (impedance and contact geometry). There is also concern that the brain tissue can adapt to the electrical stimulation. Farakhor et al. (2019), found that stimulation of the MFB habituates as a function of the number of pulses, and the frequency and amplitude of the pulse widths. They found that 170 microamps at a frequency of 400 Hz with a pulse width of 1,500 microseconds resulted in the least habituation. It is interesting that in a study by McMurray et al. (2017), they found that electrical stimulation of the MFB was more rewarding to rats, in a decision-making task, than food. Electrical stimulation of the brain (ESB) can act as either a reinforcer or a punisher: Bower and Miller (1958) showed that stimulating a single site in the rat brain could produce approach (reward) or avoidance (punishment) effects depending on stimulation conditions, thereby supporting both kinds of learning. In a classical-conditioning study, Linseman and Olds (1973) recorded single-unit activity in the hypothalamus, preoptic area, and striatum and found training-related changes in firing—responses time-locked to conditioned stimuli and other trial events—indicating that associative learning is reflected in these forebrain circuits.

The concept of brain reward systems, a subject of research for decades Routtenberg (1978), centers on the principle that reinforcement—a process promoting learning—can be driven by stimuli considered rewarding and motivating. A key demonstration of this was the work of Milner (1991) showing that electrical stimulation of specific brain areas could itself function as a reward. The therapeutic potential of this phenomenon has been explored for conditions like severe depression. In early foundational work by Heath, stimulation of the septal area in depressed patients was found to be desired by them, yet they did not describe the sensation as explicitly pleasurable (Frank, 2018). Supporting the safety and efficacy of such approaches, Furlanetti et al. (2015) used a rat model for depression to demonstrate that electrical brain stimulation was not only safe but also helped normalize behaviour.

Electrical stimulation of the medial forebrain bundle is positively reinforcing likely because it stimulates dopamine release. Electrical stimulation of the MFB can be used as a reward for bar pressing and the rate of bar pressing is increased when rats are given amphetamine, as opposed to saline or controls subjects, Schaefer et al. (1987). Carlson (1981) used micro dialysis and found that electrical stimulation of the medial forebrain bundle caused the release of dopamine from the nucleus accumbens (NAc). Coenen et al. (2018) analyzed DTI images from human subjects and determined projections from the MFB reward system connect to the prefrontal cortex (PFC) and orbital frontal cortex (OFC). Wise (1980b) reports that the ascending dopamine axons are in highest concentration in the MFB, the ventral tegmental area and the substantia nigra areas.

Stimulation techniques in animal research has developed rather slowly, largely due to the interest in ESB providing help for humans. Still, studying electrical stimulation of the brain of rats does provide a convenient animal model to learn about the behaviour of ultrasonic vocalization as a measurable index of hedonic response (Lozano et al., 2019). While ESB in animal research is not as popular as it was in the decade after the discoveries of Olds and Milner, there still is some research using these methods being done (about 40–60 papers; published Oct 19, 2020, to Oct 19, 2025; searches across PubMed/Google-Scholar for results of “medial forebrain bundle + rat + stimulation + DBS”).

There is much evidence that electrical stimulation of specific brain structures has dramatic effects on behaviour in both humans and rats. The exact sites in the brain which are most influenced by stimulation, and optimal stimulation parameters are still a matter of some debate and research. That ESB can be used as a reinforcer and is reinforcing has been established in associative learning experiments. I have chosen the MFB as it historically has been the most effective site for producing strong and reliable reinforcement and because it contains a large number of ascending dopaminergic axons. What is needed is an animal model of a positive affective state where ESB creates USVs, produced by rats in response to stimulation, in a controlled learning paradigm.

Calorie Restriction

Food deprivation and calorie restriction are widely utilized motivational tools in behavioural neuroscience, particularly in studies involving associative learning in rats. Associative learning, the process whereby animals learn to associate a stimulus with a specific outcome or response, forms the basis for understanding various cognitive and emotional processes (Balcombe et al., 2004).

Associative learning in rats typically involves classical or operant conditioning, both of which rely on the animal's capacity to form meaningful connections between environmental cues and outcomes. Motivation plays a pivotal role in enhancing the efficiency and speed of this learning process. Among the common motivational manipulations, reducing access to food via deprivation or calorie control is effective in increasing the drive to obtain a food reward, which in turn strengthens learned behaviours.

Food deprivation generally involves withholding food for a specified period before learning sessions, often reducing the rats’ body weight to approximately 80-90% of their free-feeding weight to induce hunger without causing health detriments. It creates a motivational state that heightens the saliency of food rewards. Conversely, calorie restriction entails long-term limitation of caloric intake while ensuring nutrient sufficiency.

The motivational influence of hunger is crucial because it directly affects reward-processing neural circuits, including dopaminergic pathways in the mesolimbic system. These pathways modulate the release of neurotransmitters that underlie reward anticipation and reinforcement learning. Higher motivation levels typically result in improved attention, increased interaction with task stimuli, and enhanced memory consolidation.

Appetitive 50-kHz ultrasonic vocalizations provide a sensitive, noninvasive index of rats’ motivational state—especially hunger—and track the affective processes that support associative learning. For instance, rats under food deprivation often exhibit increased emissions of 50- kHz calls during the anticipation or consumption of food, reflecting heightened positive motivation and arousal (Brudzynski, 2013). These vocal signals can serve as non-invasive measures of the internal emotional states that drive associative learning performance.

Experimental paradigms studying associative learning under conditions of food deprivation have often observed that the presence and frequency of appetitive USVs correlate with enhanced learning outcomes. For example, when a cue reliably predicts food, rats show a robust rise in 50-kHz USVs during the cue period, and growth in calling across training typically mirrors acquisition of the conditioned response (Knutson et al., 2002).

It is important to consider ethical aspects when implementing calorie restriction in animal studies so that practices align with the principles of humane animal research.

Dopamine and Reward

Rat 50-kHz calls are associated with dopamine level changes in the central nervous system. Specific brain nuclei have been found to be involved in the release of dopamine and the nucleus accumbens is associated with 50-kHz USVs in rats (Burgdorf et al., 2007). Further, positive-affect USVs closely track mesolimbic dopamine function: repeated cocaine enhances (sensitizes) 55-kHz calling in parallel with locomotor sensitization (Mu et al., 2009). Stimulants such as cocaine and amphetamine reliably increase both the number and peak frequency of positive-affect USVs. Frequency-modulated 50-kHz calls often reach 55–70 kHz, and sensitization enhances these higher-frequency emissions.

Drugs associated with dopamine influence the amount of USVs rats make. Mu et al. (2009) showed that rats bred for higher production of 50-kHz USVs exhibit higher levels of cocaine induced sensitization than rats selectively bred for lower levels of USV’s. Ahrens et al. (2009) found that 50-kHz FM USVs are increased by repeated intravenous amphetamine, but flat calls were not increased. Not only are the number of calls influence by drugs but specific subtypes of calls can also change. The elevated number of FM calls, specifically trill calls, compared to flat calls suggests a positive association with dopaminergic levels because amphetamine is a strong dopamine reuptake inhibitor. Further support for a dopamine-vocalization link comes from the fact that 50-kHz USV's are increased over time by repeated amphetamine injections in parallel with increased signs of behavioural activation. Simola et al. (2012) found that methylphenidate, another dopamine reuptake inhibitor, increased the number of 50-kHz USVs emitted by rats but MDMA, morphine, and nicotine did not. Their research shows that stimulants are more influential in eliciting 50-kHz vocalizations in rats than are other drugs. In looking for an animal model that mimics mania, a symptom of psychosis-related disorders, Pereira et al. (2014) found that rats produce more FM calls (step and trills) when treated with D-amphetamine. They found that antimanic drugs such as lithium reversed this effect, reducing FM calls. Their research implies that while flat calls may serve as a social communication function, FM calls indicate an appetitive, high-dopamine state. Sanchez et al. (2022) found that cocaine increases locomotion and markedly elevates appetitive 50-kHz USVs, and that diazepam given after cocaine blocks the USV increase and attenuates the hyperlocomotion. Their research suggests that Diazepam may be useful in treating manic effects of dopaminergic stimulating drugs. Simola et al. (2010) showed that caffeine does not cause an increase in the number of 50-kHz USVs in rats, which is evidence that dopamine specifically, and not just behavioural activation, is key to vocalizations. Further supporting the association between dopamine and 50-kHz vocalizations in rats, Brudzynski et al. (2012) injected Quinpirole, a D2/D3 dopamine agonist, into the shell of the nucleus accumbens and found that it increased 50-kHz vocalizations.

While increasing dopamine is associated with 50 kHz calls, reducing dopamine via antagonists or selective destruction of dopaminergic cells causes a decrease in 50-kHz vocalizations in rats. Burgdorf et al. (2007) showed that both the dopamine D1/D2 receptor antagonist flupentixol and 6-

hydroxydopamine (6-OHDA) lesions reduced 50-kHz vocalizations. In addition, Ciucci et al. (2009) showed that two rat Parkinsonian models—one produced by unilateral infusions of 6-OHDA and the other by a dose of haloperidol (a dopamine antagonist)—resulted in rats emitting fewer FM calls and more flat calls.

One key locus for the effect of dopamine on vocalizations is the nucleus accumbens. Burgdorf et al. (2007)demonstrated that microinjections of amphetamine into the nucleus accumbens greatly increased the number of 50-kHz USV's emitted by rats. This effect was further localized to the nucleus accumbens shell rather than core. This latter finding was confirmed by Thompson et al. (2006), again using microinjections of amphetamine. These findings are important because they localize the effect of dopamine on vocalizations to specific brain areas known to be involved in dopamine release and activation.

The 50-kHz vocalizations of peer rats can elicit dopamine release in nucleus accumbens. Willuhn et al. (2014) tested the hypothesis that 50-kHz USV's, but not 22-kHz USV's, would elicit nucleus accumbens dopamine release by analysis of subjects who were presented with 50- or 22-kHz playback vocalizations. Their findings show that dopamine release in nucleus accumbens is associated with 50-kHz USV's, but not with the alarming 22-kHz USV's, which supports the idea that the two USV types are processed by separate brain regions.

Dopamine is released both tonically (slowly) and phasically (quickly), with the former often associated with motivation and the latter with learning. Schultz and Dickinson (2000) have suggested that VTA neurons encode reward prediction error (the error between the amount of reward you expect and what you get). The dopamine reward response is greater the greater the discrepancy between the expected reward and the reward received. Initially, in a classical conditioning paradigm, the reward elicits an increased phasic dopamine response (unexpected reward, positive prediction error). During learning, the repeated pairing of the stimulus and the reward elicits an increased dopamine response to the stimulus, which is the first indicator of the upcoming reward amount. Meanwhile, the VTA response to the reward itself decreases because, once the cue stimulus is known, the amount of reward is known and hence there is no reward prediction error. Daw and Tobler (2014) review evidence that transient (phasic) changes in dopamine neuron firing—and related striatal signals—encode reward-prediction errors that update value estimates during learning.

Another line of research demonstrates tonic release of dopamine in the ventral striatum tied to both the expectation of reward and response vigor. Howe et al. (2013) showed that dopamine signals ramp up when rats are expecting to attain a reward. Tonic activity is related to the delay to the reward as well as the size of the reward. They suggest that the tonic dopamine levels are indicative of maintaining a motivational state. Salamone et al. (2016) reviewed research that shows that dopamine is required for effort-based reward choices. Nucleus accumbens lesions or dopamine depletion lead to lack of willingness to pursue reward in face of physical effort. This evidence supports the idea that dopamine levels in nucleus accumbens are related to response vigor. In a recent attempt to reconcile the roles of tonic and phasic dopamine, Berke (2018) pointed out that phasic, reward-prediction signals are seen in the firing of VTA cells whereas measurements of tonic dopamine are typically done in the ventral striatum using methods such as micro dialysis. This raises the possibility that the phasic signal is carried broadly by VTA neuronal projections but tonic dopamine in the striatum is caused by pre-synaptic modulation of the dopaminergic VTA terminals in the striatum. Hence, dopamine may serve both as a teaching signal for learning as well as a motivational signal based on tonic levels in the ventral striatum.

That the transmitter dopamine is involved in more than the reward system, Schreiner (2019) suggests it has purpose in motivation, perception, attention, movement, and the release of hormones is not to underestimate its importance as a chemical involved in reward. Schultz (2010) defines rewards, "as objects or events that generate approach and consummatory behaviour, produce learning of such behaviour, represent positive outcomes of economic decisions and engage in positive emotions and hedonic behaviour”. Based on his review he states that phasic dopamine signals are related to reward based on lesion and pharmacological research, and dopamine neurons show phasic response to external stimuli.

Linking dopamine to 50-kHz calling, Garcia et al. (2015) show that novelty modulates rats’ ultrasonic vocalizations in a context-dependent way, with different call subtypes emerging during reward anticipation versus reward receipt. When a new environment became a familiar place where the rat expected a reward (like being tickled), the rat made more high-pitched, positive vocalizations overall. However, looking closer revealed two different stories. The rats made more complex, harmonic calls just from being in the place where they expected the reward, showing they were anticipating something good. On the other hand, they made more frequency-modulated calls (trills, specifically) when they were actually being tickled. So, novelty itself doesn't just increase all calls; it specifically boosts the types of calls linked to anticipating a reward in a familiar place.

Berke (2018), in studying what dopamine release means, suggests that it is a critical modulator in motivation and learning. Dopamine is thought to be involved in motivation and reward, and in this research, it may be expected that reinforcement associated with learning would involve the release of dopamine in the rats. Kringelbach and Berridge (2012) suggest that an increase in dopaminergic activity is associated with wanting something, as opposed to just liking it. They suggest that electrical stimulation of various midbrain structures may not produce pleasure but is desired and rewarding. Using in vivo microdialysis, Ahn and Phillips (1999) found that dopamine efflux in the medial prefrontal cortex and nucleus accumbens rose during consumption of a palatable food, declined with sensory-specific satiety, and increased again when a different palatable food was offered—indicating that dopamine in these circuits tracks current motivational value.

Associative Learning

Associative learning is a fundamental psychological process whereby an organism learns the relationship between two stimuli or between a stimulus and a response and is widely studied through experimental tasks involving animal models, particularly rats.

Classical conditioning, first systematically studied by Ivan Pavlov, is a process where a neutral stimulus becomes associated with an unconditioned stimulus to elicit a conditioned response. In rats, this is often demonstrated through tasks such as the Pavlovian fear conditioning, where a tone (neutral stimulus / CS) is paired with a mild foot shock (unconditioned stimulus / US), resulting in the rat showing freezing behaviour (conditioned response / CR) upon hearing the tone alone (Kim & Jung, 2006). This method allows researchers to probe the neural circuits underlying fear and memory formation, with the amygdala playing a central role (Maren, 2001).

Operant conditioning is a type of instrumental conditioning that emphasizes how consequences shape the future probability of a behaviour. A reward is any stimulus that is experienced as desirable or positive, whereas a reinforcer is defined by its effect on behaviour—specifically, whether it increases the likelihood of a response. A stimulus can be rewarding without reinforcing behaviour, but a reinforcer must, by definition, change behaviour.

Trace conditioning is a form of classical conditioning in which the conditioned stimulus (CS) ends before the unconditioned stimulus (US) begins, leaving a stimulus-free gap (the “trace interval”) between them. For example, a light (CS) might be illuminated for several seconds, followed by a brief pause with no stimulus, after which an electrical stimulation (US) is delivered. The subject must maintain a memory trace of the CS across this interval to associate it with the US.

Much of the behaviour of humans and other animals is directed toward seeking out rewards (Steinberg et al., 2013). Learning to identify environmental cues that provide information about where and when natural rewards can be obtained is an adaptive process that allows this behaviour to be distributed efficiently. Theories of associative learning have long recognized that simply pairing a cue with reward is not sufficient for learning to occur. In addition to contiguity between two events, learning also requires the subject to detect a discrepancy between an expected reward and the reward that is actually obtained.

Experimental Overview

The primary purpose of this experiment was to determine when 50 kHz vocalizations are emitted relative to the primary rewards of brain stimulation and food. A secondary goal was to determine which sub-types of 50 kHz calls were used in any reward-related calling.

The dependent variable is the amount and type of 50-kHz vocalizations, and whether vocalizations are time-locked in any way to the time of reward (electrical stimulation in the medial forebrain bundle or sugar pellet delivery in the case of calorie restricted subjects). One question is, when do vocalizations occur: before, during, or after the stimulation or sugar pellet delivery. An escalation of vocal counts leading up to reward would be consistent with a link between dopamine in ventral striatum and 50-kHz vocalizations. Vocalizations occurring immediately after reward would be consistent with a link between the phasic, reward-prediction error signal and vocalizations. Further, if vocalizations are tied to the offset of reward (and hence perhaps tied to a reward-prediction error signal) it would indicate that the VTA dopamine cell firing rates, during learning, post-reward vocalizations should decrease while vocalizations tied to the offset of the first cue predictive of reward will increase.

Identifying call subtypes data can be used to address the larger question, is there a specific call subtype, or group of calls, associated with reward and learning. Video observation and data were collected about the subject's behaviour to see what they were doing during the experiment.

Experimental question: Test to separate the relationship between reward, cues, and instrumental behaviour to obtain electrical stimulation reward or a sugar pellet. If vocalizations are tied to reward anticipation, we would expect to see the number of 50-kHz calls increase and be of specific call types as rats learn when to expect reward.

Another question I have from doing a literature review on these topics is: do organisms ‘anticipate’ based on the time of events, an associated reward? When conditioned, instinct may be involved, to life or environmental events, and reinforcement occurs, we act or anticipate doing. A central question raised by the literature is whether animals learn to anticipate rewards solely from the temporal structure of events — that is, whether the passage of time itself becomes a predictive cue. Conditioning interacts with innate motivational systems such that, once reinforcement contingencies are learned, animals begin to anticipate the required action or upcoming reward and adjust their behaviour accordingly. These experiments test to see if bilateral electrical stimulation of the medial forebrain bundle or delivery of sugar pellets to a calorie restricted rat influence the production of 50-kHz ultrasonic vocalization behaviour.

In sum, this work uses ultrasonic vocalizations to clarify the moment-to-moment dynamics of reward processing by identifying which 50-kHz call subtypes are emitted and when. We employed two complementary paradigms—a predictable fixed-delay (~90 s) delivery and a Pavlovian cue presented 8 s before reward—under both food reward and medial forebrain bundle stimulation. Calls were classified into established subtypes (e.g., complex, trill, trill-with-jumps, flat), and we constructed peri-event time histograms aligned to cue onset and reward delivery to determine whether each subtype was emitted anticipatorily, consummatory, or was unrelated to the event. By time-locking specific call categories to defined task epochs, the study addresses a key gap in the literature and provides a clear framework for interpreting USV subtypes as noninvasive readouts of motivational state across natural and electrical reinforcers.

Chapter 2: Experiment 1 – Fixed Time Interval

Rationale

Theories of associative learning emphasize that contiguity by itself is not enough; learning depends on a signal's predictive value. Under a fixed-time schedule (e.g., reward every 20 s), the passage of time functions as a cue, and animals develop anticipatory responses that grow as the expected reward time approaches. In Experiment 1, to establish that ESB are associated with USV's in rats, I replicate Burgdorf et al.'s (2000) experiment 1. In that experiment they used an un-cued single pulse of stimulation (1 second) to the VTA on a fixed time schedule, every 20 seconds, for 10-minute sessions and recorded the subject vocalizations. The subjects were tested every second day for 4 days of trials.

In the Burgdorf experiment they analyzed the number of 50-kHz vocalizations. They tested nine Long-Evans rats and saw an increase in the number of vocalizations. They looked at the mean number of vocalizations per minute in 5 seconds time bins leading up to the VTA stimulation.

In this experiment I also analyzed when the vocalizations occur, relative to the stimulation.

Replicating the experiment by Burgdorf, Knutson, and Panksepp, all leading researchers in rat ultrasonic vocalizations, allowed us to validate our behavioural paradigm as well as test whether our experimental set-up—sound equipment, video recording equipment, my Matlab code, and my surgical procedures—were working properly.

Hypotheses

It is hypothesized that rats exposed to a fixed-time 20-second schedule of electrical brain stimulation (ESB) of the medial forebrain bundle (MFB) will exhibit an increase in ultrasonic vocalizations (USVs) across successive trials and days. Essentially, on fixed-time-20 schedule rats receiving ESB of the MFB will produce more USVs as the trials progress over days.

Method

Three male Long-Evans rats weighing 420 to 484 grams were the subjects.

To record the ultrasonic vocalizations rats produced; a soundproof wooden box was created and lined with 5 centimeters (cm) thick soundproof foam. The wooden box (a) is 63.5 cm (25 inches) wide by 91 cm (36 inches) long and 71 cm (28 inches) deep. A Plexiglass box (b) was created which fits inside the wooden box and can be placed inside or removed for cleaning. The plexiglass box is 35.5 cm (14 inches) wide by 48 cm (19 inches) long and 45.7 cm (18 inches) high and made with 0.63 cm (¼ inch) plexiglass bonded together with epoxy. Figure 2 shows the four box configurations. Figure 2-c shows the soundproof foam and figure 2 – d the plexiglass box with pellet feeder attached. See Appendix 1 for details about the testing chambers.

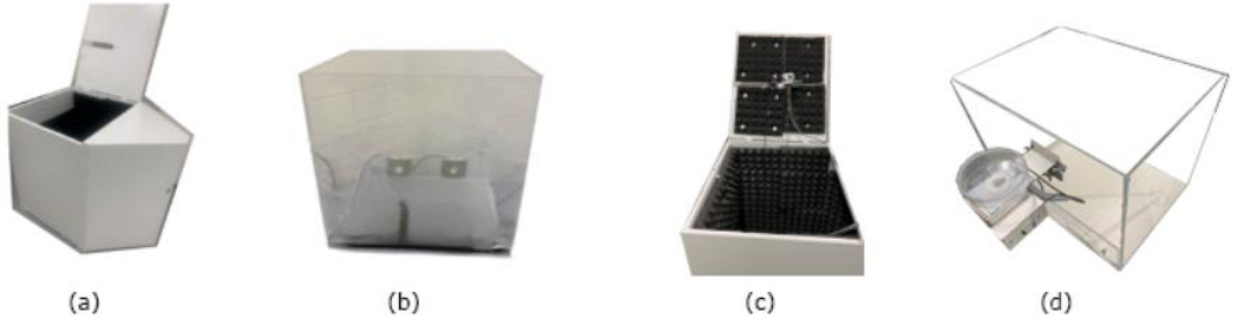


Figure 2. (a) wood soundproof box, (b) plexiglass testing box, (c) sound proofed box, (d) pellet dispenser on plexiglass box

Attached to the sound-proof wooden painted exterior chamber are an ultrasonic microphone, two video recording cameras, infrared lights, a commutator to connect electrode wires to the subject which allows free movement of the rat while tethered, and a LED light and beeper to signal when a trial series starts and ends. See Appendix 2 for a description of the attachment of the microphone, start/stop beeper/light device, and the commutator.

Electrical stimulation consisted of a biphasic pulse of 200 µs per phase separated by a 20 µs inter-phase interval. Each double-pulse train was triggered either by a button press or by MATLAB control code. Following the completion of a pulse train, a 6.5 ms inter-stimulation interval was imposed before the system could accept another trigger. During this 3-s refractory period, the output voltage was held at 0 V to prevent further stimulation.

The stimulation protocol was implemented using an Arduino Uno (R3) microcontroller running custom code (Appendix 3), which communicated with a WPI A-365 stimulator via serial interface. The stimulator was set to bipolar mode, delivering a 1 mA range at 50 % output (0.5 mA actual current). The objective was to produce a biphasic waveform consisting of a 200 µs positive phase followed by a 200 µs negative phase, separated by a 6.5 µs gap, yielding an effective pulse-train frequency of approximately 150 Hz. Figure 3 shows the WPI A-365 stimulator (a), a pulse captured on an oscilloscope (b), and the Arduino Uno with push button (c).

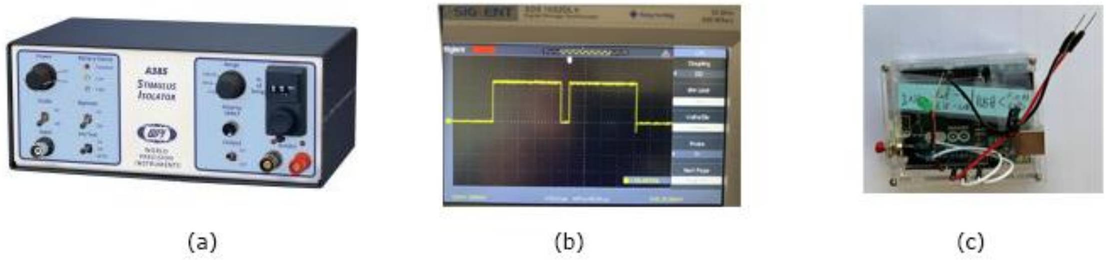


Figure 3. Stimulation (a) WPI A-365, (b) Oscilloscope Recorded Pulse, (c) Arduino Uno

Each stimulating electrode consisted of two, Teflon-coated, stainless steel wires (coated diameter of 0.0045 inches; 316SS3T; MedWire, Mt. Vernon, NY) twisted together with 0.5mm of insulation removed from one tip as described by Euston and McNaughton (2006).

To test the efficacy of the stimulation procedure relative to the surgical placement of the electrodes, the subjects were placed in the test chamber and stimulated with the experimenter and assistant observing and recording the subject behaviours. The procedure looks at how the subject responds to stimulation as an indication of the most optimal electrode combinations and polarity. Specifically, I looked for bouts of sniffing and exploratory head movements elicited by the stimulation as indications of efficacy.

Stimulation wiring to test electrode placement efficacy, numbering of electrode wires shown in Figure 4.

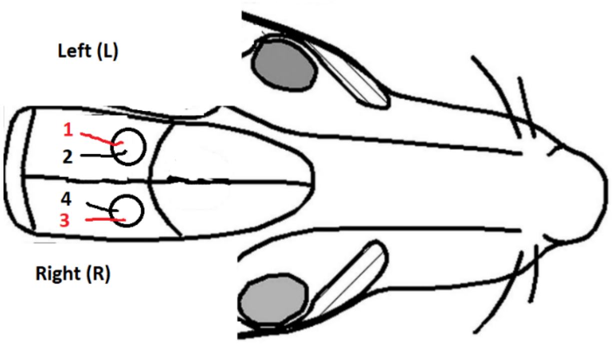


Figure 4. Stimulation Efficacy Wiring Numbers

Once the best electrode configuration was determined, that is, the electrodes which give maximum behavioural response, the amplitude-response rate was tested using 50, 60, 70, 80, and 90 microamp stimulation looking at the maximum response rate to each level of stimulation.

Table 1. Electrode Combinations to Test Efficacy

Red - hot	Black - ground	Matlab Code	Comment (and do over Stim microamps)
Lateral			
L1	L2	1	
R3	R4	2	
L2	L1	3	
R4	R3	4	
Across Hemispheres			
L1	R3	5	
L2	R4	6	
L1	R4	7	
L2	R3	8	

The most effective electrode wiring was determined individually for each rat by testing different combinations of wire tips and observing behavioral responses. In all cases, the optimal configuration involved across-hemisphere (left–right) electrode pairings, which produced bilateral stimulation of the medial forebrain bundle. Bilateral stimulation reliably elicited stronger and more consistent behavioral activation, indicating more effective engagement of reward-related dopaminergic pathways.

Matlab code was written to determine what microamp stimulation produced the greatest number of nose pokes (see Appendix 4). The data from the number of nose pokes (see Appendix 10 for nose poke box description) was used to set the stimulation amount for the experiment. The subjects were shaped for 15 minutes by experimenter provided ESB, manually push-button delivered, as the rat approached the node poke box. After shaping the rats were tested for between 5 and 9 days, until the best microamp stimulation amount was determined.

Prior to conducting these studies, a Standard Operating Procedure was created and approved by the Animal Care Department at the University of Lethbridge (see Appendix 5 - SOP #330 Electrical Stimulation of Deep Brain Structures Procedure in an Adult Rat).

All procedures were conducted in accordance with the Canadian Council on Animal Care guidelines. This research was approved by the University of Lethbridge Animal Welfare Committee.

Thirty minutes pre-op the rat was injected subcutaneously with buprenorphine, (0.3 mg/ml). Each rat was anesthetized with isoflurane (1–1.5% by volume in oxygen at a flow rate of 1.5 L/min; Holocarbon Laboratories, River Edge, NJ); placed in a stereotaxic holder and injected along the incision site with a lidocaine/epinephrine combination to control pain and reduce bleeding.

The skull was cleared of skin and fascia using sterile Q-tips, and craniotomies were opened for two stimulating electrodes targeting the medial forebrain bundle (MFB). To hold the dental acrylic skull cap in place 4 holes were drilled in the skull and screws secured to the depth of the dura.

The rats had electrodes implanted in the MFB (AP −3.25 mm, ML ±1.65 mm, DV −8.5 mm from bregma. During the surgery an Arduino with the same code used for stimulation during behavioural experiments was connected to a WPI A365 stimulator and current was applied to the electrodes on each side. The subject was observed for sniffing behaviour which has been indicated as a behaviour associated with optimal placement for stimulation of the VTA (Rossi & Panksepp, 1992).

Following surgery, the rats were monitored in a recovery chamber twice per day and given Metacam (5 mg/ml; 1 mg/kg) subcutaneously once a day, every day for 5 days.

After surgery the rat was allowed a minimum of two days to recover before testing began.

Ultrasonic vocalization recording done with a microphone (Model 4939, Brüel& Kjaer, Denmark) with a frequency response of 4-Hz to 100-kHz. The microphone was located in the ceiling of the chamber approximately 15 cm above the middle of the enclosure. The microphone was connected to a Soundconnect™ amplifier (Listen, Inc, Boston, MA) and sound waves were recorded at 195,313 Hz using 16-bit resolution via a multifunction processor (model RX6, Tucker-Davis Technologies, Alachua, FL). Data from the RX6 connected via fiber optic cable to sound analysis dedicated computer running Matlab script. See Figure 5 for the computers' layout for sound and video capture.

Figure 5. Computers for Sound and Video Recording

Vocalizations were scored by one of two methods. DeepSqueak (authors; Kevin R. Coffey, Ruby E. Marx, and John F. Neumaier) software running in Matlab (Version: 24.1.0.2578822 R2024a, update 2) to automatically analyze sound file recordings and manually using the Raven Pro software (Cornell Lab of Ornithology; K. Lisa Yang Center for Conservation Bioacoustics) to identify the number of calls, the call categories, and the timing of the calls. The data collected was converted to Excel spreadsheet files and analyzed in terms of the number of calls and when the calls occurred as well as the types of calls from the manual data analysis. Frequency modulated call patterns (trills, and trill with jumps, complex, composite, inverted U, etc.) were of particular interest because of their association with positive affective states. Data were analyzed by various statistical and graphing techniques using Excel, Matlab and Python coding.

Initially videos were recorded using two cameras. One was located directly above the test chamber. This was a USB Microsoft webcam with the infrared filter removed. The other was a Sony digital video camera which has infrared sensitivity and was set up at the animal level view on the end of the test chamber. Illumination was provided by an array of 16 100mW infrared (IR) LEDs mounted on the ceiling of the exterior inside of the test chamber (see figure 6).

To record behaviour in dark conditions the webcam had its IR filter removed and a UV light source was provided. The Sony camera can capture movement in the provided light.

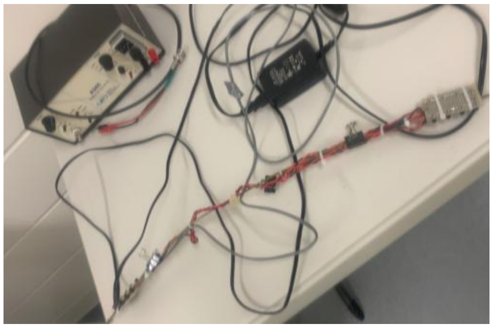


Figure 6. Infrared Light Source

This camera/light technology was used for experiments 1 and 2 using ESB as a reinforcer, but it was determined that analyzing the captured videos was less than ideal. For the calorie restricted rat experiments the cameras were replaced with two, one at the top and one at the side, cameras with built-in infrared lighting. These cameras (Arducam 1080P Day & Night Vision USB Camera for Computer, 2MP Automatic IR-Cut Switching All-Day Image USB2.0 Webcam Board with IR LEDs) produced much clearer video for video file analysis.

After behavioural testing, the subjects were sacrificed and perfused, then histology performed to identify the location of simulation electrodes. After behavioural testing, the subjects were sacrificed and perfused, then histology performed to identify the location of simulation electrodes. Paxinos and Watson, The Rat Brain, was used to verify the electrode placements.

Two cohorts of male Long-Evans rats were used as subjects. The Long-Evans rats weighing between 260 and 650 grams and between 8 months and 1.2 years were used in the three experiments. The subjects were on reverse light cycle with darkness from 11:00 a.m. to 11:00 p.m. It has been found by other researchers at the University of Lethbridge that the rats on this schedule are most active for two hours after they are going into the dark cycle and then again for an hour just before the lights come on for them. Testing was commonly started at 11:00 a.m.

To replicate the study of Burgdorf, Knutson, Panksepp (2000), where stimulation was provided 3 times per minute for 10 minutes every second day (FT-20), I created a Matlab script that operated an Arduino Uno, to run the WPI A-365 stimulator, and an Arduino MEGA that controlled the timing of the experiment and data recording to an Excel data file (see Appendix 6.). The ultrasonic vocalization sound files were analyzed using the DeepSqueak program (see Appendix 7.). The Excel data files were manually scored, and the data were graphed to show the trends of the subject's performance. Peri-Event Time

Histograms (PETH) were created to show the individual performances of the subjects around stimulation events.

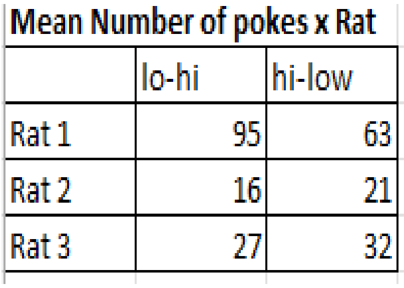
To prepare the rats for histological analysis they were sacrificed and perfused to acquire their brains. In the Burgdorf, et al. (2000) they state, “Brains were then frozen and sliced into 80-µm coronal sections with a freezing microtome. Sections through the tips of the electrodes were mounted on microscope slides, stained with cresyl violet”. I used cryogenic slicing but at 40-µm.

Viewing the slice showed the electrodes were in the medial forebrain bundle, bilaterally. I used the work of Rautio et al. (2024) for my reference to my electrode location.

Results

The mean number of nose pokes made by each rat was determined by starting a low stimulation (from 50% to 99%) and then going up and repeated starting at high stimulation (99% to 50%) and going to low stimulation. Table 2 shows the averages over days. While all three animals showed evidence of sustained self-stimulation, rat 1 showed the most robust rate of self-stimulation.

Table 2. Self-stimulation Data to Determine Stimulation Efficacy. Mean Number of Nose Pokes Averaged Over Days with Amperage Going Low Then High and Vice Versa



Mean Number of pokes x Rat

	lo-hi	hi-low
Rat 1	95	63
Rat 2	16	21
Rat 3	27	32

Burgdorf, et al, (2000) reported mean (± SEM) 50-kHz ultrasonic vocalizations per minute in 5-s time bins leading up to ventral tegmental (VTA) electrical stimulation of the brain for day 1 only (see figure 7). In experiment 1 I replicated the methods used in Burgdorf, et al (2000) experiment 1.

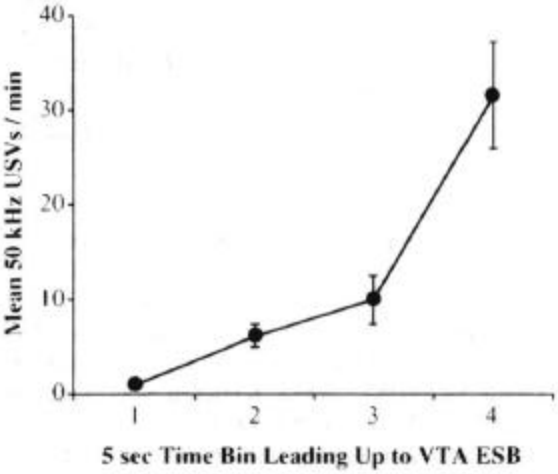


Figure 7. Burgdorf (2000) Experiment 1

To see the effect of days on call rate, Figure 8 shows the mean number of calls of all rats and all days.

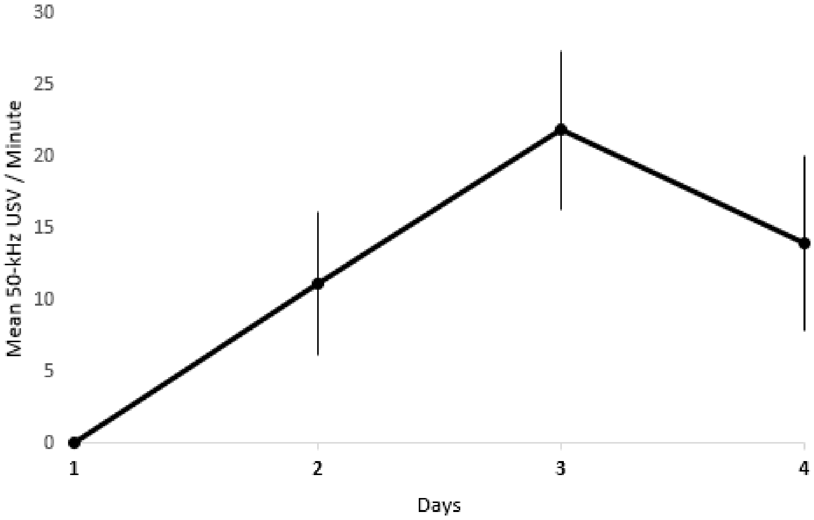


Figure 8. Mean (± SEM) total number of 50-kHz ultrasonic vocalizations (USVs) emitted per day across all rats during Experiment 1. This graph summarizes overall daily calling without reference to event timing

To better see the individual difference of the rat's performance around the time of the stimulation, Figure 9 shows the rates of calls the individual rats were making around the presentation of the ESB over the four days of testing. The event (dashed red line) is when the ESB came on and the x-axis is the time scale for 8 seconds before through 8 seconds after the stimulation

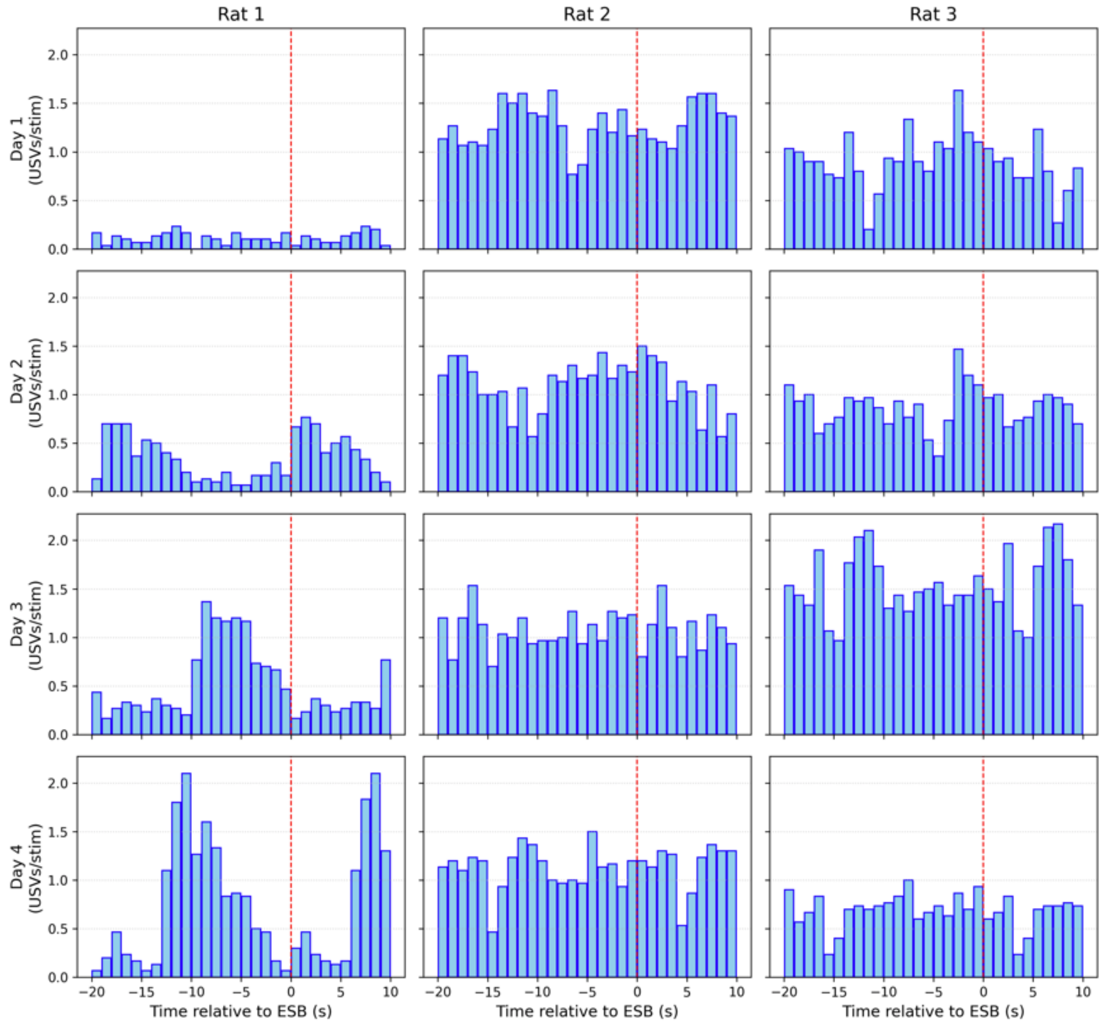


Figure 9. Peri-event time histogram (PETH) of mean (± SEM) 50-kHz ultrasonic vocalizations aligned to medial forebrain bundle (MFB) stimulation at 0 s (red dashed line). The histogram shows call rates from 20 s before stimulation to 10 s after, averaged across rats and sessions. A clear suppression of vocalizations occurs immediately following stimulation

In examining the peri-event time histogram (Figure 9), a distinct reduction in vocal behaviour is observed immediately following MFB stimulation. In the 20-s period preceding stimulation, rats produce intermittent 50-kHz calls at a modest but consistently non-zero rate, reflecting the baseline level of spontaneous vocal activity. At the moment of stimulation (time 0) and for several seconds thereafter, the call rate drops to near zero across animals, producing a "quiet" post-stimulation window. This reduction is evident in the markedly lower—or absent—post-event histogram bars relative to the pre-event period. Although absolute calling rates are low overall, the comparison of pre- versus post-stimulation epochs indicates a reliable transient suppression of vocalizations following each stimulation pulse. Thus, the interpretation of "clear suppression" reflects a relative decrease in calling immediately after stimulation, rather than a large absolute change in call number.

Histological analysis showed that, at least in one rat, the electrodes were in the medial forebrain bundle, bilaterally. I used the work of Rautio et al. (2024) for my reference to my electrode location (see Figure 10). I perfused rat 1 and 2 but rat 3 developed an infection around the skull cap so he was sacrificed. The brain of rat 1 did not fix well and when I sliced it the tissue was soft and bunched up, consequently not appropriate for mounting. Rat 2 did fix properly, and I mounted his brain slices on gel coated slides.

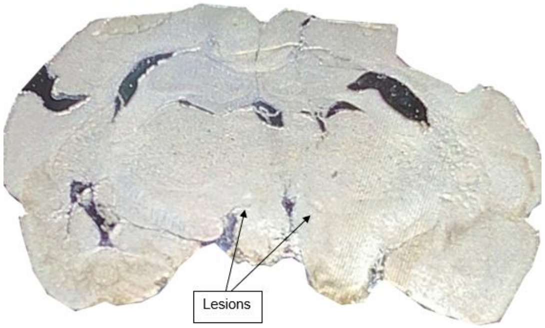


Figure 10. Representative coronal section showing bilateral electrode placements within the medial forebrain bundle (MFB) for Rat 2. Electrode tracks were verified using Paxinos and Watson atlas coordinates (AP −3.25 mm, ML ±1.65 mm, DV −8.5 mm from bregma)

Discussion

The small sample size in Experiment 1 precluded formal statistical testing; therefore, interpretation is necessarily qualitative. Rat 1 showed the most robust medial forebrain bundle (MFB) self-stimulation behaviour and provides the clearest signal. Across the final two training days, his ultrasonic vocalization (USV) rate steadily decreased as the stimulation time approached, suggesting that vocalizations decrease as anticipation of reward increases. In contrast, Rats 2 and 3 did not show consistent within-session or across-day patterns, making their results largely inconclusive. Nevertheless, all rats displayed a general day-to-day increase in total USV production, likely reflecting familiarization with the testing apparatus and reduced anxiety in a previously novel environment.

These results differ from those of Burgdorf et al. (2000), who reported a clear rise in 50-kHz calls preceding MFB stimulation. Several methodological factors could account for this discrepancy. Small differences in electrode placement may have altered which fiber tracts within the MFB were activated, producing variable engagement of dopaminergic versus non-dopaminergic components of the reward circuitry (Cossette et al., 2016). Additionally, the use of a fixed-time (FT-20 s) schedule may have promoted habituation or reduced arousal. The most important reason our findings differ from Burgdorf et al. (2000) may be that we stimulated the medial forebrain bundle using high-frequency pulse trains, whereas they stimulated VTA cell bodies with brief single pulses. These two methods activate different neural elements, and this may explain why Burgdorf observed increased USVs while our stimulation produced post-stimulation suppression. Also, individual differences in the rat's stimulation sensitivity or learning rate could also contribute to the variability observed here.

From a neurobiological perspective, the gradual reduction in calling seen in Rat 1 as stimulation expectancy increased suggests that 50-kHz USV production is not always positively coupled with reward anticipation, there may be other factors. Dopamine activity is known to ramp up as a reward approach

(Howe et al., 2013), yet in this case, vocalizations decreased across the same period. This inverse pattern raises the possibility that under predictable or overtrained conditions, vocal expression of positive affect may diminish even as dopaminergic expectancy signals increase. Such dissociation underscores that 50-kHz USVs, while often correlated with positive motivational states, do not provide a one-to-one measure of mesolimbic dopamine activity.

In summary, Experiment 1 demonstrated that fixed-interval MFB stimulation engages reward circuits but yields heterogeneous behavioural outcomes across animals. The pattern for Rat 1—reduced calling as the time of stimulation approached — suggests that temporal regularity may attenuate affective arousal rather than enhance it. These findings highlight the importance of individual analyses and variable-interval designs in future work and establish a technical and methodological foundation for the associative-learning paradigms explored in the subsequent experiments.

Chapter 3: Experiment 2 - Classical Conditioning (ESB)

Rationale

As with Experiment 1, the purpose of Experiment 2 was to determine the types of 50 kHz calls most closely associated with brain stimulation reward. In this case, it employs a Pavlovian (classical) conditioning framework. In this paradigm, an initially neutral stimulus (a brief, unpredictable light) is systematically paired with the presentation of an unconditioned stimulus (stimulation), which presumably elicits a reflexive, unconditioned response (USVs). Through repeated pairings, the neutral light acquires predictive value and becomes a conditioned stimulus. As a result, the rat begins to exhibit a conditioned response in anticipation of the stimulation. This design enables the investigation of associative learning processes, specifically the extent to which a temporally uncertain, randomly presented, cue can serve as a reliable predictor of a salient event (ESB). More importantly, it allows an investigation of the relationship of 50 kHz vocalizations to cues predictive of reward, anticipation of reward, and the reward itself. In experiment 2, as in experiment 1, I will examine the USVs using DeepSqueak to determine the number of calls and when they occur. I will not analyze the USV call subtypes as the volume of call data is prohibitive to analyze manually using Raven Pro.

Hypotheses

It is hypothesized that pairing an LED light (conditioned stimulus) with electrical brain stimulation (unconditioned stimulus) will result in an increased number of ultrasonic vocalizations (USVs) across repeated trials and days, reflecting the formation of a conditioned association. Behaviourally, animals should show decreased latency to attain the food as they learn the cue-reward association.

Method

All methods were the same as experiment 1. The only differences to report are details of the learning protocol (cue duration, intervals between cues, time from cue offset to stim start, etc.) and details on the timing of sessions and number of sessions.

USVs were recorded for the duration of the cue-reward presentation. Behaviour of the subjects was also recorded by video capture with two IR capable cameras. The timing of LED cue lights and MFB stimulation was unpredictable but occurred on average every 90 seconds, ensuring that the cue, not the timing, was predictive of reward. Further, a 0 second delay between LED cue offset and MFB onset ensured rapid associative learning. Using average intertrial intervals between 5 and 90 seconds where the LED lights come on for 8 seconds and the stimulation immediately follows the lights going off, should result in strong acquisition of the cue-stimulation association (trace classical conditioning: a classical conditioning procedure in which the conditioned stimulus is presented, and remains present, for a fixed period, before the unconditioned stimulus is introduced). In this trace conditioning the conditioning stimulus (lights/LEDs) come on for 8 seconds with the unconditioned stimulus (electrical stimulation) coming on immediately after the light goes off. The 8 seconds is the duration the CS is present, not the interval between the CS offset and UCS onset such that the ESB immediately follows the termination of the 8 seconds CS (light).

The length of time of the trials and the intervals between the trials (inter-trial interval), as determined by Lattal (1999), was chosen as the most optimal and were used in this experiment.

Data were gathered from the subjects in cohort 1.

The audio recordings were analyzed using DeepSqueak software in Matlab.

Results

The same rats used in experiment 1 were used for experiment 2.

Data were analyzed by counting the number of calls, for each rat for each day. The calls were counted in 8 second windows before the LEDs came on, while the LEDs were on and for 8 second window after the stimulation came on.

The performance of the rats over the 4 days shows the number of calls they made. After the LED goes off the subjects may have made an association between the light and the stimulation. Figure 11 shows a comparison of the number of calls before, during, and after the stimulation over days.

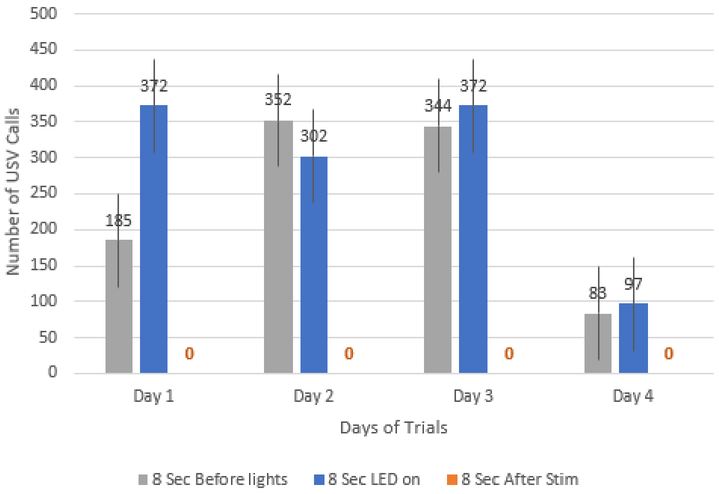


Figure 11. Mean number of ultrasonic vocalizations (USVs) during the 8-second period before, during, and after medial forebrain bundle (MFB) stimulation across training days. Each point represents the mean number of calls across all rats for that condition. Error bars indicate the standard error of the mean (SEM)

A paired-samples t-test compared the mean number of ultrasonic vocalizations (USVs) emitted during the 8 s preceding the LED cue (M = 241.00, SD = 133.53) with those emitted while the LED was illuminated (M = 285.75, SD = 149.75), collapsing across all four testing days. The difference between the pre-cue and LED-on conditions was not statistically significant, t (3) = −0.89, p = .44, 95%. These results indicate that the LED cue itself did not significantly alter the rate of USV production compared with the immediately preceding period.

A paired-samples t-test compared the number of ultrasonic vocalizations (USVs) emitted during the 8-s LED-on cue period with those emitted during the 8-s window immediately following stimulation. USV production was substantially higher during the cue (M = 285.75, SD = 130.09) than after stimulation (M = 0.00, SD = 0), and this difference was statistically significant, t (3) = 4.39, p = .022.

In Experiment 1, stimulation occurred against a background of low and variable baseline calling, so the post-stimulation decrease appeared modest. In Experiment 2, however, stimulation followed an 8-s cue that reliably elicited strong anticipatory calling, and the transition from this high-vocalization state to the immediate behavioural interruption caused by MFB stimulation produced an apparent complete suppression of USVs in the post-stimulation window.

Discussion

Experiment 2 examined whether pairing a discrete visual cue with medial forebrain bundle (MFB) stimulation would evoke conditioned anticipatory ultrasonic vocalizations (USVs) in the 50-kHz range. Across animals, USVs were consistently present prior to stimulation, indicating that the subjects were capable of producing reward-related vocalizations under the testing conditions. However, the pattern of results suggests that the cue failed to acquire meaningful predictive value, and the stimulation itself produced a strong and reliable suppression of vocal behaviour.

A key finding was the dramatic contrast between the LED-on and post-stimulation periods. Rats produced substantial numbers of USVs during the 8-s LED cue but emitted no calls at all during the 8 s following stimulation. This effect was statistically significant, t(3) = 4.39, p = .022, confirming that MFB stimulation reliably suppressed vocal output. Importantly, this post-stimulation silence persisted far longer than would be expected from a momentary motor disruption caused by the 300-ms electrical pulse. Instead, the extended absence of calling suggests a shift in motivational state: once the stimulation was delivered, the anticipatory drive underlying vocal production appeared to collapse, leaving a brief consummatory period in which USVs were not generated.

The absence of conditioned increases in USVs during the cue period likely reflects two interacting factors. First, only one of the three rats showed robust self-stimulation behaviour in Experiment 1, indicating that stimulation parameters were at or below reinforcing thresholds for most subjects. Inconsistent or weak reinforcement makes it difficult for a cue to acquire predictive value and limits the opportunity for conditioned anticipatory responses to develop. Second, the large variability in baseline call rates across subjects suggests that the animals differed substantially in their sensitivity to stimulation, their motivational state, or both. Such variability could mask subtle conditioning effects, especially with a small sample size.

Despite the lack of evidence for conditioned anticipation, the strong and reliable post-stimulation suppression provides an important clue about the functional meaning of 50-kHz USVs in this context. Rather than reflecting the hedonic impact of stimulation itself, the calls appear to track the anticipatory phase of reward processing. Once the expected reward is delivered, calling ceases, consistent with theories proposing that 50-kHz USVs index motivational approach or “wanting” rather than consummatory pleasure. This interpretation aligns with prior work showing that MFB stimulation can satisfy appetitive drive states, thereby reducing the incentive conditions that normally support vocalization.

In summary, although the cue failed to elicit conditioned anticipatory USVs, Experiment 2 revealed a robust and prolonged suppression of vocalizations immediately after MFB stimulation. This effect underscores the importance of distinguishing between anticipation and receipt of reward when interpreting USVs. Future studies using individualized stimulation thresholds, larger samples, and extended training may help clarify whether visual cues can reliably acquire motivational significance when paired with electrical brain stimulation. More broadly, the results reinforce the notion that 50-kHz USVs primarily reflect anticipatory reward processes, with vocalization sharply curtailed during the consummatory aftermath of stimulation.

The planned analysis of specific 50-kHz call subtypes in Experiment 2 could not be carried out because the data did not contain enough usable calls outside the cue period to support subtype classification. Although rats produced many USVs during the cue, the stimulation itself caused a complete suppression of calling in the post-stimulation window, and very few calls were emitted immediately before stimulation. Because subtype analysis requires a sufficient number of calls in each time period being compared, the near absence of pre- and post-stimulation vocalizations made it impossible to evaluate whether different call categories were associated with MFB reward or whether call types changed as conditioning progressed.

Chapter 4: Experiment 3 – Classical Conditioning (Calorie Restricted)

Rationale

Experiments 1 and 2 examined the effect of anticipation and receipt of brain stimulus reward on vocalizations. In Experiment 3, we sought to replicate Experiment 2, doing cue-reward associative training, but this time using food reward. The idea was to see if the anticipation or receipt of food reward by a hungry animal would elicit vocalizations. If an increase in vocalizations was observed, we also sought to determine which sub-types of calls were most associated with anticipatory or post-reward vocalizations. Again, the emphasis of our analysis was on the timing of calls relative to the cue and food reward.

Hypotheses

An LED light (CS) can be paired with sugar pellet delivery (US) to produce more USV calls and calls of specific types (CR) as the association is repeated over trials and days.

Subjects will be quicker to the feeder after the light onset as trials and days progress.

Method

Twelve male Long-Evans male rats were initially used. After running all rats in the experiment only 6 rats vocalized more than 10 calls per day so there data were used, and the 6 non-vocalizing rat data were discarded. When the animals were at least 100 days old, we restricted their food from the normal intake of 29 g per day to 16 gm for 3 days and then 14 gm until they reach a target weight of 85% of their starting weight. During this time, they were weighed at least every 2 days. Once they hit 85% of their free feeding body weight, they got a consistent amount of food every day (approximately 8 gm) plus whatever they earned during the task (typically 7 gm), and their weight was checked at least weekly to ensure that they did not lose any further weight. If an animal did lose weight, their daily portion of food was increased. In practice, all our rats under this protocol showed small decreases in weight over time. Scheduling overview is in Table 3.

Testing: In the sound protected dark chamber subjects were tested in a classical conditioning paradigm where they receive sugar pellets delivered by a Med-Associated ENV-203 dispenser for 15 trials with random 10 to 40 second inter-trial intervals on consecutive days for up to three weeks. On each trial, an LED light will flash for 5 seconds, and this will be followed immediately by delivery of a 45 mg sucrose pellet.

The ultrasonic vocalizations were recorded as well as video of behaviour from two IR cameras, each having its own IR light source. One camera was above and one on the side. Twelve Long-Evans male rats were used.

Table 3. Rat Handling and Testing Scheduling

Test (weeks)	Activity
1	Acclimatization and some handling
2-4	Food restriction beginning and handling for tameness
5-6	Familiarize rats with testing room; Light-food training and vocalization recording
7	Familiarization of rats in pairs in testing chamber (together for 5 minutes/day)
8-14	Experiment 3 with behaviour, vocalization, and video recording
15	Rats return to ad-lib feeding
16	Experimental end point (sacrificed)

Monitoring Schedule: During testing animals were checked daily and weighed every second day while on calorie/food restriction to check for general health conditions. Once a week a welfare check was performed and recorded to ensure no excess signs of distress or other issues.

The plexiglass chamber for testing the rats was modified to support a pellet dispenser (Med-Associates ENV-203 Pellet Dispenser) and deliver 45 mg sugar pellets via a 2 cm diameter glass tube into a glass 8 cm petri dish. Glass was chosen as rats will chew plastic, and the glass is easy to clean. The cameras used in experiments 1 and 2 were updated with two Arducam 1080P Day & Night Vision USB Cameras, 2MP automatic IR-Cut switching with IR LEDs. One camera was mounted on the top and centered on the wooden box and the other camera mounted on the bottom at the side.

Matlab code to run the experiment (15 trials with 10-40 second random inter-trial intervals) was uploaded to an Arduino MEGA which operated a 1 channel relay module control board with optocoupler isolation high- and low-level trigger (5V) to drive the pellet dispenser.

An LED light flashed 3 times, and sound board beeped 3 times at start and end of experiment to provide references to the start and end of the experiment for audio and video analysis.

The LED cue light came on and flashed for 5 seconds (500 milliseconds on/off). At termination of the cue light, a pellet was dispensed. Upon completion of a set of trials for a subject the Matlab code wrote an Excel file which stored the data of all events occurring (start and end times, times of LED on and when the pellet was delivered). See Appendix 9 for Matlab Live Script.

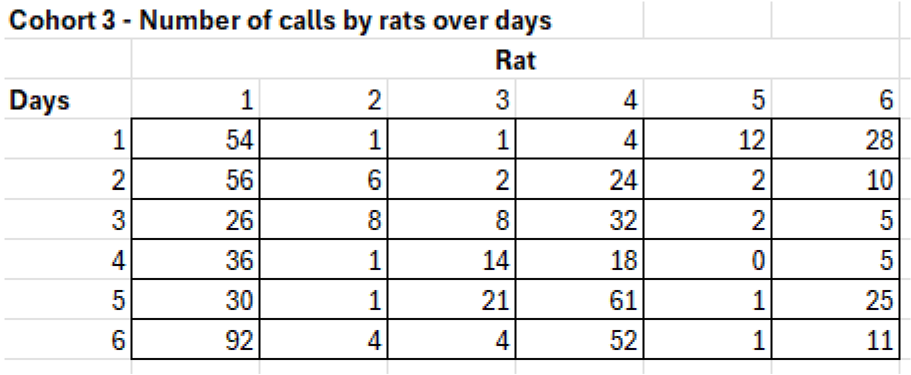
USV analysis was manually scored using Raven Pro by an independent experimenter and data analysis was done with Excel, Matlab, and Python to identify when USVs occurred, how many calls were emitted, and the types of calls. Video behaviour was analyzed using python code to determine the latency time from when the LED came on and then off to time if the rat was within centered 8 square cm of the end of the glass pellet dispensing tube.

Rats will optimistically seek out reward (Rygula et al., 2012) and classical conditioning where sugar pellets are used for a food restricted rat can be used as an effective reward (Burgdorf et al., 2007). The cohort 3 rats were handled for 5 days by a female student as an assistant. The same assistant also handled rats during testing procedures. There has been research that suggests that rats in experimental settings are calmer when handled by a female than with a male experimenter (Faraji et al., 2022).

Results

After testing the rats for a total of 15 days the pairing of the LED (CS) and the sugar pellet delivery should be well established. To see the subjects' performance, I looked at the number of calls the rats made over the last 6 days of the experiment and that behavioural analysis is shown in Table 4 and that data is represented graphically in Figure 12.

Table 4. Number of Calls Over Days



Cohort 3 - Number of calls by rats over days

	Rat					
Days	1	2	3	4	5	6
1	54	1	1	4	12	28
2	56	6	2	24	2	10
3	26	8	8	32	2	5
4	36	1	14	18	0	5
5	30	1	21	61	1	25
6	92	4	4	52	1	11

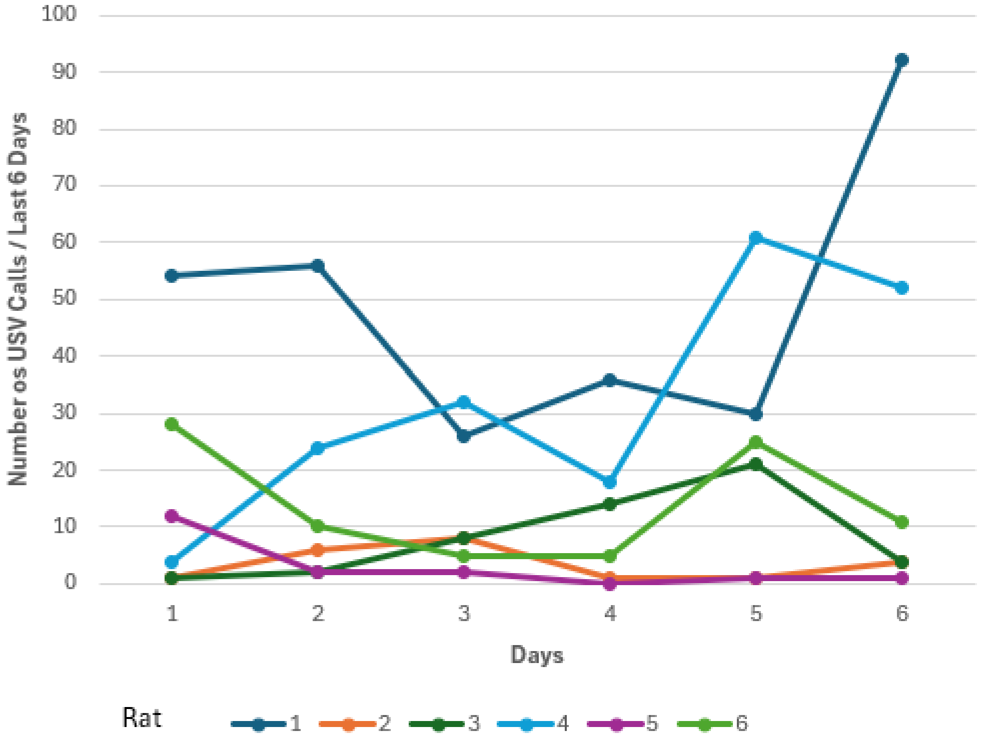


Figure 12. Number of USVs over Days by Each Rat for Last 6 Days of Experiment 3

As can be seen, there are wide disparities in average call rates, but relative consistency within an animal. Low callers remained low callers and high callers generally made many calls across days. Among the high callers was Rat 1, who made many more calls in the last 6 days of the experiment than any other rat. In contrast, Rats 2 and 5 had very low USV rates over the 6 days.

A paired-samples t-test was conducted to compare the mean number of ultrasonic vocalizations (USVs) emitted during the 8-s period immediately before each event (pre-event window) to the number emitted during the 8-s period immediately following the LED on event (post-event window).

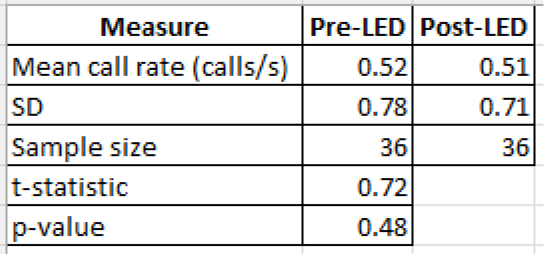
For analyses aligned to the LED cue, the pre-cue window comprised the 8 s immediately preceding cue onset (−8 to 0 s), and the post-cue window encompassed the 8 s during which the LED was illuminated (0 to +8 s).

For analysis, the pre-pellet window covered the 8 s immediately before pellet release, and the post-pellet window the 8 s immediately following delivery.

Call counts within each window were averaged across trials for each rat and entered into paired t-tests to assess changes in vocalization rate associated with cue presentation and reward receipt.

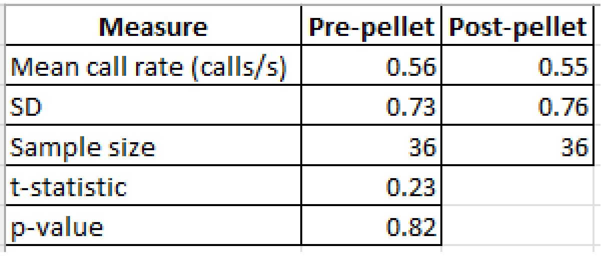
The paired t-test comparing pre- and post-LED call rates across all rats and for the last 6 days shows no statistically significant difference between conditions (p = 0.48). See Table 5. This indicates that, overall, there was no significant change in the mean number of calls from pre- to post-LED across all rats and days. A paired t-test comparing pre- and post-pellet call rates across all rats and days shows no significant difference (p = 0.82). See Table 6.

Table 5. Mean ultrasonic vocalization (USV) call rates (calls per second) in the 8-s windows before and after LED onset, with corresponding paired t-test statistics



Measure	Pre-LED	Post-LED
Mean call rate (calls/s)	0.52	0.51
SD	0.78	0.71
Sample size	36	36
t-statistic	0.72	
p-value	0.48	

Table 6. Mean ultrasonic vocalization (USV) call rates (calls per second) in the 8-s windows before and after pellet delivery, with corresponding paired t-test statistics



Measure	Pre-pellet	Post-pellet
Mean call rate (calls/s)	0.56	0.55
SD	0.73	0.76
Sample size	36	36
t-statistic	0.23	
p-value	0.82	

This indicates that, overall, the mean number of calls did not significantly change from pre- to post-LED or pellet delivery across all rats and days over the last 6 days of testing.

The total number of calls does not indicate that there is a relationship to the LED time events or pellet delivery events as there was seen large variability in individual performance which has been reported by others (Sangarapillai et al., 2021). That specific call types my relate to classical conditioning learning is a possibility as specific call subtypes have been associated with specific behaviours. To see if a relationship exists I looked at some FM calls to see if specific call subtypes are related to events. In analyzing the USV data it was noticed that specific FM calls tend to be predominant in the USV data.

The number of trill, complex and composite call subtypes the subjects made over the last 6 days of testing are shown in Table 7. The call subtypes are graphically shown in Figure 13.

Table 7. Trill, Complex, and Composite Call Subtypes over the Last 6 Days by All Rats

Days	Rat					
	1	2	3	4	5	6
1	23	1	0	4	4	9
2	4	1	0	4	1	0
3	7	0	2	4	2	1
4	4	1	10	0	0	3
5	4	0	9	12	0	12
6	19	1	4	15	1	5

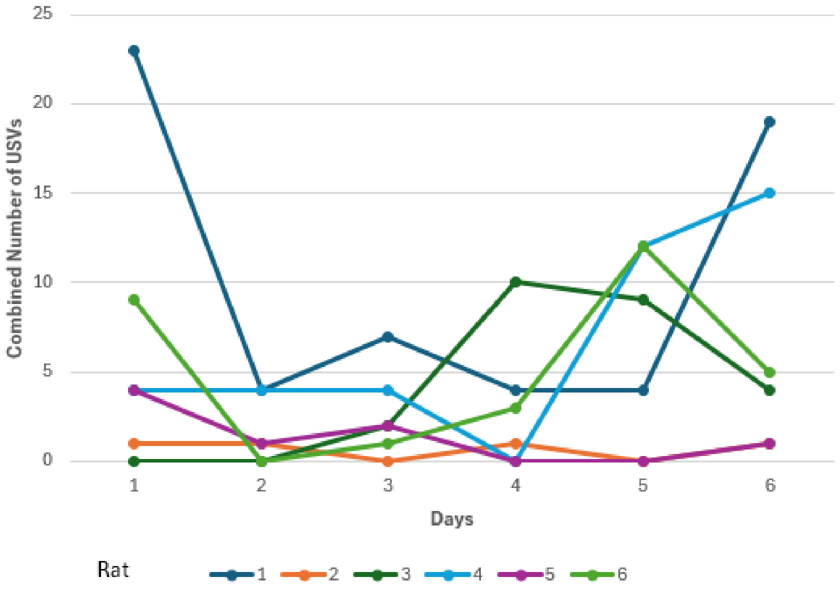


Figure 13. Mean number of frequency-modulated (trill, complex, and composite) 50-kHz ultrasonic vocalizations (USVs) produced by all rats across the final six testing days. Data are averaged across animals (± SEM)

Across all rats and testing days, frequency-modulated (FM) ultrasonic vocalizations remained low throughout the peri-event intervals surrounding both LED onset and pellet delivery. When aligned to the cue, FM call rates showed a small, brief increase within one to two seconds of LED illumination, although the overall magnitude of calling was minimal across the full ±8-second window. In contrast, PETHs aligned to pellet delivery revealed a modest reduction in call rate immediately before and after time 0, followed by a return to low baseline levels. Subtype-specific analyses demonstrated that trill, complex, and composite calls exhibited similar temporal profiles for both event types. Although minor fluctuations were present—such as slight elevations near LED onset and brief dips around pellet delivery—these patterns were small in magnitude, and SEM bands indicated limited variability across rats, days, and events. See figures 14 through 17.

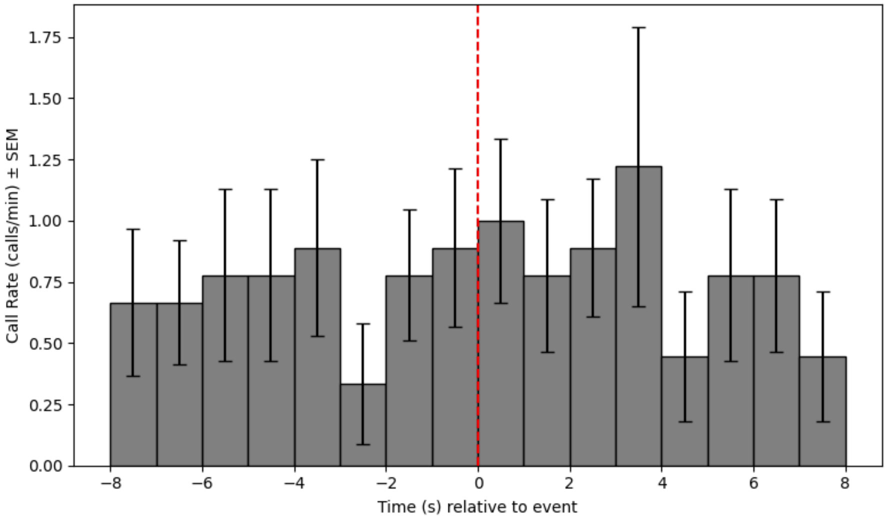


Figure 14. Peri-event time histogram (PETH) showing frequency-modulated (FM) ultrasonic vocalizations (USVs) aligned to LED onset across all rats and sessions. Mean call rate (calls per minute ± SEM) is plotted in 1-s bins across an 8-s window preceding and following the cue (LED) onset. FM calls include trill, complex, and composite subtypes. Time 0 represents LED illumination, marking the beginning of each trial

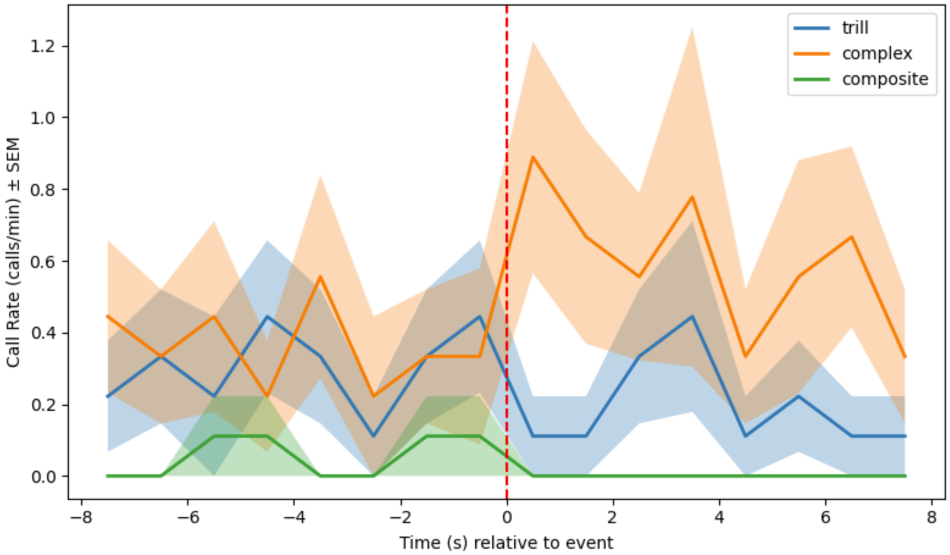


Figure 15. Peri-event time histogram (PETH) showing frequency-modulated (FM) ultrasonic vocalizations aligned to LED onset across all rats and sessions. Mean FM call rate (calls per minute ± SEM) is displayed in 1-s bins for an 8-s window centered on the moment of LED onset (time 0). FM calls comprise trill, complex, and composite subtypes

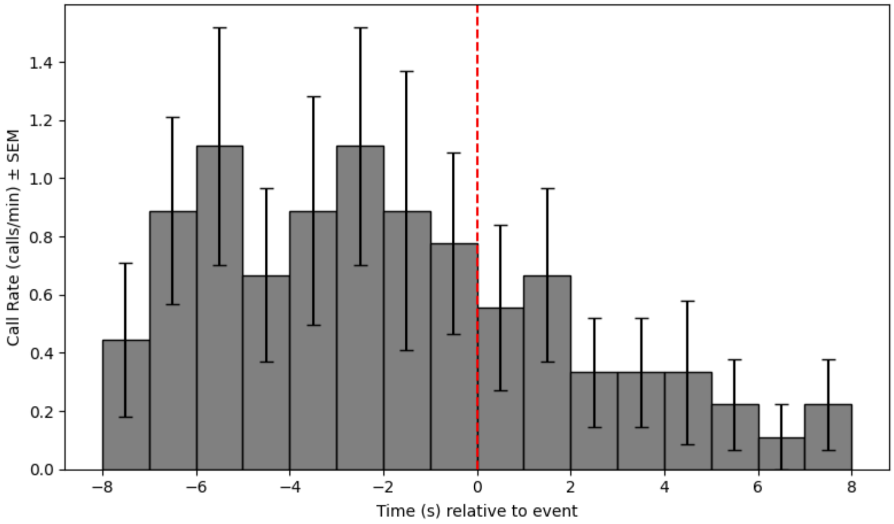


Figure 16. Frequency-modulated (FM) ultrasonic vocalizations aligned to pellet delivery, separated by call subtype. Mean call rate (calls per minute ± SEM) for trill, complex, and composite FM calls is shown in 1-s bins across an 8-s window around cue onset (time 0)

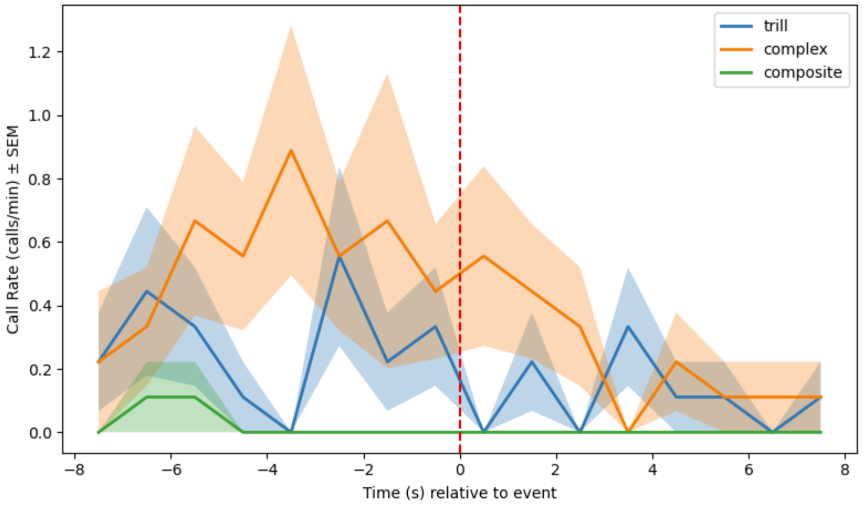


Figure 17. Frequency-modulated (FM) ultrasonic vocalizations aligned to pellet delivery, separated by call subtype. Mean call rate (calls per minute ± SEM) for trill, complex, and composite calls is plotted in 1-s bins for an 8-s peri-pellet window

Call-type analyses were not conducted for Experiments 1 and 2 because the pattern and distribution of ultrasonic vocalizations did not provide a sufficient dataset for subtype classification. In both experiments, overall calling was sparse and, critically, calls were not consistently time-locked to the stimulation events. In Experiment 2, post-stimulation calling was completely absent in the 8-s analysis window, leaving no data available for comparison of call categories before and after stimulation. Due to these limitations, subtype analysis could not be meaningfully applied.

In contrast, Experiment 3 produced enough frequency-modulated (FM) calls across repeated cue–reward trials to permit descriptive examination of trill, complex, and composite call types. Although calling remained low overall, the presence of FM calls across multiple days allowed for graphical exploration of subtype patterns (Figures 15 and 17). These analyses were therefore included for Experiment 3 but were not feasible for Experiments 1 and 2.

The initial presumption was that over 15 days of trials the subjects would make more USV calls and would get to the sugar pellet delivery quickly. This would indicate both learning and that as "happy" rats getting a sugar pellet they would vocalize more. The more conclusive results that would support my hypothesis would be of how they performed at the start of the experiment verses how they performed at the end of the experiment.

Mean latencies from cue onset to feeder arrival were very small across all trials. This was apparent from the video analysis where the rats were often by the feeder tube most of the time.

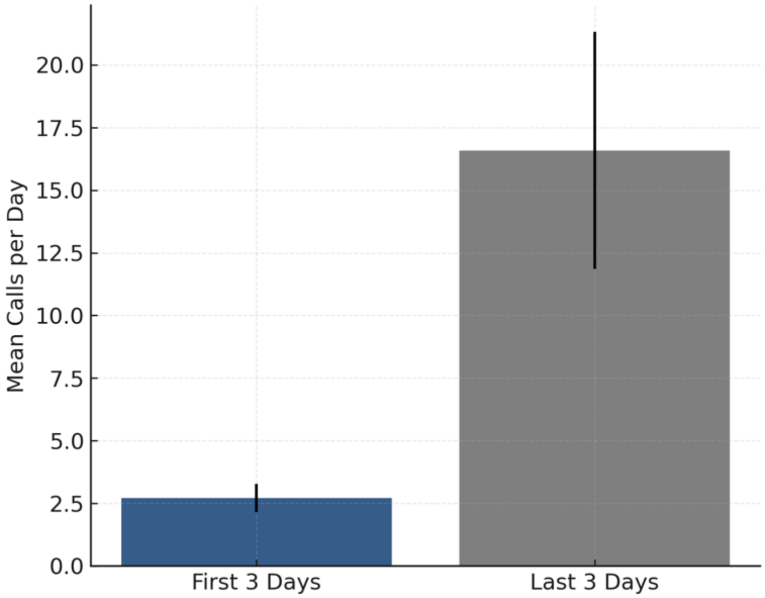


Figure 18. Number of ultrasonic vocalizations (USVs) during the first and last three days of testing. Each box represents the interquartile range of total calls across rats; the horizontal line indicates the median, and whiskers show the full range of observations

The mean number of USVs made in the last 3 days versus the first 3 days of testing is shown in Figure 18. This shows that the subject vocalized very little in the first 3 days and by the end of the 15 days they were vocalizing more. This may be related to novelty or anxiety in the experiment, but the number of calls made is very different between the starting and ending days. The response of the rats to pellet delivery was focused and deliberate always getting the pellet.

A two-sample t-test was conducted to compare the mean number of ultrasonic vocalizations (USVs) between the first and last three days of testing. The difference was statistically significant, t (46) = –2.91, p = .006, 95 %.

Rats produced significantly fewer calls during the first three days (M = 2.71, SD = 2.76, n = 24) than during the last three days (M = 16.58, SD = 23.17, n = 24), indicating a marked increase in calling across sessions.

Discussion

Experiment 3 investigated whether mild calorie restriction and repeated pairings of an LED cue with sucrose pellet delivery would elicit measurable changes in 50-kHz ultrasonic vocalizations (USVs). Across all subjects, call counts were low, and there was no clear increase in calling either in anticipation of, or immediately following, food delivery over the last six days of testing.

Over the six days of Pavlovian training, rats did not show reliable increases in 50-kHz vocalizations in response to either the LED cue or pellet delivery. Peri-event analyses revealed very low call counts overall, and neither cue onset nor food arrival produced a robust, time-locked change in vocal output. The brief dip in calls immediately before and after pellet delivery is more consistent with momentary behavioural pauses—such as orienting, retrieving the pellet, or adjusting posture—than with affective suppression or a prediction-error-related signal. Latency measures support this interpretation: rats were often already positioned at the feeder at cue onset, resulting in near-zero approach latencies on most trials. This proximity to the food source likely reduced the motivational demands of the task and may have diminished any anticipatory arousal that would otherwise manifest as elevated 50-kHz calling. Overall, the behavioural measures indicate that animals learned the temporal structure of the task but did not express this learning through vocal output.

Call-subtype analyses further show that the extremely low levels of frequency-modulated calls (trill, complex, and composite) placed strong constraints on detecting meaningful learning-related patterns. Daily totals and event-related averages indicate that FM calls were emitted only sporadically, with no consistent increases across training days and no selective enhancement around the predictive cue. Given that FM subtypes are typically the clearest markers of positive affect and reward anticipation in the USV literature, their scarcity here suggests that the calorie-restricted food reward may not have been sufficiently salient to generate robust appetitive vocalizations. Food restriction itself may also reduce calling, as low energetic state can blunt exploratory and affective behaviours. Together, these findings imply that while rats did acquire the cue–reward association—as shown behaviourally through feeder-zone proximity and short latencies—the USV system was not strongly engaged under these conditions.

The scarcity of food-related calling contrasts sharply with reports that electrical stimulation of the medial forebrain bundle evokes strong affective responses. By comparison, natural food reward appears to be a much weaker elicitor of 50-kHz calls, consistent with earlier findings that rats emit relatively few USVs during feeding itself (Wöhr & Schwarting, 2013). Several factors could explain this difference. The caloric restriction used here was mild—designed to maintain motivation without distress—and may not have produced the heightened dopaminergic drive that more severe deprivation evokes.

Comparisons between the first and last three days of training further clarify how learning and motivational engagement evolved over the course of the task. Latency measures showed that rats were already approaching the feeder rapidly during the initial sessions and maintained these near-zero latencies across the final sessions, indicating that the cue–reward association was acquired early and remained stable.

Overall, these data provide limited support for the hypothesis that mild calorie restriction or food reward alone produces strong positive-affective vocal output. If anything, the results indicate that food reinforcement exerts subtle and inconsistent effects on ultrasonic vocalization behaviour. For now, the most conservative interpretation is that rats did not acquire a robust cue–reward association detectable in their vocal behaviour, and that hunger state and reward salience play critical roles in modulating 50-kHz vocal output.

Chapter 5: General Discussion

The present set of experiments examined how rat 50-kHz ultrasonic vocalizations (USVs) relate to reward processing and associative learning when reinforcement is produced either by electrical stimulation of the medial forebrain bundle (MFB) or by food reward under mild calorie restriction. Collectively, the findings suggest that the relationship between reinforcement and vocalization behaviour is more nuanced than previously assumed. Electrical stimulation was reliably reinforcing but suppressed vocal output, whereas natural food reward produced few measurable changes in USV rate or subtype. Across experiments, total calling tended to increase modestly over days, most plausibly reflecting habituation or reduced novelty rather than the formation of learned cue–reward associations.

Electrical Stimulation of the Brain

Consistent with prior research, MFB stimulation strongly engaged reward circuitry but produced an immediate reduction in 50-kHz calling during the stimulation period. This transient silence contrasts with the assumption that positive reinforcement should heighten vocal expression. One interpretation is that the stimulation evokes intense dopaminergic activation that temporarily disrupts motor output or shifts affective processing inward, resulting in behavioural quiescence. Burgdorf et al. (2000) reported increased 50-kHz calling prior to stimulation, and they did not analyze the post-stimulation period. Rather, vocal behaviour reflects a balance between motivational drive and competing behavioural or neural processes at the moment of reward delivery. Individual variability in stimulation sensitivity and electrode placement likely contributed to differences in response magnitude, underscoring the heterogeneity of affective outcomes even within a single reinforcement modality.

Food Reward and Calorie Restriction

Food reward under mild calorie restriction produced only limited changes in 50-kHz vocalizations. Calling rates were low across animals, which is consistent with evidence that mild food restriction can suppress USV production, as rats shift behaviour toward food seeking and consumption rather than vocal expression. This reduced baseline calling substantially limits statistical power, making it difficult to detect subtle cue- or reward-evoked effects even if associative learning is occurring behaviourally. Stronger anticipatory calling has been reported under more intense deprivation, suggesting that the motivational state induced here was insufficient to reliably engage dopaminergic systems that generate FM calls. Therefore, the weak vocal responses observed likely reflect low vocal propensity under mild hunger rather than a true absence of learning.

Integrating Findings Across Reinforcement Types

Together, the results highlight that electrical and natural reinforcers affect vocalization through distinct mechanisms. Electrical stimulation directly activates mesolimbic dopamine neurons and is experienced as intensely rewarding, yet it can paradoxically suppress overt vocal expression. Food reward, by contrast, engages these circuits indirectly and elicits only modest vocal changes unless accompanied by strong motivational states. The comparison underscores that 50-kHz USVs cannot be interpreted as simple readouts of dopamine release or positive emotion; their expression depends on contextual, physiological, and procedural factors—including predictability, reinforcement magnitude, and internal state.

From a neurobiological standpoint, these findings fit within current models distinguishing phasic and tonic dopamine functions (Schultz & Dickinson, 2000). Phasic dopamine bursts signal reward-prediction errors that drive learning, whereas tonic levels modulate motivation and response vigor. The lack of strong cue-evoked calling in the present data may reflect weak or inconsistent phasic signaling, whereas the gradual rise in total calling across days could correspond to slow changes in tonic motivation. Thus, 50-kHz vocalizations appear to integrate multiple aspects of dopaminergic function but are not a direct proxy for either.

Methodological and Conceptual Considerations

Future studies could employ larger cohorts, automated cue delivery, and within-subject comparisons across multiple deprivation levels to better isolate the contribution of hunger and reinforcement salience to vocal behaviour. Increasing sample size would reduce the high inter-individual variability that characterized the present data and strengthen statistical power for detecting subtle anticipatory or cue-evoked changes in USVs. Automating cue timing and reward delivery would eliminate experimenter-imposed variability, improve temporal precision for PETH analyses, and reduce the possibility that incidental contextual cues overshadow the light cue itself. Critically, systematically varying deprivation levels within the same animals—such as testing each rat under mild, moderate, and more substantial food restriction—would make it possible to determine whether calling increases in a graded, motivationally dependent manner. Such within-subject manipulations would also help distinguish true motivational effects from stable individual differences in vocal propensity. Together, these refinements would clarify whether low calling in the present study reflected insufficient motivational drive, weak cue–reward associations, or inherent variability in 50-kHz vocal behaviour, thereby providing a clearer understanding of how hunger state and reinforcement intensity shape the expression of appetitive ultrasonic vocalizations.

Conclusions

Through three experiments, electrical and natural rewards produced divergent effects on ultrasonic vocalizations. MFB stimulation, though highly reinforcing, suppressed calling, whereas sucrose reward under mild calorie restriction elicited few calls and little evidence of associative learning. Modest increases in calling across days are best attributed to familiarization with the apparatus rather than conditioning. These results refine the view that 50-kHz USVs are indicators of positive emotion by showing that such calls are not automatically produced by rewarding stimulation or reward receipt but instead depend strongly on behavioural context and anticipatory engagement. Rather than reflecting a simple hedonic response, 50-kHz USVs appear more closely tied to expectation, arousal, and task structure, indicating a more nuanced relationship between vocalization and positive affect.

Chapter 6: Limitations and Future Directions

Limitations

Although this project successfully developed and validated the technical methods required to record ultrasonic vocalizations (USVs) during reinforcement tasks, several methodological constraints substantially limited the scope and strength of the conclusions.

Sample size and statistical power

The most significant limitation was the small number of rats included in each experiment. Attrition across cohorts and incomplete datasets reduced the effective sample size to the point that the statistical power of all inferential tests was severely compromised. With so few subjects, even moderately large behavioural effects could not be detected reliably, and apparent trends must therefore be regarded as preliminary.

Effectiveness of medial forebrain bundle (MFB) stimulation

A second limitation involved the variable efficacy of MFB stimulation, likely caused by minor differences in electrode placement. Because reinforcement value depends critically on stimulation of dopaminergic fibers within the bundle, small deviations in depth or medial–lateral position can render stimulation ineffective or aversive. Several animals failed to self-stimulate reliably, further reducing usable data and complicating interpretation of between-subject differences.

Restricted number of testing days

The limited duration of behavioural testing also constrained the ability to observe learning. Especially in the classical-conditioning paradigms, more days of training might have produced stronger cue–reward associations and clearer behavioural or vocal indicators of learning. Short testing schedules, combined with individual variability, likely prevented the emergence of stable conditioned responses.

Illness and surgical complications

A substantial loss of data resulted from illness in the second surgical cohort. Post-operative infections and general health decline necessitated euthanasia before testing could be completed. The resulting reduction in subject numbers compounded the problems of low statistical power and incomplete within-subject replication.

Histology challenges.

Histological verification of electrode placement proved difficult. Tissue quality was compromised in some brains due to fixation issues, and one specimen could not be mounted for microscopic confirmation. Without complete histological verification, electrode localization could only be inferred from surgical coordinates and behavioural responses, reducing confidence in anatomical accuracy.

Impact of the COVID-19 pandemic

Finally, the COVID-19 shutdowns delayed progress by more than a year. Access to laboratory space, animals, and equipment was restricted for extended periods, interrupting data collection and preventing timely replication. These delays not only limited total data volume but also contributed to the need for smaller, sequential experimental runs rather than a single, well-powered cohort.

Implications

Collectively, these limitations indicate that the current findings should be interpreted as exploratory. The low sample sizes, incomplete histological verification, and variability in stimulation effectiveness preclude strong statistical or mechanistic conclusions. The patterns observed—such as transient suppression of vocalizations following MFB stimulation and weak cue-related responding to food reward—are intriguing but require replication with adequately powered samples before they can be considered reliable.

Future Directions

Future research should build on the methodological groundwork established here while addressing these constraints. Increasing the number of animals per condition and ensuring consistent electrode placement. Extending the number of training and testing sessions would allow more robust assessment of learning curves and conditioned responses. Larger sample sizes would also permit the use of mixed-effects modeling to account for individual variability in reinforcement sensitivity and vocal output. Subsequent studies should combine behavioural, electrophysiological, and histological verification within the same subjects to directly relate vocalization patterns to dopaminergic activation. Incorporating stricter or graded food-restriction paradigms could clarify how hunger intensity modulates appetitive USVs.

In summary, while this project demonstrates the feasibility of measuring ultrasonic vocalizations during electrical and natural reinforcement, the current results should be viewed as an initial step. Future work with larger, healthier cohorts and more precise methods will be essential to confirm whether the patterns observed here represent reliable features of reward-related affect and learning in the rat model.

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Appendix 1 (Test Chambers)

Two chambers were built to allow for the behavioural testing of ultrasonic vocalizations produced by rats in learning tasks for electrical stimulation of the medial forebrain bundle and calorie restriction sugar pellet dispenser as a reinforcer.

The external chamber, designed to be soundproof to external noise, was constructed from 1.9 cm (¾ inch) plywood glued together. The outside of the chamber was painted with urethane paint so it can be cleaned with a solution of Virkon (a disinfectant). The wooded box is 63.5 cm (25 inches) wide by 91.4 cm (36 inches) long and 71 cm (28 inches) deep. The box was lined with soundproof foam 5 cm (2 inches) thick attached to the walls with double-sided 3M sticky tape and wood screws used to hold the foam in place. A hole near is middle of the triangular part of the box was utilized for wires to pass through to stimulation equipment, the Arduino boards (Uno and Mega), and to allow the placement of a video camera to view into the box. Also, a slot in the top of the box allowed for wires to pass through for the beeper/light signal, webcam, and the wires from the stimulator to the commutator, and the wire from the ultrasonic microphone. The infrared camera with lights was attached to the inside of the box near the top and adjusted to view into the plexiglass box.

The plexiglass box was created which fits inside the wooded box and can be placed inside or easily removed. The plexiglass box is 35.6 (14 inches) wide by 48 cm (19 inches) long and 45.7 cm (18 inches) high and made with 0.64 cm (¼ inch) plexiglass bonded with epoxy.

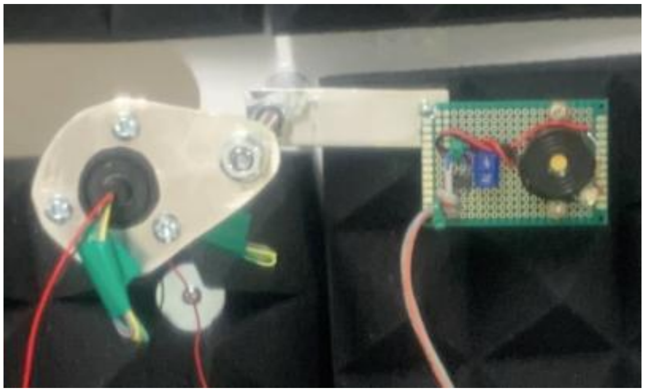
Appendix 2 (Microphone, Beeper/Light Signal Device, and Commutator)

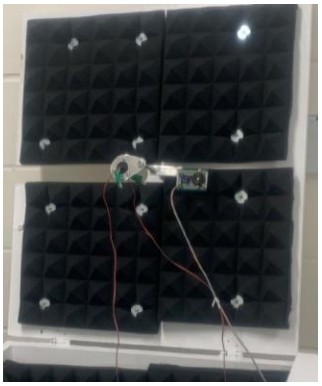
At the top and attached to the lid of the wooden housing box is a 1.9 cm(¼-inch) bolt which extends approximately 20 cm (8-inches) into the Plexiglass test box when the lid of the wooden box is lowered.

The microphone is attached to the bolt using surgical tape.

An aluminum bracket, at the bottom of the bolt, has the commutator attached to it (Adafruit Slip Ring with Flange, Product ID: 736). Two of the output wires attach to the subject and the input comes from the Arduino Uno controlling the WPI A-365 stimulator. The delivery of the stimulation is controlled through the Arduino Uno by the main Matlab code for each experiment. The main Matlab code also runs the timing of the three experiments as well as logs the time of the stimulation pulses.

The start and end times of each trial set is signaled, both in audio and video, by a beep/light device which is also attached to the center bolt. This device is controlled by the Arduino Mega and coded in Matlab for the three experiments. The light/beep is initiated at the beginning and at the end of each trial such that the information can be used for both audio and video analysis.





Appendix 3 (Stimulation Code)

```
// Generates a double digital pulse of 200 us of +5V with a 20 us gap (0V)
between - when the button is pressed.
// Once the double pulse train is generated, by button press, there is 6.5 ms
inter-stimulation gap.
// The system ignores additional button press for 3 sec with 0V output at end
of a train of pulses.
// Stim on is created by logical TRUE to pin 11
const int stimPin = 9; // to input on stimulator - pin 9 to center and GND to
Shield
const int buttonPin = 11; // logical true - to Arduino Ground

void setup()
{
  pinMode(buttonPin, INPUT_PULLUP);
  pinMode(stimPin, OUTPUT);
}

void loop()
{
  // read the button pin

  if (digitalRead(buttonPin) == LOW) {
    // if button pressed generate train of 150 Hz pulses - value 1 was value
LOW (HIGH is 0)

    for (int freqCount =0; freqCount <76.429; freqCount ++){
                                                     // take double pulse
with gap (0.42 us) plus inter-stimulation interval (6.5 ms) and repeat for
500 ms
                            // 200 us x2 plus 20 us gap is 1 pulse (so 420 us
or 0.42 ms)PLUS 6.5 ms between double pulse
                            // 500 ms total stimulation divided by 6.542 =
76.429 times for loop

    for (int i = 1; i<2; i++) {
      // generate a double pulse for bipolar (set on stimulator (WPI A365)
      digitalWrite(stimPin, HIGH);
               delayMicroseconds(200);
                 digitalWrite(stimPin, LOW);
               delayMicroseconds(20);
      digitalWrite(stimPin, HIGH);
               delayMicroseconds(200);
      digitalWrite(stimPin, LOW);
               delayMicroseconds (6500);

    }
    }

    delay(3000); // do not allow another pulse for 3 sec?
    // do not make this value 0, otherwise code can no longer generate train
pulses

  }
```

```
delay(10); // sample button state at 100 HZ frequency
}

// want 500 ms stimulation (1/2 second).
// sending 2 - 200 uS pulses with 20 uS gap between PLUS 6.5 mS between
pulses for 1 bipolar pulse
// 500 ms / 6.542 ms = 76.42 pulses (line for FreqCount)

// E=IR = 0.5 mA * 1 K ohm R = 0.5 Volts
```

Appendix 4 (Matlab Code for Testing Efficacy)

```
% STIMULATION EFFICACY TESTING
% test efficacy of stim to various wire configuration on implants
% Word doc ascribes codes to wire configurations
% light comes on in poke box 1 to signal stim on in Poke box 1
% within a 30 second block, S pokes for stimulation with 1 second time-out
% Mega & Uno plugged into computer with Mega doing code & Uno serial Stim
% store data in XLSX
% Video record behaviour and DV is ultrasonic vocalizations - Note WHEN
% Beep and Light to signal start and end trial (from Mega)

clear
clc
tic % to get total time (start)

% get Subject number and current date
prompt = 'What is the Subject number? ';
x = input(prompt);
date = datestr(now, 'mm/dd/yy');

% get the electode wires having stimulation
prompt = 'What are the wires having stimulation? ';
y = input(prompt);

m=arduino('com3','Mega2560'); % open and run Mega

% turn Light & Beep on then off 3 times to signal Start of Trial
j=0;
for j=1:3;
    writeDigitalPin (m,'D3',1);
    pause (0.3); % wait 1/3 second
    writeDigitalPin (m,'D3',0);
end

% Configure Mega for Nose Poke switch IR
configurePin (m, 'D4', 'DigitalInput'); % box 1
configurePin(m, 'D4', 'pullup');% set pullup resistor

ST1 = zeros(1,30); % set matrix to store times Box1

tic % start time

   while (toc<30) % if poke record time and lite off -then repeat
     i=0; % clear loop counter
        for i=1:30
     writeDigitalPin (m,'D7',1); % lite on in box 1
      Voltage=readDigitalPin(m, 'D4');
    if (Voltage <= 0.5)% poke box #1 is poked
            ST1(i) = toc;
            writeDigitalPin (m,'D7',0); % lite off in box 1

         % Stim ON in Poke Box 1
           % turn on Stimulator - output UNO from pin 9 + GND to A365
```

```
            s=serial('COM4','BAUDRATE',9600);   % create serial port Uno
            fopen(s);                           % open the serial port
            pause (0.5);                        % wait 1/2 second
            fclose(s);                          % close  serial port
    end % end if
        end % end for
  end % end while for box #1
     writeDigitalPin (m,'D7',0); % lite off inbox 1

% turn Light & Beep on then off 3 times to signal End Trial
j=0;
for j=1:3;
    writeDigitalPin (m,'D3',1);
    pause (0.3); % wait 1/3 second
    writeDigitalPin (m,'D3',0);
end

BE = toc; % total time end

% write xlsx data
% x is subject #, D is date, BT is total time begin to end
% ST1(i) is box 1 time, ST2(i) is box 2 time, ST3(i) is box 3 time

a = num2str(x);
A=['Subject # ',a];
AC=cellstr(A); % subject string
b = num2str(date);
B=['Date ',b];
BC=cellstr(B); % date string
c = num2str(BE);
d = num2str (y);
E = ['Wire configuration # ',d];
DE = cellstr(E);
DT=BE .'; % Total time
tt=num2str(DT);
D=['Total Trial Time ',tt];
DC=cellstr(D); % Total Time string

g=['Poke box times -- 1'];
TT=cellstr(g); % ST(1) to string

F=ST1;

xlswrite('E-Test.xlsx',AC, 'Sheet1','A1'); % Subject string
xlswrite('E-Test.xlsx',BC, 'Sheet1','A2'); % Date string
xlswrite('E-Test.xlsx',DE, 'Sheet1','A3'); % Stim wire config
xlswrite('E-Test.xlsx',DC, 'Sheet1','A4'); % Total time
xlswrite('E-Test.xlsx',TT, 'Sheet1','A5'); % Poke times
xlswrite('E-Test.xlsx',F, 'Sheet1','A6'); % Box 1 times

% Misc Notes:

% Uno - pin 9 to stim red and other to GND (black)
% Mega - D3 is start & end beep & lite + GND
```

```
% Mega pins for box 1 lite (D7) [RED]
% Mega pin to power and read IR switch broken in Box 1 (D4) [PURPLE]

% IR detector to Mega board
% Connect transmitter (2 wires) to Ground & +5V
% Connect receiver to GND, +5V and data to digital pin 4
% RED is + 5 V & BLACK is GND
```

Appendix 5 (SOP 303 - Electrical Stimulation of Deep Brain Structures in Adult Rodent)

UNIVERSITY OF LETHBRIDGE ANIMAL WELFARE COMMITTEE STANDARD OPERATING PROCEDURE

Department:
Neuroscience, CCBN

Title: Electrical Stimulation of Deep Brain Structures in Adult Rodent SOP # 330

Author: J. Walkey and D. Euston

AWC Approval Date: July 23, 2020

Purpose: The purpose of this Standard Operating Procedure (SOP) is to describe the humane and reliable method of implantation of bilateral electrical stimulation of deep brain structures in the adult rodent.

1. Frequency of Procedure

   a. Once per animal

2. Person(s) Responsible

   a. Principal Investigator (PI)
   b. Trained Laboratory Research staff

3. Documentation of Procedure

   a. Anesthesia & Surgical Data Sheet
   b. Room Procedure Log
   c. Animal cage ID card
   d. Controlled Drug Log

4. OHS Requirements

Physical Hazards

a. *Animal bites and scratches* ***Hazard:*** *skin punctures, skin irritation* ***Controls:*** transport tubs, animal training, animal handling SOP, gown, gloves.
b. *Allergies* ***Hazard:*** *skin, eye irritant, inhalation* ***Controls:*** ventilation, facility operational procedures**,** gown, gloves, cap, safety googles, and fit tested respirator.
c. *Needle poke* ***Hazard:*** *skin punctures* ***Controls:*** follow safe needle handling techniques, dispose of needles in an appropriate sharps disposal container, never recap used needles.
d. *Oxygen cylinder* **Hazard**: *gas under high pressure* **Controls**: Cylinder transport as well as removal and placement of regulator is to be completed by authorized trained personnel only. Cylinders must have the cylinder safety cap securely attached prior to transport and are only to be transported with the use of the gas cylinder transfer cart and security chain. All cylinders must be secured to the wall/benchtop during use (as indicated in SOP #201 *Isoflurane Anesthetic Machine Operation and Maintenance)*

5. Chemical Hazards:

a. *Dental Acrylic fumes* **Hazard:** *eye/lung irritation* **Controls:** appropriate usage of the anesthetic scavenging snorkel system.
b. *Isoflurane* **Hazard:** serious eye damage/eye irritation, reproduction toxicity, specific target organ toxicity (repeated exposure) **Controls:** When filling and emptying precision vaporizer, the waste gas snorkel exhaust or fit tested respirator must be used to limit inhalation. Additionally, gowns, gloves and safety glasses must be worn. Handle the Isoflurane glass bottle carefully with dry hands to avoid accidental spillage or bottle breakage. MSDS can be found in the MSDS binder located in EP1033 within the vivarium. Refer to SOP
c. #201 Isoflurane Anesthetic Machine Operation and Maintenance and SOP #202 “Rodent Anesthesia”.
d. 70% Isopropyl Alcohol Hazard eye irritation, skin irritant with prolonged exposure, flammable Controls: No sparks or flames in immediate area. All equipment used must be grounded. Avoid contact with eyes, wear gloves.

6. Specific Training Required

a. WHMIS
b. Rodent Research Facility Orientation
c. IAUTP (Part 1 and Part 2)
d. Rodent surgical training and proof of competency for aseptic surgical procedures

7. Materials Required (specific for this surgical procedure)

a. Sterile Surgical Pack
b. Bone wax and micro cautery (to control bleeding)
c. Dental acrylic
d. 2mm drill bit (rat), or 1mm drill bit (mouse)
e. high speed handheld drill
f. Compressed Air
g. Electrode guide array
h. Fine wires
i. 16-20 small stainless-steel screws (~0.75mm diameter)
j. Kwik-Sil
k. Sterile gel foam

l. Vicryl 4-0 FS-2 (rat), Vicryl 5-0 C-3 (mouse) suture material

8. Detailed Procedures

a. Surgery

a. Surgery will be performed under aseptic conditions; all surgical instruments must be autoclaved prior to surgery, and surgeons must scrub in (surgical cap, surgical mask, sterile gown and surgical gloves).

b. Administer analgesics as described in SOP #101 *"Rodent Analgesia"* and in accordance with the current approved protocol. Buprenorphine (rat: 0.03-0.05mg/kg, mouse: 0.05-0.1mg/kg) is administered 30 minutes prior to anesthesia induction and Metacam (rat: 1mg/kg, mouse: 5-10mg/kg) is administered post-operatively unless indicated otherwise.

c. Anaesthetize the animal as described in SOP #202 "*Rodent Anesthesia"* using Isoflurane anesthesia.

d. Surgically prepare the animal as described in SOP #317*"Rodent Stereotaxic Surgery"*.

e. Administer Lidocaine HCl 2% with Epinephrine (2-4 mg/kg, diluted to 0.5% is the animal's weight is less than 100g) subcutaneously at the incision site before incision to provide additional local analgesia and also reduce wound bleeding through vasoconstriction effect from the epinephrine. Refer to SOP #101 *"Rodent Analgesia"*.

f. Expose the cranium by making a surgical anterior-posterior incision with a scalpel blade, from between the eyes to the occipital crest and dissect the fascia covering the skull bluntly as indicated in SOP #317 "Rodent Stereotaxic Surgery". Control the bleeding if required using a combination of bone wax and/or micro cautery ensuring that the skull is well dried.

g. Using the handheld drill, drill the following 4 holes (remove bone dust as needed during the drilling with the compressed air):

   i. *Craniotomy #1:* Drill two 2-3mm diameter holes and perforate the dura by blunt dissection assisted by the tip of a sterile needle for implantation of the fine wires for brain micro-stimulation. Temporary cover this craniotomy with moist sterile gel foam.

   ii. *Craniotomy #2:* Drill two small secondary holes for two 0.75 mm diameter stainless steel for screws used to secure the dental cement skull cap. Temporary cover this craniotomy with moist sterile gel foam.

h. Remove the sterile moist gel foam from the craniotomy #1. Gently place the fine wire pair for brain micro-stimulation in the stereotaxic. Using the stereotaxic tower lower the wire pair into the brain structure coordinates. Apply dental acrylic around the craniotomy #1 set of stimulating electrode wires and allow a few minutes to set up.

i. Remove the sterile moist gel foam from the craniotomy #2 hole and gently place the fine wires pair for brain micro-stimulation in the stereotaxic. Using the stereotaxic tower lower the wire pair into the brain structure coordinates. Apply dental acrylic around the second set of stimulating electrode wires and allow a few minutes to set up. Proceed to apply dental acrylic around both sets of stimulating wires and allow a few minutes to set up. The dental acrylic needs to be dry enough and firm enough to hold the stimulating electrodes in place so they do not move then the wires can be disconnected from the stereotaxic tower.

j. Using a small flat-headed screwdriver, screw in jewelers' screws into Craniotomy #2 holes leaving 1 mm space gap above skull bone. The screws should be tightly set in the craniotomy and should not be screwed in too superficially or too deeply. The screw should be rotated sufficiently through the full thickness of the skull to have the bottom of the screw flush with the inner skull (not beyond as it will damage the cortex of the brain).
k. Apply a coat of dental acrylic around the jewelers' screws.
l. Shape the dental acrylic around craniotomies and jewelers' screws to create a solid ***smooth*** well-defined dome where the skin will be opposed against, ensuring that all screws and wires are well covered.
m. Clean off any blood from the animal and apply antibiotic ointment alongside of the dental acrylic and skin junction. Administer subcutaneous fluids as required (see SOP #316 "Rodent Post-Operative Care").
n. Recover the animal as described in SOP #316 "*Rodent Post-Operative Care*".
Note: rodents tend to recover from this procedure slowly as they are adjusting to the additional head weight.
o. If the animal is observed to present with any post-operative complications (i.e.: seizure activity; infection; significant functional impairments such as grooming, movement, eating; weight loss greater than 15% (rat); loss of dental acrylic or permanent fixed wires, etc.) the Animal Care Services staff must be notified.
p. Allow animals **one-week** recovery prior to beginning training.

Appendix 6 (Experiment 1 Matlab Code)

```
% EXPERIMENT 1.
% Replicate Burgdorf 2000
% Un-cued stim on a fixed-time schedule
% every 20 sec get 1 second of stim for 10 min every second day for 8 Days
% So 3 per min is 30 stimulations for 10 minutes - in one day trial
% No cues, predictive only. Rat anticipates stimulation (Fixed Time)
% Video record behaviour and DV is ultrasonic vocalizations
% Beep and Light sets to signal start and end trial (from Mega)

clear
clc
tic

m=arduino('com3','Mega2560'); % open and run Mega
% turn Light & Beep on then off 3 times to signal Start/End Trial
j=0;
for j=1:3;
    writeDigitalPin (m,'D3',1);
    pause (0.3); % wait 1/3 second
    writeDigitalPin (m,'D3',0);
end

jj=0;
for jj=1:30 % run test 30 times (every 20 sec. so 10 minutes)

% turn on Stimulator - output on arduino from pin 9 to stimulator + GND
s=serial('COM4','BAUDRATE',9600);   % to create the serial port Uno
 fopen(s);                          % open the serial port
pause (1);
 fclose(s);                         % close  serial port
 pause(2);

% wait 18 seconds then loop again
pause(18);

end

% Signal end of trial with light and beep on/off pulses
j=0;
for j=1:3;
    writeDigitalPin (m,'D3',1);
    pause (0.3); % wait 1/3 second
    writeDigitalPin (m,'D3',0);
end

toc % just to see total time in command window

% WIRING NOTES:
% Arduino Mega
% LED and BEEP signal device - connect - Pin D3 (red) & GND (black)
% Arduino Uno
% Stim Pin - Pin 9 (red) and GND (black)
```

```
% UPLOAD "Just_Run_Stim_Once" code to Arduino Uno
% Sketch Code -Just_Run_Stim_Once! Runs from serial port connection

% NOTES ON STIMULATION:
% want 500 ms stimulation (1/2 second).
% sending 2 - 200 uS pulses with 20 uS gap between
% PLUS 6.5 mS between pulses for 1 bipolar pulse
% 500 ms / 6.542 ms = 76.42 pulses (line for FreqCount)
% E=IR = 0.5 mA * 1 K ohm R = 0.5 Volts
% did to oscilloscope as test

% DATA:
% Video - to observe behaviour
% Audio - to be analyzed
```

Appendix 7 (Auditory File Analysis)

To analyze the ultrasonic vocalizations both Raven Pro software and DeepSqueak (authors; Kevin R. Coffey, Ruby E. Marx, and John F. Neumaier) code were used. DeepSqueak works well for mice but not as well for analyzing the ultrasonic vocalizations of rats.

In the research by Burke et al. (2017), the rat vocalizations were analyzed using Raven Pro software. Dr. Burke was kind enough to provide me with personal training in using the software and analyzing the vocalizations. I used her method to score my data and train an assistant.

Once the vocalization files were analyzed a table was generated which was opened in Microsoft Excel and graphs were used to see trends in the vocalization behaviour across the test groups in the three experiments.

Appendix 8 (Experiment 2 Matlab Code)

```
% Experiment 2 - Classical conditioning (ESB)
% This script generates random intervals with a
% specified average interval length. It also contains sample code to
% deliver a cue light and stim using these intervals.

% we want an average interval between of 90 seconds between cue onsets
% _this take just over 1 hour to run _
avg_interval = 90;
t = 3600; % 3600 is number of seconds in 2 hours
N = 2*t/(avg_interval); % Number of cues/stims in 2 hours if spaced 90 seconds apart
                        % We will generate this many intervals. This is
                        % twice as many as we are likely to need, but this
                        % over-preparation allows us to pick enough
                        % intervals to cover a 1 hour session without
                        % worrying about running out


% set up a stim array with one entry for every second indicating if a cue/stim will
% presented at that time point
stim_array = rand(2*t,1)<(N/(2*t));

% now find the actual intervals
intervals = diff(find(stim_array));
intervals = intervals(intervals>10 & intervals<205); % this limits extreme
                                                     % values but still
                                                     % gives a mean of
                                                     % roughly 90 seconds. I
                                                     % determined these
                                                     % values through trial
                                                     % and error


% ------------------------------------------------------------------------

% get Subject number and current date
prompt = 'What is the Subject number? ';
x = input(prompt);
date = datestr(now, 'mm/dd/yy');
% D = date;
time = datestr(now,'HH:MM:SS');

m=arduino('com4','Mega2560'); % open and run Mega
% turn Light & Beep on then off to signal trial set
j=0;
for j=1:3;
    writeDigitalPin (m,'D3',1);
    pause (0.3); % wait 1/3 second
    writeDigitalPin (m,'D3',0);
end

% ------------------------------------------------------------------------
```

```
% histogram(intervals,100)  % checks the distribution of all delays generate
% mean(intervals)  % use to verify that the mean is close that what is desired

% Deliver cues and stim with the intervals we've created
% Use an 8 second delay between cue onset and stim onset.
% The cue would remain on until the onset of stim delivery.
% A review of Pavlovian conditioning suggests that a
% 8 second delay with cue and stim coupled with a 90 second inter-trial
% interval will result in strong acquisition of the cue-stim association (see Lattal
1999).
i=1;
for pl = 1:40  % 40 is 3600/90, so average number of stims expected in an hour

  if pl == 2 || 7 % based  on 5% of 40 trials that would be 2
      % just arbitrarily picked 2 & 7 as trials for lights but no stim
   % turn on all 3 nose-poke LED's
   writeDigitalPin (m,'D5',1);
   writeDigitalPin (m,'D6',1);
   writeDigitalPin (m,'D7',1);
   pause(8); % wait 8 seconds

   % turn off all 3 nose-poke LED's
   writeDigitalPin (m,'D5',0);
   writeDigitalPin (m,'D6',0);
   writeDigitalPin (m,'D7',0);
      pause(intervals(pl));
  % Store set block data --- BB is Block begin & BE is block end

  else % end if for the test for 2 or 7
      % turn on all 3 nose-poke LED's
   writeDigitalPin (m,'D5',1);
   writeDigitalPin (m,'D6',1);
   writeDigitalPin (m,'D7',1);
   pause(8); % wait 8 seconds
   % turn on Stimulator - output UNO from pin 9 + GND to A365
             s=serial('COM3','BAUDRATE',9600);   % create serial port Uno
             fopen(s);                           % open the serial port
             pause (0.5);                        % wait 1/2 second
             fclose(s);                          % close  serial port

   % turn off all 3 nose-poke LED's
   writeDigitalPin (m,'D5',0);
   writeDigitalPin (m,'D6',0);
   writeDigitalPin (m,'D7',0);

  % disp(['current delay: ' num2str(intervals(pl))])
   pause(intervals(pl));
  % disp(''); % just creates space to make more readable

  end % end if
   % Store set block data --- BB is Block begin & BE is block end
   Block(i)=(intervals(pl));
   i=i+1;
end % end for
```

```
%------------------------------------------------------------------------
% get data and write to xlsx file

% x is subject #, D is date, BB is block begin, BE is block end,
%  NO Stim - BBN is Block begin & BBE is block end
% data = [x, D, block interval]

% xlswrite('Exp2DJ.xlsx',data); % Saves data in Matlab working folder
% A=[x D];
% B = transpose(A) is an alternate way to execute A.'
a = num2str(x);
A=['Subject # ',a];
AC=cellstr(A);
b = num2str(date);
B=['Date ',b];
BC=cellstr(B);
c=num2str(time);
C=['Start time ',c];
ST=cellstr(C);
d = Block; % interval length
CT=d .';

%warning('OFF', 'MSGID'); % stops warning to Command Window - ERROR in code
xlswrite('Exp2DJ.xlsx',AC, 'Sheet01', 'A1'); % subject number
xlswrite('Exp2DJ.xlsx',BC, 'Sheet01', 'A2'); % date
xlswrite('Exp2DJ.xlsx',ST, 'Sheet01', 'A3'); % start time
xlswrite('Exp2DJ.xlsx',CT, 'Sheet01', 'B5'); % block length

% Misc Notes: -----------------------------------------------------------
% UPLOAD "Just_Run_Stim_Once" code to Arduino Uno
% Sketch Code -Just_Run_Stim_Once! Runs from serial port connection
% maybe shape going to lite nose-pokewith sugar pellets - Criteria?
% Dr. DE suggested using stim as Rw for shaping
% Uno - pin 9 to stim red and other to GND (black)
% Mega - D3 is start & end beep & lite + GND
% Mega - D5 is nosepoke #1 lite + GND (Red light wire)
% Mega - D6, D7 are nosepoke lites #2 & #3
```

Appendix 9 (Matlab Script for Experiment 3)

Food Deprived Rats- Pellet Dispensed in Classical Conditioning Measuring Ultrasonic Vocalizations and video Behaviour

Calorie restricted Long Evans rats (Age, Weight, Gender)

Med-Associates ENV-203 Pellet Dispenser

Matlab code to run the experiment (15 trials with 10-40 second random inter-trial intervals)

Arduino MEGA to 1 Channel Relay Module Control Board with Optocoupler Isolation High and Low Level Trigger (5V)

USV's analysis using Raven Pro and also Python for Trill, and other call types

Video when the subject eats the pellet to determine the time eating

Cameras (Top & Side):

Arducam 1080P Day & Night Vision USB Camera for Computer, 2MP Automatic IR-Cut Switching All-Day Image USB2.0 Webcam Board with IR LEDs for Windows, Linux, Android and Mac OS

LED on as CS for 5 seconds and pellet dispensed when LED off

LIGHT and Beep 3 times at start and end of experiment

store data in xls Excel file

```
clear % clear data and variables
clc   % clear command window
% set the number of trials
disp('Run 15 trials (takes about 10 minutes to run) ');
```

```
Run 20 trials (takes about 10 minutes to run)
```

```
  fprintf('\n')
question='How many trials to run : ';
  z= input(question);

% get Subject number and current date
prompt = 'What is the Subject number? ';
```

```
x = input(prompt);
date = datestr(now, 'mm/dd/yy');
% D = date;
port='COM4';
board='Mega2560';
a = arduino(port, board); % open and run Mega

i=1; % sequence times
k=1; % codes

ST=tic; % to get times (start)
% turn Light & Beep on then off 3 times to signal Start/End Trial

 SE(i)=toc(ST); % start time
 SC(k)=5;
 i=i+1;k=k+1;

j=0;

 SE(i)=toc(ST); % start time
 SC(k)=6;  % code
 i=i+1;k=k+1; % increment

jj=0; % clear loop counter for trials

B=0;
% Start/End - LED & BEEP
for B=1:3
    writeDigitalPin (a,'D3',1);
    pause (0.3); % wait 1/3 second
    writeDigitalPin (a,'D3',0);
    pause(0.3);
end
cts=datetime; % computer time as start time (after beep/LED start)
B=0;
pause(5); % pause 5 seconds after start signal.
% A set of "z" - blocks
for jj=1:z
 % LED flashing loop counter
    j=0;
 % Random Time
    r=0;
    r=randi([10,40],1); % here 10/40 ca. 10 minutes (DE suggested changing to
30 to 50 - 20 min to run)

% Trial block .........................................
```

```
pause(r); % seconds random delay time between trials

     writeDigitalPin (a,'D7',1); % start time LED blinking as CS
       SE(i)=toc(ST);
       SC(k)=1;
       i=i+1;k=k+1;

   % LED flashes every 0.1 seconds for 5 seconds
   % (code for 5 sec)
        for j=1:19
            writeDigitalPin (a,'D7',1);
            pause (0.1); % wait 1/10 second
            writeDigitalPin (a,'D7',0);
            pause (0.1);
        end % end flashes

     % End of Blink & Food Pellet delivered
          SE(i)=toc(ST);
          SC(k)=2;
          i=i+1;k=k+1;
% food Pellet deliverd
            writeDigitalPin (a,'D8',0);
            pause (0.1); % wait 1/10 second
            writeDigitalPin (a,'D8',1);
            % drive pellet dispenser

end % end of z trial blocks


 SE(i)=toc(ST); % start time
 SC(k)=7;
 i=i+1;k=k+1;

pause (5); % pause 5 seconds at end before LED & BEEP

% turn Light & Beep on then off 3 times to signal Start/End Trial Set
B=0;
for B=1:3
    writeDigitalPin (a,'D3',1);
    pause (0.3); % wait 1/3 second
    writeDigitalPin (a,'D3',0);
    pause(0.3);
end

 SE(i)=toc(ST); % start time
 SC(k)=8;
```

```
ET=toc(ST); % get total time


% write xlsx data ---------------------------------------------------------

a = num2str(x); % Subject number
A=['Experiment CC Cohort 3 - Subject # ',a];
AC=cellstr(A); % subject string
b = num2str(date);
B=['Date ',b];
BC=cellstr(B); % date string

% Total time
tt=num2str(ET);
D=['Total Trial Time ',tt];
DC=cellstr(D); % Total Time string

% Events / times
e=['  Event time codes: 5/6 - 7/8 is start/end '];
CT=cellstr(e);
g=['  Start Time of LED flashing = 1'];
SOn=cellstr(g);
f=['  Pellet dispensed = 2'];
CT1=cellstr(f);


% Number of Trials
zz=num2str(z);
ZZ=['Number of trials: ',zz];
ZT=cellstr(ZZ);

% Sequence times
FS=(SE)';

% Event Code Numbers
GS=(SC)';

% Computer time as start time
CoT=datestr(cts); % start time string
COT=['Computer time start: ',CoT];
CTS=cellstr(COT);

FileName=['Exp_CC2_',datestr(now, 'dd-mmm-yyyy'), '_',a];
 % myDir   = 'C:\Users\jbw\Documents\MATLAB';
 myFile = [date '.xlsx'];
```

```
  pathAndFilename = fullfile(myFile);xlswrite(FileName,AC, 'Sheet1','A1'); %
exp# & subject #
  pathAndFilename = fullfile(myFile);xlswrite(FileName,BC, 'Sheet1','A2'); %
date
  pathAndFilename = fullfile(myFile);xlswrite(FileName,ZT, 'Sheet1','A3'); %
number of trials
  pathAndFilename = fullfile(myFile);xlswrite(FileName,DC, 'Sheet1','A4'); %
total trial time
  pathAndFilename = fullfile(myFile);xlswrite(FileName,SOn, 'Sheet1','A5'); %
Codes text - LED start
  pathAndFilename = fullfile(myFile);xlswrite(FileName,CT1, 'Sheet1','A6'); %
Codes text - Pellet dispensed
  pathAndFilename = fullfile(myFile);xlswrite(FileName,CTS, 'Sheet1','A7'); %
Compute exp total time
  pathAndFilename = fullfile(myFile);xlswrite(FileName,FS, 'Sheet1','A9'); %
Times
  pathAndFilename = fullfile(myFile);xlswrite(FileName,GS, 'Sheet1','B9'); %
Codes

 % Indicate end of script:
 clc
 fprintf ('The total time to run was %0.1f seconds\n',ET)
```

```
The total time to run was 594.7 seconds
```

```
 % Wiring Notes:

 % Mega  - D3 is start & end beep & lite + GND
 %       - D8 is run pellet relay ( red to 5 V and black to GND)
 %       - D7 flashing LED for conditioning Stimulus (CS) + GND
```

Appendix 10 (Nose Poke Switches)

Three nose poke switches were built with similar design to commercial nose poke switches. The inside of the switches was 3-D printed using Tinker Cad software and the faceplates were cut from 0.3 cm (1/8-inch) aluminum. After the shell was built IR Break Beam Sensors (Adafruit PRODUCT ID: 2168) with 5mm LED and 2 K-ohm resistor were wired into the boxes. In the image below, picture (a) is the send and receive infrared devices and the second image (b) is the sideview of the attachment of the send/receive devices. These switches detect a break in the beam faster than commercial switches at a fraction of the cost.