

Pharmacological analysis of 50 kHz vocalizations in the male rat.

Tim Woodford

Biological Sciences

(Supervisor: S. M. Brudzynski)

(Submitted in partial fulfillment of the requirements

For the degrees of Masters of Biological Sciences)

Brock University

St. Catharines, Ontario

June, 2015

© Tim Woodford, 2015

Abstract

The production of 50 kHz ultrasonic vocalizations in rats has been associated with both positive social interactions and appetitive behavioural situations. Furthermore, there is significant evidence showing that these vocalizations are controlled by the meso-limbic dopamine system. The purpose of this study was to perform a pharmacological analysis of 50 kHz calls by using dopamine and two dopamine agonists amphetamine and apomorphine, to induce calls. The acoustic parameters of the different call types were compared across each agonist. All three agonists were able to significantly induce more 50 kHz vocalizations compared to the vehicle control. Furthermore, calls elicited by apomorphine had a significantly higher bandwidth compared to those elicited by dopamine and amphetamine. All three agonists also had significantly different pharmacokinetic properties. These observations suggest that the D2 receptor sub-type is involved in the length of call bandwidths.

Table of Contents

Abstract.....	ii
List of Figures.....	v
Introduction.....	1
Animal Communication.....	1
Ultrasonic Rat Vocalizations.....	2
Ultrasonic 22 kHz Rat Vocalizations.....	3
Pharmacological Initiation of 22 kHz Rat Vocalizations.....	5
Ultrasonic 50 kHz Rat Vocalizations.....	8
Pharmacological Initiation of 50 kHz Rat Vocalizations.....	13
Mesolimbic System and Dopamine Sub-Types.....	18
Rationale and Study Hypothesis.....	20
Methods.....	23
Animals.....	23
Stereotaxic Surgery.....	23
Drugs and Injection Procedures.....	24
Vocalization Recording and Analysis.....	25

Histological Procedures.....27

Results.....29

 Localization of Injection Sites.....29

 Injection Results.....29

 Acoustic Analyses of Calls.....34

 Call Sub-Type Analysis.....37

 Drug Pharmacokinetics Analysis.....40

Discussion.....45

References.....52

List of Figures

<i>Figure 1</i> Examples of 50 kHz rat vocalizations.....	28
<i>Figure 2.</i> Localizations of injection sites.....	30
<i>Figure 3a.</i> Mean number of 50 kHz vocalizations from all injection conditions.....	31
<i>Figure 3b.</i> Mean number of 50 kHz vocalizations from missed injection sites.....	33
<i>Figure 4a.</i> Mean call peak frequencies from vocalizations.....	35
<i>Figure 4b.</i> Mean call bandwidths from vocalizations.....	36
<i>Figure 4c.</i> Mean call durations from vocalizations.....	38
<i>Figure 5a.</i> Mean percent of calls that were FM.....	39
<i>Figure 5b.</i> Mean percent of FM calls that were trills.....	41
<i>Figure 6a.</i> Pharmacokinetical analysis of time distribution of calls.....	42
<i>Figure 6b.</i> Pharmacokinetical analysis of time distribution of calls total percentage.....	43
<i>Table 1.</i> Comparison of drug dosages and moles used.....	44

INTRODUCTION

Animal Communication:

Animal communication developed through evolution mostly as a means of protection from predators and to influence and modify of the behaviour of recipients (e.g. during mating, or taking care of newborns). At any given time, an animal is receiving a vast amount of external stimuli. When these external stimuli contain information that allows the animal to predict certain outcomes from the environment, they are termed biologically significant stimuli or cues (complex stimuli). Furthermore, when biologically significant stimuli originate from an organism and have the ability to modify behaviour, they are then termed signals (Brudzynski, 2005). Signals are a combination of the actual behavioural manifestations of the communicator and the degree of information that those manifestations represent.

There are two factors that represent the degree of information that a signal can provide to a recipient. The first factor is the urgency of the call (Seyfarth and Cheney, 2003). The second factor for signals is defined by the referential specificity of the communication (Seyfarth and Cheney, 2003). Using the example of alarm calls, some species have highly specific alarm calls that refer to a specific predator. Other species, however, have one general alarm call that does not specify the type of predator. The acoustic properties of calls may also vary to signify the level of urgency (Seyfarth and Cheney, 2003). A true signal may also be referred to as a sign because the behavioural manifestation of the signal represents something significant to the recipient. The meaning of the signal is known as the semiotic content (Brudzynski,

2005) to distinguish it from semantic content of human speech. Therefore, for true animal communication to occur there must be process of signal production combined with a behavioural response from the recipient as an evidence of decoding the semiotic content in the signal.

Rat Ultrasonic Vocalizations:

Rat ultrasonic vocalizations developed for biologically adaptive benefits. Unlike audible vocalizations, which are produced by vibrating vocal folds, rat ultrasonic vocalizations are produced by a whistle mechanism. Usually, these ultrasonic vocalizations are pure tones and have no harmonics. The source for production of ultrasonic vocalizations was determined to be located at the larynx through nerve transection experiments by Roberts (1975). The vocal folds constrict to a point where they cannot vibrate but leave a 1-2 mm opening where, when air is forced through, an ultrasonic whistle-like vocalization occurs.

The 'symbolic meaning', or semiotic content behind rat calls will usually refer to general situations, conditions, or physiological states. Rat ultrasonic vocalizations also tend to have low referential specificity, although referential aspects can be possible. The semiotic content of rat ultrasonic vocalizations is related to several broad biological functions such as a locating function between rats, expressing internal emotional states, activating attention, an alarming function to external danger, agonistic function relating to escape and withdrawal, approach behaviour, and general social calls to maintain connections and cohesiveness within a social group (Brudzynski, 2005). A rat call may only have one semiotic meaning (monosemic), but usually a call will have several meanings (polysemic). So generally, rat ultrasonic vocalizations have low specific informative value as well as low referential specificity as compared to human language.

When analyzing rat ultrasonic vocalizations, there are three main physical properties: peak frequency, bandwidth, and call duration. The peak frequency of a call is defined as the frequency within the call that has the highest decibel level. The bandwidth of a call is defined as the difference between the highest frequency and the lowest frequency within a call. Finally, the call duration is defined as the time from the initiation of a call to the termination of a call. Also of importance is the magnitude of the semiotic content from the call or how important is the message (urgency). This has been shown in several mammalian communication systems such as growling in cats where the changes in cumulative growling time correlated with the magnitude of external danger (Brudzynski, 1981). Therefore it is likely that call duration in rats may play a role in the magnitude and urgency of biologically significant information in rat ultrasonic vocalizations. However, not enough data exist to date to determine which acoustic parameter carries the quantitative part of the semiotic content other than the rate of calling (Brudzynski, 2005).

However, there are two distinct types of ultrasonic vocalizations emitted by rats that represent very different semiotic content. These types of calls are differentiated by their peak frequencies and have been dubbed the 22 kHz and the 50 kHz vocalizations.

Ultrasonic 22 kHz Rat Vocalizations

The 22 kHz rat vocalizations have been associated with alarm calling within a colony of rats as well as signalling of negative affective states. These calls have a frequency range of 18 to 32 kHz and most have peak frequencies between 22 and 24 kHz. Their bandwidth is relatively narrow, ranging between 1 and 6 kHz. Finally, the duration of these calls ranges from 300 to 3400 ms, which is substantially longer than other ultrasonic calls (Brudzynski, 2005). During the

emission of 22 kHz vocalizations, a rat will physically exhibit a defensive stance, entering into a tense, motionless crouching position, when observed in a cage.

Blanchard et al. (1990) investigated a connection between the production of 22 kHz calls and the presence of a predator. The researchers established seven rat colonies, each containing three males and two females. These colonies were housed in a visible burrow system. When the colony of rats was presented to a single cat, a significant number of 22 kHz vocalizations was recorded. Behaviourally, a rat will first retreat inside of its burrow colony, and then produce a large amount of 22 kHz vocalizations for up to 30 minutes after the cat has been removed. However, when a cat is presented to a single rat, where the rat has no possible retreat, it will not produce any 22 kHz vocalizations. Therefore, these results point towards development of the 22 kHz vocalization as an evolutionary adaptation to warn fellow colony rats of nearby predators (Blanchard, Blanchard, Agullana and Weiss, 1990).

The 22 kHz ultrasonic vocalizations have also been recorded in several other situations related to negative affect. Tonoue, Ashida, Makino and Hata (1986) observed rats emitting 22 kHz for several minutes after receiving foot shocks, even while isolated from other rats. Thomas, Takahashi and Barfield (1983) recorded males producing both 50 kHz and 22 kHz during aggressive encounters between male rats. Finally, Barfield and Geyer (1975) were able to record 22 kHz calls from males during their post-ejaculatory refractory period. Therefore, there is significant converging evidence that supports the claim that rat 22 kHz ultrasonic vocalizations are indicative of a negative affective state of a rat.

Pharmacological Initiation of 22 kHz Rat Vocalizations

Researchers have extensively looked into the pharmacological mechanisms behind the initiation of 22 kHz vocalizations. The first study took place in 1990 and was done by Brudzynski and Bihari. Based on previous observations that intracerebral injections of cholinergic agonists could induce vocalizations in cats, they attempted to produce similar results in rats. The researchers injected 1 μg of carbachol, a cholinergic agonist, into the anterior hypothalamic/preoptic area of twelve conscious male rats. The injection of carbachol within the hypothalamic/preoptic area successfully induced 22 kHz vocalizations. However, injections of carbachol to adjacent structures were unable to induce any calls. The study also showed two subtypes of 22 kHz vocalizations based on call duration. The first group had durations of 110-300 ms and the second had durations of 310-1000 ms (Brudzynski and Bihari, 1990).

Subsequently, Brudzynski (1994) looked at localization and dose-response relations of carbachol when injected into the anterior hypothalamic/preoptic area. Following similar procedures, carbachol was injected at doses ranging from 0.125 to 4.0 μg in 25 brain sites located within and around the hypothalamic/preoptic area. There were two areas from which carbachol induced significant 22 kHz vocalizations, the known hypothalamic/preoptic area and areas of the lateral septum. Increasing the dosage of carbachol did not affect the peak frequencies of the calls, which remained within the range of frequencies for 22 kHz. However, increasing the dosage did increase the sound intensity of the calls but also decreased the bandwidth of the calls. Furthermore, higher dosages of carbachol decreased the number of short calls (100-150 ms), but increased the number of longer calls (300-400 ms). Finally, the total number of 22 kHz vocalizations was not affected by the dose of carbachol. These results draw some important conclusions about 22 kHz vocalizations, mainly that the intensity or the urgency

of rat 22 kHz vocalizations are not based on the amount of calls given, but on the bandwidth and the duration of individual calls (Brudzynski, 1994).

It is also important understand how the anterior hypothalamic/preoptic area connects with the system associated with aversive responses within the brain. The laterodorsal tegmental nucleus (LDT) is found within the midbrain and is one of the main cholinergic nuclei of the brainstem reticular system. This ascending cholinergic system is critically important for directly influencing limbic regions involved in regulation of behaviour, particularly aversive behaviour (Brudzynski, 2007). It is thought that mostly external stimuli are responsible for the activation of this system. Cornwall, Cooper and Phillipson (1990) used anterograde and retrograde lectin tracers and determined that the hypothalamic/preoptic area had both afferent and efferent connections with the LDT. Therefore, it seemed likely that activation of the LDT led to activation of the anterior hypothalamic/preoptic areas, which led to the behavioural effect of the rat emitting 22 kHz vocalizations.

Researchers tested this idea in a study done by Brudzynski and Barnabi (1996). The investigators performed intracerebral injections of 67.6 μg of L-glutamate, an excitatory neurotransmitter, directly into the LDT. They also performed injections of 1 μg of carbachol into the anterior hypothalamic/preoptic area to serve as a positive control. The injections of L-glutamate into the LDT, the source of cholinergic projections, were able to induce 22 kHz vocalizations. Furthermore, the acoustic parameters of the calls were not significantly different from the calls induced by the carbachol control from the target areas. To give further evidence that the 22 kHz calls induced by the activation of the LDT were produced by the ascending cholinergic system, the researchers performed another experiment. They first injected 2 μg of scopolamine, a cholinergic antagonist, into the anterior hypothalamic/preoptic area. This would

effectively block most muscarinic cholinergic receptors in the area. Next, they injected L-glutamate into the LDT. The researchers found that when the hypothalamic/preoptic area is pre-treated with scopolamine, injecting L-glutamate into the LDT was unable to induce 22 kHz vocalization as compared to the saline control. From this finding, one can conclude that the injection of L-glutamate into the LDT did activate the ascending cholinergic system, which in turn induced the rat to vocalize with 22 kHz calls (Brudzynski & Barnabi, 1996).

It is noteworthy that the previous study only investigated the efferent connections of the LDT to the hypothalamic/preoptic area and not the afferent ones. For even a greater understanding of this system, Brudzynski, Iku, and Harness (2011) performed a study to observe if the afferent connections from the LDT to the hypothalamic/preoptic area played a role during 22 kHz vocalizations. They investigated this by injecting 1 μ g of carbachol into the hypothalamic/preoptic area to induce 22 kHz calls and then killed the rats 90 minutes later. Subsequently, expression of c-Fos and ChAT were studied histochemically in the LDT. C-Fos expression is a useful marker for metabolic activity within a cell and ChAT is an enzyme used to synthesize acetylcholine and therefore an indication of cholinergic neurons. The researchers found that c-Fos expression within the LDT was 2 times higher than control, and 2.5 times more active cholinergic neurons (doubled-labeled in LDT) were observed in rats that had prolonged 22 kHz vocalizations after carbachol injections compared to rats that did not vocalize or vocalized very little from the carbachol injections or the saline control injections (Brudzynski, Iku, and Harness, 2011). In addition to the involvement of LDT cholinergic neurons in this response, this evidence points towards a possible positive feedback loop from the hypothalamic/preoptic area to the LDT. If the initial injection of carbachol into the hypothalamic/preoptic area can cause an

aversive behavioural change, then positive feedback may activate the whole aversive system, prolonging the effects of the initial drug (Brudzynski, Iku, and Harness, 2011).

Ultrasonic 50 kHz Rat Vocalizations

Unlike 22 kHz vocalizations, the 50 kHz rat vocalizations are heavily correlated with behavioural situations of positive affect. The acoustic characteristics of these calls include a peak frequency between 35 and 72 kHz, a bandwidth varying between 5 and 7 kHz, and a call duration between 30 and 50 ms. Furthermore, there are two distinct sub-types of 50 kHz calls based on a visual representation of the call structure in a sonogram. The first sub-type has been named a flat call. These calls maintain a constant frequency throughout the call, and their visual representation resembles a straight line. The second sub-type has been named a frequency modulated call. These calls have frequencies that change throughout the call. They have many different visual representations, including inverted U's, sweeping lines or a rapid step from one frequency to another with trills.

It had been hypothesized that 50 kHz vocalizations represent a state of appetitive motivation and reward anticipation in rats (Brudzynski and Pniak, 2002, Burgdorf, Knutson, and Panksepp, 2000). Studies such as one by McIntosh and Barfield (1980) have recorded 50 kHz vocalizations during sexual behaviour between rats. The production of these 50 kHz calls was correlated with the level of sexual motivation. The researchers suggested that 50 kHz vocalizations served for the coordination of the mating behaviours. However, 50 kHz vocalizations have also been implicated in many same sex social encounters.

Brudzynski and Pniak (2002) attempted to provide a strong connection between the production of 50 kHz calls and actual or potential social contact with adult rats. Three

experiments were carried out to provide evidence for such a connection. The first experiment had 28 adult male rats being placed individually in a soiled recording cage from another rat for 10 min per day over the course of four days. Vocalizations were recorded during this time. The recording cage was only roughly cleaned between rats, only removing feces and hair, and not washing the cage or removing the paper flooring, thus preserving the natural odours. The order in which the rats were placed into the cage was changed each day. A separate control group of 20 rats was also recorded over four days, with each rat being placed in a fresh clean recording cage that had not be visited by other rats. The results from the experiment showed a significant increase in 50 kHz call production from day one to day four, but only in the soiled cage. Furthermore, the control group showed a significant decrease in 50 kHz call production from day one to day four. These results suggested that the increased production of 50 kHz calls was caused by olfactory stimuli left by previously visiting rats. Therefore, traces of olfactory scent may play an important role as positive social cues (Brudzynski & Pniak, 2002).

A second experiment was carried out by Brudzynski and Pniak (2002) to further demonstrate that olfactory stimuli were the driving force behind increased 50 kHz call production. Three tests were run a week apart from each other using three different recording cage conditions with 24 adult male rats. The first test was a double recording cage condition where for the first three days each rat was individually placed in a recording cage for 10 min. There were twelve recording cages where the same two rats visited the same cage every day. The second test was a common cage condition where all 24 rats visited the same cage each day. The third test was a single cage condition where each rat visited its own recording cage each day, which required 24 separate recording cages. Again, the recording cages were not washed to allow the olfactory stimuli from other rats to remain. During the fourth day of testing under each

condition, rats were individually placed in their cage condition for 10 min and their vocalizations were recorded. During the single cage condition, the average 50 kHz call production was less than one call per 10 min. The double cage condition produced an average of 37 calls per 10 min. Finally, the common cage condition had rats calling an average of 82 times per 10 min. Since the common cage condition produced over double the number of 50 kHz calls than the double condition, it is suggested that during the anticipation of social contact through olfactory stimuli, the number of 50 kHz calls produced by rats is proportional to the number of possible social contacts. These results provided further evidence of 50 kHz calls being associated with social contact (Brudzynski & Pniak, 2002).

The third experiment by Brudzynski and Pniak (2002) looked at the possible production of 50 kHz calls during direct non-agonistic social contact. It was not known if 50 kHz calls only appeared during the anticipation of social contact. The question was whether calls would continue during direct contact between rats. The experiment divided up 36 adult male rats into two groups, host or guest. The host rat was placed in an observation cage for 10 min and his vocalizations were recorded. After 10 min had passed, the guest rat was then placed within the same observation cage with the host rat and vocalizations were recorded for another 10 min. The next day the same pairs of rats were retested; however, the guest rat became the host and *vice versa*. The results showed an increase of more than five-fold of 50 kHz vocalizations when the guest rat was placed in the cage with the host rat compared to the host rat being alone. The same increase in 50 kHz calls was also observed when the pairs of rats switched roles (Brudzynski & Pniak, 2002). During the paired vocalization recording, however, it was impossible to distinguish which rat was vocalizing. It was assumed that if both rats called equally, then both called over twice the amount when compared to their control recording. The dramatic increase in 50 kHz

calls with the addition of a guest rat into a cage provides clear evidence that the production of 50 kHz vocalizations is maintained and continued during direct social contact. Furthermore, the highest number of 50 kHz calls was recorded during the direct social contact experiment, as compared to the anticipatory olfactory stimuli experiments.

Beyond the anticipation of social contact or direct social contact, many other studies have implicated 50 kHz calls in appetitive behaviours. Knuston, Burgdorf, & Panksepp (1998) looked at possible 50 kHz calls produced by juvenile rats during rough and tumble play. It is very common for juvenile rats to play fight among themselves, and it is very rare for any injuries to occur during the play (Pellis, Field, Smith, & Pellis, 1997). Generally, the main objective during play fighting is to gain an advantage over the opponent by contacting a target area on the opponent's body and simultaneously avoid being contacted (pinned). One main hypothesis of the function of rough and tumble play is to develop adult skills needed later in life, such as actual combat skills (Pellis, Field, Smith, & Pellis, 1997). The authors recorded vocalizations of 13 pairs of juvenile rats over three days in two different conditions. During the first condition, two rats were paired together, and were allowed to play for 120 s while their vocalizations were recorded. Each day the rats faced new partners to play with. During the second condition, the rats were isolated in a control chamber for 120 s, again having their vocalizations recorded. Results showed a significant increase in the number of 50 kHz calls in days two and three during the rough and tumble play compared to the combined control calls of the rats. This provides further evidence of 50 kHz calls being involved in appetitive play behaviour (Pellis, Field, Smith, & Pellis, 1997).

Another experiment in the same study by Pellis et al. (1997) examined whether 50 kHz calls would be produced by juvenile rats if they were placed in a familiar play fighting

environment while alone. There were 20 juvenile rats that again over three days went through two trial conditions, playing with a partner for 300 s in play chamber, and being alone in a control chamber for 300 s. On the fourth day, vocalizations from rats were recorded for 30 s when they were alone in both the play and control chambers. There was a significant increase in 50 kHz vocalizations in the play chamber compared to the control chamber. These results coincide with those found in the study by Brudzynski & Pniak (2002), where 50 kHz calls were observed in the anticipation of social conditioned behaviour.

Knutson, Burgdorf & Panksepp (1998) also investigated whether motivation for play was a factor for increasing 50 kHz calls. In their experiment, 16 juvenile rats were placed into two groups. One group lived in a cage with their mother and litter mates, the other group were weaned and individually caged. The rats were paired with an unfamiliar partner within the same condition in a play chamber for 120 s while their vocalizations were recorded. The results showed that the isolated rats had significantly more 50 kHz vocalizations during play than the socially housed rats. These results point towards a connection between motivation to play and 50 kHz calls. This evidence further points to the idea that 50 kHz calls are involved in appetitive behaviour.

A review article by Knutson, Burgdorf & Panksepp (2002) on ultrasonic vocalizations being an indicator for affective states on rats also referenced several other studies that provide evidence for an increase in 50 kHz call production during appetitive behaviours. These behaviours include the presence of an estrous female rat or estrous female odor for males, tickling by familiar humans (mimicking rough-and-tumble play), tickling cues, and food cues. The review article also summarizes evidence that when rats are faced with aversive stimuli or predators, no increases in 50 kHz vocalizations are observed, and generally they are decreased

instead. The authors of the review article conclude that there is enough research and evidence to support the claim that ultrasonic vocalizations in rats are a reliable indicator for affective states. Although it is impossible to definitively know the emotional subjective state of a rat, it is clear that 50 kHz calls are correlated with appetitive and approach behaviours and that 22 kHz calls are correlated with aversive and avoidance behaviours.

Pharmacological Initiation of 50 kHz Rat Vocalizations

Researchers have also extensively looked into the pharmacological mechanisms behind the initiation of 50 kHz vocalizations. The mesolimbic dopaminergic system within the brain has been heavily connected to positive affect and reward states. Particularly, dopaminergic connections running from the ventral tegmental area (VTA) to the nucleus accumbens are involved in the rewarding and addicting effects of drugs of abuse. Since behavioural studies pointed towards the idea that the production of 50 kHz vocalizations was involved in appetitive and reward states, it was likely that the initiation of 50 kHz vocalizations could occur from the mesolimbic system. A review by Alcaro, Huber & Panksepp in 2007 suggested that the behavioural functional role of the mesolimbic dopaminergic system was to activate a seeking behaviour towards any appetitive stimuli.

The VTA has several regions inside of it. In particular, there are several distinct subnuclei that have been identified by the presence of dopamine. Staining for tyrosine hydroxylase (TH), an enzyme synthesizing dopamine, can identify rich dopaminergic cell bodies. It has been found that the middle two-thirds of the VTA contain high amounts of TH-positive cell bodies (Hause & Shammah-Lagnado, 2002). This area has been broken down into two separate zones, the paranigral nucleus (PN) and the parabrachial pigmented area (PBP) (Hause & Shammah-

Lagnado, 2002). The posteromedial VTA, mainly within the PN, seems to be highly involved in drug rewarding effects. Researchers have found that rats will learn to self-administer drugs of abuse such as nicotine, opiates, cannabinoids, cocaine and ethanol into the posteromedial VTA but not the anterior VTA. It is most likely that most of these drugs do not directly target dopaminergic neurons, but will cause increased firing of dopamine neurons within the VTA (Wise & Bozarth, 1987).

There have also been studies researching projections from the VTA and they have found connections with the nucleus accumbens. A study by Beckstead, Domesick & Nauta in 1979 delivered isotopes of proline and leucine through microelectrophoresis into the VTA to map projection patterns using autoradiography. They found that the medial forebrain bundle leaving the VTA innervated the striatum region, including the nucleus accumbens. Furthermore, a study by Phillipson and Griffiths in 1985 used the retrograde transport of unconjugated wheatgerm agglutinin to further study the inputs into the nucleus accumbens. The researchers found a mediolateral topography in the efferent connections from the VTA to the nucleus accumbens (Phillipson & Griffiths, 1985). This data is also consistent with that indicating that the anterior and posterior VTA are responsible for different functions.

The anatomy of the nucleus accumbens also has two distinct sub-structures, referred to as the shell and the core. These two areas differ in both structure and function. Neurons in the shell of the nucleus accumbens have fewer dendrites and smaller dendritic arborization, fewer terminal segments and smaller spine densities when compared to the core (Meredith, Agolia, Arts, Groenewegen & Zahm, 1992). The shell of the nucleus accumbens receives strong dopaminergic connections from the posteromedial VTA, yet little connections from the anteromedial or lateral VTA, consistent with the known dopamine reward circuitry of the VTA

(Ikemoto, 2007). The afferent connections of the core of the nucleus accumbens are more similar to the lateral shell than to the medial shell.

A study by Heimer, Zahm, Churchill, Kalivas and Wohltmann in 1991 researched the differences of the projection patterns between the core and shell of the nucleus accumbens of rats. They accomplished this by injecting leucoagglutinin, horseradish peroxidase and fluorescent tracers into both the core and shell regions of the nucleus accumbens, and observing the anterograde transport of the injection types. Both regions were found to project to ventral pallidum as well as mesencephalic areas such as the substantia nigra as well as the ventral tegmental area. However, the core only projects into parts of the lateral hypothalamus whereas the shell has diffuse projections into all of the lateral hypothalamus. Furthermore, the shell exclusively projects onto the extended amygdala (Heimer *et al.*, 1991).

An effective method for researchers to study the production of 50 kHz ultrasonic calls in rats is to pharmacologically manipulate the dopaminergic mesolimbic system. Studies such as one done by Wintink and Brudzynski in 2001 looked at the effects of systemically administered amphetamine on the production of 50 kHz vocalizations. Amphetamine is a stimulant drug commonly used for its ability to increase wakefulness, maintain mental attention, increase motivation, and decrease appetite. Amphetamine is also a commonly abused street drug due to its ability to induce euphoria in the user. Amphetamine operates on a cellular level on multiple sites and is classified as an indirect dopamine agonist (Sulzer, Sonders, Poulsen & Galli, 2005). First, amphetamine acts on the presynaptic dopamine transporter, DAT, where it reverses the flow of dopamine so that it moves out of the neuron into the synaptic cleft. Secondly, amphetamine can be uptaken into dopaminergic vesicles, displacing the dopamine from vesicle storage into the extracellular space (Sulzer, Sonders, Poulsen & Galli, 2005). The end result of these two actions

allows for release of dopamine and an excess of this transmitter within the synaptic cleft, creating increased dopaminergic activity. The researchers in the study injected amphetamine at dosages of 1.5, 2, and 2.5 mg/kg intraperitoneally. They found all three dosage caused a significantly increased number of 50 kHz calls emitted, as compared to the saline control (Wintink & Brudzynski, 2001).

Researchers also wanted to directly observe the interaction between dopaminergic agonists and the mesolimbic system. A comprehensive mapping study done by Thompson, Leonard, & Brudzynski in 2006 looked at the ability of amphetamine to induce 50 kHz calls when injected into the nucleus accumbens and neighbouring areas. The study produced a dose-response curve where amphetamine doses of 5.0, 7.0, and 10.0µg injected bilaterally into the nucleus accumbens produced a significant increase in 50 kHz vocalizations over a 5 minute period when compared to baseline. The maximal response occurred at 10.0µg, where rats called an average of 26 times higher than baseline. However, since there was no significant difference in the increase in 50 kHz calls among the 5.0, 7.0 and 10.0µg doses of amphetamine, the 7.0µg dose was determined to be the optimal dose to induce a response. A follow-up experiment within the study mapped out the degree (magnitude) of responses to 7.0 µg of amphetamine injected into many sites throughout the nucleus accumbens in 86 rats. It was found that though injections into both the core and shell of the accumbens produced a significant increase of 50 kHz calls compared to baseline, the responses induced from the shell were significantly greater than those from the core (Thompson, Leonard & Brudzynski, 2006). This result agrees with previous research that stated that the shell of the accumbens is more involved with the dopamine mesolimbic reward system than the core (Rodd-Henricks, McKinzie, Li, Murphy & McBride, 2002).

The study also carried out subsequent experiments to provide evidence that the induced 50 kHz calls occurred because of amphetamine's actions on the dopamine system within the nucleus accumbens. The caudate-putamen, a structure situated dorsal to the nucleus accumbens, contains large amounts of dopamine D1 and D2 receptors. It was a possibility that the induced 50 kHz calls from the previous experiment in the study were caused by amphetamine diffusing into the caudate-putamen. To test this hypothesis, 20 rats were injected with 7.0 µg of amphetamine into the ipsilateral caudate-putamen. The results showed no significant increases in 50 kHz calls from baseline, therefore making the caudate-putamen a very unlikely structure to be involved in the production of 50 kHz calls. To show evidence that it was amphetamine's actions on dopamine receptors within the accumbens that induced 50 kHz calls, a final experiment was performed where the injection of 7.0 µg of amphetamine into the accumbens was pre-treated with equimolar amounts of either the D1 receptor antagonist SKF-83566 or the D2 receptor antagonist raclopride. Both SKF-83566 and raclopride antagonized the response and were able to attenuate the effects of amphetamine.

The results of this study also coincide with a previous study by Burgdorf, Knutson, Panksepp and Ikemoto in 2001. In a similar experiment, rats were unilaterally injected with amphetamine into the nucleus accumbens and the caudate-putamen. A dose-dependent response to the production of 50 kHz calls was also found when amphetamine was injected into the accumbens. Furthermore, injection of amphetamine into the caudate-putamen did not induce an increase of 50 kHz call production. Combining these two studies, results gives strong evidence to the idea that the dopamine system within the nucleus accumbens is heavily involved in the production of 50 kHz calls from rats.

Mesolimbic System and Dopamine Sub-Types

In the central nervous system, dopamine is known to play a role in locomotor activity, emotion, cognition, positive reinforcement, food intake and endocrine regulation (Missale, Nash, Robinson, Jaber & Caron, 1998). There are three main dopaminergic pathways in the mammalian brain: the nigrostriatal pathway, the mesocorticolimbic, and the tuberoinfundibular pathway (Civelli, Bunzow, Grandy, Zhou & Tol, 1991). The nigrostriatal pathway originates at the substantia nigra, which supplies the striatum with dopaminergic inputs. The striatum has an important role in regulation of locomotor activity and movement. The mesocorticolimbic pathway begins within the ventral tegmental area and projects into the limbic forebrain and prefrontal cortical areas (mesocortical component) and to the nucleus accumbens (mesolimbic component). This pathway is involved in emotional stability as well as positive reinforcement and reward. Finally, the tuberoinfundibular pathway begins within the hypothalamus and connects to the pituitary. This pathway is involved in endocrine regulations and lactation (Civelli, Bunzow & Grandy, 1993).

As of now, there are five known sub-types of dopamine receptors. The first distinction of receptors was made by Keibadian and Calne (1979), mentioning two different sub-types, D1 and D2. These two receptors differed based on their pharmacology and physiology (Civelli, Bunzow, Grandy, Zhou & Tol, 1991). Many dopamine agonists and antagonists have affinities that differ between D1 and D2 receptor sub-types. Furthermore, although all dopamine receptors are coupled to G proteins, their 2nd messenger effect differs between receptor sub-types. Activation of the D1 receptor sub-type will activate the Gs complex which will lead to an increase in cAMP levels, which in turn will lead to stimulation of adenylyl cyclase activity (Missale, Nash, Robinson, Jaber & Caron, 1998). However, activation of the D2 receptor sub-type activates the

Gi complex, which leads to a decrease in cAMP levels and, therefore, an inhibition of adenylyl cyclase activity.

Division into two dopamine receptor sub-types remained until gene cloning revealed another three sub-types of dopamine receptors, D3, D4 and D5 (Missale, Nash, Robinson, Jaber & Caron, 1998). Both D3 and D4 receptors have been classified as D2-like receptors because both receptors will also lead to a decrease in cAMP levels. The D5 receptor is classified as a D1-like receptor because it increases levels of cAMP. The D3 and D4 receptor sub-types are physiologically different enough from the D2 receptor to have agonists and antagonists that can be more discriminatory towards them. However, the D5 receptor is so physiologically close to the D1 receptor that currently no agonist or antagonist exists that can discriminate between the two.

It is also possible for the different dopamine receptor sub-types to form heterodimers with each other. Perreault *et al.* (2010) observed D1 and D2 receptors forming heterodimers throughout the basal ganglia and the nucleus accumbens. This heterodimer receptor was found to be coupled to the G complex Gq/11, which in turn activated phospholipase C. Increased activity of phospholipase C leads to intracellular calcium release. Interestingly, the D1-D2 heterodimers were only found on pre-synaptic terminals. Maggio and Millan (2010) examined heterodimers formed by D2 and D3 receptor sub-types. The D2-D3 heterodimers have been detected in both the globus pallidus and in the nucleus accumbens. Furthermore, they have been localized to both pre-synaptic and post-synaptic neurons. The D2-D3 heterodimers also inhibit adenylyl cyclase like any D2-like sub-type receptor.

Rationale and Hypothesis

The current pharmacological literature on 50 kHz rat vocalizations has been focused on simply inducing 50 kHz. However, it has not been investigated if using different dopamine agonists to induce the calls could have an influence on the call characteristics as well. Studies also have been done in an attempt to classify sub-types of 50 kHz calls. Wright, Gourdon and Clarke (2010) injected 0.25-2 mg/kg of amphetamine subcutaneously into adult Long-Evans rats to induce 50 kHz ultrasonic vocalizations in order to analyse the acoustic characteristics of the calls. Furthermore, rats were also alone or paired with another rat during the injections to compare if adding a social testing condition would affect the total number of calls as well as the characteristics of the calls. The authors identified 14 different sub-types of 50 kHz vocalizations based only on their sonographic appearance. However, there was no significant difference in the overall call parameters between injection groups.

Brudzynski, Komadoski, and St. Pierre (2011) examined the drug, quinpirole, and its effects on the production of 50 kHz vocalizations. The drug quinpirole is classified as a D2/D3 agonist, with a higher selectivity to D2 receptors. A full dose-response curve was carried out for both a low dosage range and a high dosage range of quinpirole. Quinpirole was injected intracerebrally into the shell of the nucleus accumbens of male Wister rats. For the low dose range, the researchers used quinpirole doses of 0.06, 0.12, 0.25, 0.5 and 1.00 µg, and for the high dose range they used doses of 0.5, 3.0, 6.0, 10.0, 20.0 µg. Furthermore, they also used 7.0 µg of amphetamine as a positive control. The researchers found that quinpirole worked on a triphasic level, where it had two peak maximal effects on vocalizations at dosage levels of 0.25 and 6 µg. Both of these dosages caused rats to produce significantly more 50 kHz vocalizations than the

saline control. Furthermore, the responses to quinpirole were also similar to those of positive control injections of amphetamine.

Brudzynski *et al.* (2011) were also interested in the role of both D2 and D3 receptors in the production of 50 kHz calls during quinpirole injections. To test their roles, the researchers attempted to block the ability of quinpirole to elicit 50 kHz calls by pretreating the injectionsites with two different dopamine antagonists, raclopride or U-99194A maleate (UM). Raclopride has a high affinity for D2 receptors and is mainly classified as a D2 antagonist. Therefore, raclopride will block actions of any dopamine agonist onto D2 receptors. On the other hand, UM has a higher affinity for D3 receptors than D2 and is classified as a D3 antagonist, blocking actions onto D3 receptors. Interestingly Brudzynski *et al.* (2011) found that pretreating the low 0.25ug dosage of quinpirole with raclopride did not significantly reduce the number of 50 kHz vocalizations recorded from the rats. However, pretreating with UM did significantly reduce the number of 50 kHz vocalizations produced by the rats. These data suggested that at low dosages, quinpirole was mainly acting on D3 receptors to produce the increase in 50 kHz vocalizations. However, at the high quinpirole dosage (6 µg), raclopride significantly reduced 50 kHz vocalization, whereas UM was ineffective in doing so. Therefore, it is likely that at higher dosages, quinpirole was acting on D2 receptors that produce the 50 kHz calls. This study, however, was not focused on the call characteristics themselves and how they changed in response to the different drugs that induced 50 kHz calls.

The purpose of the present study is to investigate the variation in acoustic characteristics of rat 50 kHz vocalizations through the injection of dopamine agonists into the shell of the nucleus accumbens. Though it is well understood that the dopamine system within the nucleus accumbens is critical in the production of 50 kHz calls, it is not understood why there is such

variation in the acoustic characteristics of 50 kHz calls, or what role dopamine and its receptors play in this variation. To begin to investigate this variation, two general dopamine agonists, amphetamine and apomorphine, as well as dopamine itself were injected into the shell of the nucleus accumbens to induce 50 kHz vocalization. Since all drugs do not interact with receptors in the exact same way, it was predicted that even dopamine agonists with similar receptor interactions would generate variations in the acoustic characteristics of 50 kHz vocalizations.

There were three hypotheses that were formulated for this research. The first was that dopamine and two dopamine agonists would induce substantially the same type of 50 kHz vocalizations typical for the species, when directly injected into the nucleus accumbens shell. Second, application of dopamine agonists, amphetamine, apomorphine, and dopamine itself would produce 50 kHz vocalizations that differ at least in source acoustic parameters. Finally, the application of dopamine and dopamine agonists should induce 50 kHz vocalizations that differ in the proportions of subtype of calls, depending on the agonist injected.

METHODS

Animals

Thirty-four adult male Long Evans rats (*Rattus norvegicus*) were used during this study and were obtained from Charles River Laboratories in Quebec. There were two groups of twelve rats and one group of ten rats (total 34 animals). Upon entering the lab, rats were housed two per cage on a 12:12 hr light/dark cycle. The plastic cages were 460 cm long and 250 cm wide. All cages contained corn cob dust-free bedding, PVC tubing for hiding, paper towels, and a wood stick for enrichment. All rats were given rat pelleted food and water *ad libitum*. All rats were handled prior to their surgeries to habituate them to human contact and were adapted to being wrapped in a towel for future injections. Surgeries were carried out a week after the arrival of the rats to ensure that all rats were healthy and free of infections or other diseases. Pre-surgical weights of rats varied from 272 to 364 g. Post-surgical rats were moved to single cages and had a metal grate in their cages instead of bedding for a week to protect their wounds from the bedding. All procedures were approved by the Brock University Animal Care and Use Committee and followed the guidelines of the Canadian Council on Animal Care and Use.

Stereotaxic Surgery

The first group of twelve rats were anaesthetized using intraperitoneal injections of ketamine at a dosage of 40 mg/kg and xylazine at a dosage of 6 mg/kg. The subsequent two groups of rats were anaesthetized through inhalation of isoflurane at 0.25-4.0%. Prior to beginning the surgery, all rats were giving subcutaneous injections of the antibiotic Tribissen (trimethoprim and sulfadiazine, 1:5 ratio, Merck Animal Health, Whitehouse Station, NJ,) at a dosage of 40 mg/kg, and the analgesic of Metacam (meloxicam, Boeringer Ingelheim, Germany)

at the dosage of 2 mg/kg. A Kopf stereotaxic apparatus (David Kopf Instruments, Tujunga, CA) was used to hold and perform surgeries on the rats. Stainless steel guide cannulae were constructed from 23 and ½ gauge needles (Becton-Dickinson and Co., NJ, U.S.A.) and were implanted bilaterally into the shell of the nucleus accumbens 1mm above the injection site. The coordinates for the shell of the nucleus accumbens were determined to be A = 10.6 mm from the interaural plane, L = 0.8 mm from the midline, and V = 5.7 mm from the surface of the brain by using the stereotaxic atlas by Paxinos and Watson (Paxinos & Watson, 1986). Self-curing dental acrylic (DenPlus Inc., Boucherville, Quebec) and stainless-steel screws were used to construct a skull cap which held the guide cannula in place and to prevent infection. Rats were given one week for recovery before experimental injections started. Post-surgical care involved a 3 ml injection of saline subcutaneously and topical antibiotic around the open wound.

Drugs and Injection Procedures

The injection of drugs was achieved by using a Hamilton constant-rate microsyringe (model CR 700-20, Hamilton Co., Reno, NV) connected to a stainless steel injection cannula via polyethylene PE-10 tubing (Intramedic, Becton-Dickinson and Co., Mississauga, ON). The injection cannula had a length of 16.0 mm, which was 1 mm longer than the guide cannula. This ensured the diffusion of the drugs occurred into the brain region of interest. Injections were carried out as a within-subjects design. Injections were spaced out, giving four days between injections in order for the tissue to recover from the previous injection and to avoid habituation effects. All injections were given in volumes of 0.3 µl, injected at a rate of 0.5 µl/min and were given unilaterally. Furthermore, the order of injections was properly counter-balanced. The injection cannula was left in the brain for 30 s after the termination of injection to allow the drug to fully diffuse from the cannula into the brain region.

The first group of rats received drug injections of 7.0 µg of amphetamine dissolved in saline, 20.0 µg of dopamine dissolved in saline, and saline itself as a control. The dosage of amphetamine was determined by a previous study by Thompson, Leonard & Brudzynski (2006) which found that 7.0 µg of amphetamine was the optimum dosage to induce 50 kHz calls. The ability of dopamine to induce 50 kHz calls had never been fully studied. A dosage of 20.0 µg of dopamine was estimated based on previous studies (Wachtel, Ahlenius, & Andén, 1979) to overcome the action of endogenous enzymes that break down dopamine such as the extracellular COMT or to overcome rapid reuptake by the transporter DAT. The second group of rats received injections of dopamine at 15.0 µg dissolved in saline, and saline alone to serve as a control. The third group of rat received injections of 7.0 µg of amphetamine dissolved in saline, 3.0 µg of apomorphine dissolved in saline with 0.1% ascorbic acid, and saline alone or saline with 0.1% ascorbic acid to serve as a control. The 0.1% ascorbic acid was required to dissolve the apomorphine in saline and increase drug stability. The dosage of 3.0 µg of apomorphine was determined from a previous study by Silkstone & Brudzynski (2013).

Vocalization Recording and Analysis

Immediately following the diffusion of the drug from the cannula into the brain region of interest, the injection was terminated, and the rats were placed in a new clear polycarbonate cage measuring 240 cm long by 210 cm wide. Cages had corn cob bedding placed on the bottom to simulate the rat's home cage. Furthermore, each rat received a new recording cage to avoid cages being contaminated with the previous rats' odour. A metal grate was placed on top of the recording cage to prevent the rat from escaping. A CM16/CMPA ultrasonic condenser microphone (Avisoft Bioacoustics, Berlin, Germany) was mounted onto the metal grate pointing downward into the recording cage. The output was recorded on a computer through the use of

the program Avisoft SAS Lab Pro (Avisoft). The microphone was set to detect frequencies between 20 kHz and 100 kHz with a sampling rate of 500,000 Hz in a 16 bit format. Each rat was kept in the recording cage for 10 min after injection to record possible vocalizations.

The individual calls identified from the computer output were analyzed for four different parameters: call duration in ms, call peak frequency in kHz, call bandwidth in kHz and type of call (frequency modulated or flat calls) shown in Figure 1. The call peak frequency was determined at the power spectrum as the frequency that had the highest dB level. Call bandwidth was the difference in frequency from the highest frequency to the lowest within a call. The number of all 50 kHz calls was scored from 10 minutes of each recording. Furthermore, all 50 kHz calls were analyzed for their peak frequency, call bandwidth, call duration and call sub-type. Three sonographic call types were distinguished: flat vocalization (no major changes in frequency), frequency-modulated vocalizations (major steps or rapid changes in frequency), and trills (frequency-modulated calls with sine wave-like changes). One sample T-tests as well as a one-way ANOVA were run to determine if there were significant differences in the average amount of 50 kHz calls between each injection condition and the vehicle control. Two-way between-subjects ANOVAs were run to determine if there were significant differences in any of the acoustic parameters. Kolmogorov-Smirnov tests for equal distributions were run to determine if the pharmacokinetic properties of each drug were significantly different. All statistical analysis was carried using the computer program SPSS Statistics 20 (SPSS Inc., Chicago, IL).

Histological Procedures

After all injections were completed, the rats were sacrificed with an overdose of sodium pentobarbital (Euthanyl, Bimeda-MTC, Cambridge, ON). Brains were transcardially perfused using a 10% formalin solution and stored in 10% formalin for at least 24 hours. Extracted and fixed brains were sectioned using a freezing microtome (Hacker Instruments & Industries, Winnsboro, SC) producing 50 μm sections. Following a modified procedure from Skinner (1971), the brain slices were delipidized in alcohol and stained with a water solution of thionine. The preparations were then differentiated in alcohol, cleared with xylene, covered with Permount mounting medium (Fisher Scientific Canada, Ottawa, ON) and then coverslipped. Localizations of injection sites were achieved by viewing the stained slides under a projection microscope. Diagrams based on the stereotaxic atlas by Paxinos and Watson were used to record the localizations of injection sites (Paxinos & Watson, 1986).

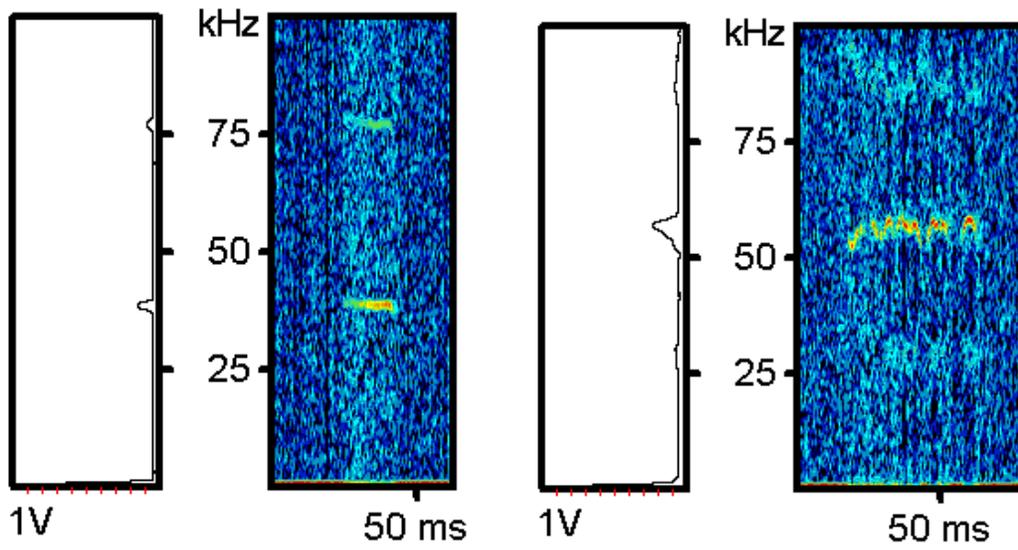


Figure 1: Example of a 50 kHz flat call (left) and of a 50 kHz FM trill call (right).

RESULTS

Localization of Injection Sites

The target brain region of interest was the shell of the nucleus accumbens. It was determined to be a successful injection location if the localization was inside or partly inside the shell of the nucleus accumbens. Out of the twenty-nine injection sites, fifteen were classified as successful injection locations. Only data from successful injection locations were used in the analysis. All localizations are shown in Figure 2.

Injection Results

The purpose of this study was to compare the acoustic characteristics of 50 kHz vocalizations from rats that were induced by three different dopamine agonists: amphetamine, dopamine and apomorphine. To be able to perform the analysis of the acoustic characteristics of calls, it must first be confirmed that the dopamine agonists were able to significantly induce more 50 kHz calls compared to vehicle controls. It was found that there were significant differences between the four injection conditions, $[F(3,38) = 8.144, p \leq 0.001]$ and these results are summarized in Figure 3a.

It was found that amphetamine was able to induce significantly more 50 kHz vocalizations compared to the vehicle control, $[t(12) = 3.013, p = 0.011]$. Amphetamine on average induced 14.00 calls/10 minutes, whereas the vehicle control average was 1.85 calls/10 minutes. Dopamine was also able to significantly induce more 50 kHz vocalizations compared to the vehicle control, $[t(5) = 3.122, p = 0.026]$. The dopamine condition contained results from six injections which had an average of 39.50 calls/10 minutes, whereas the vehicle control average

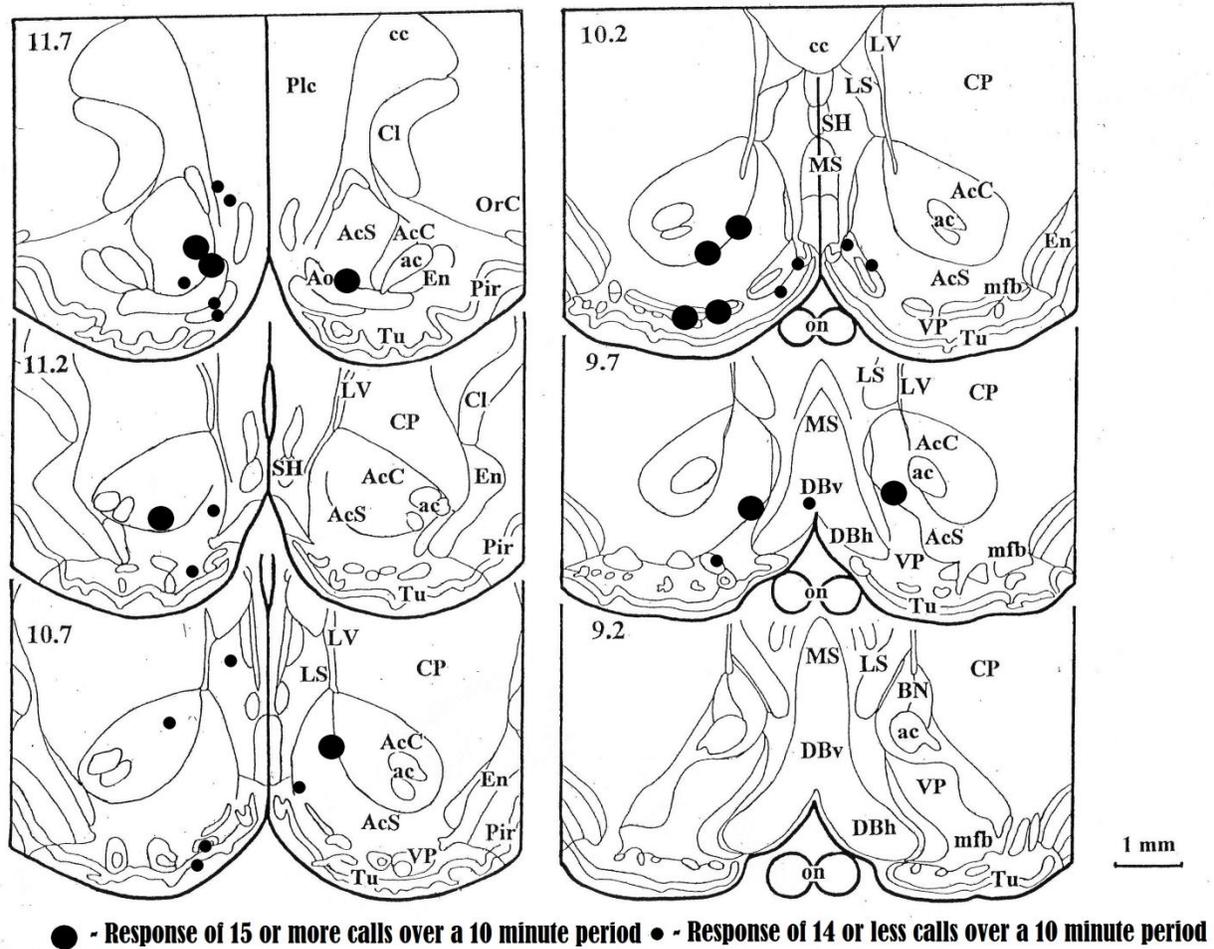


Figure 2: Localizations of injection sites of twenty five rats in the nucleus accumbens between the frontal stereotaxic planes AP = 11.7 – 9.2 mm according to the stereotaxic atlas by Paxinos and Watson (1986).

The 1 mm scale bar represents the scale for all four diagrams. ac: anterior commissure, AcC: nucleus accumbens core, AcS: nucleus accumbens shell, Ao: anterior olfactory nucleus, BN: bed nucleus of stria terminalis, cc: corpus callosum, Cl: claustrum, CP: caudate-putamen, DBh: horizontal limb of the diagonal band nucleus, DBv: vertical limb of the diagonal band nucleus, En: endopiriform nucleus, LS: lateral septal nucleus, LV: lateral ventricle, mfb: medial forebrain bundle, MS: medial septal nucleus, OrC: ventral orbital cortex, Plc: prelimbic cortex, SH: septohippocampal nucleus, Tu: olfactory tubercle, and VP: ventral pallidum.

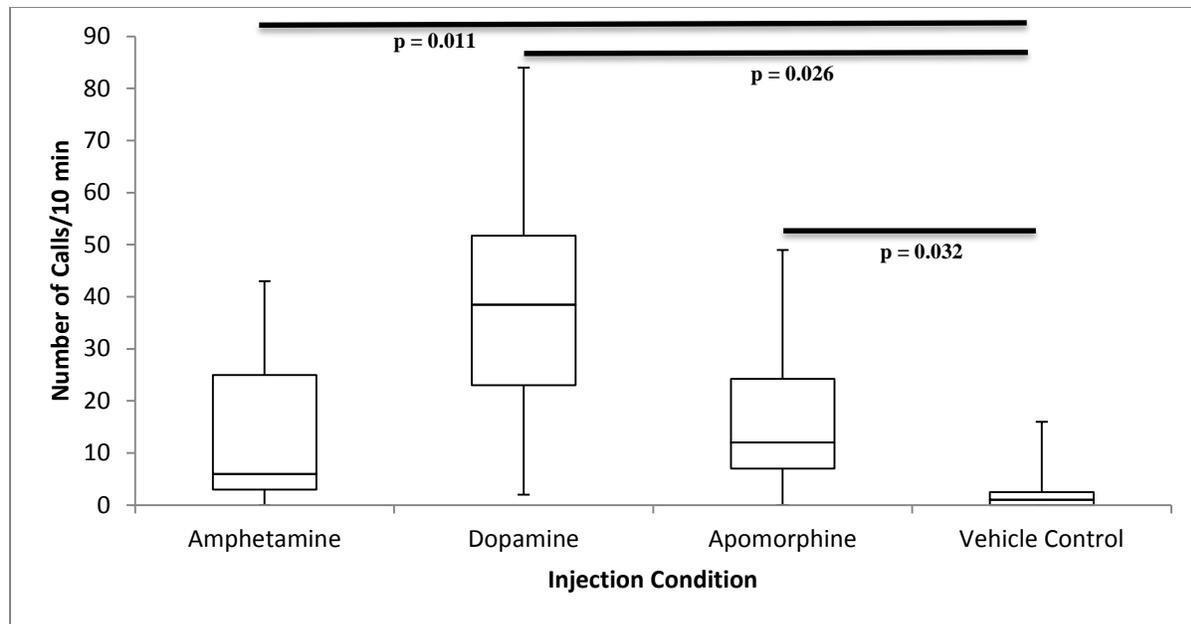


Figure 3a: Results of injections of 7 μg of amphetamine ($n=13$), 20 μg of dopamine ($n=6$), 3 μg of apomorphine ($n=8$) in the nucleus accumbens compared to vehicle controls ($n=15$) where n represents one injection with a 10 minute recording period. The total number of 50 kHz calls recorded were summed then averaged in each condition. A one-way ANOVA found a significant difference in the average call duration of 50 kHz calls between injection conditions [$F(3,38) = 8.144$, $p \leq 0.001$]. The whiskers in the figure represent the minimum and maximum values within the data. The lower line in the box represents the 25% percentile, the middle line in the box represents the median, and the upper line in the box represents the 75% percentile.

was 4.50 calls/10 min. This group, however, did contain an outlier. One rat called well over 200 times during ten minutes when injected with dopamine. The data from this dopamine injection were removed from any analysis. Finally, the apomorphine injections were also able to significantly induce more 50 kHz calls compared to the vehicle control, [$t(7) = 2.676$, $p = 0.032$]. Apomorphine on average induced 17.40 calls/10 minutes, whereas the vehicle control average was 1.63 calls/10 minutes. Therefore, since all three dopamine agonists were able to significantly induce more 50 kHz calls than the vehicle control, it is assumed the injection of the agonists was responsible for the increase in 50 kHz vocalizations.

To provide evidence, that injecting dopamine agonists only into the shell of the nucleus accumbens can induce the response, responses from the remaining fourteen trials, representing missed injection sites, were also statistically analyzed to observe if those injections were able to significantly induce 50 kHz vocalizations. Figure 3b summarizes the average number of 50 kHz vocalizations per ten minutes after injections of amphetamine, dopamine, apomorphine, and the vehicle control to missed sites. There were eleven injections of amphetamine, eight of dopamine, six of apomorphine, and fourteen of the vehicle control. No significant differences were found among any of these injection conditions (One-way ANOVA, [$F(3, 35) = 0.914$, $p = 0.444$, n.s.]). Therefore, these data provide further evidence that dopamine agonists induce 50 kHz vocalizations only when injected into the shell of the nucleus accumbens and to the neighbouring structures.

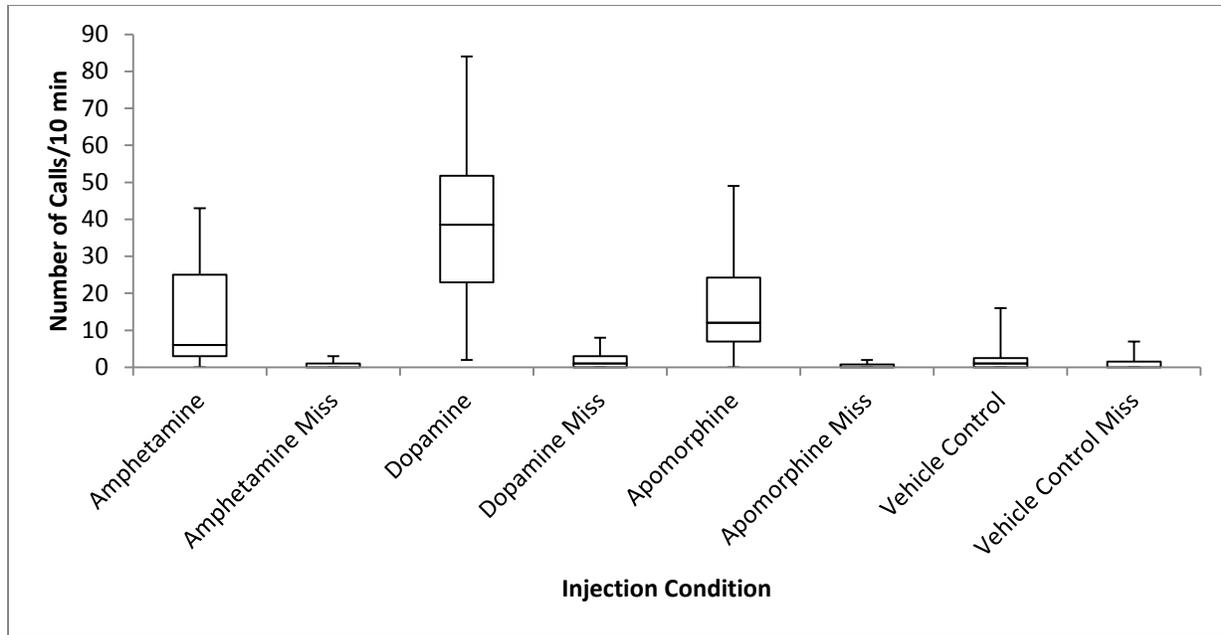


Figure 3b: Results of injections of 7 μg of amphetamine ($n=11$), 20 μg of dopamine ($n=8$), 3 μg of apomorphine ($n=6$), and the vehicle controls ($n=14$) that missed the nucleus accumbens in a side by side comparison to the injections that hit the shell of the nucleus accumbens, where n represents one injection with a 10 minute recording period.. The total number of 50 kHz calls recorded were summed then averaged in each condition. A one-way ANOVA found no significant difference in the average number of 50 kHz calls between the missed injection conditions, [$F(3, 35) = 0.914, p = 0.444, n.s.$].

Acoustic Characteristic Analysis

After confirming that dopamine and different dopamine agonists were able to induce more 50 kHz vocalizations than the vehicle control, an acoustic analysis of all the calls was carried out to test the hypothesis that injecting different dopamine agonists will alter the physical characteristics of the vocalizations. The three main physical characteristics measured were peak frequency, bandwidth, and duration. Figure 4a summarizes the average call peak frequencies for the four different injection conditions. A 4x14 (injection condition by individual rat) between-subjects ANOVA was carried out to determine if there was a significant difference among the peak frequencies of the injection conditions. Each rat was assigned a nominal value to represent each unique rat. There was a significant main effect for the individual rat [$F(13, 563) = 11.58, p \leq 0.001$], but there was no main effect for the injection condition [$F(3, 563) = 2.114, p = 0.097$]. A significant interaction was also found between the injection condition and individual rat [$F(16, 563) = 5.845, p \leq 0.001$].

Figure 4b summarizes the average call bandwidth for the four different injection conditions. Another 4x14 (injection condition by individual rat) between-subjects ANOVA was run to determine if there was a significant difference among the average bandwidths of the injection conditions. There was a significant main effect for the individual rat [$F(13, 563) = 3.409, p \leq 0.001$] and a significant main effect for the injection condition [$F(3, 563) = 2.979, p = 0.031$]. There was no significant interaction found between the injection condition and individual rat [$F(16, 563) = 1.618, p = 0.06$]. Furthermore, an LSD (Least Significant Difference) post-hoc comparison showed that calls after both the vehicle control ($p = 0.004$) and apomorphine ($p \leq 0.001$) injections had significantly higher average bandwidths compared to amphetamine. The vehicle control ($p \leq 0.001$) and apomorphine ($p \leq 0.001$) groups also had significantly higher

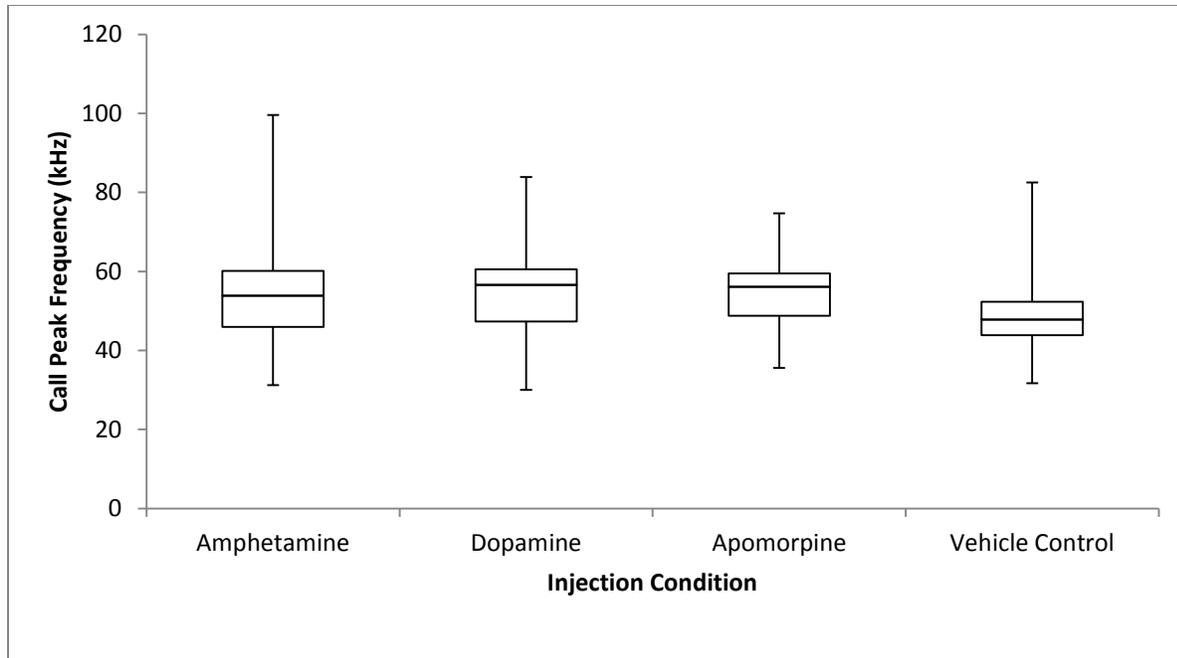


Figure 4a: Acoustic analysis of average call peak frequency in kHz for all injection conditions from recorded rat vocalizations. All peak frequencies from every 50 kHz call recorded were summed then averaged in each condition. A 4x14 (injection condition by individual rat) between-subjects ANOVA was conducted on call peak frequency. There was a significant main effect for the individual rat [$F(13, 563) = 11.58, p \leq 0.001$], but there was no main effect for the injection condition [$F(3, 563) = 2.114, p = 0.097, \text{n.s.}$]. A significant interaction was also found between the injection condition and individual rat [$F(16, 563) = 5.845, p \leq 0.001$].

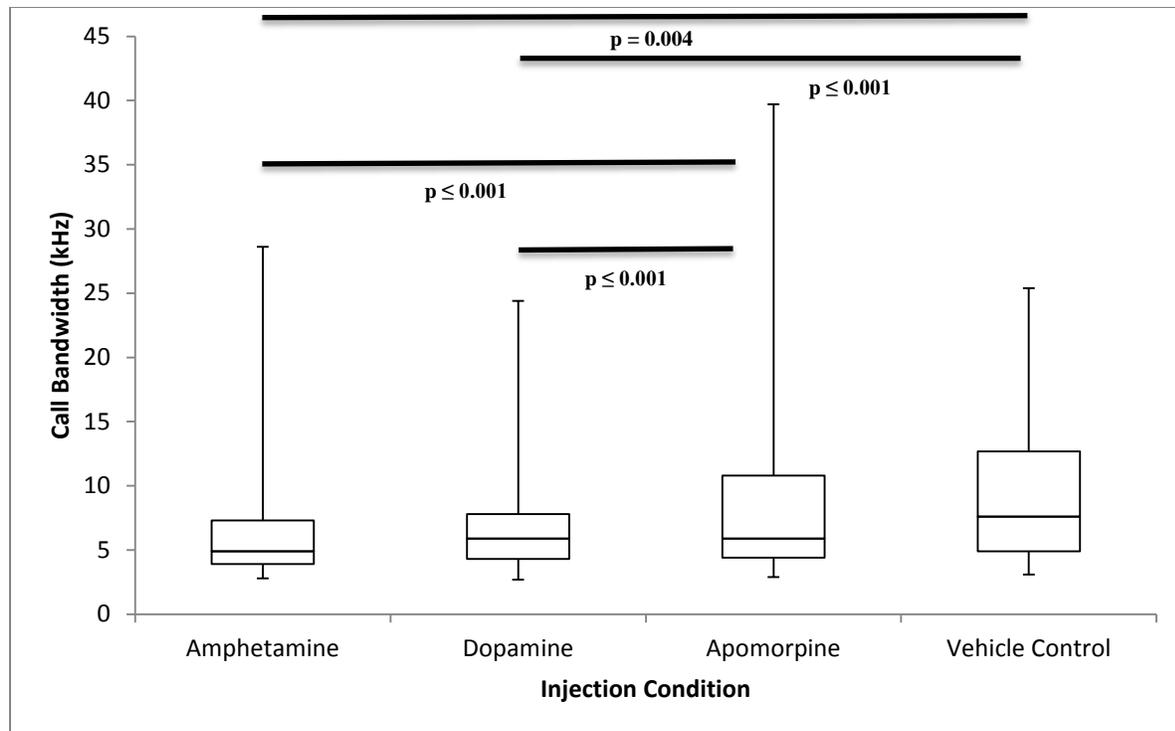


Figure 4b: Acoustic analysis of average call bandwidth in kHz for all injection conditions from recorded rat vocalizations. All call bandwidths from every 50 kHz call recorded were summed then averaged in each condition. A 4x14 (injection condition by individual rat) between-subjects ANOVA was conducted on call bandwidth. There was a significant main effect for the individual rat [$F(13, 563) = 3.409, p \leq 0.001$] and a significant main effect for the injection condition [$F(3, 563) = 2.979, p = 0.031$]. There was no significant interaction found between the injection condition and individual rat [$F(16, 563) = 1.618, p = 0.06, n.s.$]. A LSD post-hoc comparison revealed calls after both the vehicle control ($p = 0.004$) and apomorphine ($p \leq 0.001$) had significantly higher average bandwidths compared to amphetamine. Also, the vehicle control ($p \leq 0.001$) and apomorphine ($p \leq 0.001$) also had significantly higher average bandwidths compared to dopamine.

average bandwidths compared to the dopamine group. Figure 4c is a summary of the average call durations from the four different injection conditions. A final 4x14 (injection condition by individual rat) between-subjects ANOVA was carried out to determine if there was a significant difference among the average call durations of the injection conditions. There was a significant main effect for the individual rat [$F(13, 563) = 4.295, p \leq 0.001$] and a significant main effect for the injection condition [$F(3, 563) = 2.711, p = 0.044$]. There was also a significant interaction found between the injection condition and individual rat [$F(16, 563) = 2.273, p = 0.003$]. The final LSD post-hoc comparison revealed both dopamine ($p \leq 0.001$) and the vehicle control ($p = 0.004$) had significantly higher average call durations compared to amphetamine. Furthermore, dopamine ($p = 0.004$) and the vehicle control ($p = 0.028$) also had significantly higher average call durations compared to apomorphine.

Call Sub-Type Analysis

Beyond the physical characteristics of the ultrasonic vocalizations, a statistical analysis of the call sub-types was also carried out to test the hypothesis that dopamine agonists will differ in the ratio of 50 kHz call sub-types that were induced. Figure 5a is a summary of the average percent of calls that are frequency modulated compared to flat calls in each injection condition. A one-way ANOVA determined there was no significance difference in the average percent of calls that are frequency modulated among any injection conditions [$F(3, 31) = 0.313, p = 0.816$].

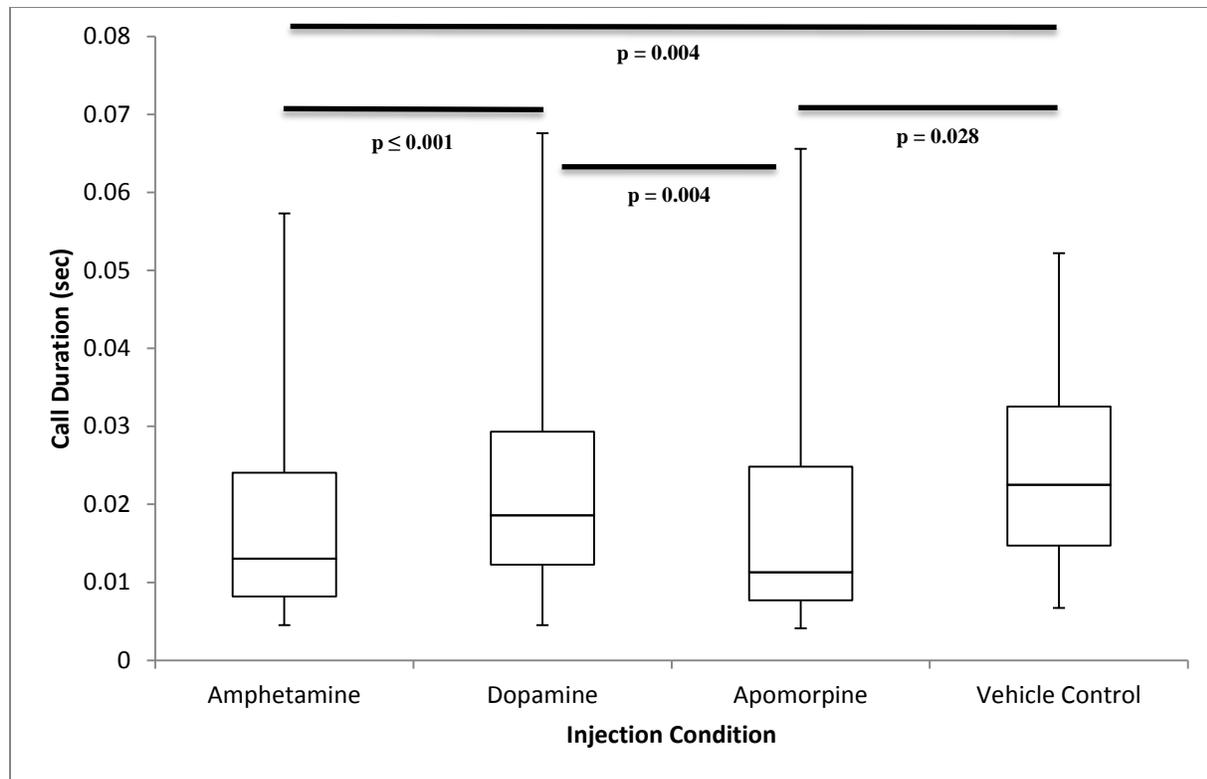


Figure 4c: Acoustic analysis of average call duration in kHz for all injection conditions from recorded rat vocalizations. All call durations from every 50 kHz call recorded were summed then averaged in each condition. A 4x14 (injection condition by individual rat) between-subjects ANOVA was conducted on call duration. There was a significant main effect for the individual rat [$F(13, 563) = 4.295, p \leq 0.001$] and a significant main effect for the injection condition [$F(3, 563) = 2.711, p = 0.044$]. There was also a significant interaction found between the injection condition and individual rat [$F(16, 563) = 2.273, p = 0.003$]. A LSD post-hoc comparison revealed both dopamine ($p \leq 0.001$) and the vehicle control ($p = 0.004$) had significantly higher average call durations compared to amphetamine. Furthermore, dopamine ($p = 0.004$) and the vehicle control ($p = 0.028$) also had significantly higher average call durations compared to apomorphine.

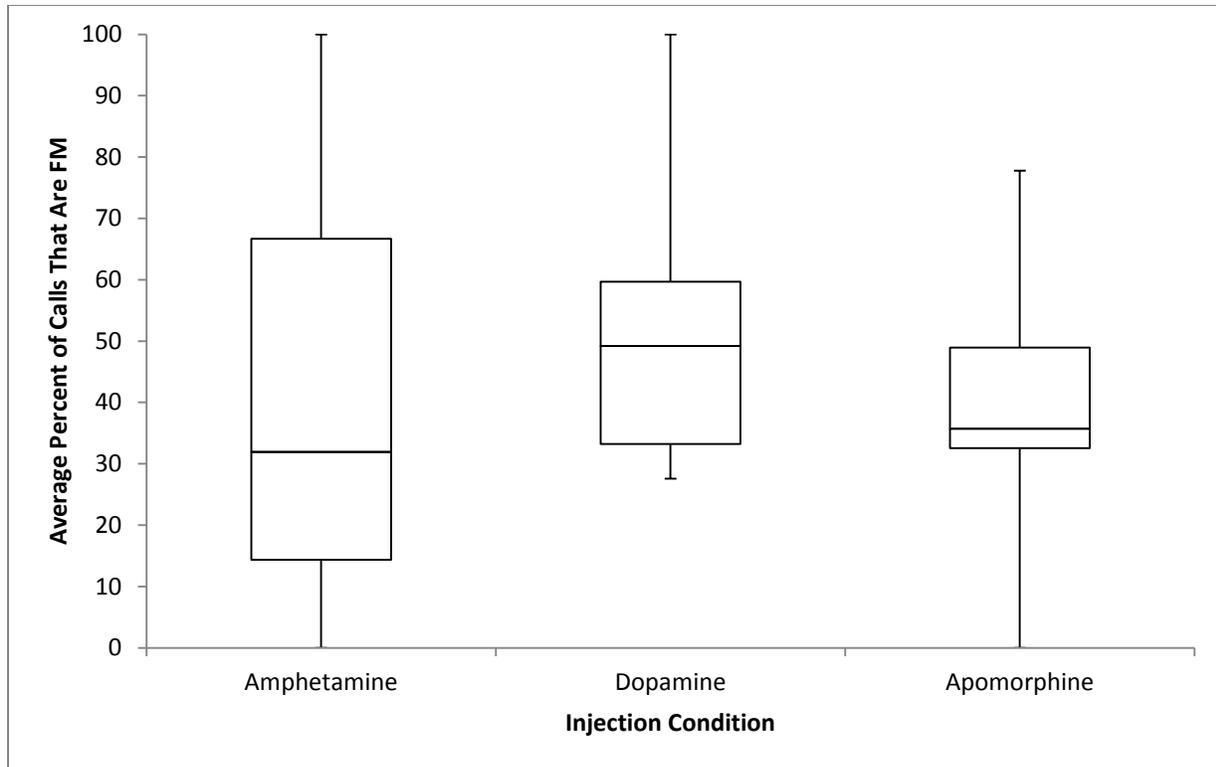


Figure 5a: Acoustic analysis of average percent of calls that are FM in all injection conditions from recorded rat vocalizations. All calls were classified into the appropriate 50 kHz sub-type, these call types were summed then averaged in each condition. A one-way ANOVA found no significance difference in the average percent of calls that are frequency modulated between any injection conditions [$F(3, 31) = 0.313, p = 0.816, n.s.$].

In an attempt to become more specific, a second one-way ANOVA was done to determine if the ratio of trill calls (any call with trill component of trill alone) within the frequency modulated sub-types differed between the injection conditions (Figure 5b). A one-way ANOVA determined there was no significance difference in the average percent of frequency modulated calls that are trills between any injection conditions [$F(2, 15) = 1.898, p = 0.184$].

Drug Pharmacokinetics Analysis

To test the final hypothesis that the injected dopamine agonists would have different pharmacokinetic properties when inducing 50 kHz calls, Kolmogorov-Smirnov tests (KS) were run to compare the time distributions of the average number of calls over the 10 minute period for each drug. Each call was placed into bins that corresponded with the appropriate time interval in 60 second bins. Then, each bin was summed to create a time distribution. KS tests determined that there were significance differences between the time distribution of 50 kHz calls between amphetamine and apomorphine ($p < 0.001$), amphetamine and dopamine ($p = 0.015$), and dopamine and apomorphine ($p < 0.001$). Figure 6a summaries the time distribution of 50 kHz calls for each drug condition. Figure 6b summaries the time distribution of 50 kHz calls based on a running total percentage of all calls within the 10 minute recording. Also, as a comparison for the relative amount of drug used in each injection condition, Table 1 indicates the number of moles used for each drug.

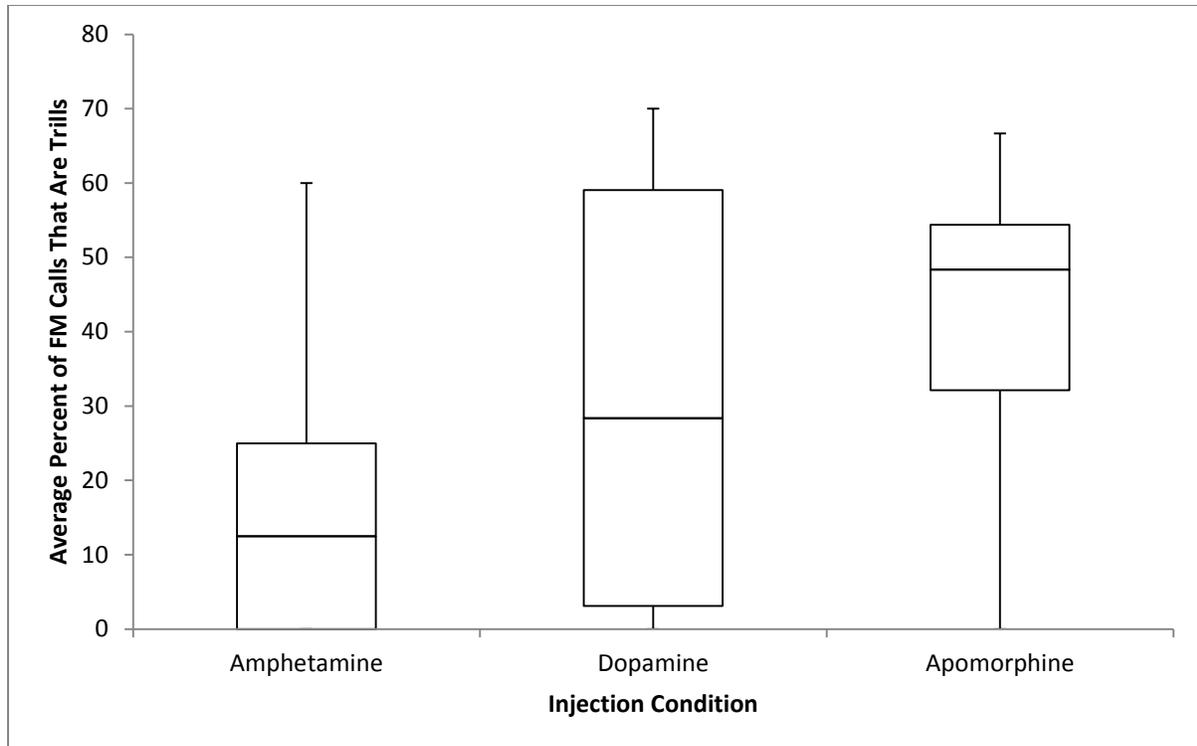


Figure 5b: Acoustic analysis of average percent of calls that are FM with trills in all injection conditions from recorded rat vocalizations. All calls were classified into the appropriate 50 kHz sub-type, these call types were summed then averaged in each condition. A one-way ANOVA determined there was no significance difference in the average percent of frequency modulated calls that have trill components or are trills between any injection conditions [$F(2, 15) = 1.898$, $p = 0.184$, n.s.].

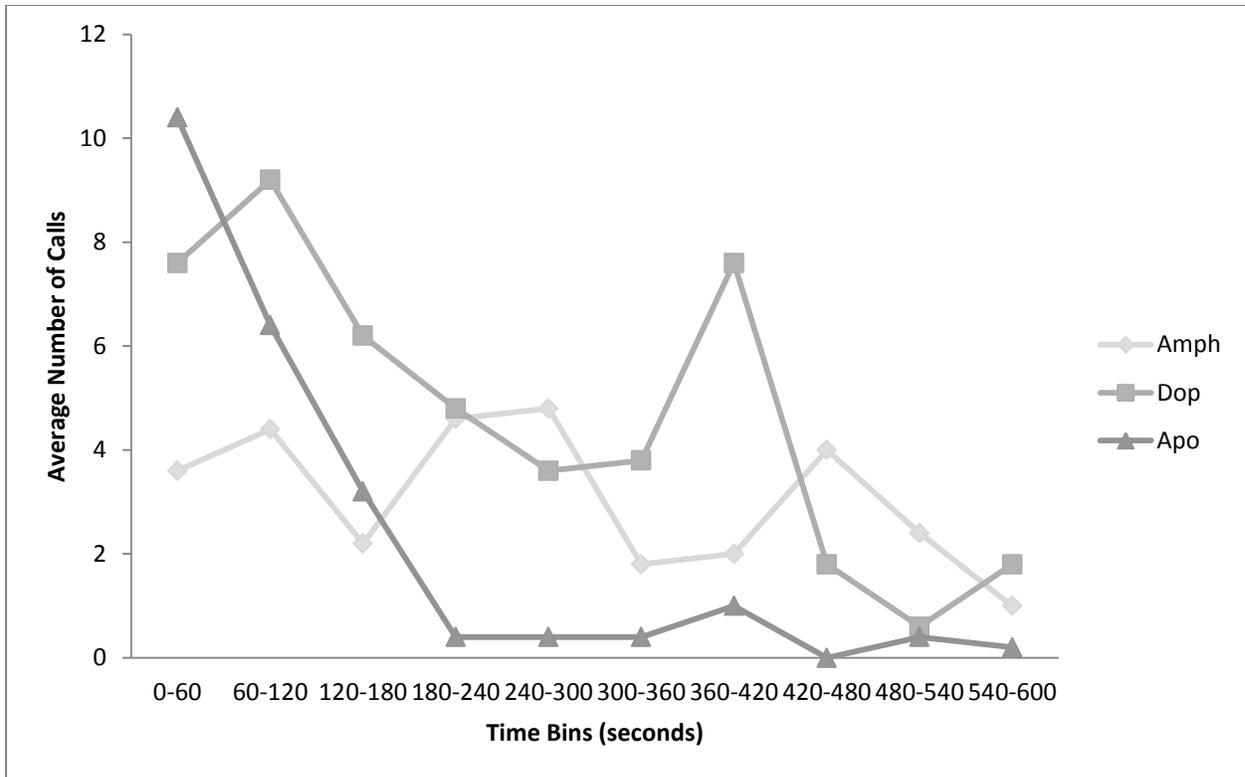


Figure 6a: Pharmacokinetical analysis of the time distribution of calls for 600 seconds.

Kolmogorov-Smirnov tests determined there were significance differences between the time distributions of 50 kHz calls for amphetamine and apomorphine ($p < 0.001$), amphetamine and dopamine ($p = 0.015$), and dopamine and apomorphine ($p < 0.001$).

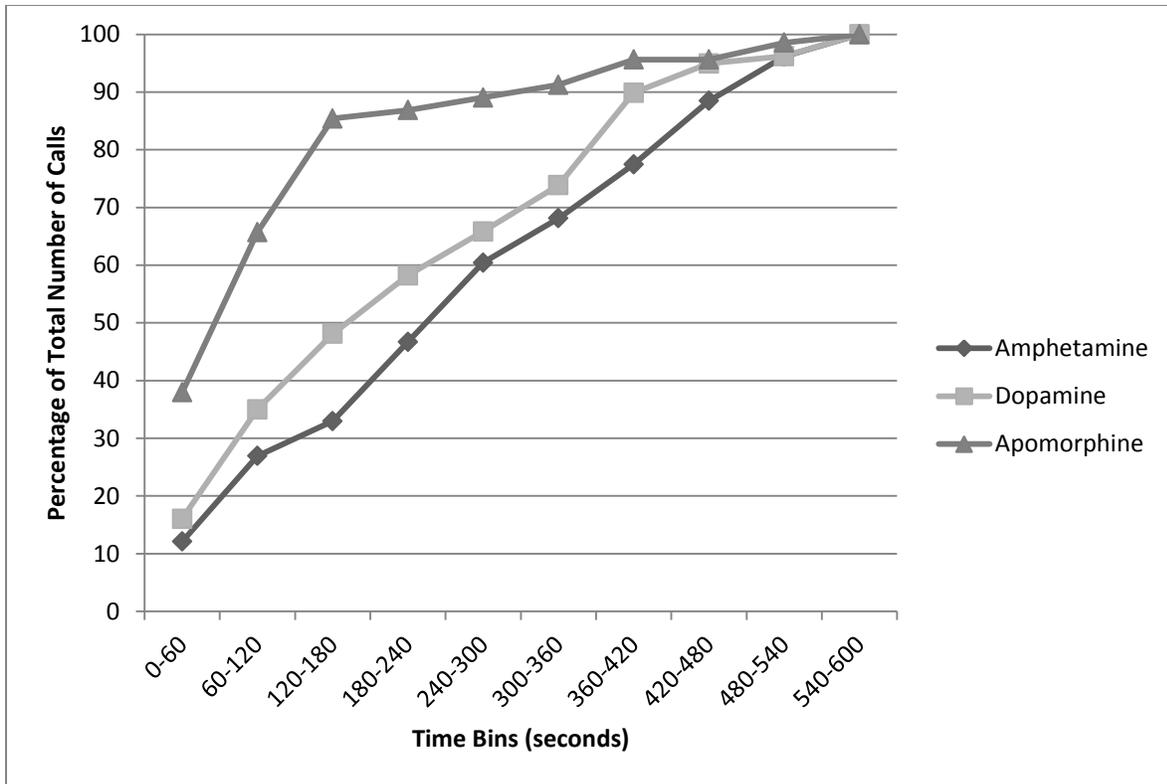


Figure 6b: Pharmacokinetical analysis of the time distribution of calls for 600 seconds based on the total percentage of calls per bin. Each bin percentage is a running percentage total of the amount of calls made during the 10 minute recording period.

Drug	Dose injected	Mol. Wt.	Micromoles
amphetamine	7 µg	135.21	0.052
apomorphine	3 µg	267.33	0.011
dopamine	20 µg	153.18	0.130
dopamine	15 µg	153.18	0.098

Table 1: Comparison of drug dosages used, their molecular weights (Mol. Wt.), as well as the amount of micromoles per injection.

DISCUSSION

Results of the present investigation have shown that three different dopaminergic agents (amphetamine, apomorphine, and dopamine) given directly into the shell of the nucleus accumbens induced significantly more 50 kHz calls than vehicle controls. This response was not present for injection sites localized outside of the accumbens. These injected agents, however, had dissimilar effects on acoustic parameters of ultrasonic calls. Apomorphine and vehicle control induced calls with significantly higher bandwidth than calls after amphetamine and dopamine, while calls after dopamine and vehicle control had significantly higher single call duration. There were no significant differences among in peak frequency or subtypes of 50 kHz vocalizations induced by the three agents.

The first hypothesis, that dopamine and two dopamine agonists would induce substantially the same type of 50 kHz vocalizations typical for the species when directly injected into the nucleus accumbens shell, was confirmed. Amphetamine (t-test, $p = 0.011$), dopamine (t-test, $p = 0.026$), and apomorphine (t-test, $p = 0.032$), when injected into the shell of the nucleus accumbens were all able to significantly increase the number of 50 kHz calls a rat emitted compared to those after the vehicle control. The results in the study also confirmed previous studies where both amphetamine ((Burdorf, Knutson, Panksepp & Ikemoto, 2001; Thompson, Leonard & Brudzynski, 2005) and apomorphine (Silkstone & Brudzynski, 2013), when injected into the shell of the nucleus accumbens, were able to induce significantly more calls than the vehicle control. This study was the first of its kind to directly inject dopamine itself into the nucleus accumbens to attempt to induce 50 kHz calls. Dopamine has never been used in previous vocalization experiments because of concerns of limited effectiveness, although it was used in previous studies of other functions. Dopamine is an endogenous neurotransmitter and therefore is

quickly metabolized in the brain by MAO and COMT enzymes. Furthermore, the dopamine will also undergo rapidly re-uptake by the DAT transporter into the pre-synaptic neuron, removing it from the action site of the synapse. It was thought that, following its injection, dopamine would be metabolized before a strong vocalization response would occur. However, this study was able to show that dopamine at dosages of 15 μg and 20 μg was sufficient enough to induce a strong 50 kHz vocalization response over several minutes. This finding allows for future experimentation in this field to use dopamine itself to induce 50 kHz vocalizations as a cost-efficient alternative to other dopamine agonists.

It was also found in this study that the average peak frequencies of calls induced by the different drugs varied within a narrow range, from 54.2 to 55.1 kHz among the drugs. Also, the average bandwidths remained between 6.1 to 8.7 kHz, and the average call duration varied from 17.3 to 22.4 ms. These call characteristics are comparable to those recorded from rats calling in a natural setting where the average peak frequency was 58.4 kHz, the average bandwidth was 6.8 kHz and the average call duration was 30.1 ms (Brudzynski & Pniak, 2002). Therefore, it can be concluded that the calls induced by the different dopamine agonists in this study are all species-typical 50 kHz vocalizations for rats.

The second hypothesis that the application of dopamine agonists amphetamine, apomorphine and dopamine itself would produce 50 kHz vocalizations that differ, at least in source acoustic parameters was also confirmed. For the average peak frequencies of the calls, there were no significant differences found in between the different injection conditions, although a significant injection condition to individual rat interaction was found. The average call bandwidth for amphetamine and dopamine was significantly higher than apomorphine and the vehicle control with no significant interaction between the injection condition and individual

rat. Finally, the average call duration was significantly higher for the vehicle control and dopamine compared to amphetamine and apomorphine with a significant interaction between the injection condition and individual rat. It should be also noted there was a main effect of the individual rat affecting all three types of acoustic parameters. This evidence suggests that each individual rat has its own distinct parameters of calling. These results are a first step towards the neuropharmacological analysis of drug action, based on the parameters of 50 kHz calls. These differences in acoustic parameters among the drugs may reflect differences in the mechanism of drug action. It is hypothesized that the acoustic characteristics that make up a 50 kHz call are related to the activation of different combinations of dopamine receptor sub-types. Studies such as one by Thompson, Leonard, and Brudzynski (2006) have shown that both D1-like and D2-like receptors require activation for the production of 50 kHz calls. That study showed this by blocking the actions of amphetamine on 50 kHz vocalizations through the pre-treatment of haloperidol (D1 antagonist) and raclopride (D2 antagonist). However, the ratio of activation between D1-like and D2-like receptors may play a key role in the acoustic parameters of 50 kHz vocalizations.

For this current study, although all the drugs used were general dopamine agonists, their actions upon dopamine receptors would differ. Apomorphine itself will activate both D1-like and D2-like receptors, but has a significantly higher affinity for D2-like receptors (Missale, Nash, Robinson, Jaber & Caron, 1998). Also, though both amphetamine and dopamine lead to dopamine itself binding to dopamine receptors, the concentration of dopamine in the synapse as well as duration of dopamine staying around within the synapse would differ between the two injections. These differences in concentration and duration may also play a role in the acoustic parameters of 50 kHz calls.

The most interesting finding was that calls elicited by apomorphine had a significantly higher average bandwidth than either dopamine or amphetamine. Calls having a higher bandwidth are usually indicative of being frequency modulated rather than flat since flat calls do not vary much in frequency. Therefore, because apomorphine has a higher affinity for D2-like receptors, these results suggest that D2-like receptors may play a more important role in the production of frequency modulated calls than flat calls. These results are also comparable to a study by Brudzynski, Komadoski, and St. Pierre (2011), where the injection of 6 µg of quinpirole, a predominately D2/D3 receptor agonist, gave a significantly higher average bandwidth compared to amphetamine and vehicle control groups. Therefore, dopamine agonists with a stronger affinity to D2-like receptors elicit calls with a higher average bandwidth than does amphetamine, a general dopamine agonist, and suggest that preferential activation of D2-like receptors is very important for the production of frequency modulated calls.

However, the third hypothesis, that the application of dopamine and dopamine agonists will induce 50 kHz vocalizations that will differ in the proportions of subtype of calls depending on the agonist injected, was not confirmed. No dopamine agonist elicited a significantly higher ratio of frequency modulated to flat calls. Furthermore, apomorphine itself showed no trend toward a higher ratio of frequency modulated calls compared to the other dopamine agonists. Therefore, these results do conflict with the previous idea that a higher average bandwidth is indicative of a higher ratio of frequency modulated calls compared to flat calls.

In an attempt to further break down the sub-types of 50 kHz calls, the average percent of frequency modulated calls that were the trill sub-type was also investigated. Again, there were no significant differences between the three drug groups. However, there was a trend towards apomorphine eliciting a higher ratio of trills compared to the overall number of frequency

modulated calls. Therefore, a new possible explanation for apomorphine eliciting calls with a higher average bandwidth would be that the frequency modulated calls themselves are more complex in nature compared to the other dopamine agonists rather than the total number of calls themselves. It should be noted however, since the number of rats used in this experiment was relatively small, attempting to dissect any changes in the sub-types of calls is statistically not very powerful. Using a larger sample size may reveal differences in the sub-types of calls that this study did not detect.

The pharmacokinetics of each drug was also investigated to have an understanding of the time course for the effect of each drug. Kolmogorov-Smirnov tests revealed that all three drugs had different time call distributions from each other over time. Interestingly, apomorphine was the most potent of the drugs (Table 1) for the first 60 s, but its ability to induce 50 kHz calls rapidly deteriorated. The action of apomorphine seemed to have disappeared 180 s after it was injected. Amphetamine had the most consistent distribution of calls across time, eliciting the high average number of calls in the 240-300 s bin. Dopamine had its strongest effect in the 60-120 s bin and then consistently slowly decreased its effect except for the 360-420 s bin where it rebounded in the amount of calls.

The results found from the pharmacokinetics analysis are consistent with the mechanisms of the drugs and the dosages used. Since amphetamine acts indirectly on dopamine receptors by stimulating release of dopamine into the synapse, it stands to reason that its potency remained somewhat constant throughout the 600 s. Apomorphine most likely had a very fast reduction in its effect because the amount of micromoles used were significantly less than the other two drugs. Finally, it is likely that dopamine had a longer effect than apomorphine because of the higher amount of micromoles used to overcome endogenous enzymes. A possible explanation to

the increased amount of calls in the 360-420 s dopamine bin may lie most probably in the VTA. If the initial injection of dopamine created a strong enough response, it is possible that a positive feedback message was sent from the nucleus accumbens to the VTA. This might have briefly caused the VTA to activate and release more dopamine into the nucleus accumbens.

This current study had several main limitations. First, the number of rats used was very small. This affected the overall amount of 50 kHz calls that were recorded. Surgical localization and lost data contributed to this low number. The analysis of the core acoustic parameters of peak frequency, bandwidth and call duration were unaffected by this, but the analysis into the 50 kHz sub-types suffered from the low sample number. It is possible that a higher sample number could reveal differences in the sub-types of 50 kHz calls. Secondly, the current study was unable to be set up in a repeated measures design. Injecting solutions of drugs into the nucleus accumbens inevitably causes local damage due to the increased volume that the injected solution adds. This limited the amount of injections to three repetitions. Since there were four injection conditions, not every rat could receive every drug condition. Therefore, the independent measures taken in this study are not as statistically powerful.

Finally, the amount of each drug injected into the nucleus accumbens was not in equimolar amounts. Since different amounts of moles were used for each drug, the relative potency of each drug cannot be compared. Apomorphine was the limiting factor in being unable to be used in equimolar amounts because after 0.011 micromoles, it no longer became soluble in the vehicle. Furthermore, it is very unlikely that either amphetamine or dopamine injected at a dosage of 0.011 micromoles would be able to induce 50 kHz calls. Therefore, this study attempted to use the dosage amount for each drug that induces the optimal effect on the production of 50 kHz calls, as known from previous studies.

There are several future studies that could be continued based on this study. Firstly, since it is now known that injecting dopamine into the nucleus accumbens can induce 50 kHz calls, a full dose-response curve should be researched for dopamine. Second, increasing the sample size used in the current study may reveal differences in the 50 kHz call sub-types that currently were not detected. Third, the addition of more specific dopamine agonists for either D1-like or D2-like may have more prolific effects on the acoustic characteristics of the 50 kHz calls. However, as the dopamine agonists become more specific to a certain type of receptor, the agonist may not be able to effectively induce production of 50 kHz vocalizations. Finally, it may be possible to design a study where the injection of dopamine is pretreated with a small amount of very specific dopamine antagonists. Ideally, the injection of dopamine should still be able to induce 50 kHz calls from the rat, but the specific antagonism of either D1-like or D2-like receptors may alter the acoustic characteristics of the calls to give a better idea about the involvement of different dopamine receptors in 50 kHz calls.

In conclusion, this study was the first to show that dopamine itself could be injected into the shell of the nucleus accumbens to induce 50 kHz vocalizations. Furthermore, all three dopamine agonists injected did differ in 50 kHz call acoustic parameters. Most importantly, apomorphine had higher average call bandwidths compared to either amphetamine or dopamine. This result suggests that since apomorphine has a higher specificity towards D2 receptors, that these receptors may play a more important role in creating complexity within a 50 kHz call. Finally, future research will need to be done with higher sample numbers to determine if apomorphine or other more specific dopamine agonists can change the amount of 50 kHz call sub-type compared to dopamine or amphetamine.

References:

- Alcaro, A., Huber, R., Panksepp, J. (2007). Behavioral functions of the mesolimbic dopaminergic system: an affective neuroethological perspective. *Brain Research Reviews*, 56(2), 283-321.
- Blanchard, R. J., Blanchard, D.C., Agullana, R., Weiss, S. M. (1990). Twenty-two kHz alarm cries to presentation of a predator, by laboratory rats living in visible burrow system. *Physiology & Behavior*, 50, 967-972.
- Beckstead, R. M., Domesick, V. B., Nauta, W. J. H. (1979). Efferent connections of the substantia nigra ventral tegmental area in the rat. *Brain Research*, 175, 191-217.
- Brudzynski, S. M. (1981). Growling component of vocalization as a quantitative index of carbachol-induced emotional-defensive response in cats. *Acta Neurobiologiae Experimentalis*, 41, 33-51.
- Brudzynski, S. M. (1994). Ultrasonic vocalization induced by intracerebral carbachol in rats: localization and dose-response study. *Behavioural Brain Research*, 63, 133-143.
- Brudzynski, S. M. (2005). Principles of rat communication: quantitative parameter of ultrasonic calls in rats. *Behavior Genetics*, 35(1), 85-92.
- Brudzynski, S. M. (2007). Ultrasonic calls of rats as indicator variables of negative or positive states: acetylcholine-dopamine interaction and acoustic coding. *Behavioural Brain Research*, 182, 261-273.
- Brudzynski, S. M., Barnabi, F. (1996). Contribution of the ascending cholinergic pathways in the production of ultrasonic vocalization in the rat. *Behavioural Brain Research*, 80, 145-152.
- Brudzynski, S. M., Bihari, F. (1990). Ultrasonic vocalization in rats produced by cholinergic

stimulation of the brain. *Neuroscience Letters*, 109, 222-226.

Brudzynski, S. M., Iku, A., Harness, A. (2011). Activity of cholinergic neurons in the laterodorsal tegmental nucleus during emission of 22 kHz vocalization in rats.

Behavioural Brain Research, 225, 276-283.

Brudzynski, S. M., Komadoski, M., St. Pierre, J. (2011). Quinpirole-induced 50 kHz ultrasonic vocalizations in the rat: role of D2 and D3 dopamine receptors. *Behavioural Brain*

Research, 226, 511-518.

Brudzynski, S. M., Pniak, A. (2002). Social contacts and production of 50-kHz short ultrasonic calls in adult rats. *Journal of Comparative Psychology*, 116, 73-82.

Burgdorf, J., Knutson, B., Panksepp J., Ikemoto S. (2001). Nucleus accumbens amphetamine microinjections unconditionally elicit 50-kHz ultrasonic vocalizations in rats. *Behavioral Neuroscience*, 115(4), 940-944.

Civelli, O., Bunzow, J. R., Grandy, D. K., Zhou, Q. Y., Tol, H. H. (1991). Molecular biology of the dopamine receptors. *European Journal of Pharmacology*, 207(4), 277-286.

Clarke, P. B. S., Wright, J. M., Gourdon, J. C. (2010). Identification of multiple call categories within the rich repertoire of adult rat 50-kHz ultrasonic vocalizations: effects of

amphetamine and social context. *Psychopharmacology*, 211, 1-13.

Cornwall J., Cooper, J. D., Phillipson O. T. (1990). Afferent and efferent connections of the laterodorsal tegmental nucleus in the rat. *Brain Research Bulletin*, 25, 271-284.

Fleckenstein, A. E., Volz, T. J., Riddle, E. L., Gibb, J. W., Hanson, G. R. (2007). New insights into the mechanism of action of amphetamines. *Annual Review of Pharmacology and*

Toxicology, 47, 681-698.

- Hasue, R. H., Shammah-Lagnado, S. J. (2002). Origin of the dopaminergic innervation of the central extended amygdala and accumbens shell: a combined retrograde tracing and immunohistochemical study in the rat. *The Journal of Comparative Neurology*, 454, 15-33.
- Heimer, L., Zahm, D. S., Churchill, L., Kalivas, P. W., Wohltmann, C. (1991). Specificity in the projection patterns of accumbal core and shell in the rat. *Neuroscience*, 41(1), 89-125.
- Ikemoto, S. (2007). Dopamine reward circuitry: two projections systems from the ventral midbrain to the nucleus accumbens-olfactory tubercle complex. *Brain Research Reviews*, 56, 27-78.
- Kebebian, J. W., Calne, D. B. (1979). Multiple receptors for dopamine. *Nature*, 277, 93-96.
- Knutson, B., Burgdorf, J., Panksepp, J. (1998). Anticipation of plays elicits high frequency ultrasonic vocalizations in young rats. *Journal of Comparative Psychology*, 112(1), 65-73.
- Knutson, B., Burdgorf, J., Panksepp, J. (2002). Ultrasonic vocalizations as indices of affective states in rats. *Psychological Bulletin*, 128(6), 961-997.
- McIntosh, T. K., & Barfield, R. J. (1980). The temporal patterning of 40 – 60 kHz ultrasonic vocalizations copulation in the rat (*Rattus norvegicus*). *Behavioral and Neural Biology*, 29, 349-358.
- Maggio, R., & Millan, M. J. (2010). Dopamine D2-D3 receptor heteromers: pharmacological properties and therapeutic significance. *Current Opinion in Pharmacology*, 10, 100-107.
- Meredith, G. E., Agolia, R., Arts, M. P. M., Groenewegen, H. J., Zahm, D. S. (1992). Morphological differences between projection neurons of the core and shell in the nucleus accumbens of the rat. *Neuroscience*, 50(1), 149-162.

Missale, C., Nash, S. R., Robinson, S. W., Jaber, M., Caron, M. G. (1998). Dopamine receptors: from structure to function. *Physiological Reviews*, 78(1), 189-225.

Paxinos, G., Watson, C. (1986). The rat brain in stereotaxic coordinates. 2nd edition. New York: Academic Press.

Pellis, S. M., Field, E. F., Smith, L. K., Pellis, V. C. (1997). Multiple differences in the play fighting of male and female rats. Implications for the causes and functions of play. *Neuroscience and Biobehavioral Reviews*, 21(1), 105-120.

Perreault, M. L., Hasbi, A., Alijaniam, M., Fan, T., Varghese, G., Fletcher, P. J., Seeman, P., O'Dowd, B.F., George, S. R. (2010). The dopamine D1-D2 receptor heteromer localizes in dynorphin/enkephalin neurons: increased high affinity state following amphetamine and in schizophrenia. *Journal of Biological Chemistry*, 285, 36625-36634.

Phillipson, O. T., Griffiths, A. C. (1985). The topographic order of inputs to nucleus accumbens in the rat. *Neuroscience*, 16(2), 275-296.

Roberts, L. H. (1975). The rodent ultrasound production mechanism. *Ultrasonics*, 13, 83-88.

Rodd-Henricks, Z. A., McKinzie, D. L., Li, T. K., Murphy, J. M., McBride, W. J. (2002).

Cocaine is self-administered into the shell but not the core of the nucleus accumbens of Wistar rats. *The Journal of Pharmacology and Experimental Therapeutics*, 303(3), 1216-1226.

Seyfarth, R. M., Cheney, D. L. (2003). Signalers and receivers in animal communication. *Annual Review of Psychology*, 52, 145-173.

Silkstone, M., Brudzynski, S. M. (2013). Induction of species-typical 50 kHz vocalizations by

- dopaminergic agents injected into the lateral septum in the rat. International Behavioral Neuroscience Society Meeting, Malahide, Dublin, Ireland, *International Behavioral Neuroscience Society Abstracts*, 22, 92.
- Sulzer, D., Sonders, M. S., Pulsen, N. W., Galli, A. (2005). Mechanisms of neurotransmitter release by amphetamines: a review. *Progress in Neurobiology*, 75(6), 406-433.
- Thomas, D. A., Takahashi, L. K., Barfield, R. J. (1983). Analysis of ultrasonic vocalizations emitted by residents during aggressive encounters among rats (*Rattus norvegicus*). *Journal of Comparative Psychology*, 97, 207-212.
- Thompson, B., Leonard, K. C., Brudzynski, S. M. (2005). Amphetamine-induced 50 kHz calls from rat nucleus accumbens: a quantitative mapping study and acoustic analysis. *Behavioural Brain Research*, 168, 64-73.
- Tonoue, T., Ashida, Y., Makino, H., Hata, H. (1986). Inhibition of shock-elicited ultrasonic vocalization by opioid peptides in the rat: a psychotropic effect. *Psychoneuroendocrinology*, 11, 177-184.
- Wachtel, H., Ahlenius, S. & Andén, N.E. (1979). Effects of locally applied dopamine to the nucleus accumbens on the motor activity of normal rats and following alpha-methyltyrosine or reserpine. *Psychopharmacology (Berl.)*, 63(3), 203-206.
- Wintink, A. J., Brudzynski, S. M. (2001). The related roles of dopamine and glutamate in the initiation of 50-kHz ultrasonic calls in adult rats. *Pharmacology Biochemistry and Behavior*, 70, 317-323.
- Wise, R. A., Bozarth, M. A. (1987). A psychomotor stimulant theory of addiction. *Psychological Review*, 97(4), 469-492.