Functional antagonism of the mesolimbic dopaminergic system on mesolimbic cholinergic system in the vocal expression of an emotional state

By

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Abstract

The overarching goal of this thesis was to determine if the initiation of a positive emotional state could antagonize the expression of a negative emotional state in rats. The hypothesis of the thesis argued that the initiation of a positive emotional state would ameliorate the vocal expression of a negative emotional state. The subjective emotional state of the rat was indexed by the quantity and type of pharmacologically induced ultrasonic vocalizations (USVs). Adolescent and adult rats can emit vocalizations above the upper threshold of human hearing (>20 kHz) termed ultrasonic vocalizations (USVs). These USVs are broadly divided into 50-kHz, reflective of a positive emotional state, and 22-kHz USVs, reflective of a negative emotional state. Pharmacologically, injection of dopamine agonists into the nucleus accumbens shell is sufficient for the initiation of 50-kHz USVs, while injection of cholinergic agonists into the anterior hypothalamic-medial preoptic area (AH-MPO) or the lateral septum (LS) can initiate 22-kHz USVs. In chapter two of the thesis, I demonstrated that microinjection of the dopamine agonist, apomorphine, into the medial shell of the nucleus accumbens attenuated the extent of carbachol-induced 22-kHz USVs from the AH-MPO. I also demonstrated that this effect was dependent upon the microinjection of apomorphine into the central region of the nucleus accumbens shell. In chapter three, I demonstrated that apomorphine could also decrease the extent of carbachol-induced 22-kHz USVs from the LS providing evidence that the effect reported in chapter two was not isolated to the AH-MPO, but rather extending along the medial cholinoceptive vocalization strip. In the third chapter. I also demonstrated that the magnitude of the reduction in the number of 22-kHz USVs was correlated to the number of emitted frequency-modulated (FM) 50-kHz USVs induced by apomorphine. In the fourth chapter, I investigated whether blocking dopamine receptors, either systemically using the typical D_2-antipsychotic agent, haloperidol, or microinjection of the D_2 antagonist, raclopride, into the nucleus accumbens shell could increase

the emission of carbachol-induced 22-kHz USVs from the LS. The results showed that antagonism of dopamine receptors, either systemically or intracerebrally, did not increase the number of 22-kHz USVs. Interestingly, it was also observed that after the prolonged recording of carbachol-induced 22-kHz USVs, some 50-kHz USVs spontaneously appeared after roughly 300 s into the recording. I argued that these 50-kHz USVs, which I defined as "rebound 50-kHz USVs" are not initiated by carbachol since they occurred when the carbachol-response weaned. It was also demonstrated these rebound 50-kHz USVs were dependent upon dopamine release within the nucleus accumbens since both systemic, and intracerebral application of dopamine antagonists into the central division of the nucleus accumbens shell blocked the occurrence of rebound 50-kHz USVs. Altogether, the data supports the thesis that activation of a positive emotional state decreases the expression of the negative emotional state in rats when measured using ultrasonic vocalizations.

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Publications

Chapter 1: Fragments published as Brudzynski, S. M., Silkstone, M. J., & Mulvihill, K. G. (2018). Ascending activating systems of the brain for emotional arousal. In: Brudzynski, S.M., (Ed.), *Handbook of Ultrasonic Vocalization. A Window into the Emotional Brain.* Academic Press/ Elsevier: Amsterdam, pp. 239-251.

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Silkstone, M., & Brudzynski, S. M. (2019). Intracerebral injection of R-(-)-Apomorphine into the nucleus accumbens decreased carbachol-induced 22-kHz ultrasonic vocalizations in rats. *Behavioural Brain Research*, 364: 264-273.

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Silkstone, M., & Brudzynski, S. M. (2019). The antagonistic relationship between aversive and appetitive emotional states in rats as studied by pharmacologically-induced ultrasonic vocalization from the nucleus accumbens and lateral septum. *Pharmacology Biochemistry and Behavior*, 181: 77-85.

Chapter 4 has been submitted to Pharmacology, Biochemistry and Behavior.

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List of Abbreviations

5-HT	Serotonin
ACH	Acetylcholine
AChE	Acetylcholinesterase
AH-MPO	Anterior Hypothalamic – Medial Preoptic Area
AMPH	Amphetamine
ChAT	Choline Acetyltransferase
cAMP	Cyclic adenosine monophosphate
CPA	Conditioned Place Aversion
CPP	Conditioned Place Preference
CTL	Control Group
CRE	Cre Recombinase
CV	Cyclic Voltammetry
DA	Dopamine
DAG	Diacylglycerol
DAT	Dopamine Transporter
DAergic	Dopaminergic
DOPA	Dihydroxyphenylalanine
fr	fasciculus retroflexus
FSCV	Fast-Scan Cyclic Voltammetry
GAD	Generalized Anxiety Disorder
GLUT	Glutamate
ICSS	Intracranial self-stimulation
IN	Interfascicular Nucleus
IP	Intraperitoneal
LHb	Lateral Habenula
LN	Linear Nucleus (c – caudal; r – rostral)
LS	Lateral Septum

LTDg	Laterodorsal Tegmental Nucleus
MDMA	3,4-methylendioxy-methamphetamine
MFB	Medial Forebrain Bundle
MSN	Medium Spiny Neurons
NA	Noradrenaline
NAc	Nucleus Accumbens, core
NAs	Nucleus Accumbens, shell
PBP	Parabrachial Pigmented Nucleus
PFC	Prefrontal Cortex
PIP_3	Phosphatidylinositol (3,4,5)-triphosphate
PN	Paranigral Nucleus
PR	Parafasciculus Retroflexus
RTMg	Rostromedial Tegmental Nucleus
SAL	Saline
SC	Subcutaneous
TH	Tyrosine Hydroxylase
TH:CRE	Tyrosine Hydroxylase: Cre recombinase
USV	Ultrasonic Vocalization
VEH	Vehicle
VP	Ventral Pallidum (l – lateral, m – medial divisions)
VTA	Ventral Tegmental Area

Chapter 1: Introduction and Literature Review

Fragments published as: Brudzynski, S. M., Silkstone, M. J., & Mulvihill, K. G. (2018). Ascending activating systems of the brain for emotional arousal. In: Brudzynski, S.M., (Ed.), *Handbook of Ultrasonic Vocalization. A Window into the Emotional Brain.* Academic Press/ Elsevier: Amsterdam, pp. 239-251.

1.0 General Introduction

Mental health is a vital component to the overall wellness of an individual and is impacted by variables such as emotions, other psychological processes, and social well-being (Galderisi et al., 2015). Mental health helps predict coping strategies, how individuals accommodate and handle stress, and the formation of interpersonal relationships (Reblin & Unchino, 2008). Factors that contribute to the overall status of mental health include biological factors such as neurochemistry, neuroanatomy, and genetics, as well as environmental factors such as familial relationships and social factors. Deficits in these biological and environmental factors can precipitate psychiatric diseases like generalized anxiety disorder, schizophrenia, obsessive-compulsive disorder, bipolar disorder, major depressive disorder, psychosis, etc. (Mala et al., 2015). The impact of these psychiatric disorders limits the quality of life of the afflicted, but also increases the economic burden of Canadians due to lost taxes, increased health care costs, increased disability utilization, and legal costs (Lim et al., 2008)

It is unfortunate that psychiatric disorders cannot be entirely cured using today's medical treatments, rather people suffering from these diseases try and manage their illness using a variety of pharmaceuticals or social therapies. (Bower & Gilbody, 2005). Thus, in order to increase the effectiveness of treatment of patients suffering from a psychiatric disorder, and to ultimately better improve the quality of life of the afflicted, a better understanding of the neurobiological basis of emotion is required.

The investigation into the biological basis of emotion in humans has been divided into neuroimaging studies and biochemical studies. Neuroimaging studies are appealing because they are non-invasive, while biochemical studies use more invasive methods to try and correlate

biochemical markers to mood disturbances. For instance, endogenous molecules like somatostatin (Rubinow, 1986), tau proteins (Paquet et al., 2016), or homovanillic acid (Maas et al., 1997) have been analyzed from cerebral spinal fluid of normal and psychiatric patients to see if correlations exist between the concentration of these endogenous molecules and psychiatric disturbance. However, due to ethical concerns, the scope and invasiveness of human studies are limited, and thus a strong emphasis is placed on animal models to try and develop experiments that mimic both behavioural and physiological manifestations of different psychiatric illnesses. It is the hope that probing the networks responsible for emotional initiation and regulation in the rat will translate to human studies, and eventually, better healthcare management for the psychiatric community.

Now, emotions and emotional behaviour should be defined.

Although no formal definition of emotion exists, this thesis will adopt the definition proposed by Kleinginna & Kleinginna (1981), that defines emotion as an emergent property arising from the subjective interpretation of physiological changes integrated with sensory information from the environment. This integration of information from viscera and environment (the subjective experience of emotion) energizes the animal motorically and autonomically to deal with complex issues associated with fitness and survival. The emotionally driven changes in the animal motor system that tries to solve goal-directed behaviour can be defined as emotionally driven behaviour. Emotionally driven behaviour can range from approach behaviour, avoidance behaviour to vocal behaviour. These types of emotionally-driven behaviours can be initiated unconditionally or initiated via classical conditioning (Kleinginna & Kleinginna, 1981).

An investigation into the biological basis of emotion has led to two prominent hypotheses: the constructionist hypothesis and basic hypothesis. Research using a constructionist lens argues that emotions like *anger, sadness, happiness, disgust,* and *fear* arise from common and overlapping neural systems (Posner et al., 2008; Russell, 1980; Watson et al., 1990). However, the basic view of emotions hypothesizes that each emotion is evolutionary conserved and hard-wired within unique and separable brain circuit that, when activated, produces a unique physiological, psychological and behavioural pattern of activation (Barret & Wagner, 2006; Ekman, 1972; Nesse & Ellsworth, 2009; Panksepp, 1982).

1.1: Cross-mammalian comparison of subcortical structures involved in emotional regulation: emphasis on rats and humans.

In order to use rats in studying the neurobiology of emotion, the argument first must be made that rats are capable of the expression of emotional states. In this thesis, I will take the explicit assumption that rat ultrasonic vocalizations (USVs) are forms of emotional expression and that USVs can be initiated by the stimulation of specific circuits that are organized within the brain. Stimulation of these brain nuclei initiates USVs in rats that have the same sonographic features observed when rats emit USVs in negative or positive natural contexts. Likewise, these subcortical brain circuits are evolutionary conserved neural circuits that are also found in humans which, when electrically stimulated, produce self-reported measures of positive or negative emotional states. In rats, two different types of USVs are reflective of emotional states: 50-kHz USVs reflect a positive emotional state and 22-kHz USVs reflect a negative emotional state (see Figure 1)

Investigations into the neurobiology of emotions in humans come from neuroimaging studies, and intracranial self-stimulation (ICSS) studies combined with verbal feedback. For example, human participants readily self administer electrical current (intracranial self-stimulation) into the septal nucleus because of the reported feelings of intense pleasure, sexual excitation, euphoria, relaxation, sexual vigor and motivation they experience from it (Heath, 1963; Moan & Heath, 1972). The adjectives used by the patients are presumed to be associated with positive emotional states since the subjective feeling resulting from ICSS produces and reinforces the subject to repeat the behaviour. Other brain areas that support ICSS in humans are the nucleus accumbens, and the ventral tegmental area (VTA). For example, Heath (1972) described a patient where ICSS of the VTA produced the most pleasurable sensation the subject has ever experienced in their life (Heath, 1972).

Imaging and recording studies also support the role of subcortical nuclei that are associated with a positive effect. Electroencephalogram recordings from the human septum show increased activity during heterosexual intercourse and orgasm (Heath, 1972; Moan and Heath, 1972). Likewise, the activity of dorsal/ventral striatal regions has been shown to be activated by laughter (Osaka & Osaka, 2005) and electrical stimulation of these regions have been shown to induce mirth in patients with treatment-resistant anxiety disorders (Greenberg et al., 2006; 2010). Thus, stimulation of subcortical brain structures like the lateral septum, nucleus accumbens, hypothalamus, and ventral tegmental area have all been able to induce measurable levels of positive emotional states in humans. These same brain nuclei, when artificially stimulated in rats can also initiate positive or negative emotional states. In this thesis, the expression of an emotional state will be measured via the emission of USVs.

During adolescences and adulthood, rats are capable of emitting vocalizations above the upper threshold of human hearing (>20 kHz); these vocalizations have been defined as USVs and are categorized as either 50-kHz and 22-kHz USVs (See Figure 1; Brudzynski, 2013; Takahashi et al., 2017). These USVs are hypothesized to serve as social communication signals (Brudzynski & Chiu, 1995) and/or the expression of affective states (Knutson et al., 1999; 2002).

The social communication hypothesis of USV production in rodents suggests that, contained within the 50-kHz or 22-kHz USVs, are social signals that inhibit or facilitate interactions with conspecifics. Evidence that supports this hypothesis primarily comes from playback studies in which naturally recorded or artificially generated 50-kHz and 22-kHz USVs are played back through a speaker to conspecifics, and the behaviour of the rat is concurrently recorded. Recorded 50-kHz USVs were studied under two different contexts: sexual contexts and non-sexual contexts.

In socio-sexual contexts, 50-kHz USVs serve as a communication tool to coordinate sexual activity between rats. The proceptivity of a female is correlated to behaviours, such as orientation to the male, ear wiggling, and darting (Beach, 1976; McIntosh et al., 1978). The sensory cue that appears to initiate sexual solicitation from a male rat to an oestrus female is the emission of 50-kHz USVs by the male (White & Barfield, 1989). In studies where the inferior laryngeal nerve was severed in rats, estrus females preferred to spend time with vocalizing males that emitted USVs as opposed to devocalized males (Pomerantz et al., 1983).

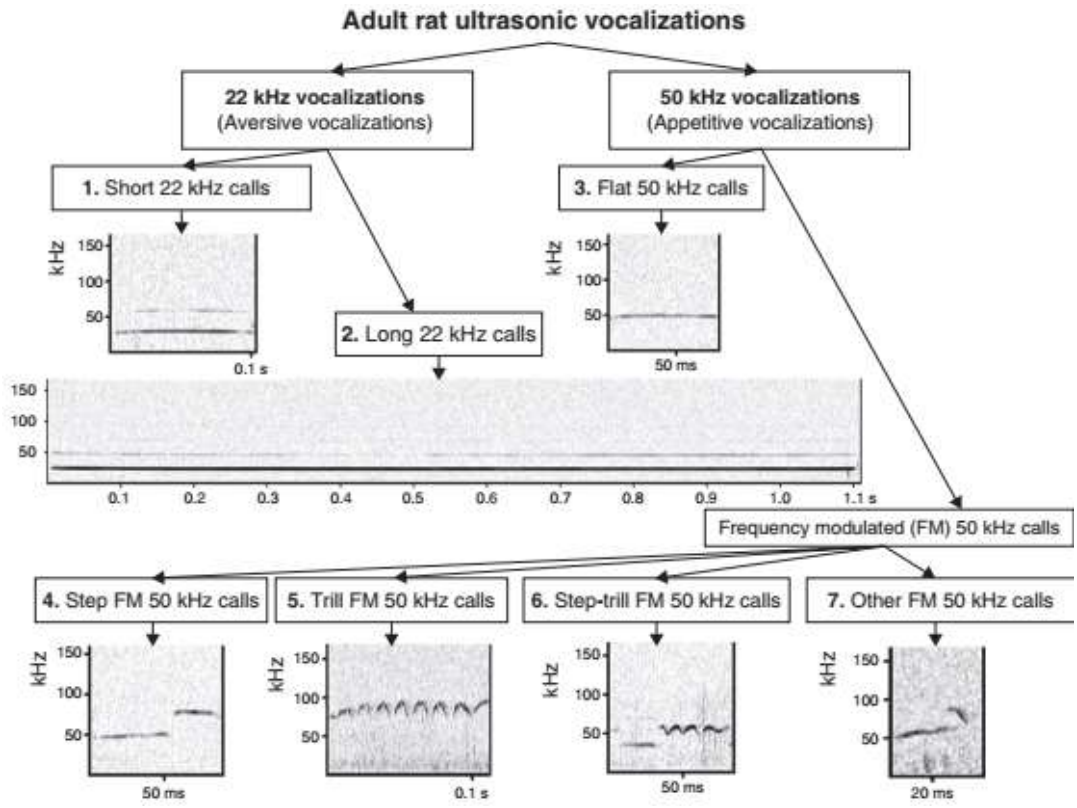


Figure 1: Sonographic representations of ultrasonic vocalizations in rats. The sonograph displays the variety of different types of USVs that rats are capable of producing. The x-axis shows time (in ms or s) and y-axis sound frequency (in kHz). 22-kHz USVs are divided into short and long calls. Short 22-kHz USVs are hypothesized to reflect an internal anxiety-driven state while longer vocalizations are hypothesized to signal proximity to predators. Typically, 22 kHz USVs, both long and short, have a peak frequency of ~22-kHz. The duration of short 22 kHz USVs range from roughly 20-100 ms, while long 22-kHz USVs have a duration that ranges from 100-3000 ms. 50-kHz USVs can be divided into flat and frequency modulated (FM) calls. Flat vocalizations have a peak frequency between 50-58 kHz and duration between 20-100 ms. FM 50 kHz USVs can have a duration ranging between 20-80 ms and a peak frequency ranging from 56-80 kHz. Picture was taken from: Brudzynski, S. M. (2013). Ethotransmission: communication of emotional states through ultrasonic vocalization in rats. *Current Opinion in Neurobiology*, 23(3), 310-317.

Similarly, deafening an oestrus female does not dramatically change copulatory contacts between males and females, but rather significantly decreases sexual solicitation in females regardless of the amount of 50-kHz USVs emitted by the male (Barfield et al., 1979). The decreasing solicitation of females and the decreased preference of females to mute males suggest that the production of 50-kHz USVs is critical to the proper socio-sexual interaction and mating between male and female rats.

Rats are a gregarious species and choose to spend time with conspecifics as opposed to solitude life (Latane, 1969). A critical measure of social interaction in rats and a predictor of aberrant social behaviour in later years is adolescent play (Argue & McCarthy, 2015; Barale et al., 2015). Adolescent play is the earliest form of non mother-oriented social behaviour in rats and is characterized as "rough-and-tumble" play with the emissions of large numbers o 50-kHz USVs (Vanderschuren et al., 1997; Panksepp 1986). The play behaviour is characterized by both somatosensory contact via pinning behaviour, as well as the emission of 50-kHz USVs. The facilitation and engagement in play behaviour seem to be dependent upon emission and receiving 50-kHz USVs. Rats that have had their auditory canals sealed with bone-wax engage in fewer bouts of play behaviour compared to rats that have somatosensation reduced via subcutaneous lidocaine injections suggesting that vocal cues are more important for coordinating and engaging in bouts of play behaviour than direct contact (Panksepp, 1986)

Playback studies also suggested a communicative role for 50-kHz and 22-kHz USVs. During the playback of naturally occurring 22-kHz USVs, rats exhibited defensive responses such as freezing (Brudzynski & Chiu, 1995; Sales, 1991; Wöhr & Schwarting, 2007). However, some experiments do report rats will engage in running and jumping, which are defensive/escape-like behaviours in response to the presentation of either artificially generated

22-kHz USVs or naturally recorded 22-kHz USVs (Commissaris et al., 2000). The duration of emitted 22-kHz USVs seems to be critical for the signaling of aversive cues, as opposed to the temporal patterns and peak frequencies observed during the communication with 50-kHz USVs. For example, during cocaine, alcohol, or opioid withdrawal, the duration of recorded 22-kHz USVs are short and lasting between 300-1000 ms (Berger et al., 2013). However, longer vocalizations ranging between 1000-3000 ms possibly communicates dangers, such as proximity to predators (Blanchard et al., 1991) since long 22-kHz USVs are observed being emitted by rats in proximity to a predator (Blanchard & Blanchard, 1991). These vocalizations have been dubbed "alarm cries" (Litvin et al., 2007).

Evidence suggesting that USVs can also signal emotional states comes from pharmacological studies. Injection of anxiolytics, drugs that reduces anxiety in humans and can decrease the number of emitted 22-kHz USVs in rats in response to aversive contexts such as anticipation to foot shocks, air puff to the snout, or acoustic startle (Jelen et al., 2003; Naito et al., 2003; Vivian & Miczek, 1993). Likewise, injection of anxiolytics into rats decreased the number of emitted 22-kHz USVs in response to withdrawal from drugs that are abused by humans such as morphine, nicotine or cocaine (Berger et al., 2003; Gawin, 1991; Knapp et al., 1998; Lembke et al., 2007; Miczek & Barros, 1996; Mutschler & Miczek, 1998; Moy et al., 2000). Typically, the withdrawal from morphine, cocaine, or nicotine in humans is associated with emotional instability and anxiety (American-Psychiatric Association, 2007). These negative emotional states can be attenuated with pharmacological compounds in humans and can also decrease 22-kHz USVs in rats.

Contrary to the punishing circumstances that can initiate 22-kHz USVs, frequency modulated FM 50-kHz USVs are typically emitted during positive pro-social encounters or

during anticipation of rewarding stimuli. These positive circumstances which can initiate FM 50-kHz USVs suggesting these vocalizations signal a positive emotional state. For example, in anticipation of electrical stimulation of areas that are reported to cause pleasure in humans, social play with conspecifics or with experimenters will increase the number of emitted of frequency modulated (FM) 50-kHz USVs (Burgdorf et al., 2000). Likewise, when paired with an environment that the rat had previously been exposed to addictive pharmacological agents such as amphetamine, morphine, nicotine or cocaine, rats will increase the number of FM 50-kHz USVs (Knutson et al., 1995). These pharmacological drugs that increase the number of FM 50-kHz USVs have a unique propensity to increase the concentration of dopamine within the mesolimbic dopamine system.

1.2. Anatomy and physiology of the mesolimbic dopamine system

The initial description of the ventral tegmental area was reported by Tsai (1925) in a series of investigations that examined the pathway of the optic tracts through the opossum brain. Tsai documented a group of cells lying medial to the optic tectum called the nucleus tegmenti ventralis (Tsai, 1925), which was later defined as the ventral tegmental area of Tsai by Nauta (Nauta, 1956;1958). Tsai's description of the VTA was strictly anatomical and did not speculate as to the chemical identity of the neurons localized within this region of the opossum brain.

The initial neurochemical identification of monoamine-containing neurons within the VTA was visualized using Falck and Hillarp method. In this method, sections of frozen neural tissue, when exposed to formaldehyde vapor, converts derivatives of phenylalanine, and phenylethylamine (such as dopamine, serotonin, and noradrenaline) into 1,2,3,4-tetrahydroisoquinoline, which subsequently emits green or yellow fluorescence (Falck et al., 1962). Using this histochemical method, Dahlstroem and Fuxe (1964) identified locations of

nuclei within the brain that contain dopamine, noradrenaline, and serotonin. They introduced a nomenclature of individual nuclei, A1-12 and B1-9, with A10 being classified as the ventral tegmental area of Tsai (Dahlstroem and Fuxe, 1964) and being neurochemically consistent with catecholamine-containing neurons.

As histochemical and fluorescence techniques developed, the VTA has been understood as a composite collection of dopamine cells that span several zones. These zones are called the paranigral nucleus (PN), parabrachial pigmented nucleus (PBP), parafasciculus retroflexus (PR), caudal linear nucleus (cLN), rostral linear nucleus (rLN), interfascicular nucleus (IN) and rostromedial tegmental nucleus (RTMg) (the tail of the VTA). These zones can be further grouped based on dopamine-rich and poor regions, as well as the specificity of their connections to other subcortical and cortical structures (Ikemoto, 2007). The most-dense dopamine regions within the collection of subnuclei are the PN and PBP, while dopamine-poor zones are the PR and RTMg (Morales & Margolis, 2017).

The dopamine-rich and dopamine poor regions of the VTA are associated with distinct ascending circuits identified as the mesolimbic dopamine system, mesocortical system, and the nigrostriatal system (Björklund & Dunnett, 2007). The term ”meso” was described by the anatomist Ungerstedt (1971) to separate the pathway of dopamine neurons that innervate subcortical structures other than the nigrostriatal system. Since the fibers originated from the mesencephalon, the term “mesolimbic” describes dopamine neurons within the ventral tegmental area that innervate subcortical structures such as the olfactory tubercle, nucleus accumbens, amygdala, and lateral septum while the term “mesocortical” identifies a subset of dopamine neurons within the VTA that innervates the prefrontal cortex of the rat (Ungerstedt, 1971; see Ikemoto, 2007 for review). This thesis will focus on the projections of the mesolimbic dopamine

system to the nucleus accumbens and its role in the initiation of 50-kHz USVs in rats (See Figure 2).

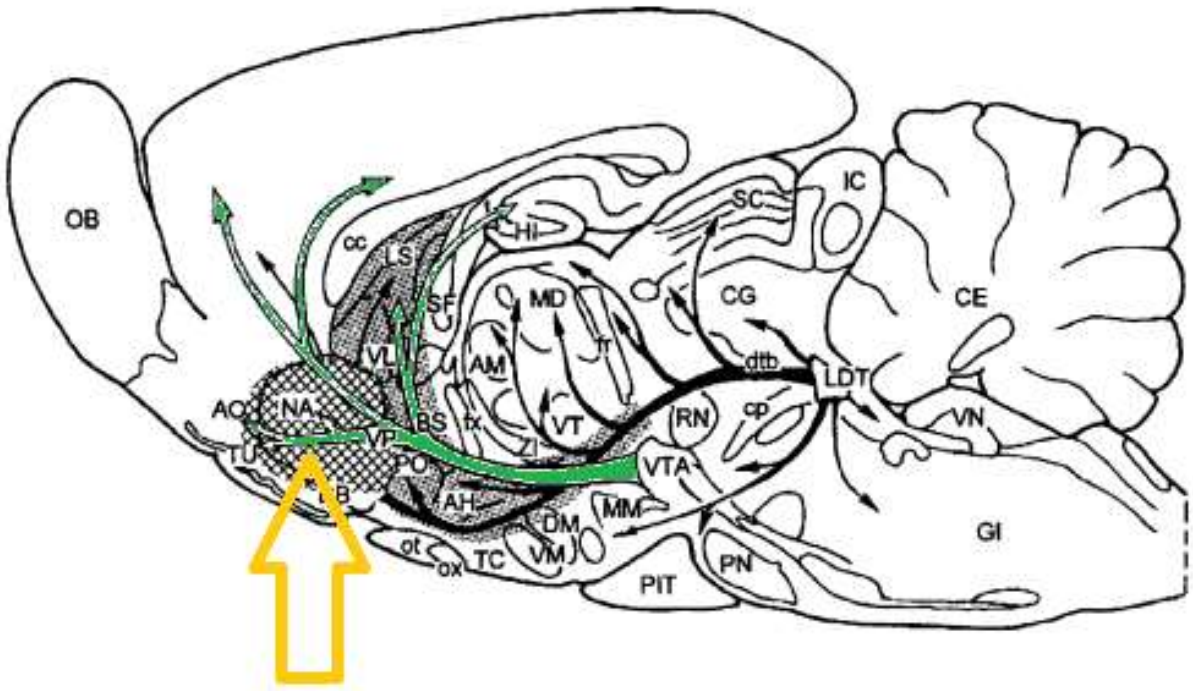


Figure 2: Parasagittal cross-section of the rat brain diagramming both the cholinoceptive vocalization strip and the mesolimbic dopamine system (green pathway). Injection of dopamine agonists into the nucleus accumbens (yellow arrow) induces species-typical 50-kHz USVs. The figure is taken from Brudzynski, 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(2), 261-273.

The initial anatomic description of the nucleus accumbens was introduced by the anatomist Ziehen (1904) who described the nucleus as a ventromedial extension of the striatum extending dorsally into the lateral parts of the septum. (Ziehen, 1904; Salgado & Kaplitt, 2015). Although initially, the nucleus accumbens seemed separate from the dorsal striatum because of

its relationship with the lateral septum, later, with the development of tracing techniques, the nucleus accumbens became a distinct striatal region separate from the dorsal striatum (Zahm & Brog, 1992). The introduction of autoradiographic tracing methods by Weiss and Holland (1967) provided significant improvements on the classical anatomical tracing methods of tract-tracing and visualization of degenerating neurons (Swanson, 1981; Weiss & Holland, 1967). This technique, used by a variety of different experimenters, delineated three efferent systems from the nucleus accumbens: extrapyramidal connection, striatopallidal connections, and striatomesencephalic connections (Nauta et al., 1978; Zahm & Brog, 1992).

The striatopallidal projection system is the efferent system originating from the nucleus accumbens and projecting to the ventral pallidum (VP.) The core and shell projects to the VP in distinct patterns. The core projects to the lateral division of the VP (VPl) while the medial division of the shell projects to the medial division of the ventral pallidum (VPm) (Usuda et al., 1998; Voorn et al., 2004). The output of the lateral and medial VP bifurcates to target different mesencephalic structures. The VPm feedbacks onto dopamine-rich zones on the VTA, completing what has been called a long-feedback loop (see Figure 3). The VPl, however, projects to motor regions such as the cholinergic pedunculopontine tegmental nucleus, as well as, the substantia nigra pars compacta (Rahman et al., 2001).

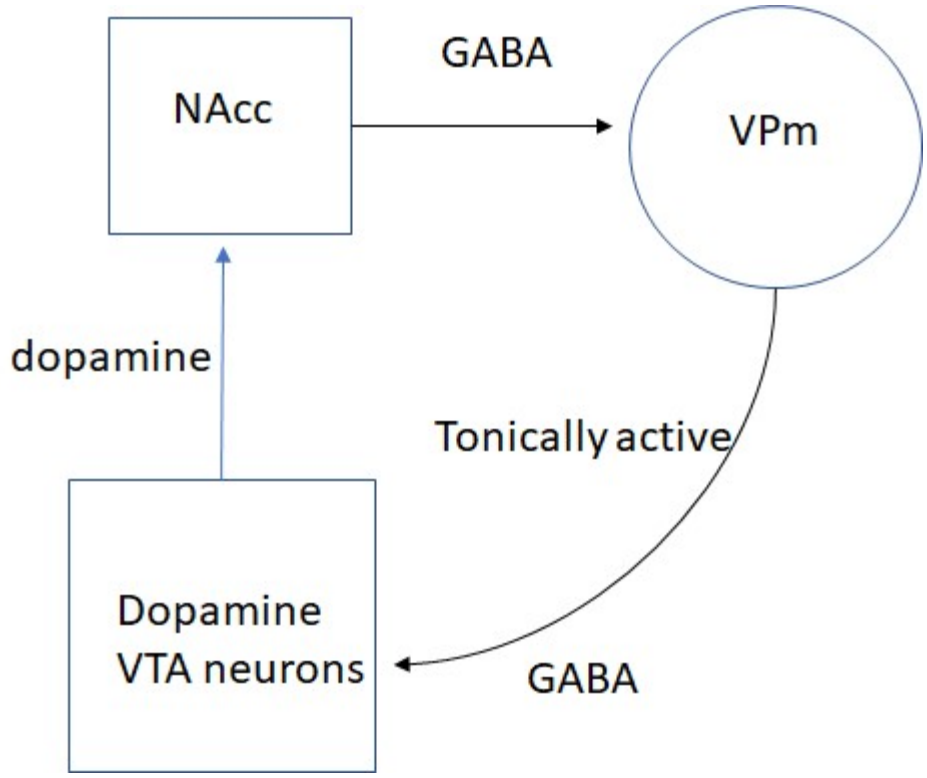


Figure 3: Long loop feedback from the nucleus accumbens to dopamine neurons in the VTA. Activation of dopamine neurons in the VTA excites GABAergic projection neurons of the nucleus accumbens (NAcc) to inhibit VPm GABAergic neurons. Since the VPm tonically inhibits dopamine neurons in the VTA, inhibition of these neurons by NAcc release VTA dopamine neurons from tonic inhibition. This causes irregular spontaneous firing leading to the tonic release of dopamine in the extracellular space. Figure adapted from: Grace, A. A., Floresco, S. B., Goto, Y., & Lodge, D. J. (2007). Regulation of firing of dopaminergic neurons and control of goal-directed behaviors. *Trends in Neurosciences*, 30(5), 220-227. Abbreviations: GABA: Gamma-aminobutyric acid; NAcc: Nucleus accumbens shell; VPm: medial division of the ventral pallidum.

The extrapyramidal connections of the nucleus accumbens shell encompass forebrain projections to subcortical nuclei that are separate from basal ganglia nuclei. Using tract-tracing methodology, it has been demonstrated that the nucleus accumbens sends medium spiny neuron (MSN) projection neurons to the lateral hypothalamus, anterior hypothalamus-medial preoptic

area, lateral septum, cingulum, and thalamus (Zahm & Heimer, 1993). Finally, the striatomesencephalic projections encompass projections that terminate in regions of the mesencephalon such as the medial and lateral divisions of the VTA, substantia nigra pars compacta and pars reticulata, periaqueductal grey and raphe nucleus (Conrad & Pfaff, 1976; Groenewegen & Russchen, 1984; Nauta et al., 1978). These findings, amongst others, had led to the argument that the nucleus accumbens is a key nodal point that integrates subcortical information related to emotions and translates this to motivated behaviours with its connections to the PPTg and pars compacta (see Figure 4; Floresco, 2015; Heimer & Wilson, 1975; Mogenson et al., 1980).

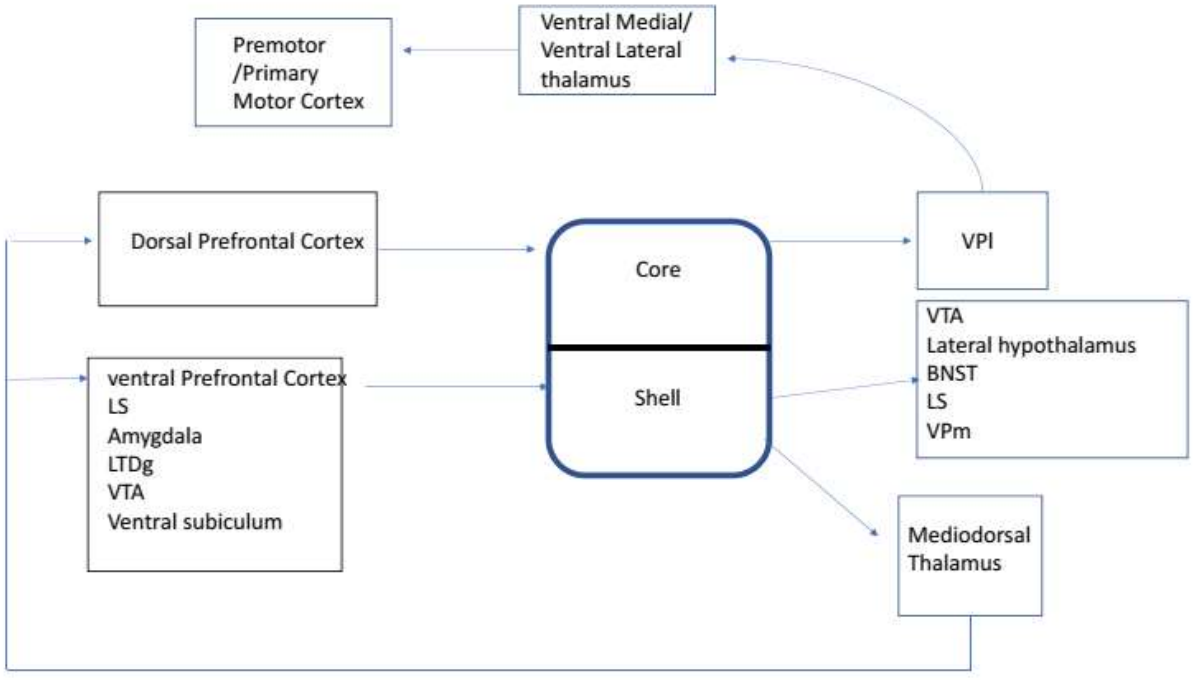


Figure 4: Diagram of the afferent (arrows leading to) and efferent (arrows leading away) connections of the nucleus accumbens. The nucleus accumbens core receives dense projections

from the dorsal prefrontal cortex. The output of the core primarily targets the lateral division of the ventral pallidum which targets one of the final common targets of the basal ganglia, the thalamus. The shell of the nucleus accumbens receives input from a variety of structures that are related to homeostatic maintenance and emotional regulation. The figure is adapted from studies by: Pennartz, Groenewegen, & da Silva (1994). The nucleus accumbens as a complex of functionally distinct neuronal ensembles: an integration of behavioural, electrophysiological and anatomical data. *Progress in Neurobiology*, 42(6), 719-761; Usuda, Tanaka, & Chiba, (1998). Efferent projections of the nucleus accumbens in the rat with special reference to subdivision of the nucleus: biotinylated dextran amine study. *Brain Research*, 797(1), 73-93; and Zahm, & Brog, (1992). On the significance of subterritories in the "accumbens" part of the rat ventral striatum. *Neuroscience*, 50(4), 751-767.

The release of dopamine into the nucleus accumbens has been categorized as occurring in either tonic or phasic patterns (Goto et al., 2007; Grace, 1991). The tonic release of dopamine into the nucleus accumbens shell results from the disinhibition of dopamine neurons within the VTA by inhibition of GABAergic projection neurons within the medial division of the ventral pallidum. This causes a subset of dopamine neurons within the VTA to fire irregularly and provide a bassline dopamine level within the extrasynaptic space of the nucleus accumbens (Zhang et al., 2009). During rewarding stimulation, dopamine neurons within the VTA are observed to undergo synchronized burst firing. This burst firing causes dopamine to be released within the synaptic space at high concentrations to stimulate postsynaptic dopamine receptors and stimulate the dopamine reuptake transporter (DAT). This spatially restricts dopamine within the synaptic compartment opposed to the extrasynaptic space. (Blaha et al., 2006; Floresco et al., 2003; Grace, 2000; Sombers et al., 2009; Schultz, 1998). The phasic release of dopamine into the nucleus accumbens requires not just disinhibition of dopamine neurons within the VTA but is

also dependent upon glutamatergic input on dopamine neurons from areas like the subthalamic nucleus and the prefrontal cortex (Smith & Grace, 1992) (See Figure 5).

The phasic release of dopamine within the nucleus accumbens can be artificially recreated by intracerebral injection of dopamine agonists into the nucleus. Injection of dopamine agonists can bind to postsynaptic dopamine receptors and augment the activity of GABAergic projection neurons of the nucleus accumbens.

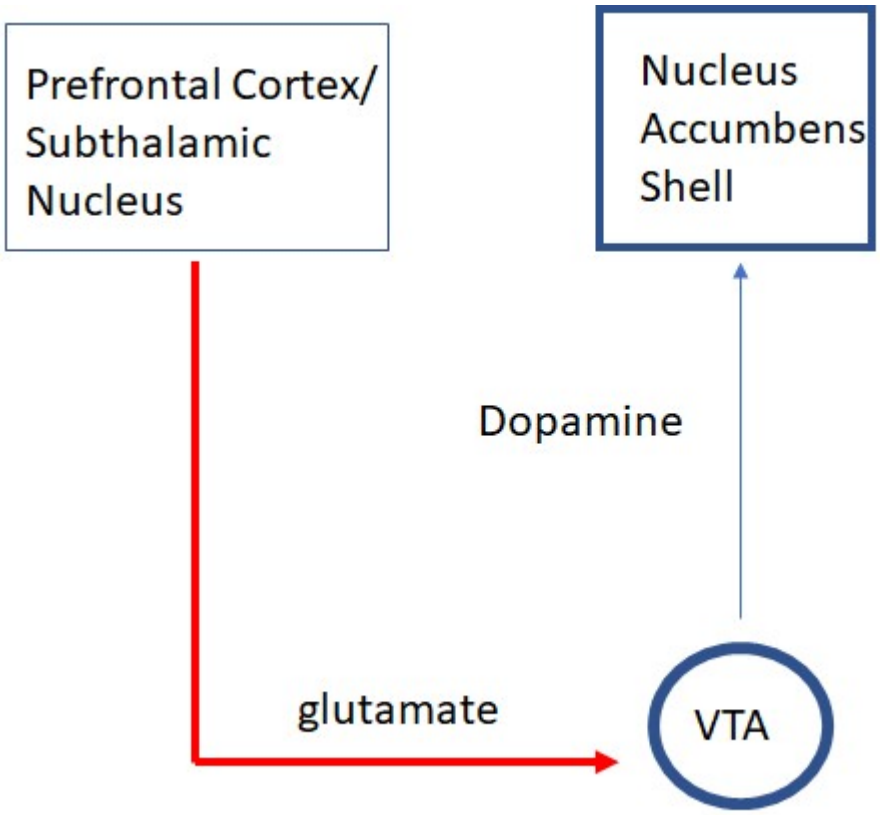


Figure 5: Phasic release of dopamine within the nucleus accumbens. Dopamine neurons within the VTA are inhibited by tonically active GABA neurons from the VPm. Activation of dopamine neurons in the VTA, leading to the phasic release of dopamine within the nucleus accumbens shell, can be accomplished by release of glutamate onto dopamine cells within the VTA from areas such as the prefrontal cortex or the subthalamic nucleus in combination with the inhibition of tonically active GABA cells from the VPm (Schultz, 1998).

1.3: Involvement of the ascending mesolimbic dopamine system in pro-social 50-kHz USVs in rats

Dopamine is a catecholamine neurotransmitter that is synthesized from the amino acid tyrosine. Tyrosine is converted to dihydroxyphenylalanine (DOPA) by the enzyme tyrosine hydroxylase. DOPA is then converted to dopamine via the enzyme aromatic amino acid decarboxylase (Daubner et al., 2012). Within the brain, there are three major neural pathways that synthesize and release dopamine. The nigrostriatal dopamine pathway innervates the dorsal striatum and is important for locomotor activity and sensory integration. The mesocorticolimbic dopamine system originates within the VTA and innervates the olfactory tubercle, nucleus accumbens shell, amygdala and the prefrontal cortex (PFC) (Ranaldi, 2014; Sun, 2011); and the tuberoinfundibular system originates within the arcuate nucleus of the hypothalamus and travels to the median eminence (Dawson, 1985).

The action of dopamine on receptors is neither directly excitatory or inhibitory. Instead, dopamine functions as a neuromodulator that can alter the electrophysiological properties of neurons via five distinct G-protein coupled receptors: D_1, D_2, D_3, D_4, and D_5 (Beaulieu & Gainetdinov, 2011). The five different receptors are classified into two groups: D_1-like and D_2-like groups. The grouping of the five distinct dopamine receptors was based on biochemical studies showing D_1/D_5 receptors stimulate the formation of the intracellular signaling molecule adenyl cyclase (D_1-like group) while the D_2-like group inhibits the formation of adenyl cyclase (D_2, D_3, D_4) (Jackson & Westlind-Danielsson, 1994).

Initial evidence that was put forth arguing that dopamine transmission within the shell of the nucleus accumbens was responsible for the initiation of 50-kHz USVs was from the intracerebral application of the indirect dopamine agonist amphetamine. Burgdorf and colleagues

(2001) showed a reliable dose-dependent effect of amphetamine (ranging from 0.3µg-10.0µg) on the increased emissions of FM 50-kHz USVs. The emission of 50-kHz USVs was not observed when the same doses of amphetamine were injected into the caudate-putamen or core of the nucleus accumbens shell. (Burgdorf et al., 2001). Despite amphetamine blocking the reuptake of dopamine, noradrenaline, and serotonin, optogenetic experiments investigating VTA neurons provided substantial information on the importance of dopamine release in the nucleus accumbens shell and its association with behaviours reflecting positive emotional states.

Optogenetic tools use genetically modified light-gated proteins for the excitation or inhibition of neural tissue. Some of the most commonly used light-gated proteins are channelrhodopsin-2 (ChR2), which can be activated by 450-500 nm light pulses (1-7 ms) (Ye & Kaszuba, 2017). These light pulses cause a conformation change in the 7-transmembrane protein located in the neural tissue that allows for large influxes of calcium (Ca^{2+}) or sodium (Na^{+}) ultimately leading to neuron depolarization (Boyden et al., 2005).

To assess the role of dopaminergic neurons involved in reward-related behaviour Adamantidis and colleges (2011) was able to express ChR2 in dopamine-containing neurons of the VTA using a viral vector. The results demonstrated two important consequences of stimulating dopamine neurons in the VTA: 1) Optogenetic excitation of dopamine neurons at 5 ms light-pulses delivered at a frequency of 25 Hz in the VTA resulted in phasic release of dopamine signals within the shell of the nucleus accumbens measured via fast-scan cyclic voltammetry (FSCV); 2) Optical stimulation that results in the release of phasic dopamine within the nucleus accumbens was sufficient to promote self-stimulation-like behaviour (Adamantidis et al., 2011).

Consistent with the rewarding-like effects of optical stimulation of dopamine neurons in the VTA that promotes self-stimulation behaviour, Scardochio and colleagues (2015) showed a comparison between electrical stimulation of the VTA and optical stimulation of dopamine neurons in the VTA and their ability to induce 50-kHz USVs. Electrical stimulation of the medial forebrain bundle, which contains dopamine axons originating from the VTA, was sufficient to induce intracranial self-stimulation, increased the release of dopamine within the nucleus accumbens and increased the emissions of 50-kHz USVs (Fiorino et al., 1993; Scardochio et al., 2015) The authors further demonstrated that optogenetic activation of dopamine neurons in the VTA that is sufficient to release dopamine in the nucleus accumbens shell in a phasic manner could initiate FM 50-kHz USVs (Scardochio et al., 2015)

1.4: Acetylcholine and negative emotional states

Acetylcholine (ACh) contains two prominent projection system: a basal forebrain system that innervates the entirety of the cortical mantle and a pontomesencephalic system that innervates subcortical structures (Woolf, 1990). Like the catecholamine system, the pathways in the brain that contain acetylcholine synthesizing neurons have been labeled Ch1-Ch6, which are segregated into basal forebrain systems and ponotomesencephalic systems (Mesulam et al., 1983). Ch1-Ch2 systems are located in the medial septal nucleus, and vertical limb of the diagonal band of Broca and subsequently provide the major acetylcholine input into the hippocampus. Ch3 is located in the horizontal limb of the diagonal band of Broca which innervates the olfactory bulb. Ch4 contains cholinergic neurons that provide the major cholinergic innervation to the cortical mantle. Ch5 group cholinergic neurons are located within the pedunculopontine tegmental (PPTg) nucleus and innervate locomotor regions of the forebrain. Ch6 contains neurons located within the laterodorsal tegmental nucleus (LTDg) which

provides cholinergic input to a variety of different brain nucleus such as the VTA, nucleus accumbens, anterior hypothalamus, and lateral septum (Mesulam et al., 1983; Woolf, 1991; Woolf et al., 1983; Woolf et al., 1985; Woolf et al., 1985).

Once released into the synapse, the effects of acetylcholine are mediated by two different classes of receptors: metabotropic muscarinic receptors and ionotropic nicotinic receptors (Taylor et al., 1980). The muscarinic metabotropic receptors contain five distinct classes of receptors (M_1-M_5) and are G-protein coupled receptors that signal through different intracellular pathways. For example, M_1, M_3, and M_5 receptors signal through $G_{\alpha q}$ pathway which leads to activation of phospholipase C and eventually the formation of diacylglycerol (DAG) and inositol triphosphate (IP_3) via hydrolysis of 4,5-bisphosphate (PIP_2). Conversely, muscarinic receptors M_2 and M_4 signal through the $G_{\alpha i}$ pathway that inhibits the production of cyclic AMP (cAMP) via suppression of the enzyme adenylate cyclase (Wess, 1996; van Koppen & Kaiser, 2003). Acetylcholine can also exert an excitatory influence on neurons via ionotropic nicotinic receptors. These receptors are pentameric ion channels that can be homomeric channels composed of multiple α subunits (α_{2-7}) or heteromeric channels that are composed of a mixture of α (α_{2-7}) and β (β_{2-4}) subunits (Higley & Picciotto, 2014; Paterson & Nordberg, 2000). The termination of acetylcholine within the synapse is mediated by the enzyme acetyolcholinesterase (AChE), which hydrolyzes the neurotransmitter into choline and acetate (Čolović et al., 2013).

External or endogenous stimuli that impose a demand on the body can be defined as a "stress." Stress can impact almost all divisions of a human's physiological systems, including gastrointestinal, cardiovascular, endocrine, immunological, etc. These responses to stress have been termed stress responses. Depending on the system that is being investigated, stress can be measured by quantifying cardiovascular changes such as increased heart rate, vasodilation, or

increased respiration rate (Yuenyongchaiwat, 2017). Stress can also be quantified using self-report from patients themselves while under a stressor. Self-report is assumed to reflect psychological changes indicative of mood and behaviour and can include anxiety, depression, hostility, agitation, paranoia, and emotional malaise (Julian, 2014). In general, stressors that decrease the fitness of the animal can induce negative emotional states (Brudzynski, 2013).

Hypotheses speculating of the involvement of acetylcholine in negative emotional states involved the evaluation of effects of organophosphates which are, by design, anticholinesterase agents (anti-AChE). Organophosphates like sarin, EA-1701, diisopropylfluorophosphate exert their influence by binding to acetylcholinesterase (AChE), the main enzyme that is responsible for the breakdown of the neurotransmitter acetylcholine (Taylor & Brown, 1999). The inability of ACh to be hydrolyzed by AChE results in an abnormally increased availability of acetylcholine within the synapses that is capable of interacting with both pre- and post-synaptic acetylcholine receptors, which can induce a variety of different physiological results.

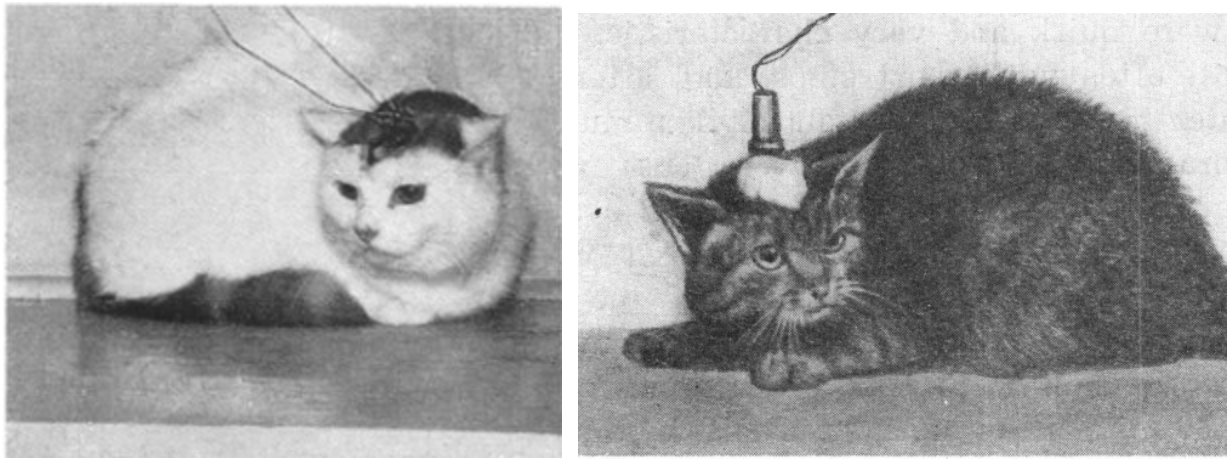
The behavioural manifestations of patients within the clinical setting in response to anti-AChE compounds paralleled the affective changes noted by Holmes and Gaon (1956) in a population of roughly 600 people (mostly factory workers and farmers) that were exposed to the anti-AChE compounds paraoxon, diisopropyl fluorophosphate or tetramethyl pyrophosphate. In the study, patients reported marked increase in confusion, lethargy, irritability, insomnia, paranoia, anxiety, anger and depression upon exposure (Bowers et al., 1964; Holmes & Ganon, 1956; Grob & Harvey, 1958; Janowsky et al., 1973; Levin et al., 1976). Since organophosphates increase the synaptic concentration of acetylcholine within the nervous system, the identity of the receptor system or pathway mediating the psychiatric symptomology of organophosphate exposure was obscure.

Evidence that specifically began to narrow the involvement of acetylcholine receptors in the negative emotional states emerged with clinical experiments using drugs that bind to muscarinic receptors but do not induce any biology effect. These drugs are known as muscarinic antagonists and include the compound scopolamine. Scopolamine's influence on psychiatric symptomatology was reported by Khajavi and colleagues (2012). They evaluated the efficacy of oral scopolamine (oral ingestion of 1mg/day) in forty patients suffering from a major depressive disorder in a randomized, double-blind placebo-controlled study. They reported that scopolamine-treated group increased their self-reported measure of positive emotional state and that their overall energy and motivation levels (Khajavi et al., 2012). Similar improvements in mood were observed when scopolamine was given to clinically depressed patients suffering from alcoholism. Intramuscular injection (0.4 mg i.m.) of scopolamine decreased self-reported measures of tension, anxiety, frustration, anger, fatigue, confusion, and depression (Gillin et al., 1991). Similar results of scopolamine's anti-depressant and anti-anxiety effects were reported in other clinical cases (see Drevets et al., 2010; Furey et al., 2006; Furey et al., 2010). The positive effects of scopolamine on emotional states have been mimicked by other muscarinic antagonists such as hydroxyzine (Guaiana et al., 2010), promethazine (Jalbout et al., 1994), and tofenacin (Capstick & Pudney, 1976) suggesting that an aspect of negative emotionality is mediated by the muscarinic family of receptors.

1.5: The neurobiology of the ascending mesolimbic cholinergic system: a comparison between rats and cats

Experimental methods that have been used to investigate the neurobiological systems that can initiate emotional responses include electrical stimulation and injection of drugs into brain nuclei or ventricles. For example, electrical stimulation of the hypothalamus in the cat can

produce a prototypical emotional defense response consisting of defensive postures, retraction of ears, an extension of the tail, hissing, spitting, pupil dilation, and piloerection (Brown et al., 1969; Hess & Akert, 1955). A similar defensive response could be initiated from cats via injection of the muscarinic agonist (a muscarinic agonist is a drug that binds to the muscarinic receptor and acts like acetylcholine) carbachol into the same region that was electrically stimulated by Brown and colleagues (1969). The defensive response induced by carbachol injections resulted in protraction of claws, growling vocalization, hissing, pupil dilation, arching of the back, and piloerection (see Figure 6; Baxter, 1967; Brudzynski, 1981). The strip of tissue that can initiate defensive responses in cats begins at the tegmental region and extends along the neuraxis to the lateral septum (Decsi, 1974) (see Figure 7).



A) Electrical stimulation of AH-MPO B) Chemical stimulation of AH-MPO

Figure 6: Development of defensive behaviour in the cat in response to electrical (Figure A) and chemical stimulation of the AH-MPO with the cholinergic agonist carbachol (Figure B). In both cases, stimulation of the AH-MPO produces a growling response, piloerection, pupillary dilation, and slight retraction of the ears. Figure A taken from Brown, Hunsperger, & Rosvold (1969). Defense, attack, and flight elicited by electrical stimulation of the hypothalamus of the cat. *Experimental Brain Research*, 8(2), 113-129. Figure B was taken from Brudzynski (1981).

Carbachol-induced agonistic behavior in cats: aggressive or defensive response. *Acta Neurobiologiae Experimentalis*, 41, 15-32.

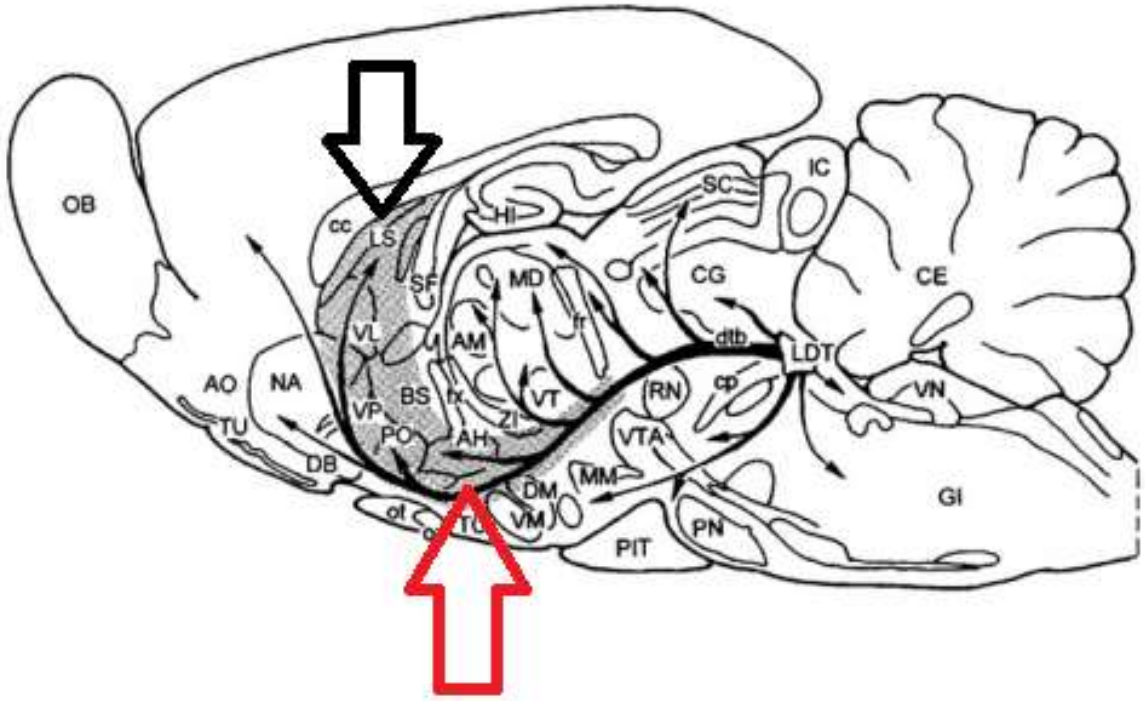


Figure 7: A parasagittal cross-section of the rat brain displaying the medial cholinoceptive vocalization strip. The dark lines emanating from the LTDg represent cholinergic axons innervating various areas of the midbrain and forebrain. The regions that are shaded from the hypothalamus in the caudal division (red arrow) to the LS (black arrow), the most rostral division represents the medial cholinoceptive vocalization strip. Injection of carbachol into the AH-MPO (red arrow) or the LS (black arrow) induced species-typical 22-kHz USVs. Sime abbreviations: AH – anterior hypothalamus; LDT – laterodorsal tegmental nucleus; LS – lateral septum. The figure is taken from: 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(2), 261-273.

The strip of neural tissue that is sensitive to cholinergic stimulation that results in the initiation of defensive behaviours in cats is also found in rats. In rats, the laterodorsal tegmental nucleus (LTDg) is a prominent cholinergic nucleus within the pons that innervates nuclei along the neuraxis. Projections of the LTDg overlap extensively with stimulation points that have been reported to induce defense responses in cats observed upon cholinergic or electrical stimulation (Woolf et al., 1984; Wolf & Butcher, 1986). This strip of tissue has been defined as the medial cholinoceptive vocalization strip since it is a strip of neural tissue composed of medially positioned nuclei that, upon stimulation with muscarinic agonists, induce species-typical vocal responses.

In rats, injection of the excitatory amino acid, glutamate, into the LTDg activated cholinergic neurons and induced species-typical 22-kHz USVs without the occurrence of 50-kHz USVs. (Brudzynski & Barnabi, 1996). These glutamate-induced 22-kHz USVs from the LTDg can be significantly attenuated when the muscarinic antagonist scopolamine is injected into the anterior hypothalamus-medial preoptic area, an area that can initiate growling responses in cats when stimulated (Brown et al., 1969; Brudzynski & Barnabi, 1996). Consistent with a scopolamine-induced decrease of glutamate-induced 22-kHz USVs from the LTDg, direct injection of carbachol into the AH-MPO can induce species-typical 22-kHz USVs suggesting muscarinic receptors within the medial cholinoceptive vocalization strip are important for the initiation of 22-kHz USVs in rats (Brudzynski, 2007).

The most rostral division of the medial cholinoceptive vocalization strip contains the lateral septum (LS); a structure that is involved in the processing of fear and anxiety. For example, foot-shocks and air-puffs, both conditions, which induced emission of 22-kHz USVs (Brudzynski & Holland, 2005; Inagaki & Sato, 2016; Knapp & Pohorecky, 1995), can increase

the neural activity within the LS measured by the activation of the protooncogene *c-fos (*Duncan et al., 1996). Likewise, pharmacological injection of carbachol into the LS can induce species-typical 22-kHz USVs. The increase in 22-kHz USVs upon carbachol microinjection into the LS was correlated with an increase in cholinergic cell activity within the LTDg (Brudzynski et al., 2011). The observations that glutamate injection into the LTDg induced 22-kHz USVs that were attenuated by scopolamine, a muscarinic antagonist, and 22-kHz USVs can be directly initiated by direct application of the muscarinic agonist, carbachol, into the AH-MPO and the LS, suggests that: 1) muscarinic receptors are involved in the initiation of species-typical 22-kHz USVs and that 2) the emission of 22-kHz USVs is an indirect measure of acetylcholine release within the medial cholinoceptive vocalization strip (Brudzynski, 2007).

1.6: Summary

Rats can emit 50-kHz and 22-kHz USVs that signal distinct, mutually exclusive emotional states; 50-kHz USVs, specifically FM 50-kHz USVs, signals a positive emotional state while 22-kHz USVs signal a negative emotional state. The initiation of 50-kHz USVs is induced by phasic dopamine release within the shell of the nucleus accumbens, while 22-kHz USVs is induced by acetylcholine being released along the medial cholinoceptive vocalization strip. The occurrence of the expression of each type of USVs is mutually exclusive with 22-kHz USVs only being observed in situations that initiate a negative emotional state, while 50-kHz USVs are only observed in situations that are associated with positive emotional states. Despite the exclusivity of these two types of USVs, there has been no direct investigation of whether the systems that initiate these USVs are antagonistic to each other. However, there is indirect evidence briefly summarized below:

1) The anticipation of drugs of abuse, or natural rewards like food, increase the phasic release of dopamine within the nucleus accumbens. Phasic release of dopamine is associated with the initiation of 50-kHz USVs in rats. However, failure to deliver an expected reward, conditions that initiate 22-kHz USVs, inhibit dopamine neurons in the VTA indirectly by activating GABAergic neurons in the tail of the VTA that projects onto dopamine cells in the VTA (Barrot et al., 2012).

2) The anticipation of foot-shock or air-puff increases fos immunoreactivity in brain structures that can initiate 22-kHz USVs upon electrical or cholinergic chemical stimulation (Brudzynski et al., 2011; Duncan et al., 1996; Kroes et al., 2007; Singewald et al., 2003)

3) Drugs that decrease anxiety in humans like fluoxetine or benzodiazepine, decreases nucleus accumbens shell acetylcholine release (Chau et al., 2011).

4) Tickling adolescent rats, which has been shown to increase both phasic releases of dopamine into the nucleus accumbens and increase the emission of FM 50-kHz USVs decreases audible pain vocalizations (Cloutier et al., 2014)

5) Rats selectively bred for low levels of 50-kHz USVs have increased cortical measurements of the peptide cholecystokinin (CCK), which is correlated to increased 22-kHz USVs during the social defeat in rats (Burgdorf et al., 2006; Panksepp et al., 2004).

6) Expected electrical stimulation of the VTA increases the emission of FM 50-kHz USVs, but failure to the expected electrical stimulation shifts the emission of FM 50-kHz USVs to 22-kHz USVs (Burgdorf et al., 2000). A similar result is observed in cocaine self-administration studies. Self-administration of cocaine at doses lower than expected shift the emissions of FM 50-kHz USVs to 22-kHz USVs (Barker et al., 2000).

7) Induced morphine withdrawal using the pharmacological agent naloxone, a condition which increases 22-kHz USVs, decreases nucleus accumbens dopamine concentration while increasing nucleus accumbens acetylcholine concentration (Pothos et al., 1991; Rada et al., 1991).

8) Electrical stimulation of the nucleus accumbens relieves drug-resistant depression, anxiety and obsessive-compulsive disorder in men (Bewernick et al., 2010; Bewernick et al., 2012; Grubert et al., 2011; Taghva et al., 2013).

1.7: Purpose of experiment and hypotheses

The purpose of this thesis is to examine whether the initiation of a positive emotional state can decrease the magnitude of expression of a negative emotional state. In this thesis, the positive emotional state will be induced by microinjections of a dopamine agonist R-(-)-apomorphine in the nucleus accumbens shell and the magnitude of the emotional state will be reflected by the number of emitted FM 50-kHz USVs. The negative emotional state will be induced by microinjections of the muscarinic agonist carbachol into the AH-MPO and the most rostral division of the medial vocalization strip, the LS. The magnitude of the negative emotional

state will be indexed by the number of emitted 22-kHz USVs. The overarching hypothesis of this thesis is that the initiation of a positive emotional state inhibits the initiation of a negative emotional state.

In order to achieve the intended purpose of the thesis, three questions will be asked, which are divided into three chapters:

1) Can initiation of a positive emotional state, induced by apomorphine injection into the medial nucleus accumbens shell, decrease carbachol-induced 22-kHz USVs from the AH-MPO? (Chapter 2). The first hypothesis was that initiation of positive pro-social FM 50-kHz USVs via injection of the dopamine agonist apomorphine into the nucleus accumbens would be able to decrease the number of emitted 22-kHz USVs initiated by injection of carbachol into the AH-MPO. To validate the hypothesis, apomorphine was injected into the shell of the nucleus accumbens; then carbachol was injected into the AH-MPO a few minutes later. The number of 22-kHz USVs were automatically recorded, then manually analyzed.

2) Can initiation of a positive emotional state, induced by apomorphine injection into the medial nucleus accumbens shell, decrease carbachol-induced 22-kHz USVs from the LS? (Chapter 3). To answer this question, apomorphine was injected into the medial division of the nucleus accumbens shell followed by carbachol injection into the LS. The number of 22-kHz USVs were then automatically recorded and manually analyzed. The second hypothesis was that apomorphine would be capable of decreasing carbachol-induced 22-kHz USVs from the LS. The purpose of the

experiment was to demonstrate that the antagonism of the positive emotional state on a negative emotional state was not restricted to the anterior hypothalamus. The methodology used to examine the second hypothesis was similar to the first experiment. However, carbachol was injected into the LS instead of the AH-MPO.

3) Can pharmacological antagonism of dopaminergic neurons done by systemic haloperidol or microinjection of the D_2 antagonist raclopride, increase carbachol-induced 22-kHz USVs induced from the LS? (Chapter 4). The final hypothesis of the thesis was that blocking dopamine receptors within the mesolimbic system would increase the magnitude of the negative emotional state induced by carbachol injection into the LS. The increased magnitude of the negative emotional state would be hypothesized to be reflected in the increased quantity of emitted 22-kHz USVs. This question was divided into two experiments. In the first experiment, haloperidol, a D_2 receptor antagonist, was injected systemically followed by injection of carbachol into the LS. The number of 22-kHz USVs were then recorded. In the second experiment, raclopride, a D_2 receptor antagonist, was injected directly into the nucleus accumbens shell followed by carbachol injection into the LS. The number of 22-kHz USVs were then automatically recorded.

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Chapter 2: Intracerebral injection of R-(-)-Apomorphine into the nucleus accumbens decreased carbachol-induced 22-kHz ultrasonic vocalizations in rats

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2.0: Abstract

Rats can produce ultrasonic vocalizations (USVs) in a variety of different contexts that signal their emotional state to conspecifics. Under distress, rats can emit 22-kHz USVs, while during positive pro-social interactions, rats can emit frequency-modulated (FM) 50-kHz USVs. It has been previously reported that rats with increasing emission of FM 50-kHz USVs in anticipation of rewarding electrical stimulation or positive pro-social interaction decrease the number of emitted 22-kHz USVs. The purpose of the present investigation was to determine, in a pharmacological-behavioural experiment, if the positive emotional arousal of the rat indexed by the number of emitted FM 50-kHz USVs can decrease the magnitude of a subsequent negative emotional state indexed by the emission of 22-kHz USVs. To induce the positive emotional arousal, an intracerebral injection of a known D_1/D_2 agonist R-(-)-apomorphine (3.0µg/0.3µl) into the medial nucleus accumbens shell was used, while the negative emotional arousal was

induced by intracerebral injection of carbachol (1.0µg/0.3µl), a known broad-spectrum muscarinic agonist, into the anterior hypothalamic-medial preoptic area. Our results demonstrated that the initiation of a positive emotional state was able to significantly decrease the magnitude of subsequently expressed negative emotional state measured by the number of emitted 22-kHz USVs. The results suggest the neurobiological substrates that initiate positive emotional state antagonize the brain regions that initiate negative emotional states.

2.1: Introduction

In the past few decades, it has been documented that rats can vocally express their emotional states through the emission of ultrasonic vocalizations (USVs) (Cuomo et al., 1992; Knutson et al., 2002; Brudzynski, 2009; 2013; 2015). These vocal signals are valence-specific and directed to other rats (Brudzynski, 2007; Takahashi et al., 2010; Laplagne & Costa, 2016). Prototypical USVs are classified into two different categories based on their sonographic features (Brudzynski, 2007; Portfors, 2007).

22-kHz USVs are a category of USVs that are emitted by rats during defensive or aversive contexts (Brudzynski, 2007). For example 22-USVs are emitted in response to proximity to a predator (Blanchard et al., 1991; 1992), anticipation of electrical shock (Borta et al., 2006; DeVry et al., 1993; Jelen et al., 2003), anticipation of an air-puff directed to the head or nape of the rat (Brudzynski & Holland 2005; Knapp & Pohorecky, 1995), in response to acoustic startle response (Kaltwasser, 1991), or withdrawal from prolonged treatment of drugs of abuse like cocaine (Mutschler & Miczek, 1998). Structurally, 22-kHz USVs are unmodulated or

flat frequency calls with a duration ranging between 100-3000 ms, and a peak frequency between 18-32-kHz (Brudzynski et al., 1993; Brudzynski., 2007). The number of 22-kHz USVs that are emitted during conditioned anxiogenic circumstances can be attenuated by administration of anti-anxiety medications (Sánchez, 1993; Tomazini et al., 2006) further supporting the argument that 22-kHz USVs are reflective of a negative emotional state.

In contrast to the 22-kHz USVs, rats can also emit 50-kHz USVs in positive and pro-social contexts. 50-kHz USVs are divided into flat (F) and frequency modulated (FM) 50 kHz USVs although F calls seem to mediate a form of social coordination (Biały et al., 2000; Brudzynski and Pniak, 2002; Schwarting et al., 2007; Wöhr et al., 2008). Rats usually emit higher numbers of FM 50 kHz USVs than F 50 kHz USVs during heterospecific play (Burgdorf et al., 2008; Brugdorf et al., 2011; Burgdorf & Panksepp, 2001; Panksepp & Burgdorf 2000), drug-reward cues (Meyer et al., 2012), or in anticipation of rewarding electrical brain stimulation of brain regions mediating reward (Burgdorf et al., 2000; Burgdorf et al., 2007; Scardochio et al., 2015).

Emission of 22-kHz or 50-kHz USVs is initiated by two separate neurotransmitter systems arising from two separate brain nuclei located in the midbrain tegmentum of the rat brain. The initiation of 50-kHz USVs has been reported to be dependent on the phasic release of dopamine within the nucleus accumbens shell from terminals originating from the ventral tegmental area (VTA) (Burgdorf et al., 2001; Thompson et al., 2006; Brudzynski et al., 2012), while the initiation of 22-kHz USVs has been reported to be dependent on the release of acetylcholine into the medial cholinoceptive vocalization strip, including the anterior hypothalamus-medial preoptic area (AH-MPO) and lateral septum (LS), by activity of cholinergic cell bodies located in the laterodorsal tegmental nucleus (LTDg) (Brudzynski & Bihari, 1990; Brudzynski, 2001; 2007; Brudzynski et al., 2011; Brudzynski & Barnabi, 1996).

Results of behavioural-pharmacological investigations of the neurotransmitter systems involved in addiction suggested a possible dynamic relationship between the VTA and the LTDg in promoting behaviours associated with positive or negative emotive states. 22-kHz USVs are reliably recorded from rats during withdrawal from opiate drugs like morphine (Vivian & Miczek, 1991), alcohol (Berger et al., 2013), cocaine (Miczek et al., 1996; Simmons et al., 2018) and nicotine (Rada et al., 1991); all conditions, which decreased the concentration of dopamine within the nucleus accumbens shell (Hildebrand et al., 1998; Rossetti et al., 1991; Rada et al., 2004; Liu & Jun 2004). Likewise, drugs of abuse that increase the concentration of dopamine within the nucleus accumbens increase the propensity of rats to emit 50-kHz USVs (Brudzynski 2007; 2013; Simola et al., 2018; Wright et al., 2010).

The purpose of the present experiment was to further investigate the role of acetylcholine-dopamine interactions during pharmacologically induced expression of emotional states. We chose to examine the emission of USVs since it is a sensitive measure of the rat's emotional state (Brudzynski, 2007). In this experiment, the non-selective dopamine agonist R-(-)-apomorphine was injected into the medial shell of the nucleus accumbens to induce a positive emotional state, while carbachol was subsequently injected into the AH-MPO to induce a negative emotional state. If there is an antagonistic relationship between the neurochemical system, the initiation of a positive emotional state by R-(-)-apomorphine will reduce the magnitude of 22-kHz USVs emission. If there is no antagonistic relationship between the two neurochemical systems, then the initiation of a positive emotional state by R-(-)-apomorphine will not change.

2.2: Methods and Procedure

2.2.a: Recording of 50-kHz USVs after intracerebral injection of R-(-)-apomorphine

This group of rats was used to demonstrate the effects of R-(-)-apomorphine on the emission of 50-kHz USVs after its injection into the nucleus accumbens shell alone.

2.2.b: Subjects and Surgery

Fifteen adolescent male Long-Evans rats (Charles River Laboratories, Saint-Constant, QC, Canada) with body weights ranging from 270-350 g at the time of surgery served as the experimental subjects. All animals were housed in polycarbonate cages (48 x 27 x 20 cm) in a room with a constant temperature (23° C ± 2° C) and humidity settings. Rats were on a 12:12 h light-dark cycle with *ad libitum* access to standard food pellets and filtered tap water.

Rats underwent stereotaxic surgery for unilateral implantation of guide cannula into the nucleus accumbens shell in the left hemisphere. Briefly, rats were anesthetized with gaseous isoflurane at a concentration of 3% and placed in a Kopf stereotaxic apparatus (Model 900, David Kopf Instruments, Tujunga, CA). While in the apparatus, burr holes were drilled into the skull using an electric drill. A guide cannula (O.D. = 650 µm) was implanted into the left hemisphere. Guide cannulae were constructed from a 23 G stainless steel syringe needles (Beckton-Dickinson Canada, Mississauga, ON) and were implanted 1 mm above the intended injection site. Stereotaxic coordinates for implantation measured from the interaural plane, were as follows: A-P: 9.8-11.7; L: 0.8-1.8; D-V: 5.8-6.4 mm. The guide cannula was permanently attached to the skull with jeweler's screws and methyl methacrylate resin (Perm Resin, Hygenic Corporation of Canada Inc., St. Catharines, ON). Rats recovered for 5 days after the surgery

before they began 72 hours of habituation, for further details see Fornari et al. (2012). All research protocols were approved by Brock University Animal Care and Use Committee and complied with guidelines and policies set forth by the Canadian Council on Animal Care.

2.2.c: Pharmacological agent and intracerebral injection procedure

R-(-)-apomorphine hydrochloride hemihydrate (Sigma Chemicals Co., St Louis MO) was diluted with warm sterile 0.9% saline and 0.1% ascorbic acid. Vehicle injection conditions consisted of 0.9% saline combined with 0.1% ascorbic acid. Fresh R-(-)-apomorphine and vehicle were prepared for each injection day.

2.2.d: Intracerebral injection procedure and USV recording

Intracerebral injection of R-(-)-apomorphine was accomplished with a constant rate Hamilton® CR-700-20 micro-syringe (Hamilton Company, Reno, NV) at a rate of ~4.5 nl/s to a volume of 0.3µl. Once the injection of the drug or vehicle was finished, the injection cannula was left in place for 60 s to allow for the diffusion of the substance, the injection cannula was removed, and the guide cannula was closed with a sterile plug-pin. The rat was then placed in its home cage for approximately 90 s to recover from the handling and injection process. After the time spent in the home cage, the rat was placed in a Plexiglass recording chamber (25 cm x 18 cm x 18 cm). On top of the recording chamber, an Avisoft® CM16/CMPA condenser microphone (frequency range 2-250 kHz, Avisoft® Bioacoustics, Berlin, Germany) was placed with an average distance of 25 cm to the rat's head. Recording of the USVs was stored in a 16-bit format for later analysis. Analysis of USVs was done off-line using Avisoft® SAS LabPro program.

Identification of 22-kHz and 50-kHz USVs was followed as described in previous studies (Brudzynski et al. 1991, Brudzynski 2007, Thompson et al. 2006). Briefly, vocalizations that had a peak frequency that fell between 19-29 kHz and had a duration longer than 100 ms were classified as 22-kHz USVs while calls that had a peak frequency that fell between 39-80 kHz and had a duration less or equal to 100 ms were classified as 50-kHz USVs. USVs with peak frequency from 30-40 kHz were very rare and were not taken for analysis. Subsequent classification of 50-kHz into frequency modulated (FM) and flat (F) calls, i.e., unmodulated USVs, was based on morphological characteristics of calls on the sonograms consistent with the study by Burgdorf (2007). Recording of USVs took place for 10 min. After that time, the rat was placed back into its home cage. Each rat received a clean cage for recording time, and each soiled cage was removed from the test room. Before each additional animal was tested, the table was wiped down with Virox® (Virox Technologies Inc., Oakville, ON) then further cleaned with a diluted ethyl alcohol solution.

After the rat had received the final injection, it was anesthetized with an overdose of sodium pentobarbital. Before removal of the brain, an India-ink solution was prepared (1:100 dilution) and injected into the brain for histological determination of injection sites.

2.2.e: Histology and Localization of Injection sites

After injections were finished, animals underwent transcardial perfusion with 10% formalin. The brains were postfixed with formalin solution for 24 h and were removed and coronally sectioned on a freezing microtome (Cryo-Histomat, Hacker Instruments and Industries, Fairfield, NJ) to a thickness of ~40 μm. Sections were placed on 1% poly-lysine-coated slides, then underwent Nissl staining procedure with buffered thionin. Slides were then coated with

Permount™ mounting medium (Fisher Scientific Co., Ottawa, ON) and were coverslipped. Details of the histological procedures were followed as described by Lindroos & Leinonen (1983).

2.2.f: Statistics

A non-parametric repeated measures ANOVA (Friedman's ANOVA followed by Sign-ranked post hoc test) was used to assess the statistical difference between the number of 50-kHz USVs induced by R-(-)-apomorphine or vehicle from the nucleus accumbens shell. Analysis of sonographic features (call duration and peak frequency) was done using repeated measures ANOVA. A Shapiro-Wilks test was used to assess the normality of sonographic features to ensure the appropriate statistical procedure. All statistics were done using SPSS v 17.0 (SPSS Inc, Chicago, U.S.A). Multiple comparisons were corrected with Bonferroni method. Reported means are followed by the standard error of the mean (S.E.M).

2.2.i: Stereotaxic implantation of cannula into the left nucleus accumbens shell and left anterior hypothalamic-medial preoptic area (AH-MPO)

This group of rats was used for a double injection of pharmacological agents into two different brain areas that induce either 50-kHz USVs or 22-kHz USVs. R-(-)-apomorphine was injected into the medial shell of the nucleus accumbens to induce positive emotional arousal while carbachol was injected into the AH-MPO to induced negative emotional arousal. The intensity of emotional states was measured by the type and number of USVs emitted.

2.2.ii: Subjects and Surgery

Thirty-three adult male Long-Evans rats (Charles River) with body weight ranging from 300-350 g at the time of surgery served as the experimental subjects. All animals were housed in polycarbonate cages in constant room temperature and humidity conditions as described in 2.1.a. Rats were on a 12:12 h light-dark cycle with *ad libitum* access to food and water. After a 5-day recovery period from stereotaxic surgery, animals were handled and acclimated to the experimental procedure for 3 days before the start of the experiment.

Rats underwent stereotaxic surgery for unilateral implantation of guide cannula into the left hemisphere. Briefly, rats were anesthetized with gaseous isoflurane at a concentration of 3% and placed in a Kopf stereotaxic apparatus. While in the apparatus, burr holes were drilled into the skull and two guide cannula (O.D. = 650 µm) were implanted, one into the AH-MPO (stereotaxic parameters from the interaural line ranged from A-P: 8.04-7.44; L: -2.0-0 from the midline, and D-V: -6.4 to -8.2 from the surface of the skull), and the other cannula into the left nucleus accumbens shell (parameters from the interaural line ranged from A-P: 9.8-11.7; L: 0.8-1.8; D-V: 5.8-6.4). Cannulae were permanently secured to the skull by methyl methacrylate resin. For further details see subsection 2.1.a, above, and Fornari et al. (2012).

2.2.iii: Drugs and Injection Procedure

Carbachol (carbamylcholine chloride, Sigma Chemical Co., St. Louis MO.) was dissolved in 0.9% sterile saline and was injected unilaterally into the AH-MPO by a constant rate Hamilton® CR 700 micro-syringe in a dose of 1.0 µg/0.3µl at a rate of ~4.5 nl/s. R-(-)-apomorphine hydrochloride (Sigma, St. Louis, MO) was dissolved in saline with a 0.1% ascorbic acid and injected in a concentration of 3.0 µg/0.3µl at the same rate. Other details and vehicle preparation was described in section 2.1.b.

R-(-)-apomorphine (3.0 µg/0.3µl) was first injected into the shell of the nucleus accumbens. After injection of the drug or vehicle was finished, the injection cannula was left in place for an additional 60s to allow for proper drug diffusion, and after that, the rat was placed in its home cage. After 90 s, the rat was taken out of his cage, and carbachol was injected into the AH-MPO at the same rate and volume as R-(-)-apomorphine. After the injection of carbachol was finished, the injection cannula was left in place for an additional 60 s to allow for proper diffusion of the drug after which the injection cannula was removed, and the guide cannula was closed using a plug-pin. The rat was then immediately placed in the recording chamber, and vocalizations were recorded for 10-minutes.

2.2.iv: Recordings of USVs

The procedure for recording and analyzing USVs after intracerebral injections were the same as described in section 2.1.d.

2.2.v: Histology and localizations

After the experiment, animals underwent transcardial perfusion with 10% formalin. Brains were removed, postfix with formalin solution for 24 h, and coronally sectioned on a freezing microtome to a thickness of ~40 µm. Sections were placed on 1% poly-lysine coated slides, then underwent Nissl staining and were coverslipped. Details of the histological procedure were described in subsection 2.1.d, above, and in Lindroos & Leinonen (1983).

2.2.vi: Statistics

The statistical analysis for analyzing the number of calls and the analysis of spectrographic features of USVs was the same as described in section 2.1.f.

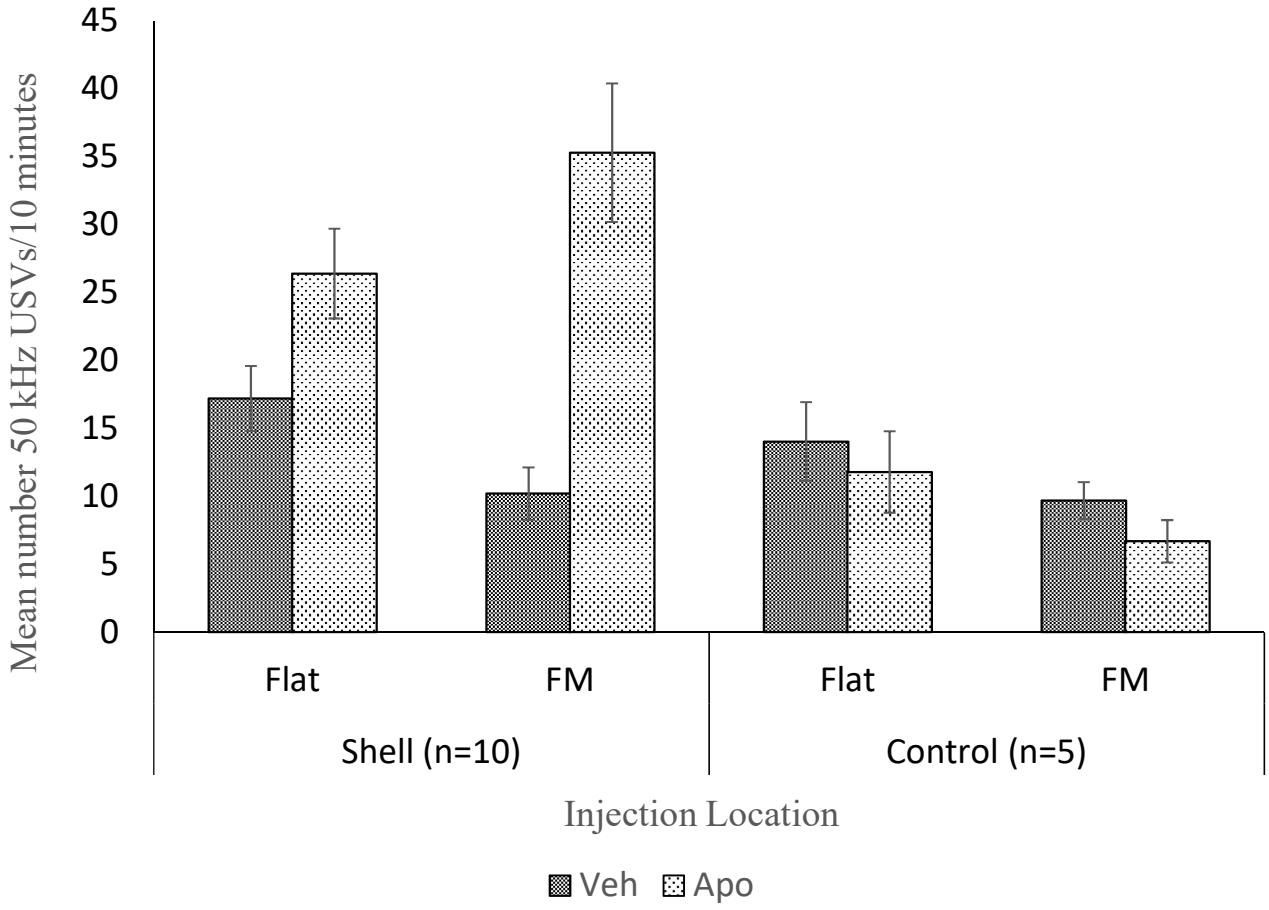
2.3: Results

2.3.a: Injection of R-(-)-apomorphine alone into the medial shell of the nucleus accumbens but not other striatal regions, significantly increased the number of frequency-modulated (FM) 50 kHz USVs.

Injection of R-(-)-Apomorphine into the nucleus accumbens shell increased the emission of frequency modulated (FM) 50-kHz USVs ($\chi^2(3) = 16.7$, $p < 0.05$, $n = 10$, see Figure 8A) when compared to saline injection in the same region. There was no difference in the number of emitted flat (F) 50-kHz USVs when comparing R-(-)-apomorphine or saline injection into the same brain region ($p > 0.95$, $n = 10$; Table is at the end of the text). For localization of injection into the nucleus accumbens shell see Figure 8B.

Injection of R-(-)-apomorphine into the ventral caudate-putamen, dorsal accumbens core, and lateral nucleus accumbens shell did not induce a change in the number of recorded F or FM 50-kHz USVs ($\chi^2(3) = 3.96$, $p = 0.27$, $n = 5$; figure 8C and Table 1).

A


45
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B **C**

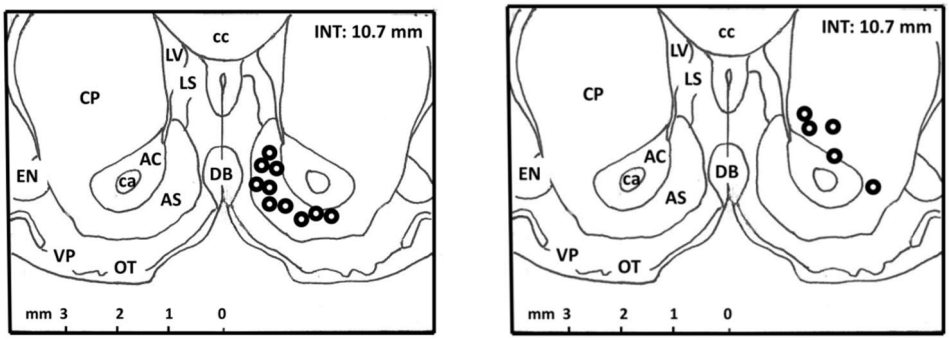

Figure 8: The mean number of F (Flat) and FM (frequency modulated) 50-kHz USVs recorded after injection of apomorphine into the centro-medial shell of the nucleus accumbens (n = 10) or regions outside the shell (Control, n = 5). Injection of apomorphine into the shell was able to increase the number of FM 50-kHz USVs in a statistically significant manner when compared to vehicle injection (Veh) ($\chi^2(3) = 16.7$, $p < 0.05$, n=10). There was no statistically significant change in the number of recorded F 50-kHz USVs between apomorphine or vehicle [$p > 0.95$, n = 10]. Injection of vehicle or apomorphine into regions outside accumbens (ventral caudate-putamen, dorsal accumbens core, and far-lateral shell labeled as "control") did not induce a statistically significant change in either F or FM 50 kHz USVs ($\chi^2(3) = 3.96$, $p = 0.266$, n = 5). Localization of injection sites (circles, n = 10) in the shell of the accumbens is shown in **B**, and outside of the accumbens in **C** (n = 5). The frontal planes are 10.7 mm from the interaural plane (INT). *Abbreviations*: AC – core of the nucleus accumbens, AS- shell of the nucleus accumbens, ca – anterior commissure, cc – corpus callosum, CP – caudate-putamen, DB – diagonal band, EN – entopeduncular nucleus, LS – lateral septum, LV – lateral ventricle, OT – olfactory tubercle, VP – ventral pallidum.

2.3.b. Injection of R-(-)-apomorphine into the central division of the medial shell of the nucleus accumbens significantly increased the number of FM 50-kHz USVs when followed by injection of the vehicle into AH-MPO (Figure 9).

Rats in this experiment had unilateral implantation of cannula into the medial nucleus accumbens shell and the AH-MPO. Rats underwent four injections: veh+veh, rats received vehicle injection into the medial shell of the nucleus accumbens followed by vehicle injection into the AH-MPO; veh+carb, vehicle injection into the medial shell of the nucleus accumbens followed by carbachol injection into the AH-MPO; apo+carb, R-(-)-apomorphine into the medial shell of the nucleus accumbens, followed by carbachol injection into the AH-MPO, apo+veh, R-(-)-apomorphine injection into the medial shell of the nucleus accumbens followed by vehicle injection into the AH-MPO.

Injection of R-(-)-apomorphine into the central medial region of the nucleus accumbens shell (A-P: 10.4-10.9) was able to significantly increase the mean number of FM 50-kHz USVs compared to veh+veh injection condition [$\chi^2(3) = 28.55$, $p < 0.05$, $n = 14$; See Figure 9, for values see Table 2]. There was no difference in the ability of R-(-)-apomorphine or vehicle to induce F 50-kHz USVs from the central division of the medial nucleus accumbens shell ($p = 0.54$, see Figure 9; Table 2) for localization of injection sites see Figure 10 B.

R-(-)-apomorphine failed to increase both F and FM 50-kHz USVs when injected in the rostral and caudal divisions of the nucleus accumbens shell ([$\chi^2(3) = 1.96$, $p = 0.59$, $n = 10$; see Figure 1A). For localization of injection sites see Figures 10 D, and E.

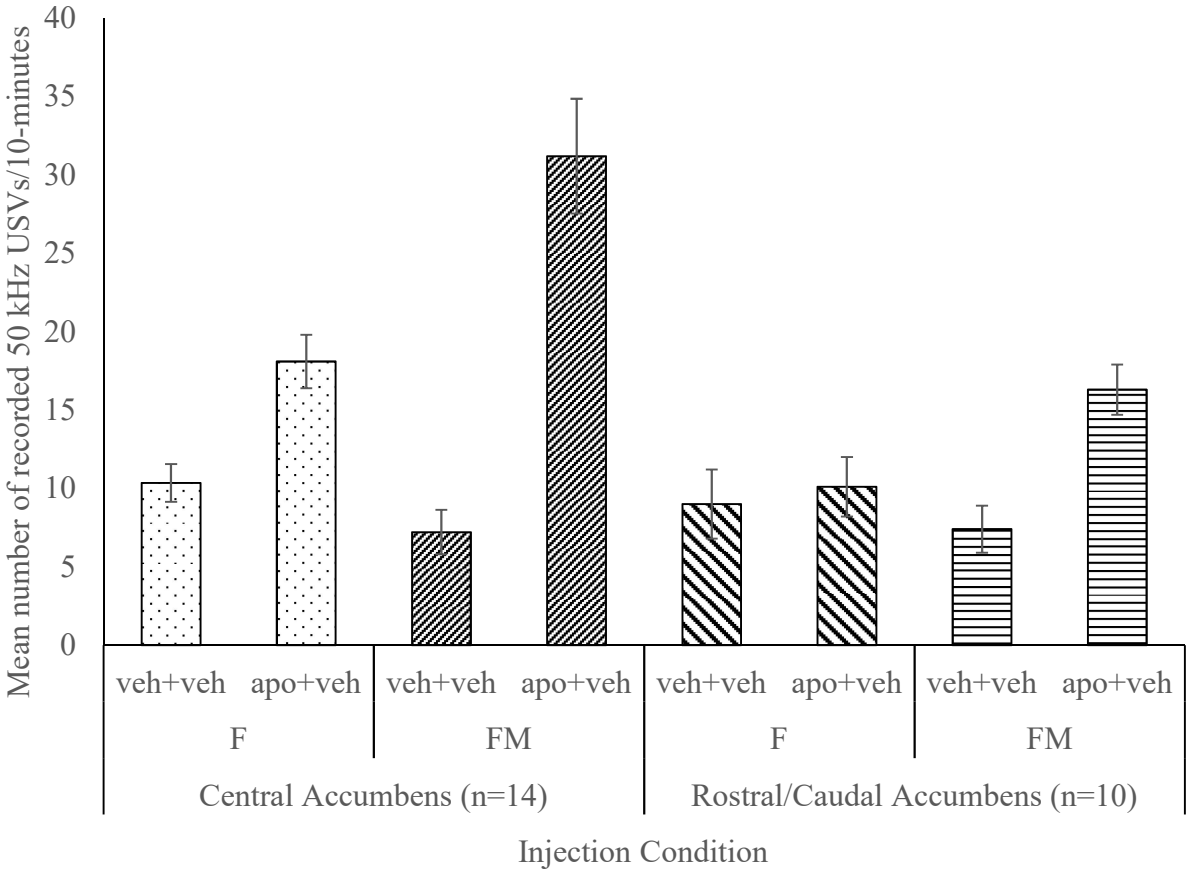

Figure 9: Injection of R-(-)-apomorphine (apo) into the centro-medial shell of the nucleus accumbens (labeled "Central," left four bars) significantly increased the number of FM 50 kHz USVs as compared to vehicle result [$\chi^2(3) = 28.55$, $p < 0.05$]. The mean number of F 50-kHz USVs was not significantly changed [$p = 0.54$, $n = 14$]. Injection of the vehicle (veh) or

apomorphine into the rostral and caudal division of the medial nucleus accumbens did not significantly change the number of F or FM 50 kHz USVs [$\chi^2(3) = 1.96$, $p = 0.58$, $n = 10$]. *Abbreviations*: veh+veh: vehicle-injected into the nucleus accumbens followed by vehicle-injected into the AH-MPO; veh+carb: vehicle injection into the nucleus accumbens followed by carbachol injection into the AH-MPO; apo+carb apomorphine injected into the nucleus accumbens followed by carbachol injection into the AH-MPO; apo+veh: apomorphine injected into the nucleus accumbens followed by vehicle injected into the AH-MPO. Localization of the injection sites is shown in Fig. 10: **10B.** Localization of injection sites (circles, n = 14) in the frontal section of the centro-medial accumbens 10.7 mm from the interaural plane. Localization of injection sites in the rostral medial shell (**10D**, 11.5 mm from the interaural plane, n = 7) and in the caudal medial shell (**10E**, 9.9 mm from the interaural plane, n = 3). *Abbreviations*: CX – cortical regions, fcc – forceps of the corpus callosum, MS – medial septum.

2.3.c. Injection of R-(-)-apomorphine into the medial shell of the nucleus accumbens decreased the mean number of recorded carbachol-induced 22-kHz USVs initiated from the AH-MPO

Injection of R-(-)-apomorphine into the centro-medial division of the medial shell of the nucleus accumbens (A-P: 10.4-10.9 mm) was able to significantly decrease the mean number of subsequent carbachol-induced 22-kHz USVs (apo+carb condition) when compared to vehicle injection (veh+carb condition) [$\chi^2(3) = 39.46$, $p = 0.013$, $n = 14$]; see Figure 10A; for localization of nucleus accumbens injection sites, see Figure 10B; for localization of AH-MPO injection sites see Figure 10C See Table 2 for values).

Injection of R-(-)-apomorphine into the rostral or caudal division of the accumbens (for localizations see figure 10 D, 10E) was unable to decrease the mean number of recorded 22-kHz

USVs (Figure 10A) caused by subsequent injection of carbachol into the AH-MPO (for localization of injection sites in the AH-MPO, see Figure 10F) (apo+carb condition, $p = 0.73$, $n = 10$; see Table 2 for values).

Injection of R-(-)-apomorphine outside the medial shell of the nucleus accumbens did not significantly increase the mean number of recorded FM 50-kHz USVs (($\chi^2(3) = 0.831$, $p = 0.842$, $n = 10$, see Figure 11) and was unable to significantly decrease the mean number of 22-kHz USVs recorded after carbachol was injected into the AH-MPO [$\chi^2(3) = 24.9$, $p > 0.95$, $n = 9$; see figure 12A]; localization of injection sites correspond to figure 12 B,C; n = 9, See Table 2 for values).

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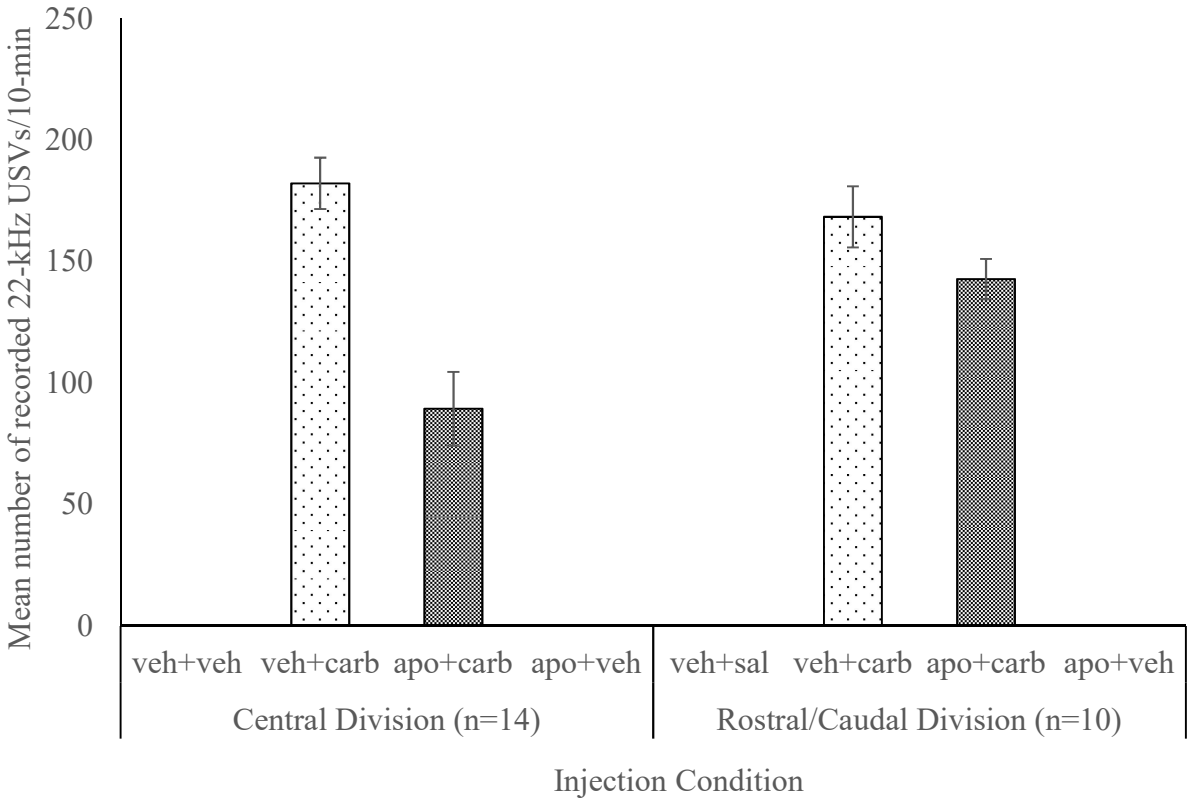

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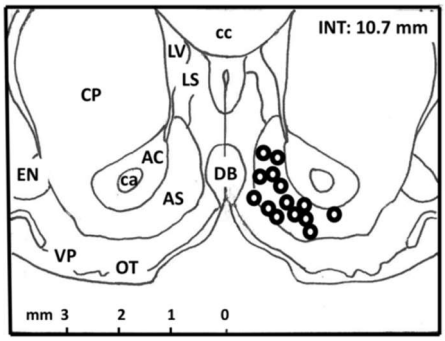
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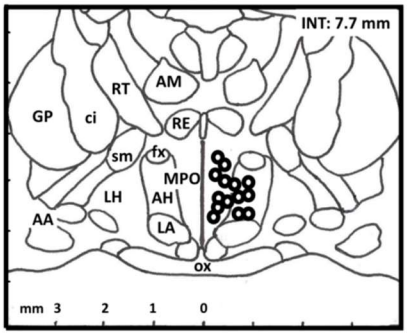
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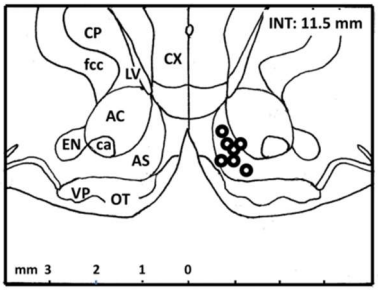
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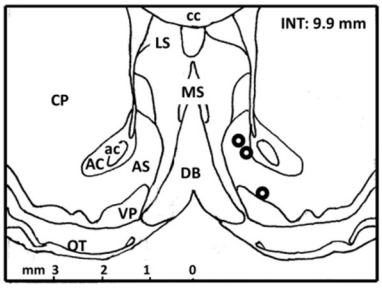
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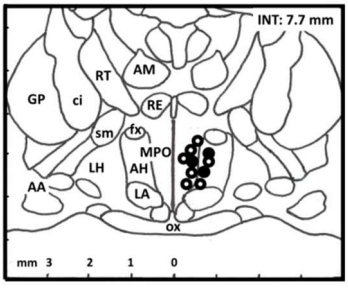
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Figure 10. A. Injection of apomorphine into the centro-medial division of the nucleus accumbens *(labeled "Central," two left bars) significantly attenuated* emission of 22-kHz USVs induced by subsequent carbachol injection into the AH-MPO ($\chi^2(3) = 39.46$, $p = 0.01$, $n = 14$). Injections of apomorphine into the rostral and caudal divisions of the shell did not significantly change the mean number of recorded 22 kHz USVs ($p = 0.73$, $n = 10$). All injections were double-injections. *Injection Abbreviations*: See Figure 1. **B.** Localization of injection sites (circles, n = 14) into the centro-medial shell of the accumbens. **C.** Localization of injection sites in the AH-MPO (n = 14) in the frontal section 7.7 mm from the interaural plane. Localization of injection sites in the rostral (**D**, n =7) and caudal (**E**, n =3) divisions of accumbens. **F.** Localization of injection sites in the AH-MPO in the frontal section 7.7 mm from the interaural plane. Injection sites coupled with the rostral shell are labeled with open circles and with caudal shell with filled circles. Abbreviations are explained in Figure 1 and 2. *Abbreviations*: AA – anterior amygdaloid area, AM – anteromedial thalamic nucleus, AH – anterior hypothalamic area, ci – internal capsule, fx – fornix, GP – globus pallidus, LA – lateroanterior hypothalamic nucleus, LH – lateral hypothalamic area, MPO – medial preoptic area, ox – optic chiasm, RE – nucleus reuniens, RT – reticular thalamic nucleus, sm – stria medullaris.

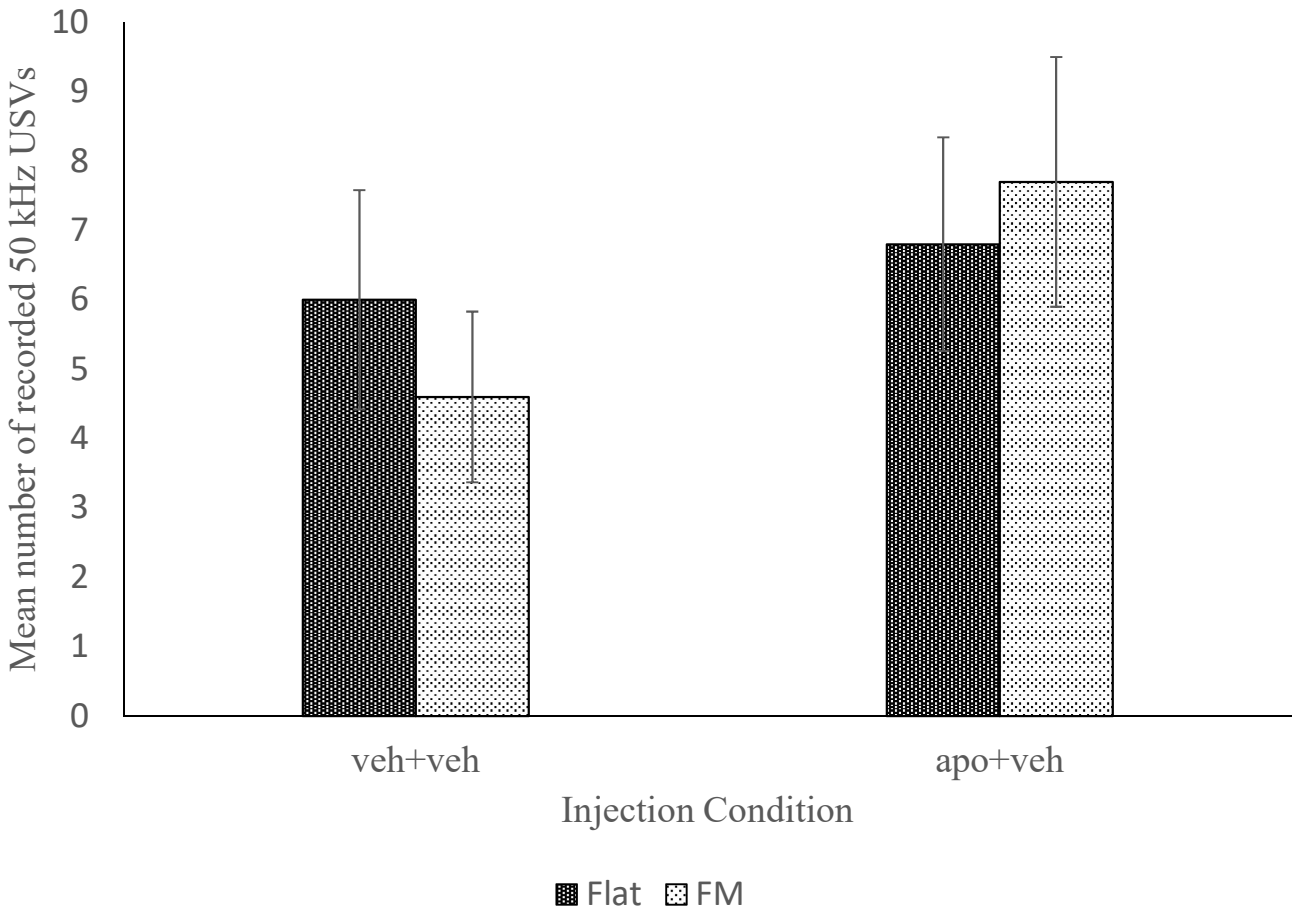

Figure 11. A. Mean number of 50-kHz USVs emitted after R-(-)-apomorphine was injected outside the medial shell of the nucleus accumbens. These areas include the ventral caudate-putamen, dorsal, and central core of the accumbens and peri-commissural areas. There was no statistical difference in the ability of apomorphine to induce either F or FM 50-kHz USVs when compared to the vehicle injection [($\chi^2(3) = 0.831$, $p = 0.842$, $n = 10$]. *Abbreviations*: veh+veh – injection of the vehicle into the shell of accumbens followed by injection of the vehicle into the AH-MPO; apo+veh – injection of apomorphine into the shell followed by injection of the vehicle into the AH-MPOA. **12.B**. Localization of injection sites (circles, n = 9) outside of the medial shell of the accumbens shown on the frontal section of the centro-medial accumbens 10.7 mm

from the interaural plane. Abbreviations the same as in Figure 1. Localization of corresponding injection sites for the vehicle (n = 9) in the AH-MPO 7.7 mm from the interaural plane corresponds to figure **12C**. Abbreviations the same as in Figure 9.

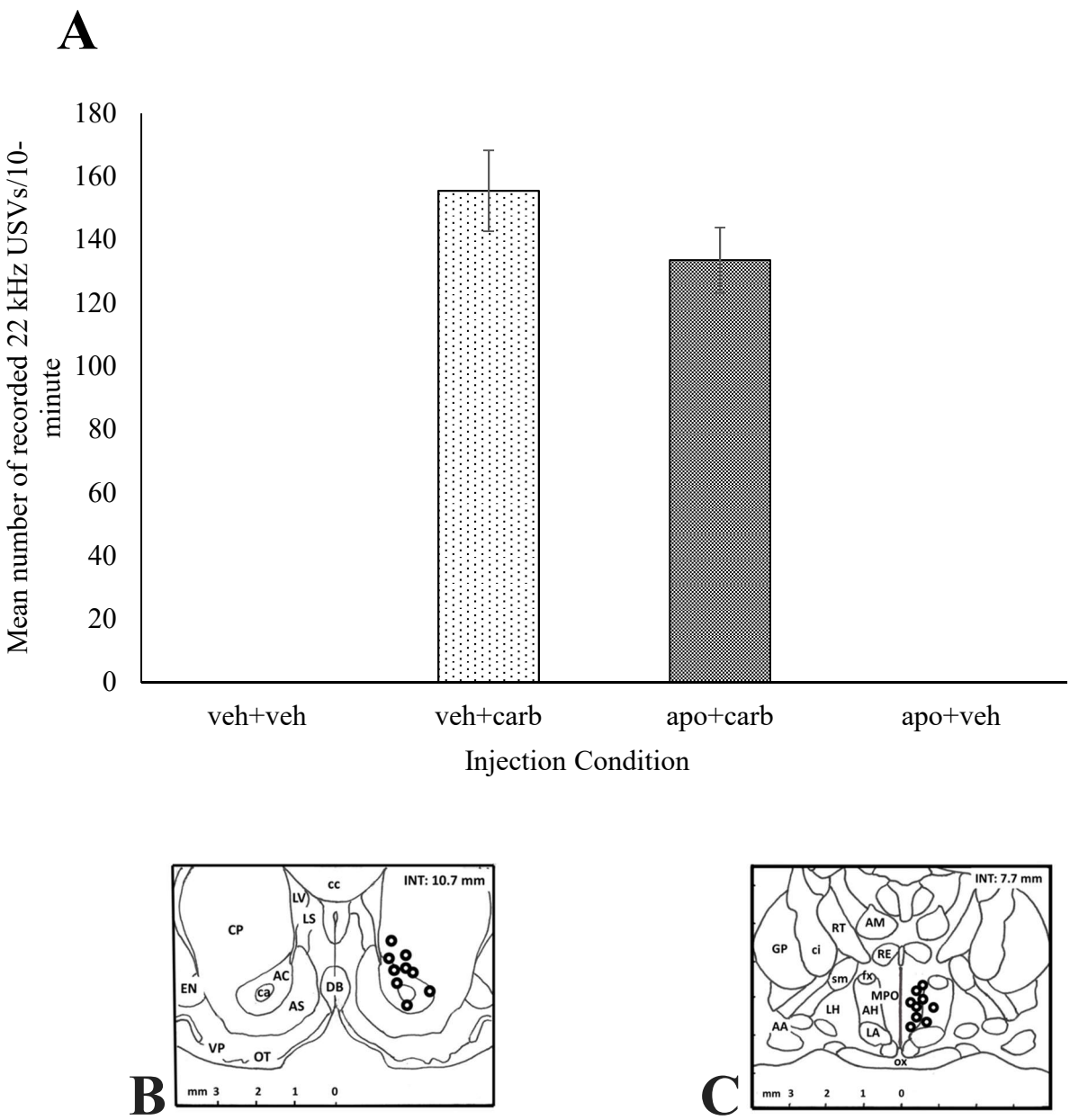

Figure 12. A. Injection of apomorphine outside the nucleus accumbens shell failed to reduce the emission of 22-kHz USVs induced by carbachol injection into the AH-MPO. Injection of apomorphine into areas outside the medial shell of the nucleus accumbens failed to increase the mean number of recorded F and FM 50-kHz USVs compared to control injections. Injection of apomorphine outside the nucleus accumbens shell also failed to significantly decrease the mean number of 22-kHz USVs emitted after carbachol was injected into the AH-MPO compared to control injection [$\chi 2(3) = 24.9$, $p > 0.95$, n=9]. *Injection Abbreviations*: See Figure 9. No 22 kHz USVs were observed under the veh+veh or apo+veh injection condition. **B**. Localization of injection sites (circles, n = 9) outside of the medial shell of the accumbens shown on the frontal section of the centro-medial accumbens 10.7 mm from the interaural plane. Abbreviations the same as in Figure 10. **C**. Localization of corresponding injection sites for the vehicle (n = 9) in the AH-MPO 7.7 mm from the interaural plane. Abbreviations are the same as in Figure 10.

2.3.d: There was no statistical difference in the duration or peak frequency of USVs across injection conditions

Injection of apomorphine failed to alter the duration of recorded 22-kHz USVs induced by subsequent carbachol injections into the AH-MPO [apo+carb; $F(1.95, 62.41) = 1408.1$, $p = 0.62$] compared to veh+carb injections. The peak frequency of recorded 22-kHz USVs did not significantly change when comparing veh+carb and apo+carb injection conditions [$F(1.76, 56.35) = 2955.95$, $p = 0.34$; see Figure 13 and Table 3 for values].

The spectrographic features of the recorded 50-kHz USVs were consistent with parameters reported elsewhere (see Brudzynski, 2007). There was no statistically significant change in the duration of recorded F or FM 50-kHz USVs [(F (2.7, 35.8) = 4.52, p > 0.95] or peak frequency of flat or FM 50-kHz USVs [F(1.8, 26.2) = 159.3, p > 0.95; see Figure 14 and Table 3 for values] when compared to vehicle controls.

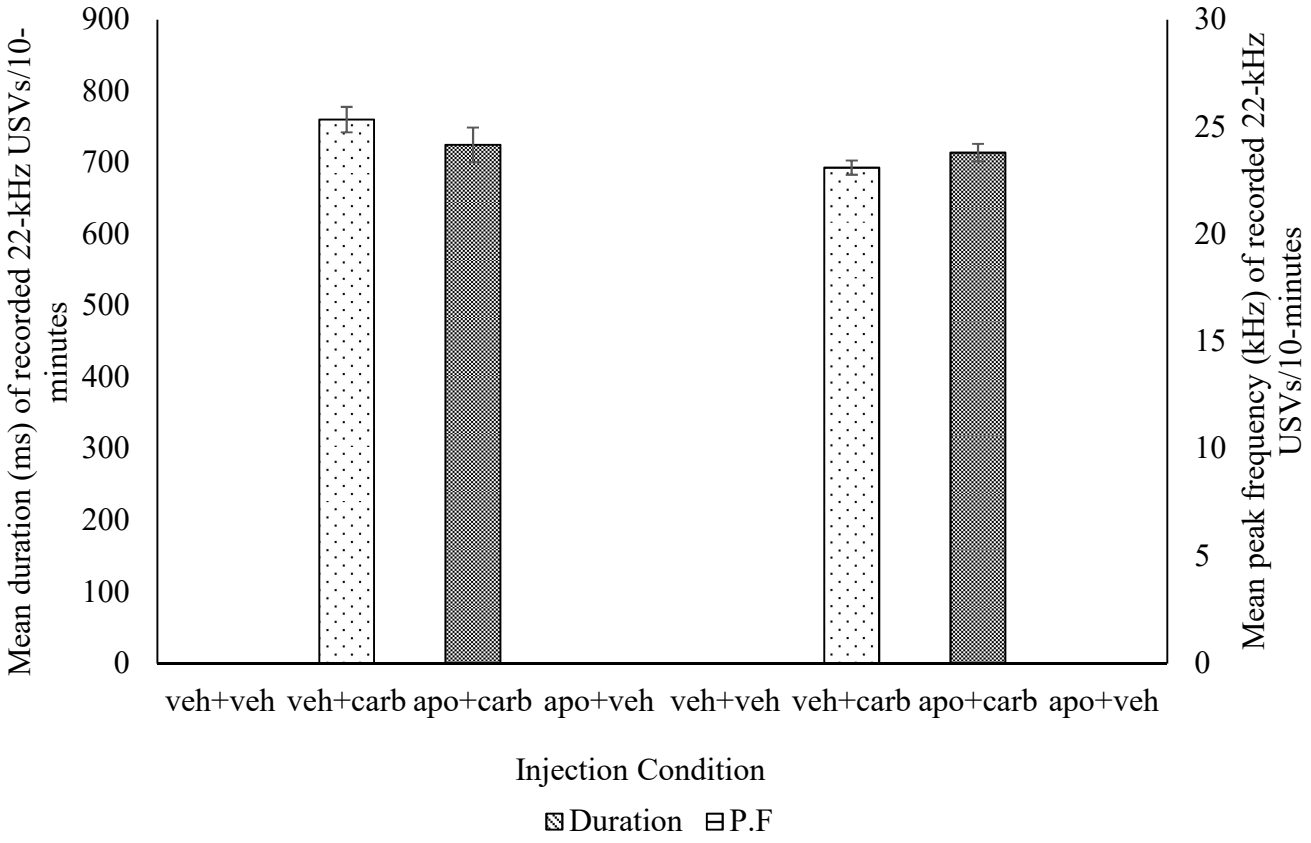

Figure 13. Spectrographic features of recorded 22-kHz USVs. No 22-kHz USVs were recorded during the veh+veh or the apo+veh conditions. Duration of vocalizations (Duration in ms, dark-shaded bars) relates to the scale on the left vertical axis, while peak frequency (P.F. in kHz, horizontally shaded bars) relates to the scale on the right vertical axis. There was no difference in the duration [$F(1.95, 62.41) = 1408.1$, $p = 0.62$] or peak frequency [$F(1.76, 56.35) = 2955.95$, $p = 0.34$] of recorded 22-kHz USVs under the various injection conditions. In the conditions of veh+veh and apo+veh, 22-kHz USVs were not emitted. For abbreviation of different conditions, see Figure 8

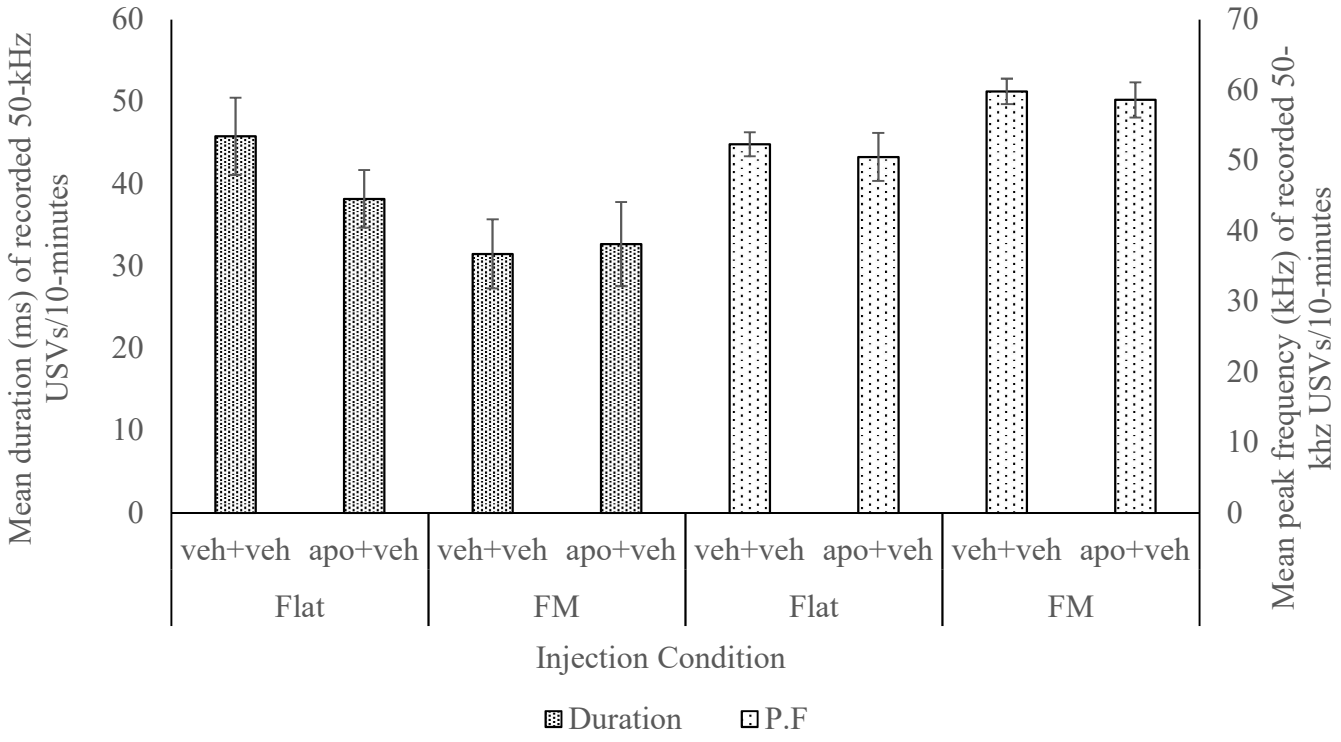

Figure 14. Spectrographic features of recorded 50-kHz USVs in different conditions. The mean duration (Duration) is shown by densely stippled bars, and peak frequency (P.F.*)* is shown by sparsely stippled bars for F 50-kHz USVs (Flat) and FM 50-kHz USVs (FM). There was no

statistical difference in the mean duration among F 50-kHz USVs [$F(2.03, 65.09) = 2162.66$, $p = 0.552$] or FM 50-kHz USVs ($p > 0.95$). There was also no significant difference when comparing the peak frequency among injection conditions ($p>0.95$). *Injection Abbreviations:* See Figure 8.

2.4: Discussion

The main purpose of the experiment was to investigate if the initiation of a positive emotional state could decrease the magnitude of a subsequent negative emotional state. In this experiment, the initiation of a positive emotional state was achieved via intracerebral injection of R-(-)-apomorphine into the medial shell of the nucleus accumbens and a negative emotional state was initiated by intracerebral injection of carbachol into the AH-MPO. The expression of the type of emotional state was gauged by the type of USV that was emitted, and the magnitude of the emotional state was gauged by the quantity of USVs emitted.

Our results suggest that upon initiation of a positive emotional state, there is a reduction in the magnitude of a negative emotional state induced by carbachol. This was demonstrated by the significant decrease in the number of emitted 22-kHz USVs when comparing veh+carb and apo+carb injection conditions. This decrease in recorded 22-kHz USVs was not due to an increase in the length of 22-kHz USVs since there was no statistical difference found between any injection conditions with regards to sonographic features. The evidence suggests that the initiation of a positive emotional state has an inhibitory effect on the initiation of a negative emotional state.

2.4.a: R-(-)-apomorphine increased the mean number of emitted FM 50-kHz USVs when injected into the central region of the nucleus accumbens shell, but not in the rostral or caudal divisions of the nucleus accumbens shell.

The nucleus accumbens shell is a forebrain structure that receives dopaminergic input primarily from the ventral tegmental area (Ikemoto, 2007). Phasic dopamine release into the medial shell of the nucleus accumbens is thought to be associated with the initiation of behaviours that reflect positive emotional states (Panksepp et al., 2002; Wanat et al., 2009; Willuhn et al., 2010). Consistent with this line of reasoning, injection of a mixed dopamine agonist R-(-)-apomorphine into the medial shell of the nucleus accumbens was able to significantly increase the mean number of FM 50-kHz USVs, which is reflective of a positive emotional state (Brudzynski, 2007; Knuston et al., 2002). Our results are consistent with other reports of significantly increased 50-kHz USVs in response to intracerebral injections of dopamine agonists into the central medial shell of the nucleus accumbens (Burgdorf et al., 2001; Brudzynski et al., 2012; Thompson et al., 2006).

An interesting finding in the experiment was that the initiation of FM 50-kHz USVs was not consistent across the whole rostral-caudal extension of the medial nucleus accumbens shell, but was rather localized to a region bound between A-P: 10.2-10.9 mm from the interaural plane. The regional selectivity of the response within the nucleus accumbens to initiate the production of 50-kHz USVs may involve the formation of D_1/D_2 heterodimers. Indirect evidence for the involvement of 50-kHz USVs in response to D_1/D_2 heterodimer formation was demonstrated with the selective reduction of amphetamine-induced 50-kHz USVs with the anti-psychotic clozapine (Wright et al., 2013). Using FRET, it has been suggested that the anti-psychotic

property of Clozapine stems from the uncoupling of D_1R-D_2R heterodimers (Faron-Górecka et al., 2008).

Systemic injections of amphetamine in rats has been reported to increase D_1-D_2 heterodimer expression within the nucleus accumbens (Perreault et al., 2010), as well as the emissions of 50-kHz USVs (Mulvihill & Brudzynski., 2018). The signaling cascades initiated via activation D_1-D_2 oligomers are distinct from those of D_1 or D_2 receptors. While D_1 receptors are coupled to $G_{\alpha/olf}$ subunit and indirectly generate the second messenger cAMP through activation of PKA, D_2 receptors are coupled to $G_{i/o}$ and negatively regulate the production of cAMP decreasing the intracellular activity of PKA (Beaulieu & Gainetdinov, 2011). In contrast, activation of the D_1-D_2 heterodimer hydrolyzes phosphatidylinositol phosphate to diacylglycerol and inositol triphosphate (IP_3). Formation of IP_3 increases the cytoplasmic concentration of $Ca^{2+,}$ leading to a myriad of different signals. One important enzyme that is activated via increased cytosolic calcium is calcium/calmodulin-dependent protein kinase II (CaMKII) by way of Gq/11, independent of cAMP function (Ng et al., 2010).

Drugs that have been known to increase the prevalence of 50-kHz USVs, such as cocaine and amphetamine (Barker et al., 2014) have shown increased expression of the protein calcium/calmodulin-dependent protein kinase II (CaMKII) in the shell but not the core of the nucleus accumbens (Anderson et al., 2008; Loweth et al., 2013; Robison et al., 2013). In some cases, the expression of CaMKII is dependent on the formation of D_1/D_2 heterodimers (Ng et al., 2010).

The importance of CAMKII in relation to emotional arousal became apparent during overexpression and deletion experiments. Overexpression of CaMKII in the nucleus accumbens

by herpes simplex viral vectors caused enhanced locomotor activity in response to both systemic and intracerebral injection of amphetamine. (Loweth et al., 2010). Conversely, injection of the CaMKII inhibitor KN-93 into the nucleus accumbens impairs the locomotor effects of amphetamine and the phasic dopamine release within the nucleus accumbens shell in response to amphetamine intake. Likewise, KN-93 significantly decreased i.v. self-administration of amphetamine when measured on a progressive ratio schedule of reinforcement (Loweth et al., 2008). It is interesting to note that intracerebral injection of amphetamine into the nucleus accumbens shell, but not the core, significantly increased 50-kHz USVs in rats (Burgdorf et al., 2001). The effects of KN-93 on the emission of FM 50-kHz USVs should be investigated.

The co-expression of D_1 and D_2 receptors within the nucleus accumbens shell has previously been shown on a subset of nucleus accumbens shell GABAergic medium spiny neurons using fluorescence resonance energy (FRET) (Perreault et al., 2011), complemented donor-acceptor resonance energy transfer (CODA-RET) (Urizar et al., 2011), and transgenic mice expressing GFP (Gangarossa et al., 2013). Recently, the regional selectivity of D_1/D_2 co-expressing neurons was found in abundance within the central region of the nucleus accumbens shell (Gagnon et al., 2017). Although the co-expression of D_1/D_2 receptors is modest, this may indicate that a subset of GABAergic medium spiny neurons participates in a unique ventral basal ganglia circuit distinct from GABAergic neurons expressing either D_1 or D_2 receptors (Frederick et al., 2015; Ikemoto et al., 2015). This third unique pathway would be akin to the novel pathway within the dorsal striatum (Perreault et al., 2011). The co-localization of D_1/D_2 receptors within the central region of the accumbens may explain why rats are more sensitive to self-administration of D_1/D_2 agonists than D_1 or D_2 agonists alone (Ikemoto et al., 1997). Further investigation should determine if the formation of D_1/D_2 heterodimers and subsequent activation

of CaMKII, within the central division of the medial shell of the nucleus accumbens is sufficient or necessary for the production of 50-kHz USVs and if GABAergic neurons expressing D_1/D_2 receptors participate in unique circuits.

2.4.b. R-(-)-apomorphine decreased the mean number of recorded 22-kHz USVs induced by subsequent intracerebral application of carbachol.

Emotional states were indexed by the type and number of USVs emitted by rats. The positive emotional state was initiated by R-(-)-apomorphine injection into the medial shell of the nucleus accumbens (Williams & Undieh, 2010; Simola et al., 2016), and the negative emotional state was initiated by carbachol injections into the AH-MPO (Brudzynski, 2001; 2007). The main finding of the experiment was that pharmacological initiation of a positive emotional state (increased FM 50-kHz USVs) decreased the magnitude of the subsequent negative emotional state (decreased 22-kHz USVs).

In addition to results of the present experiment that documented a decrease in the negative emotional state by prior initiation of a positive emotional state, other studies have also shown a potential antagonism between both these emotive states. For example, injection of 1.0 mg/kg of R-(-)-apomorphine was able to selectively increase the time spent in open arms of the elevated-plus maze without concurrent changes in motor activity (Garcia et al., 2005) suggesting an anxiolytic effect induced by activation of the dopaminergic systems. Likewise, systemic injection of R-(-)-apomorphine was able to ameliorate startle potentiation during nicotine withdrawal and was able to decrease conditioned aversion associated with morphine withdrawal, two conditions known to increase the emission of 22-kHz USVs (Radke & Gewirtz, 2012).

These results, along with our own, provide support for the hypothesis that activation of a positive emotional state antagonizes a subsequent negative emotional state in rats.

Although our data suggest an antagonistic interaction between the positive and negative emotional systems, the results are not in agreement with conclusions from some other reports. For example, it was postulated that dopamine release within the nucleus accumbens seems to play an important role in mediating fear/anxiety response (Albrechet-Souza et al., 2013; Salamone, 1994; Wenzel et al., 2014; Yorgason et al., 2013). Although some of our results showed that injection of R-(-)-apomorphine into rostral and caudal portions of the shell of the accumbens failed to decrease the number of 22-kHz USVs, they also failed to *increase* the number of emitted 22-kHz USVs, suggesting that dopamine within the nucleus accumbens, at the very least, cannot potentiate a state of anxiety. Dopamine release in these regions in response to environmental stimuli could be related to sensorimotor systems and not necessarily related to emotional processing (Mannella et al., 2103; Wan & Swerdlow, 1996).

2.5: Conclusion

Rats produce different types of USVs in a variety of situations that index their emotional states. Two main classes of USV signals exist. Positive emotional arousal is signaled by the emission of FM 50-kHz USVs, while negative emotional arousal is signaled by the emission of 22-kHz USVs. It has been hypothesized that the initiation of given emotional arousal will antagonize the development of the opposite emotional state (Brudzynski, 2007). Our results lent support to this hypothesis and showed that an initial increase in a positive emotional state decreased the magnitude of a subsequent negative emotional state.

Table 1: Mean (± S.E.M) number of emitted Flat (F) and Frequency Modulated (FM) 50-kHz USVs recorded after injection of vehicle or R-(-)-Apomorphine (Apo) into the medial shell of the nucleus accumbens, and inside the rostral/caudal division of the shell.

	Medial Shell		Rostral/Caudal Shell	
Category	Vehicle	Apo	Vehicle	Apo
F	17.2 ±2.3	26.4 ± 3.3	14.04 ± 2.9	9.7 ± 1.3
FM	10.2 ± 1.9*	35.3 ± 5.1*	11.8 ± 3.0	6.7 ± 1.6

Note: Statistically significant differences in the post-hoc analysis for the number of emitted F or FM 50-kHz USVs during different injection conditions are denoted by the superscript asterisk ($p < 0.05$).

Table 2: Mean (± S.E.M) number of emitted 22-kHz, Flat (F) 50-kHz and Frequency Modulated (FM) 50-kHz USVs recorded during injection conditions.

	Medial Shell + AH-MPO				**Outside the Shell + AH-MPO**			
Category	veh+veh	veh+carb	apo+carb	apo+veh	veh+veh	veh+carb	apo+carb	apo+veh
22	0	182.2 ± 10.6	89.4 ± 15.2[a]	0	0	168.4 ± 12.8	142.8 ± 8.3	0

F	10.3 ± 2.1	N.A	N.A	18.7 ± 1.7	6 ± 1.6	N.A	N.A	6.8 ± 1.5
FM	7.2 ± 1.4	N.A	N.A	29.3 ± 3.7[a]	4.6 ± 1.2	N.A	N.A	7.7 ± 1.8

Note: Statistically significant differences in the post-hoc analysis for the number of emitted F or FM 50-kHz USVs during different injection conditions are denoted by the superscript letter "a" ($p < 0.05$). *Abbreviations:* veh+veh, rats received vehicle injection into the medial shell of the nucleus accumbens followed by vehicle injection into the AH-MPO; veh+carb, vehicle injection into the medial shell of the nucleus accumbens followed by carbachol injection into the AH-MPO; apo+carb, R-(-)-apomorphine into the medial shell of the nucleus accumbens, followed by carbachol injection into the AH-MPO, apo+veh, R-(-)-apomorphine injection into the medial shell of the nucleus accumbens followed by vehicle injection into the AH-MPO. N.A - Not Applicable.

Table 3: Mean (± S.E.M) spectrographic features of recorded USVs during various injection conditions.

		Pooled Injection data			
		veh+veh	veh+carb	apo+carb	apo+veh
22-kHz	Duration (ms)	0	760.1 ± 17.8	724.9 ± 24.1	0
	P.F (kHz)	0	23.1 ± 0.33	23.8 ± 0.41	0

F 50-kHz	Duration (ms)	45.8 ± 4.7	N.A	N.A	38.2 ± 3.5
	P.F (kHz)	52.3 ± 1.7	N.A	N.A	50.5 ± 3.4
F.M 50-kHz	Duration (ms)	31.5 ± 4.2	N.A	N.A	32.7 ± 5.1
	P.F (kHz)	59.8 ± 1.8	N.A	N.A	58.6 ± 2.5

Note: There was no statistical difference within groups when comparing the sonographic features of duration (ms) and peak frequency (P.F). *Abbreviations:* veh+veh, rats received vehicle injection into the medial shell of the nucleus accumbens followed by vehicle injection into the AH-MPO; veh+carb, vehicle injection into the medial shell of the nucleus accumbens followed by carbachol injection into the AH-MPO; apo+carb, R-(-)-apomorphine into the medial shell of the nucleus accumbens, followed by carbachol injection into the AH-MPO, apo+veh, R-(-)-apomorphine injection into the medial shell of the nucleus accumbens followed by vehicle injection into the AH-MPO. N.A- Not Applicable.

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Chapter 3: Inhibition of carbachol-induced 22-kHz USVs from the LS by apomorphine injection into the nucleus accumbens shell

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3.0: Abstract

Rats can emit ultrasonic vocalizations (USVs) in a negative, as well as positive contexts and USVs are reflective of the emotional state of the signaler. 22-kHz USVs are emitted during aversive contexts and can be initiated by activation of the ascending cholinergic pathways originating from the laterodorsal tegmental nucleus or initiated pharmacologically by injection of cholinergic agonists into target areas of these pathways (medial cholinoceptive vocalization strip). Conversely, 50-kHz USVs are emitted during positive pro-social contexts and can be initiated by stimulation of ascending dopaminergic pathways originating from the ventral tegmental area or by injection of dopamine agonists into target areas of these pathways (nucleus

accumbens shell). Recently, we have shown an inhibitory effect that a positive emotional state has on a negative emotional state reflected in the emission of carbachol-induced 22-kHz USVs from the anterior hypothalamic/medial preoptic area (AH-MPO). However, this structure is a fragment of that cholinoceptive vocalization strip. We wanted to examine if we could observe similar effect when the aversive state is induced from the lateral septum, the most rostral division of the cholinoceptive vocalization strip. The results have confirmed that the initiation of positive emotional arousal by injection of R-(-)-apomorphine into the medial shell of the nucleus accumbens significantly decreased emission of carbachol-induced 22-kHz USVs from the lateral septum. The second finding was that the positive emotional arousal was expressed by frequency-modulated 50-kHz vocalizations and not by flat 50-kHz calls. Thus, the decrease in the number of recorded 22-kHz USVs was proportional to the emission of FM 50-kHz USVs and not the emission of F 50-kHz USVs. This research provides further support to the hypothesis that the initiation of a positive emotional state functionally antagonizes initiation of a negative emotional state in rats.

3.2: Introduction

Adolescent and adult rats communicate by producing two different categories of ultrasonic vocalizations (USVs) identified as 22-kHz USVs and 50-kHz USVs, which can be further subdivided into flat (F) and frequency modulated (FM) USVs (Knutson et al., 2002; Brudzynski, 2007; 2009; 2013; Burgdorf et al., 2008). In sonographic analyses, 22-kHz USVs have a flat temporal pattern, a long duration between 100-3000 ms, a peak frequency between 19-27 kHz and bandwidth of 3-5 kHz, while 50-kHz USVs have a duration ranging from 20-100

ms, a peak frequency ranging between 48-70 kHz and a bandwidth of 7-25 kHz (Brudzynski, 2001; Wright et al., 2010).

Both types of USVs have been hypothesized to be an inseparable component in signaling emotional states (Brudzynski, 2007; 2013). Ethological and pharmacological experiments provided evidence that 22-kHz USVs reflect a negative emotional state that can be initiated either conditionally or unconditionally. For example, emission of 22-kHz USVs can be initiated by presence of fox or lion urine (Fendt et al., 2018), by presentation of a cat to rats living in a visible burrow system (Blanchard et al., 1991), by social defeat (Kroes et al., 2007), or by anticipation of foot-shock (Jelen et al., 2003).

Pharmacological evidence supporting the thesis that 22-kHz USVs are associated with negative emotional states comes from investigations using anxiolytics and anxiogenics. Anxiolytics, drugs that decrease the self-reported measure of anxiety in humans, can decrease the emissions of 22-kHz USVs (Cullen & Rowan, 1994; Jelen et al., 2003; Miczek et al., 1995; Sánchez & Meier, 1997; Sun et al., 2010; Vivan et al., 1994). Likewise, pentylenetetrazole, an anxiogenic drug, has been shown to increase the number of cue-emitted 22-kHz USVs (Jelen et al., 2003) as well as increase the duration of immobility (Willadsen et al., 2018). Further evidence that supports the argument that 22-kHz USVs reflect a negative emotional state comes from studies reporting emission of 22-kHz USVs during withdrawal from drugs of abuse or in the absence of expected rewards (Barker et al., 2015; Covington & Miczek, 2003; Vivian & Miczek, 1991). These contexts are associated with self-reported measures of negative affect in humans (Barr et al., 2002; Corr, 2002; Pelchat, 2002)

Conversely, the emission of 50-kHz USVs by rats has been argued to be indicative of a positive emotional state. Rats will increase the number of FM 50-kHz USVs in play behaviour with other conspecifics (Burke et al., 2017) or with the experimenter (heterospecific play or ticking) (Burgdorf et al., 2008; Panksepp & Burgdorf, 2000). 50-kHz USVs can also be initiated both pharmacologically and by anticipation of delivery of rewarding electrical brain stimulation (Burgdorf et al., 2000; Scardochio et al., 2015), in response to intravenous amphetamine application (Ahrens et al., 2009), or in anticipation of cocaine consumption (Browning et al., 2011).

Cholinergic cell bodies located within the laterodorsal tegmental nucleus (LTDg) and dopamine neurons located in the ventral tegmental area (VTA) play a vital role in the initiation of 22-kHz or 50-kHz USVs, respectively (for review see Brudzynski, 2007; 2014). The ascending cholinergic pathways (mesolimbic cholinergic system) to medial mesencephalic and diencephalic structures reaching up to the lateral septum (LS) are responsible for the initiation of aversive arousal, while the ascending mesolimbic dopaminergic pathways reaching to ventral striatal regions and nucleus accumbens are responsible for the initiation of appetitive arousal. These two anatomical systems are important for the initiation of USVs in rats as an expression of emotional states. In addition to that, indirect evidence supports an antagonistic relationship between the action of acetylcholine and dopamine during the initiation of an emotional state. Systemic injection of the dopamine agonist amphetamine, a condition that reliably initiates the production of 50-kHz USVs (Mulvihill & Brudzynski, 2019) decreases brain acetylcholine levels (Domino & Olds, 1972; Vasko et al., 1974). Likewise, systemic injection of morphine, which can induce conditioned place-preference (a measure of an appetitive state) in mice (Cole et al., 2013), increases the extracellular levels of dopamine in the nucleus accumbens (Leone et al.,

1991) while decreasing basal acetylcholine levels in the nucleus accumbens (Rada et al., 1991). The morphine effect on basal dopamine and acetylcholine levels in the nucleus accumbens is reversed upon pre-treatment with the opioid antagonist naloxone (Rada et al., 1991). Pre-treatment with naloxone also induces withdrawal-like symptoms, conditioned place-aversion (a measure of anxiety) (Lin et al., 2018) and emission of 22-kHz USVs (Vivian & Miczek, 1991). Thus, the initiation of 22-kHz and 50-kHz USVs are not only indirect measures of emotional states in the signaler, but they are also an indirect measure of dopamine/acetylcholine ratios in selected forebrain areas.

Recently, we have also shown an antagonistic interaction of dopamine on acetylcholine-induced initiation of vocal expression of an emotional state. Our result showed decreased emissions of carbachol-induced 22-kHz USVs from the anterior hypothalamic-medial preoptic (AH-MPO) area after R-(-)-apomorphine was injected in the medial shell of the nucleus accumbens (Silkstone and Brudzynski, 2019). However, the AH-MPO is only one of the nuclei in the medial cholinoceptive vocalization strip (Brudzynski, 2010). This strip, which stretches from the LTDg to the most rostral extent of the basal forebrain is involved in the initiation of aversive vocalization in both cats and rats.

The purpose of the current experiment was to investigate if the injection of the dopamine agonist R-(-)-apomorphine into the nucleus accumbens could decrease carbachol-induced 22-kHz USVs from the LS, a nucleus located in the most rostral extent of the medial cholinoceptive vocalization strip (Bihari et al., 2003; Brudzynski et al., 2011). This will help clarify if the reduction in emission of 22-kHz USVs in response to R-(-)-apomorphine injection into the medial shell of the nucleus accumbens, is a response localized to AH-MPO stimulation or if the

reduction in the expression of a negative emotional state is a general response across the medial cholinoceptive vocalization strip.

3.3: Methods and Procedure

3.3.a: Stereotaxic implantation of the cannula into the left nucleus accumbens shell and left LS.

Twenty-five Long-Evans rats were used for double injections of pharmacological agents into two different brain areas that induce either 50-kHz USVs or 22-kHz USVs. R-(-)-apomorphine was injected into the medial shell of the nucleus accumbens to induce positive emotional arousal signaled by the emission of FM 50-kHz USVs, while carbachol was injected into the LS to induce negative emotional arousal reflected by the emission of 22-kHz USVs. The intensity, or magnitude, of emotional states, as measured by the number of emitted USVs.

3.3.b: Subjects and Surgery

Twenty-five adult male Long-Evans rats (Charles River) with bodyweight ranging from 280-320 g at the time of surgery served as the experimental subjects. All animals were housed in polycarbonate cages (48 cm x 25 cm x 20 cm high) with constant room temperature (23°C ± 1°C), controlled humidity conditions and in a 12:12 h light-dark cycle. Animals were housed in pairs with a dust-free corn cob bedding (Fisco Enterprises, Bolton, ON) with black polyvinyl tubing for hiding, wooden blocks for play with *ad libitum* access to water and pelleted Rodent Lab Diet (#5001, Ren's Feed & Supplies Limited, Oakville, ON). After five days of acclimation, rats underwent stereotaxic surgery.

Rats underwent stereotaxic surgery for unilateral implantation of guide cannula into the left hemisphere. Briefly, rats were anesthetized with gaseous isoflurane at a concentration of 3% and placed in a Kopf stereotaxic apparatus (Model 900, David Kopf Instruments, Tujunga, CA)

in a flat skull position. While in the apparatus, burr holes were drilled into the skull and two guide cannula (constructed from 23 G syringe needles with O.D. = 650 µm, Beckton-Dickinson Canada, Mississauga, ON) was implanted according to the coordinates from the Paxios & Watson (2005) stereotaxic atlas. One cannula was implanted into the lateral septum (LS, stereotaxic parameters from the interaural line ranged from A-P: 9.12-8.6; L: 0.6-1.2 from the midline, and D-V: -4 to -4.6 mm from the surface of the skull), and the other cannula was implanted into the left shell of the nucleus accumbens (parameters from the interaural line ranged from A-P: 10.4-10.8; L: 0.8-1.8; D-V: 5.8-6.4). Cannulae were permanently secured to the skull by stainless steel jeweler's screws and methyl methacrylate resin (Perm Resin, Hygenic Corporation of Canada Inc., St. Catharines, ON). For further details, see Fornari et al. (2012). Rats were placed in the study upon five days of recuperation from surgery and subsequent inspection of their condition by the veterinarian.

3.3.c: Drugs and Injection Order

Carbachol (carbamylcholine chloride, Sigma Chemical Co., St. Louis MO.) was dissolved in 0.9% sterile saline and was injected unilaterally into the LS by a constant rate Hamilton® CR 700 micro-syringe (Hamilton Company, Reno, NV) in a dose of 1.0 µg/0.3µl at a rate of ~4.5 nl/s. R-(-)-apomorphine hydrochloride (Sigma, St. Louis, MO) was dissolved in the vehicle and injected in a concentration of 3.0 µg/0.3µl at the same rate of carbachol. The vehicle was prepared by adding 0.1% ascorbic acid to sterile saline and buffered to a pH at about 5. The vehicle (veh) served as a control for apomorphine, while saline (sal) was the control for carbachol.

R-(-)-apomorphine or vehicle was first injected into the shell of the nucleus accumbens. After injection of the drug or vehicle was finished, the injection cannula was left in place for an additional 60 s to allow for proper drug diffusion. After 60 s the injection cannula was slowly withdrawn and a sterile plug-pin was used to seal off the cannula, the rat was then placed in its home cage for 60 s. After 60 s, the rat was taken out of his cage, and carbachol or saline was injected into the LS at the same rate and volume as R-(-)-apomorphine. After the injection was finished, the injection cannula was left in place for an additional 60 s to allow for proper diffusion. After 60 sec, the injection cannula was removed, the guide cannula was then closed using a sterile plug-pin. The rat was then immediately placed in the recording chamber and recorded for 10-minutes.

3.3.d: Recording Ultrasonic Vocalizations

Recording of ultrasonic vocalizations took place in a Plexiglass recording chamber (25 cm x 18 cm x 18 cm). On top of the recording chamber, an Avisoft® CM16/CMPA condenser microphone (frequency range 2-250 kHz, Avisoft® Bioacoustics, Berlin, Germany) was placed with an average distance of 25 cm to the rat's head. Recording of the USVs was done in real-time and stored in a 16-bit format for later analysis. Analysis of USVs was done off-line using Avisoft® SAS LabPro program. Spectrograms were created using Fast Fourier transform (length: 552; Frame: 100%; Window: Hamming; Overlap: 75%).

Identification of 22-kHz and 50-kHz USVs was followed as described in previous studies (Brudzynski et al. 1991, Brudzynski 2007, Thompson et al. 2006). Briefly, USVs that had a peak frequency that fell between 19-29 kHz and had a duration longer than 100 ms were classified as 22-kHz USVs while calls that had a peak frequency that fell between 39-80 kHz and had a

duration less or equal to 100 ms were classified as 50-kHz USVs. USVs with peak frequency from 30-40 kHz were very rare and were not taken for analysis. Subsequent classification of 50-kHz into frequency modulated (FM) and flat (F) calls, i.e., unmodulated USVs, was based on morphological characteristics of calls on the sonograms consistent with the study by Burgdorf et al. (2008). Recording of USVs took place for 10 min. After that time, the rat was placed back into its home cage. Each rat received a clean cage for recording time, and each soiled cage was removed from the test room. Before each additional rat was tested, the table was wiped down with Virox® (Virox Technologies Inc., Oakville, ON) then further cleaned with a diluted ethyl alcohol solution followed by distilled water.

After the rat had received the final injection, it was anesthetized with an overdose of sodium pentobarbital. Before removal of the brain, an India-ink solution was prepared (1:100 dilution) and injected into the brain for histological determination of injection sites.

3.3.e: Histology and Localization

After the experiment, animals underwent transcardial perfusion with 10% solution of formalin. Brains were removed, postfix with formalin for 24 h, and coronally sectioned on a freezing microtome (Cryo-Histomat, Hacker Instruments and Industries, Fairfield, NJ) to a thickness of ~40 μm. Sections were placed on 1% poly-lysine coated slides, then underwent Nissl staining and were coated with Permount™ mounting medium (Fisher Scientific Co., Ottawa, ON) and coverslipped. For further details of the histological procedure, see Lindroos & Leinonen (1983).

Localizations were performed using a projection microscope. Small depositions of India ink before perfusion were used to confirm injection sites. After marking localization of all

injection sites, they were transferred according to their stereotaxic coordinates on a selected medial stereotaxic section. The medial stereotaxic section was used as a composite diagram of all injection sites for a given group.

3.3.f: Statistics

A non-parametric repeated measures ANOVA (Friedman's ANOVA followed by Sign-ranked post hoc test was used to assess the statistical difference between the number of 50-kHz USVs induced by R-(-)-apomorphine or vehicle from the nucleus accumbens shell. Analysis of sonographic features (call duration and peak frequency) was done using repeated measures ANOVA. A Shapiro-Wilks test was used to assess the normality of sonographic features to ensure the appropriate statistical procedure. All statistics were done using SPSS v 17.0 (SPSS Inc, Chicago, U.S.A). Multiple comparisons were corrected with Bonferroni method. Reported means are followed by the standard error of the mean (S.E.M). A Pearson correlation was used to assess the relationship between the change in recorded 22-kHz USVs between injection conditions, and the number of recorded F or FM 50-kHz USVs.

3.4: Results

3.4.a. R-(-)-apomorphine was able to increase the mean number of recorded F and FM 50-kHz USVs compared to control injection.

Injection of R-(-)-apomorphine into the medial shell of the nucleus accumbens, followed by injection of saline into the LS (apo+sal), significantly increased the mean number of F 50-kHz USVs ($\chi^2[3] = 20.5$, $p < 0.008$), and FM 50-kHz USVs ($\chi^2[3] = 32.9$, $p < 0.001$) compared to veh+sal controls (see Figure 15, see Table 4 for values, and for localizations see Figure 17; n = 16). However, when R-(-)-apomorphine was injected outside the medial shell of the nucleus accumbens, the emission of 50-kHz USVs was very low and sporadic, and there was no

difference in the number of recorded F or FM 50-kHz USVs as compared to veh+sal condition (see Figure 18 and localizations of injection sites in Figure 19, n = 9). Thus, apomorphine was effective in initiating 50 kHz USVs but only from the medial division of the nucleus accumbens shell.

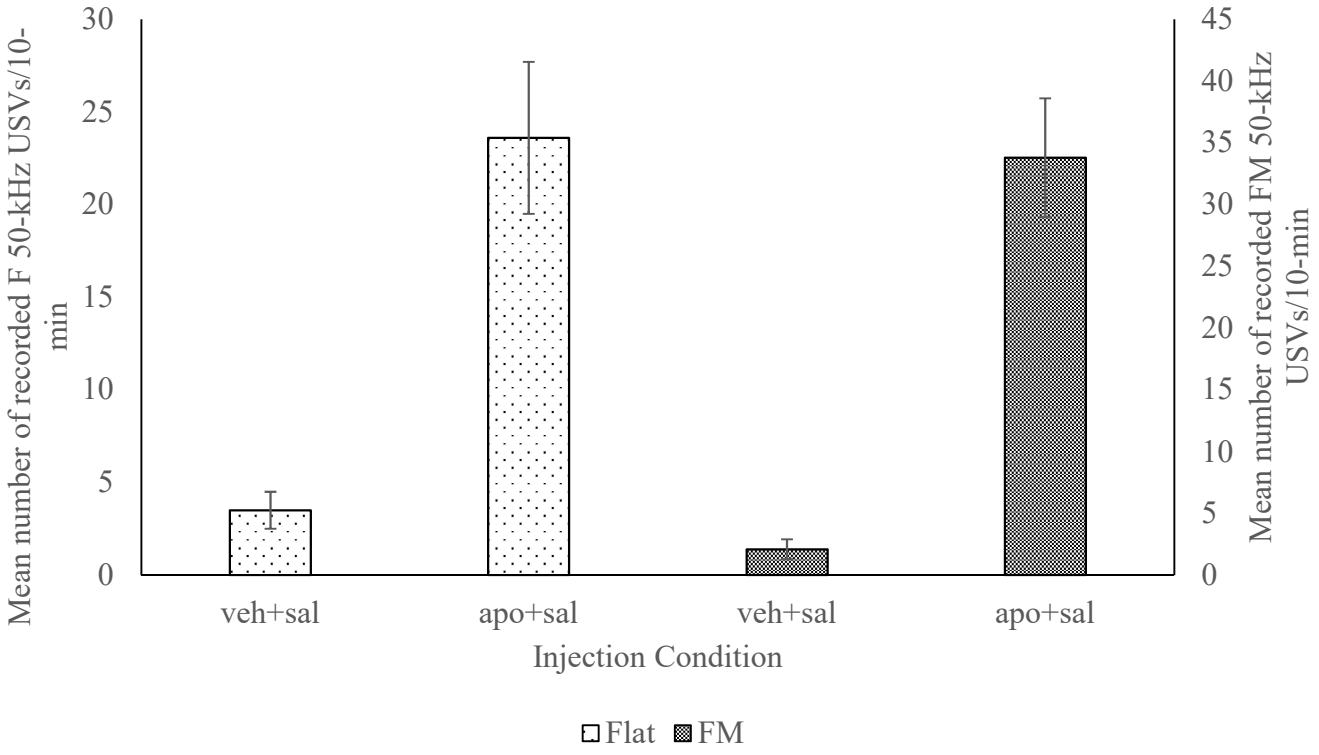

Figure 15: Mean (±S.E.M) number of flat (F) and FM (FM) 50-kHz USVs recorded during various injection conditions. There was a significant difference in the number of recorded F 50-kHz USVs when comparing the number of recorded vocalizations between veh+sal and apo+sal injection conditions ($\chi^2[3] = 20.5$, $p < 0.008$, lightly stippled bars). There was also a significant increase in the number of recorded FM 50-kHz USVs when comparing veh+sal and apo+sal ($\chi^2[3] = 32.9$, $p < 0.001$, densely stippled bars). Injection conditions: : veh+sal – injection of vehicle into the accumbens followed by saline in the LS; veh+carb – injection of vehicle into the accumbens followed by carbachol in LS; apo+carb – injection of apomorphine into the

accumbens followed by carbachol in LS; apo+sal – injection of apomorphine into the accumbens followed by saline in LS.

3.4.b: Intracerebral injection of R-(-)-apomorphine into the medial shell of the nucleus accumbens decreased carbachol-induced 22-khz USVs from the LS

This set of experiments was designed to investigate if R-(-)-apomorphine injections into the nucleus accumbens shell could decrease subsequent carbachol-induced 22-kHz USVs from the LS. Injection of vehicle to the shell of the accumbens followed by carbachol into the LS (condition veh+carb) induced robust emission of 22 kHz USVs with 127.4 ± 11.3 per 10-minute recording (Figure 16, left bar). Injection of R-(-)-apomorphine into the accumbens shell followed by carbachol injected to LS (condition apo+carb) significantly attenuated the mean number of recorded 22-kHz USVs as compared to vehicle condition ($\chi^2[3] = 44.7$, $p = 0.037$, see Figure 16, and see Figure 17 for localizations, n=16). There were no 22-kHz USVs recorded during the veh+sal or the apo+sal injection conditions (see Figure 16).

Thus, R-(-)-apomorphine injection into the medial shell region of the nucleus accumbens significantly attenuated emission of 22 kHz USVs. However, when R-(-)-apomorphine was injected outside the medial shell of the nucleus accumbens , there was no difference in the mean number of recorded 22-kHz USVs between conditions veh+carb vs. apo+carb ($\chi^2[3] = 24.9$, $p > 0.95$; see Figure 18, for localizations see Figure 19, n=9). Again, no 22-kHz USVs were recorded during the veh+sal or the apo+sal injection conditions. Thus, apomorphine injection into the medial shell of the nucleus accumbens was effective at attenuating emissions of 22 kHz USVs from the LS.

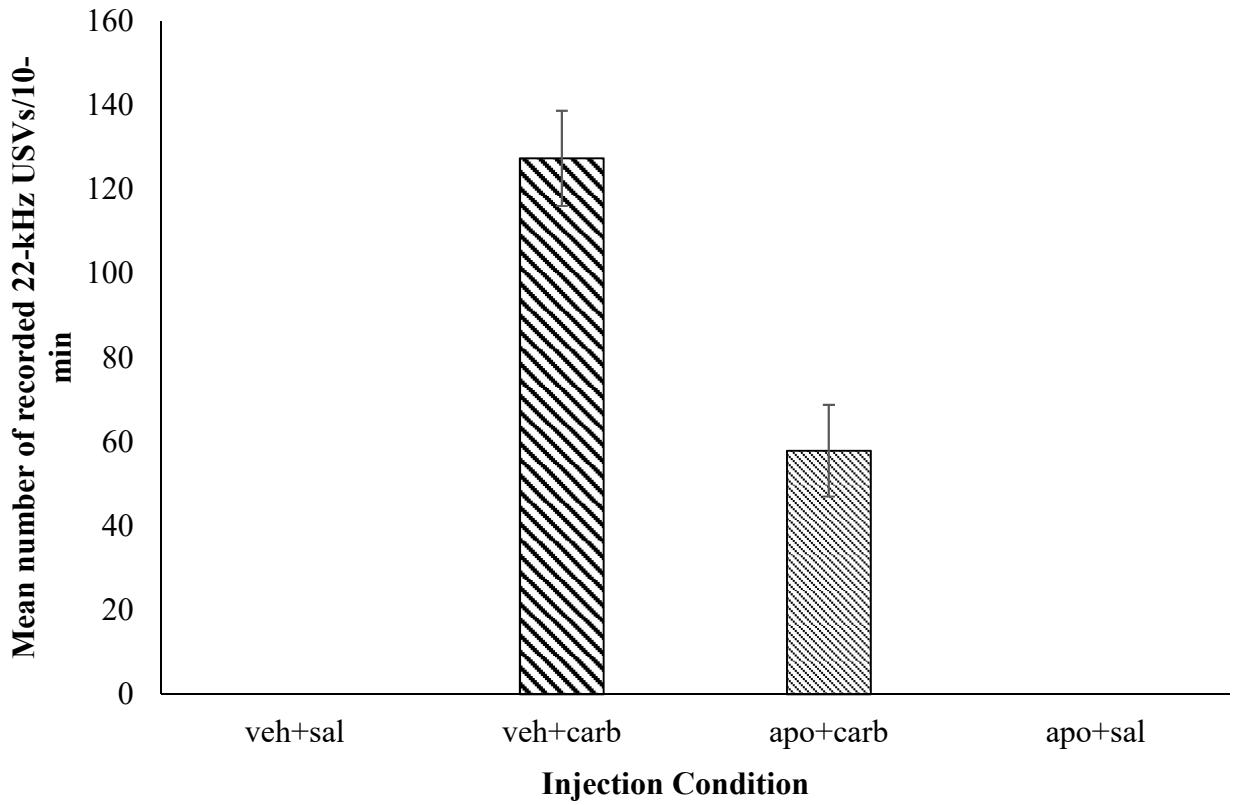

Figure 16. Mean (±S.E.M) number of 22-kHz USVs recorded during four different injection conditions. Injection of R-(-)-apomorphine into the shell of the nucleus accumbens and followed by carbachol in the LS (apo+carb) was able to significantly decrease the mean number of recorded 22-kHz USVs when compared to veh+carb injection condition ($\chi^2[3] = 44.7$, $p = 0.037$, $n = 16$). There was no recorded 22-kHz USVs under the veh+sal or the apo+sal injection conditions. For explanation of injection conditions, see Figure 15 and for localization of injection sites see Figure 17

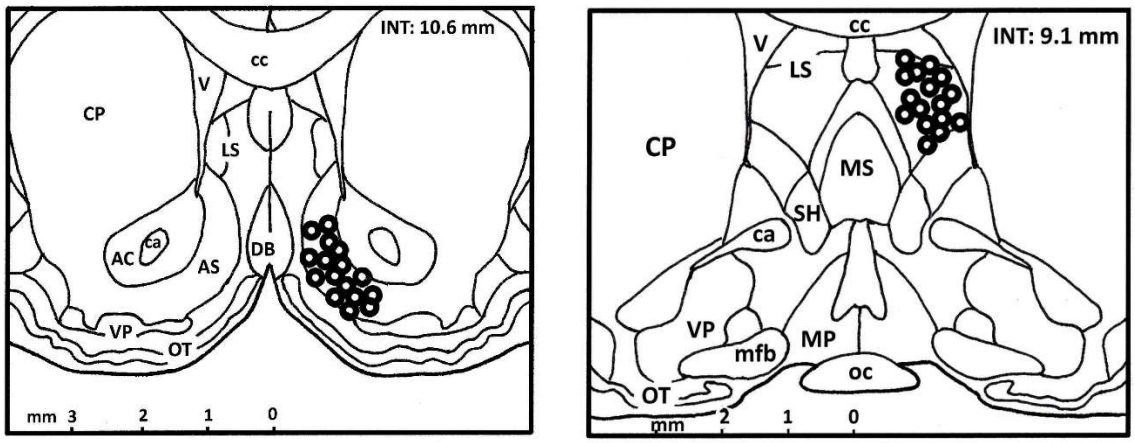

Figure 17. Localization of injection sites (dark circles) in the medial shell of the nucleus accumbens (n = 16) and corresponding localization of injection sites in the LS (n = 16). Each site was injected four times with different combination of vehicles or drugs. Coronal sections of the rat brain at the interaural (INT) stereotaxic planes 10.0 and 9.1, respectively, have been based on the stereotaxic atlas by Paxinos and Watson (2005). *Abbreviations*: AC – core of the nucleus accumbens; AS – shell of the nucleus accumbens; ca- anterior commissure; cc – corpus callosum; CP – caudate-putamen; DB – diagonal band; LS – lateral septum; mfb – medial forebrain bundle; MP – medial preoptic area; MS – medial septum; oc – optic chiasm; OT – olfactory tubercle; SH – striohypothalamic nucleus; V – lateral ventricle; VP – ventral pallidum.

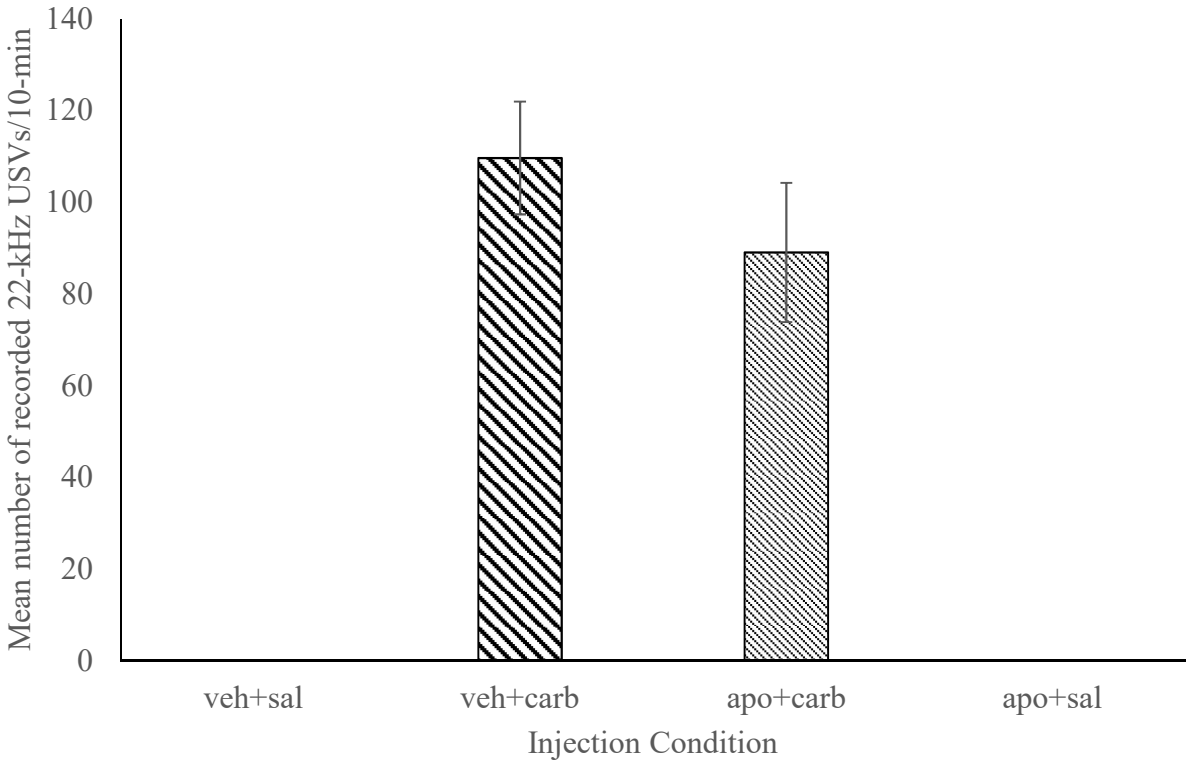

Figure 18: Mean (± S.E.M) number of 22-kHz USVs recorded during four different injection conditions. Injection of R-(-)-apomorphine outside the medial nucleus accumbens shell, followed by carbachol injection into the LS was unable to significantly reduce the mean number of recorded 22-kHz USVs ($\chi 2[3] = 24.9$, $p > 0.95$). There were no recorded 22-kHz USVs during the veh+sal and the apo+sal injection conditions. For the sequence of injection conditions and abbreviations, see Figure 19 for localizations.

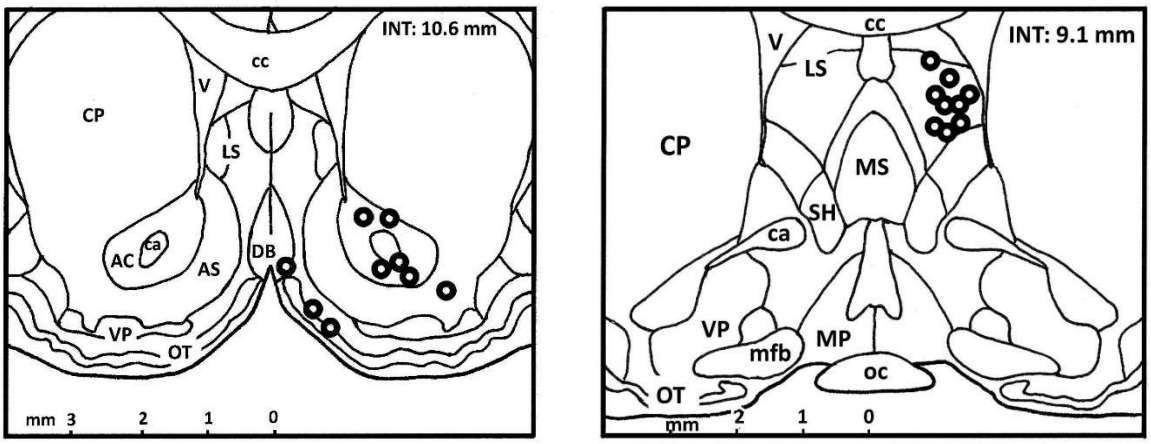

Figure 19: Localization of injection sites (dark circles) outside of the medial shell of the nucleus accumbens (n = 9) and corresponding localization of injection sites in the LS (n = 9). Coronal sections of the rat brain at the interaural (INT) stereotaxic planes 10.6 and 9.1, respectively, have been based on the stereotaxic atlas by Paxinos and Watson (2005). For list of abbreviations, see legend to Figure 17.

3.4.c: Increased emission of FM 50-kHz USVs, during apo+sal injection condition, is correlated with a reduction in the number of recorded 22-kHz USVs during the apo+carb injection condition

To investigate the relationship between the number of emitted F and FM 50-kHz USVs and the subsequent decrease in the number of emitted 22-kHz USVs, emitted F and FM 50-kHz calls were plotted against the magnitude of the subsequent decrease in 22-kHz USVs. The x-axis in Figure 20 and Figure 21 are the total number of F or FM 50-kHz USVs, respectively, that were recorded per rat (n = 16) during the apo+sal injection condition. The y-axis in Figure 20 and Figure 21 depict the standardized change (z-score) in the number of emitted 22-kHz USVs between veh+carb and apo+carb injection conditions.

No correlation between the number of recorded F 50-kHz USVs and the change in the number of recorded 22-kHz USVs between veh+carb and apo+carb injection conditions was observed ($r_s[14] = -0.39$, $p = 0.133$, Figure 20.). However, there was a significant negative correlation between the number of FM 50-kHz USVs emitted and the subsequent decrease in recorded 22-kHz USVs ($r_s[14] = -.54$, $p = 0.030$, Figure 21). Thus, the stronger the positive emotional state signaled by FM 50 kHz USVs, the greater the suppression of the negative emotional state reflected by the smaller number of emitted 22 kHz USVs.

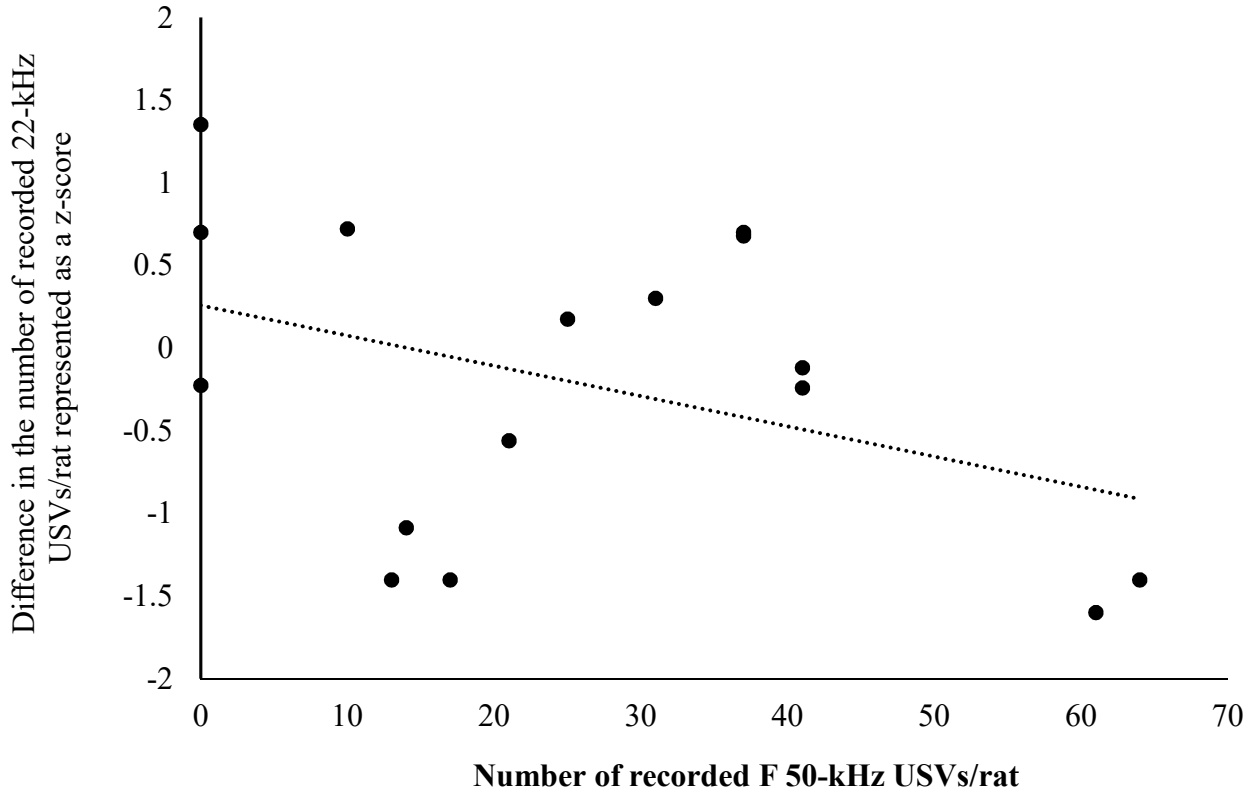

Figure 20. Difference in the number of recorded 22-kHz USVs between veh+carb and apo+carb injection conditions expressed as a function of z-score (y-axis) and plotted as a function of the number of recorded F 50-kHz USVs during a 10-minute recording session (x-

axis). There was no significant correlation between the number of F 50-kHz USVs and the change in number of recorded 22-kHz USVs between veh+carb and apo+carb injection conditions ($r_s[14] = -0.392$, $p = 0.133$, n=16). Each data point represents an individual rat.

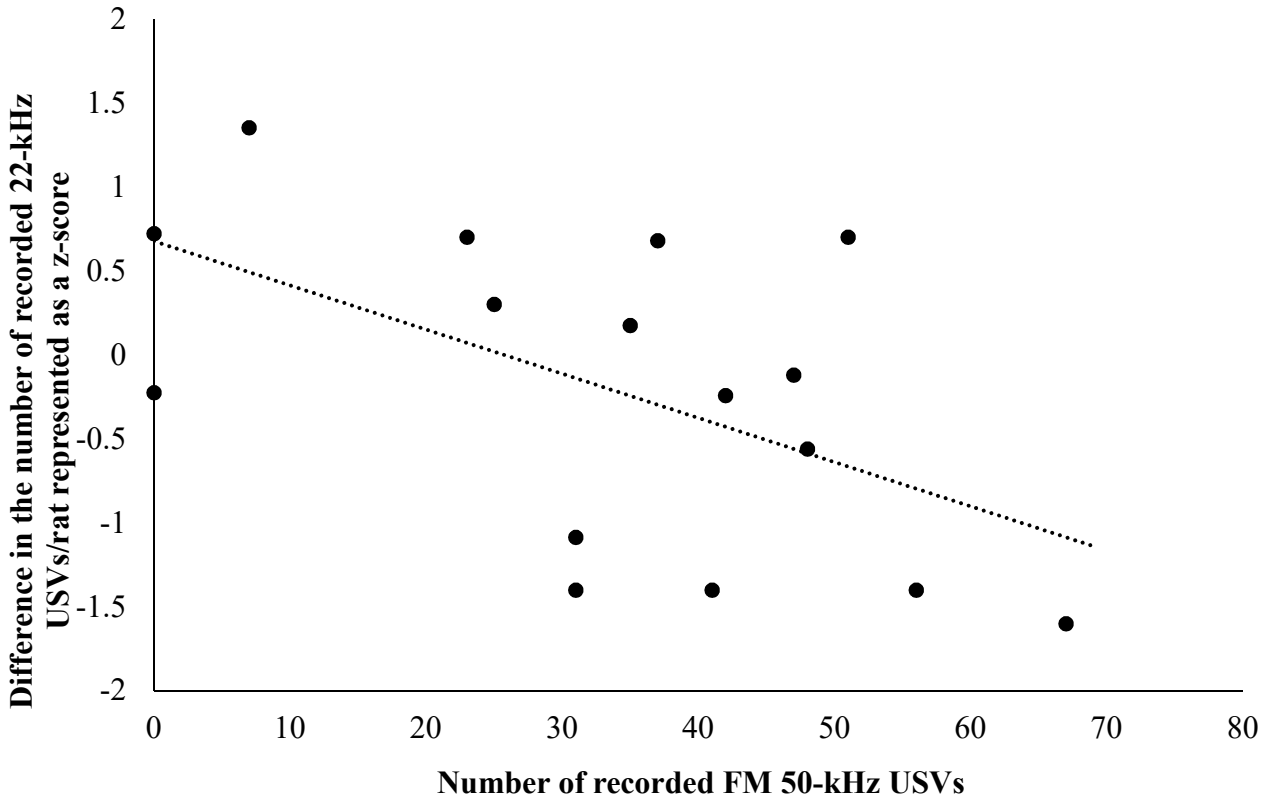

Figure 21: Difference in the number of recorded 22-kHz USVs (standardized, y-axis) as a function of the number of FM 50-kHz USVs recorded during 10-min (x-axis). There was a statistically significant correlation between the number of emitted FM 50-kHz USVs during the apo+sal injection condition and the change in the number of recorded 22-kHz USVs between veh+carb and apo+carb ($r_s[14] = -0.541$, $p = 0.030$; n=16).

3.4.d: Time-course of changes in the number of recorded 22-kHz USVs during veh+carb and apo+carb injection conditions

Plotting the number of recorded 22-kHz USVs as a function of time displays a slow decay of emitted 22-kHz USVs over the duration of the recording. Maximal responses occurred within the first three minutes and decayed to a minimum response between the 9-10 min mark during the veh+carb injection condition (see Figure 22, n = 16). The overall pattern of recorded 22-kHz USVs after apomorphine, i.e., during the apo+carb injection condition, was dissimilar to veh+carb injection condition. The maximal response occurred in the first minute, reaching a minimum between the 5^{th}-6^{th} minute and finally terminating between the 8^{th}-9^{th} minute (Figure 5). Thus, recording of 22-kHz USVs during the apo+carb injection condition showed suppression of aversive vocalizations and a faster decay of recorded 22-kHz USVs. The initial injection of R-(-)-apomorphine changed the calling profile of the carbachol response and shortened its total response.

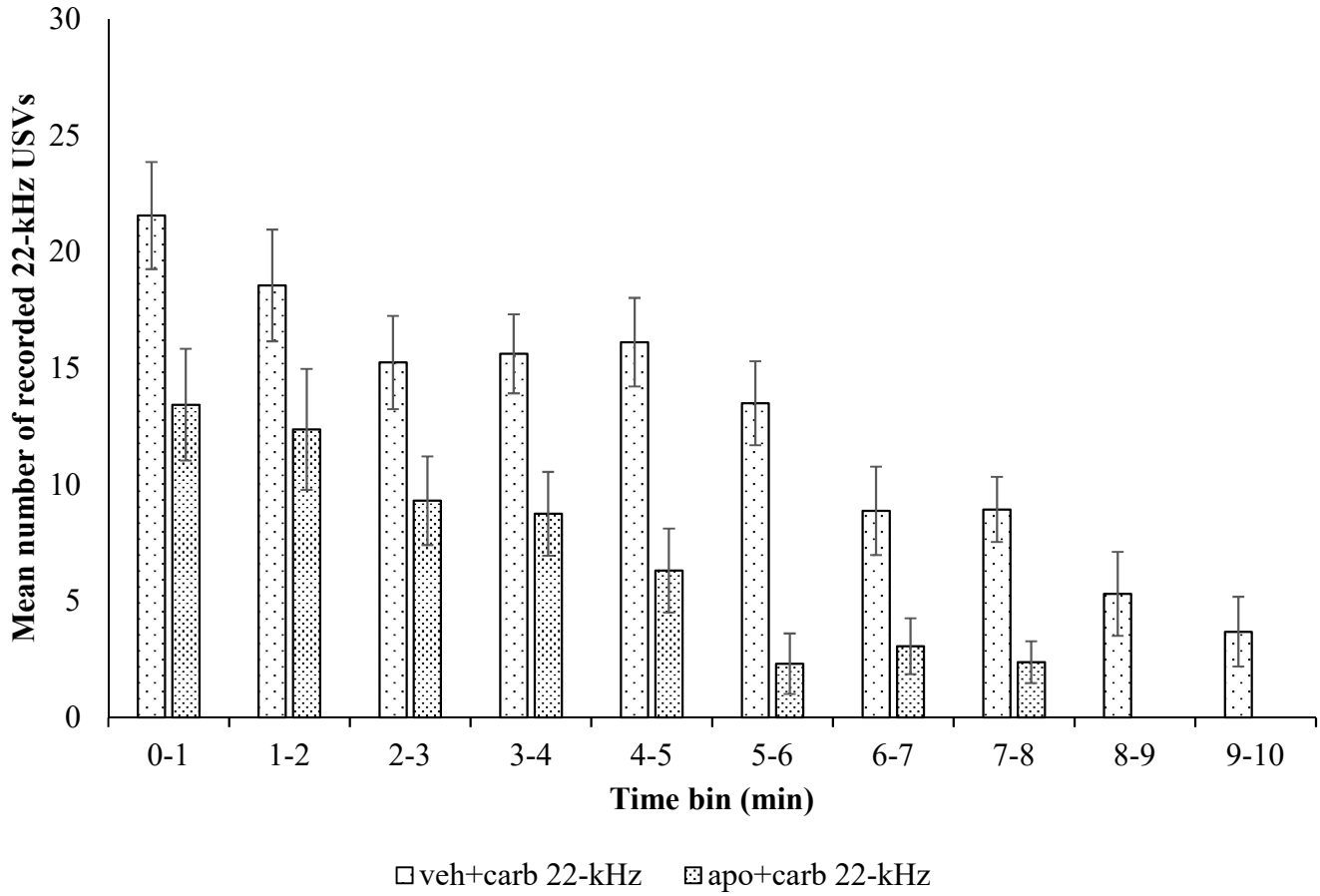

Figure 22. Time-course of the responses shown in 1 min bins for the number of recorded 22-kHz USVs during the veh+carb and apo+carb injection condition. Overall, injection of apomorphine into the shell of the nucleus accumbens prior to injection of carbachol into LS decreased the quantity of 22-kHz USVs and changed the dynamics of the response by increasing the decay of emitted 22-kHz USVs.

3.4.e: Acoustic parameters of recorded USVs

There was no statistical difference in the call duration of recorded 22-kHz USVs ($F[1.9, 46.5] = 0.368$, $p = 0.11$, partial $\eta^2 = 0.015$) or the peak frequency of recorded 22-kHz USVs ($F[1.9, 46.8] = 1.5$, $p = 0.32$, partial $\eta^2 = 0.059$) (see Figure 23 and Table 5 for values).

There was no difference for any injection condition to significantly alter the duration of F 50-kHz USVs ($F[2.6, 63.8] = 4.83$, $p = 8.84$, partial $\eta^2 = 0.168$) or FM 50-kHz USVs ($F[2.4, 58.7] = 8.4$, $p = 0.97$, partial $\eta^2 = 0.259$). There was also no difference in the peak frequency of F 50-kHz USVs ($F[2.3, 56.4] = 3.0$, $p = 0.61$, partial $\eta^2 = 0.11$) or FM 50-kHz USVs ($F[2.6, 62.2] = 2.4$, $p = 0.53$, partial $\eta^2 = 0.091$) across the injection conditions (Figure not shown; see Table 4 for values). These results confirmed that all emitted 22 kHz USVs and 50 kHz USVs were species-typical calls and were not modified by intracerebral injections.

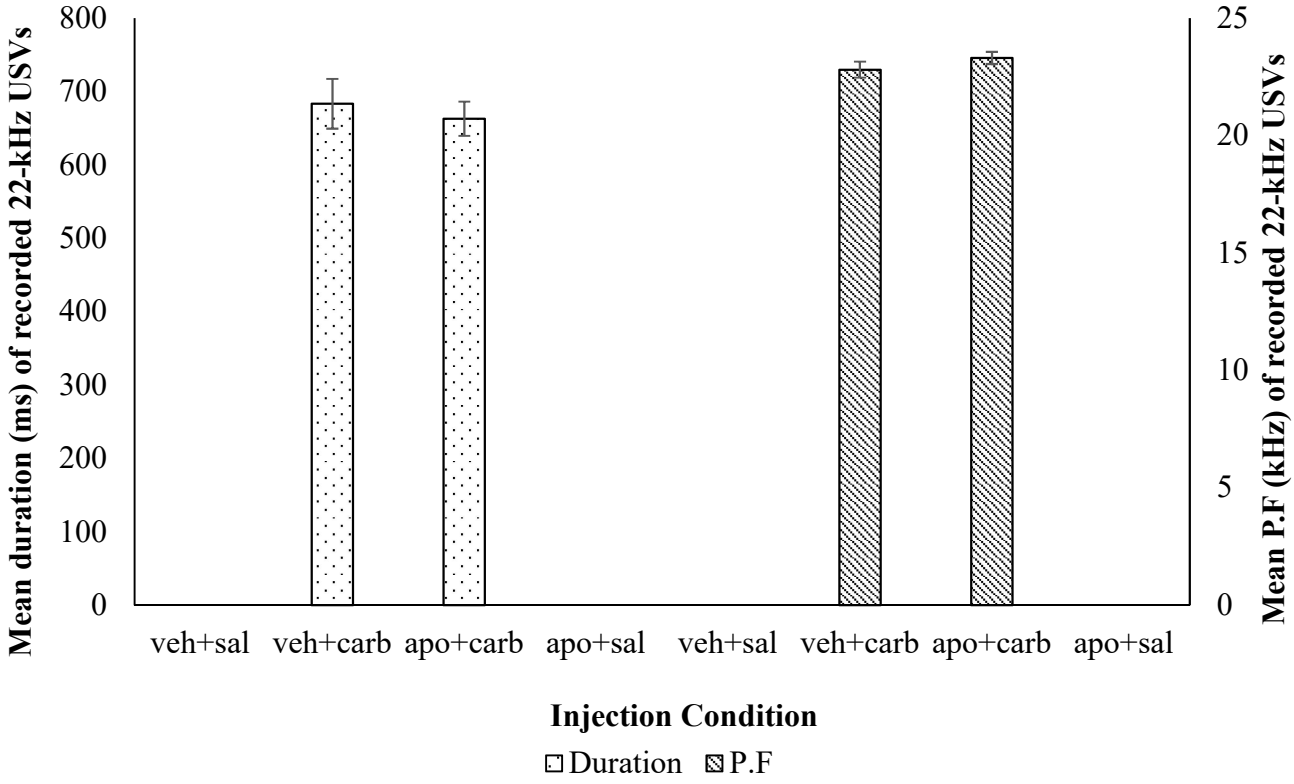

Figure 23. Mean (±S.E.M) values of spectrographic features of recorded 22-kHz USVs across injection conditions. There was no statistical difference in the single call duration (Duration) of recorded 22-kHz USVs ($F[1.9, 46.5] = 0.368$, $p=0.11$, partial $\eta2 = 0.015$) or in the peak frequency (P.F.) of recorded 22-kHz USVs ($F[1.9, 46.8]=1.5$, $p = 0.32$, partial $\eta2 = 0.059$).

3.5: Discussion

The purpose of the experiment was to determine if the initiation of a positive emotional state with 50 kHz USVs induced by injections of R-(-)-apomorphine into the medial shell of the nucleus accumbens, could supresses the vocal expression of 22-kHz USVs induced by carbachol injection into the LS.

3.5.a: Injection of R-(-)-apomorphine into the nucleus accumbens shell increased the number of F and FM 50-kHz USVs

Injection of apomorphine into the medial shell of the nucleus accumbens produced species-typical F and FM 50-kHz USVs. These results were consistent with our previous reports, and with other publications, showing increased F and FM 50-kHz USVs after intracerebral injection of dopamine agonists into the medial shell of the nucleus accumbens, or systemic injection of dopamine agonists like amphetamine (Burgdorf et al., 2001; Wintink & Brudzynski, 2001; Thompson et al., 2006; Ahrens et al., 2009; Burgdorf & Moskal, 2010; Brudzynski et al., 2011; 2012; Brudzynski, 2015; Mulvihill & Brudzynski, 2019). Emission of 50-kHz USVs, and particularly FM 50-kHz calls seem to be indicative of the initiation of a positive emotional state since rats will emit these types of vocalizations during amphetamine-induced conditional place preference (Ahrens et al., 2014; Knutson et al., 1999), during the anticipation of consumption of cocaine or sucrose (Browning et al., 2011), or during mating or during juvenile play in rats (Burke et al., 2017).

Other studies using emotional modulation of the startle reflex, which expresses a negative state, have also reported that maximal startle amplitudes were decreased by systemic apomorphine (Martin-Iverson & Stevenson, 2005). Our results corroborate their findings and

further provide evidence that suggests an antagonistic influence that a positive emotional state has on the expression of a negative emotional state.

3.5.b: Injection of carbachol into the lateral septum increased species-typical 22-kHz USVs

Our results indicated that injection of carbachol into the LS was able to significantly increase the mean number of 22-kHz USVs compared to control injections. Anatomically, the LS is located rostrodorsally to the anterior commissure and caudally to the nucleus accumbens and is involved in the expression of defensive behaviours, anxiety, and fear (Treit & Menard., 1997; Ouagazzal et al., 1999; Singewald et al., 2003; Reis et al., 2010). It is the most rostral portion of the medial cholinoceptive vocalization strip (Brudzynski, 2001; 2010; 2013; 2014), i.e., the area from which cholinergic agonists can induce aversive arousal with the emission of 22 kHz USVs.

The medial cholinoceptive vocalization strip is a strip of neural tissue that originates within the rostral division of the laterodorsal tegmental nucleus (LTD) (Brudzynski, 2010; 2014; 2015). The cholinergic neurons within the LDT form ascending pathways through the brain (mesolimbic cholinergic system) and terminate in extensive areas of the midbrain and forebrain. The release of acetylcholine from a limited subset of these pathways that terminates in the medial cholinoceptive vocalization strip will initiate aversive arousal and emission of 22-kHz USVs (Brudzynski, 2010; 2014). Pharmacological activation of the areas of the strip by muscarinic acetylcholine agonists induced species-typical aversive 22-kHz USVs in rats. In the current study, intracerebral injection of carbachol into the LS, as the rostral part of the medial cholinoceptive strip, produced species-typical 22-kHz USVs that did not differ from those reported in previous studies (Bihari et al., 2003; Brudzynski et al., 2011).

3.5.c: R-(-)-apomorphine significantly decreased carbachol-induced 22 kHz USVs from the lateral septum

Our current results have confirmed that the initiation of a positive state by apomorphine significantly reduced the development of the subsequent aversive state. The number of emitted F 50-kHz USVs was not correlated with a reduction in the number of recorded 22-kHz USVs caused by apomorphine. However, there was a significant negative correlation between the number of emitted FM 50-kHz USVs and the change in recorded 22-kHz USVs after apomorphine. This result suggests that, unlike F 50 kHz calls, emission of FM 50 kHz USVs can antagonize the expression of a negative emotional state in rats. Hence, the more FM 50 kHz calls, the greater the magnitude of the positive arousal and the deeper is suppression of the carbachol-induced aversive response. F 50 kHz vocalizations are primarily used in social-contact settings and might not be directly involved in outward expression of an internal positive emotional state (Brudzynski & Pniak, 2002; Snoeren & Ågmo, 2014; Wöhr et al., 2008). Since 50-kHz is indirectly reflective of increased dopamine concentration in the shell of the nucleus accumbens and 22-kHz USVs is indirectly reflective of acetylcholine concentration along the medial cholinoceptive vocalization strip, decreased vocal expression of 22-kHz USVs in response to apomorphine could indirectly reflect decreased levels of acetylcholine along the medial cholinocpetive vocalization strip.

Despite apomorphine's antagonism of the aversive state induced by carbachol, apomorphine was unable to eliminate emitted 22-kHz USVs. This could be a result of the pharmacokinetic profile of apomorphine or the efficacy of the drugs. Pharmacokinetic data in humans demonstrated a half-life time of apomorphine lasting approximately five minutes with clearance time around 4 hours (Gancher et al., 1989). Given the design of the current experiment,

there is a 120 s delay between the injection of apomorphine and carbachol. Thus it is possible there was a decay of apomorphine action before carbachol was injected into the LS. Another explanation could simply be attributed to the fact that carbachol could be more efficacious at initiating a negative emotional state than apomorphine is at initiating a positive emotional state.

3.6: Conclusion

It has been hypothesized that rat may signal only one of the emotional states at any given time, suggesting that emissions of 22-kHz or 50-kHz calls become mutually antagonistic (Brudzynski, 2007). The main purpose of the present experiment was to determine if the initiation of positive emotional state by apomorphine microinjection into the nucleus accumbens could decrease the expression of a negative emotional state reflected in the number of emitted 22-kHz USVs. Our current findings showed that FM 50-kHz USVs, which signal positive emotional states, were correlated with reducing the effects of carbachol-induced 22-kHz USVs from the LS. This results further extends previous working showing apomorphine injection into the nucleus accumbens shell decreased 22-kHz USVs induced from the AH-MPO. Our results thus far show that apomorphine can reduce carbachol-induced 22-kHz USVs from the caudal and rostral divisions of the medial cholinoceptive vocalization strip.

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Table 4: Mean number (±S.E.M) of recorded 22-kHz, F 50-kHz and FM 50-kHz USVs across different injection conditions for R-(-)-apomorphine injected into the medial shell of nucleus accumbens (left side of the Table) or outside of the medial shell of accumbens (right side of the Table).

	Medial nucleus accumbens shell + LS (n=16)				Outside medial nucleus accumbens shell + LS (n=9)			
USVs	veh+sal	veh+carb	apo+carb	apo+sal	veh+sal	veh+carb	apo+carb	apo+sal
FM 50-kHz	2.1 ± 0.8[a]	N.A	N.A	33.8 ± 4.8[a]	2.6 ± 1.1	N.A	N.A	4.4 ± 1.5
F 50-kHz	3.5 ± 1.0[b]	N.A	N.A	23.6 ± 4.1[b]	6.2 ± 1.4	N.A	N.A	5.5 ± 0.9
22-kHz	0	127.4 ± 11.3[c]	57.9 ± 10.8[c]	0	0	109.7 ± 12.3	89.1 ± 15.2	0

Note: Statistically significant differences in the post-hoc analysis for the number of emitted F or FM 50-kHz USVs during different injection conditions are denoted by the superscript letters.

Superscript letters and significance: a:$p=0.037$; b:$p<0.001$; c: $p=0.008$;d: $p=0.024$..

Abbreviations: veh+sal, rats received vehicle injection into the medial shell of the nucleus accumbens followed by vehicle injection into the LS; veh+carb, vehicle injection into accumbens shell followed by carbachol injection into the LS; apo+carb, R-(-)-apomorphine into the medial shell of the nucleus accumbens, followed by carbachol injection into the LS, apo+sal, R-(-)-apomorphine injection into the medial shell of the nucleus accumbens followed by vehicle injection into the LS. N.A - Not Applicable.

Table 5. Pooled sonographic features of recorded USVs during injection conditions.

	Duration (ms) of recorded USVs				Peak Frequency (kHz) of recorded USVs			
USVs	veh+sal	veh+carb	apo+carb	apo+sal	veh+sal	veh+carb	apo+carb	apo+sal
22-kHz	0	683.1 ± 33.8	662.9 ± 23.3	0	0	22.8 ± 0.34	23.3 ± 0.26	0
F 50-kHz	40.1 ± 1.1	N.A	N.A	32.6 ± 3.1	47.1 ± 1.4	N.A	N.A	53.2 ± 1.8
FM 50-kHz	28.5 ± 1.6	N.A	N.A	30.8 ± 2.1	58.2 ± 1.3	N.A	N.A	56.1 ± 2.9

Note: Mean (±S.E.M) spectrographic parameters of recorded 22-kHz and 50-kHz USVs. There were no statistically significant differences between the duration or peak frequency of recorded 22-kHz USVs or 50-kHz USV across injection conditions. For abbreviations, see note to Table 1.

Chapter 4: Dissimilar interaction between dopaminergic and cholinergic systems in the initiation of emission of 50-kHz and 22-kHz vocalizations

This chapter has been submitted to Pharmacology, Biochemistry and Behavior in 2019.

4.0: Abstract

Rats emit 22-kHz or 50-kHz ultrasonic vocalizations (USVs) to signal their emotional state to other conspecifics. The 22-kHz USVs signal a negative emotional state while 50-kHz USVs reflect a positive affective state. The initiation of 22-kHz USVs is dependent on the activity of cholinergic neurons within the laterodorsal tegmental nucleus that release acetylcholine along the medial cholinoceptive vocalization strip. Emission of 50-kHz USVs is dependent upon the activation of dopaminergic neurons located within the ventral tegmental area that release dopamine into the medial shell of the nucleus accumbens. There have been reports that showed an antagonistic interaction between acetylcholine and dopamine during the expression of emotional states, and dopamine agonists decreased carbachol-induced emission of

22-kHz USVs. The current study tests the hypothesis that initial antagonism of dopamine receptors by systemic haloperidol or microinjection of raclopride into the nucleus accumbens shell should increase the subsequent emission of 22 kHz USVs induced by carbachol from the lateral septum. Our findings showed that antagonism of dopaminergic signaling either via systemic haloperidol or via intracerebral raclopride did not alter the number of emitted 22-kHz USVs. Thus, inhibition of the mesolimbic dopamine system did not increase the magnitude of a negative emotional state. It was found, however, that prolonged emission of 22-kHz USVs initiated by carbachol caused a delayed rebound emission (R) of 50-kHz USVs appearing after 300 s of emission of 22-kHz USVs, i.e., when the response was subsiding. The R-50-kHz USVs were predominantly frequency modulated (FM) USVs and their number was directly proportional to the number of recorded 22-kHz USVs. The emission of R-50-kHz USVs was inhibited by systemic pretreatment with haloperidol or intraacumbens injection of raclopride. We argue that these the R-50-kHz USVs represent a rebound emotional state that is opposite in emotionality from carbachol-induced 22-kHz USVs. Importantly, prolonged emission of amphetamine-induced 50 kHz USVs failed to show any vocalization rebound effect.

4.1: Introduction

Adolescent and adult rats can emit 22-kHz ultrasonic vocalizations (USVs) and 50-kHz USVs that signal their emotional state to conspecifics. 22-kHz USVs are emitted typically in close proximity to a predator (Blanchard & Blanchard, 1991), in response to contact with unfamiliar humans (Brudzynski et al., 1993), in anticipation to air-puff (Knapp & Pohorecky, 1995), and in response to fox urine (Fendt et al., 2018). These calls are also induced by anticipation to foot shock (Kim et al., 2013), withdrawal from drugs of abuse (Berger et al.,

2003; Miczek & Barros, 1996), or failure to deliver expected rewards (Baker et al., 2011). Since the emission of 22-kHz USVs are emitted during negative contexts and can be significantly decreased using benzodiazepines (Nielsen & Sánchez, 1995; Jelen et al., 2003), or selective serotonin reuptake inhibitors (SSRIs) (Sánchez, 1993) these vocalizations have been hypothesized to reflect a negative emotional state similar to anxiety (Brudzynski, 2007; 2013)

The initiation of 22-kHz USVs is dependent upon the activation of the ascending mesolimbic cholinergic system that originates within the laterodorsal tegmental nucleus (LTDg) (Brudzynski et al., 2011). The axons of the LTDg ascend and innervate a variety of limbic structures such as the medial hypothalamus, lateral habenula, and lateral septum (LS) (Cornwall et al., 1990). Intracerebral injection of carbachol into the anterior hypothalamic-medial preoptic region (AH-MPO) or the LS easily induces species-typical 22-kHz USVs. Also, intracerebral injection of glutamate into the LTDg, which activated local neurons, produced robust emission of species-typical 22-kHz USVs (Brudzynski & Barnabi, 1996). This emission could be significantly decreased with intracerebral injection of scopolamine, a muscarinic antagonist, into the anterior hypothalamus (Brudzynski & Barnabi, 1996; Brudzynski et al., 1996). These pharmacologically induced 22-kHz USVs have comparable duration, sound pressure levels, peak frequency and bandwidth as non-pharmacologically induced 22-kHz USVs (for review see Brudzynski, 2007).

Unlike 22-kHz USVs, emission of 50-kHz USVs signal a positive emotional state in rats (Brudzynski, 2007; 2013; Burgdorf et al., 2007; Burgdorf et al., 2008). Emission of FM 50-kHz USVs appears during the anticipation of rewarding interactions among rats like heterospecific play (Burgdorf et al., 2008; Hori et al., 2013; Knuston et al., 1998), during sexual behavior (Barfield et al., 1979), in positively conditioned place preference (Knutson et al., 1999) and

anticipation of rewarding electrical brain stimulation of the medial forebrain bundle (Burgdorf et al., 2007).

Evidence from numerous studies suggests that the initiation of 50-kHz USVs is dependent upon the release of dopamine into the nucleus accumbens shell from the ventral tegmental area (Ikemoto, 2007). For example, optical stimulation of dopamine cell bodies within the ventral tegmental area, intracerebral application of dopamine agonists (quinpirole, amphetamine, R-(-)-apomorphine) into the nucleus accumbens, or systemic injection of dopamine agonists (amphetamine) have been demonstrated to be sufficient for the initiation of 50-kHz USVs (Brudzynski et al., 2012; Burgdorf et al., 2001; Engelhardt et al., 2017; Engelhardt et al., 2018; Mulvihill & Brudzynski, 2018; Rippberger et al., 2015; Scardochio et al., 2015; Thompson et al., 2006). The emission of 50-kHz USVs can be blocked by injections of dopamine antagonists such as U-99195A, raclopride or haloperidol (Brudzynski et al., 2012; Wright et al., 2013).

Thus, the initiation of positive or negative emotional states in rats, reflected by the emission of either 50-kHz or 22-kHz USVs, is an indirect measure of dopamine release within the nucleus accumbens shell or acetylcholine release along the ascending mesolimbic cholinergic system. However, evidence suggests a possible interaction between acetylcholine and dopamine during the initiation of emotional states in rats (Brudzynski, 2007), akin to dopamine-acetylcholine interactions in the regulation of motor movement within the dorsal striatum, (Benarroch, 2012; Lester et al., 2010; Rizzi & Tan., 2017; Tarsy, 1979; Weiner et al., 1990). Thus, this hypothesis would predict that increased activity of the mesolimbic cholinergic system would decrease the activity of the mesolimbic dopaminergic system and *vice versa*.

Although indirect, some evidence for an acetylcholine-dopamine interaction during development of emotional states is evident in pharmacological and behavioural studies. For example, apomorphine has been reported to decrease whole brain acetylcholine concentrations in some conditions (Waldmeier, 193l; Ulus, 2010) as well as decrease the immobility time rats exhibit during the forced swim test (Brocco et al., 2006). Similar results are also observed when rats are injected with organophosphates that increase synaptic levels of acetylcholine, like chlorpyrifos and permethrin (López-Crespo et al., 2009; Phillips & Deshpande, 2016; Sánchez-Amate et al., 2001; Savy et al., 2015; Shaheen et al., 2014). Likewise, organophosphates have been demonstrated to decrease striatal dopamine neurotransmission as well as decrease open arm exploration in the elevated plus maze (Karen et al., 2001; Shahabi et al., 2008).

Further indirect evidence of acetylcholine-dopamine interactions is observed during application of cholinergic agonists and dopamine antagonists within the clinical population. Injection of the dopamine antagonist metoclopramide has been reported to induce panic attacks, generalized anxiety, depression and nightmares (Anfinson, 2002; Kluge et al., 2007; Shearer et al., 1984; Weddington & Banner, 1986). These clinical symptomatologies are similar to patients' reports when administered cholinergic agonists.

Antagonists of dopamine have also been reported to increase quantitative measures of anxiety in both the clinical populations. For example, the dopamine antagonist metoclopramide has been reported to induce panic attacks, generalized anxiety, depression and nightmares in the clinical population observed (Anfinson, 2002; Kluge et al., 2007; Shearer et al., 1984; Weddington & Banner, 1986). Interestingly the clinical manifestations after metoclopramide administration can be observed after administration with the acetylcholinesterase inhibitor physostigmine (Janowsky et al., 1979; 1981). Conversely, application of dopamine agonists like

piribedil has been shown to exhibit antidepressant-like properties via its affinity for D_2 receptors (Brocco et al., 2006).

Recently, we have reported a decrease in the emission of carbachol-induced 22-kHz from the LS after apomorphine injection into the medial shell of the nucleus accumbens (Silkstone & Brudzynski, 2019). If these two mesolimbic systems remain in a mutually antagonistic relationship, one may expect that the pharmacologic antagonism of the mesolimbic dopaminergic system will increase the sensitivity of the mesolimbic cholinergic system to initiate 22-kHz USVs after cholinergic stimulation. The current experiment was designed to investigate if antagonizing dopamine transmission by systemic injection of haloperidol or by intracerebral injection of the D_2 antagonist, raclopride, into the medial shell of the nucleus accumbens can increase carbachol-induced 22-kHz USVs from the LS.

4.2 Methods and Procedure

4.2.a: Investigating if systemic haloperidol increases carbachol-induced 22-kHz USVs from the LS

4.2.a. Subjects and Surgery

Twenty-four adolescent male Long-Evans rats (purchased from Charles River Laboratories, Saint-Constant, QC, Canada) with body weights ranging between 270-295 g at the time of surgery were used in the study. All animals were housed in polycarbonate cages (48 cm x 27 cm x 20 cm) in the room with constant temperature (23° C ± 2° C) and humidity control. Rats were on a 12: 12 h light-dark cycle with *ad libitum* access to standard food pellets and filtered tap water.

Rats underwent stereotaxic surgery for unilateral implantation of guide cannula into the LS of the left cerebral hemisphere. Rats were anesthetized with gaseous isoflurane at a concentration of 3% and placed in a Kopf stereotaxic apparatus (Modell 900 David Kopf Instruments, Tujunga, CA). When securely fastened into the apparatus, burr holes were drilled into the skull using an electric drill. Guide cannula (O.D.=650 μm) was implanted into the left LS. The cannula was constructed from a 23 G stainless steel needle (Beckton-Dickinson Canada, Mississauga, ON) and was implanted 1 mm above the intended injection site. Coordinates for the LS injections were taken from (Paxinos & Watson, 2007): A-P: 8.5-8.8 mm; L:0.8-1.1 mm; V:4.8 – 5.4 mm below the surface of the dura. The cannula was secured to the rat's skull using jeweler's screws and methyl methacrylate resin (Perm Resin, Hygenic Corporation of Canada Inc., St. Catharines, ON). Rats recovered for 5 days after the surgery before they began 72 hours of habituation upon approval of their condition by staff veterinarian. For further details on stereotaxic procedure see Fornari et al., 2012). All research protocols were approved by Brock University Animal Care and Use Committee and complied with guidelines and policies set forth by the Canadian Council on Animal Care.

4.2.b. Pharmacological agents and intracerebral injection procedure

Haloperidol, a D_2-antagonist, was used to determine if blocking dopamine receptors via intraperitoneal injection of a dopamine antagonist could increase carbachol-induced 22-kHz USVs from the LS.

First, haloperidol (Precision Biochemicals, Vancouver, BC) was dissolved in a 1% lactic acid solution at a dose of 1.0 mg/kg and injected intraperitoneally at a volume of 0.5 ml. After interperitoneal injection of haloperidol, the rat was placed in its home-cage for fifteen minutes.

After fifteen minutes, the rat was retrieved, and 1.0 µg/0.3 µl of carbachol was injected into the LS using a constant rate Hamilton® CR 700 micro-syringe (Hamilton Company, Reno, NV) at a rate of ~4.5 ml/s. The injection cannula was left in place for the 30 s to allow for proper drug diffusion. After the 30 s, the injection cannula was slowly withdrawn, and a sterile plug-pin was inserted into the guide cannula. The rat was then placed in the recording chamber for a duration of 10 min.

Injections were divided into four sections: veh+sal: vehicle (a control for haloperidol injection) was interperitoneally injected followed by intracerebral injection of saline (a control for carbachol injection) into the LS 15-minutes later; veh+carb: vehicle was interperitoneally injected followed by carbachol injection into the LS 15 min later; hal+sal: haloperidol was interperitoneally injected in the rat followed by saline injection into the LS 15 min later; hal+carb: haloperidol was interperitoneally injected followed by carbachol injection into the LS 15 min later.

4.2.c: Recording of ultrasonic vocalizations

Recordings of ultrasonic vocalization took place in a 25 cm x 18 cm x 18 cm plexiglass recording chamber. The floor of the recording chamber was lined with a single paper towel since corn-cobb bedding contributes to the acoustic noise and can influence the emission of USVs (Natusch & Schwarting, 2010). The top of the recording chamber contained an Avisoft® CM16/CMPA condenser microphone (frequency range 2-250 kHz, Avisoft® Bioacoustics, Berlin Germany). Although the distance from the microphone to the snout of the rat is difficult to standardize since the rat rears, the approximate distance from the microphone to the dorsal

surface of the skull was 25 cm. Recording of vocalizations was obtained in real time and stored in a 16-bit format for later analysis in the Avisoft® SASlab program (Avisoft, Germany).

Recorded vocalizations were analyzed off-line by using sonograms. Sonograms were constructed from wave files using a 512 FFT-length with a Hamming window and a 75%-time overlap. Spectrograms were produced with a 488 kHz resolution, and calls were marked for their durations, peak frequency, and category manually. Call categories consisted of flat vocalizations (F), and frequency modulated vocalizations (FM), for a full list of categorized FM calls see Wright et al., 2010.

4.2.d: Histology and Localization of injection sites

After injections were finished, animals were anesthetized with an overdose of sodium pentobarbital and received an injection of Indian ink (1:100 dilution) for histological determination of injection sites. After injection of India ink, rats underwent transcardial perfusion with 10% formalin and the brains were extracted and stored for 48 hours. The brains were then coronally sectioned on a freezing microtome (Cryo-Histomat, Hacker Instruments and Industries, Fairfield, NJ) to a thickness of ~40 μm. Sections were placed on a 1% poly-lysine coated slides then underwent Nissle staining (see Lindroos and Leinonen, 1983, for details). Slides were then coverslipped, and injection sites were histologically verified via India ink granules under projection microscope.

4.2.e: Statistical Analysis

Results are presented as means with the standard error of the mean (S.E.M). Since the total number of vocalizations followed a non-parametric distribution, differences between

injection groups were assessed using non-parametric Friedman's ANOVA followed by Wilcoxon Signed Rank test. Acoustic parameters (peak frequency and duration) were analyzed using an ANOVA. P-values less than 0.05 were considered significant. Multiple comparisons were corrected using the Bonferroni correction. To investigate the relationship between the number of rebound F and FM 50-kHz USVs, the number of recorded 22-kHz USVs were plotted against a standardized z-score of the number of recorded F and FM 50-kHz USVs.

4.2.f: Investigating if the intracerebral injection of raclopride increases carbachol-induced 22-kHz USVs from the LS.

Twenty-four adolescent male Long-Evans rats (purchased from Charles River Laboratories, Saint-Constant, QC, Canada) with body weights ranging between 275-325 grams at the time of surgery were used in the experiment. All animals were stored in polycarbonate cages (48 cm x 27 cm x 20 cm) in the room (23° C ± 2° C) with constant humidity settings. Rats were on a 12: 12 h light-dark cycle with *ad libitum* access to standard food pellets and filtered tap water.

Rats in the second experiment underwent bilateral implantation of guide cannula into the left hemisphere. One guide cannula was implanted into the medial shell of the nucleus accumbens and the second guide cannula was placed into the LS. For implantation into the nucleus accumbens shell, the coordinates were taken from (Paxinos & Watson, 2007) and were as follows: A-P: 10.4 mm – 10.8 mm; L: 0.8-1.6 mm and D-V: 5.6-6.4 mm below the dura. Injection for the LS was as follows: A-P: 8.5-8.9 mm: L:0.6-1.0 mm; D-V: 4.8-5.6 mm below the surface of the dura. Procedures for chronically securing the cannula, the O.D of the guide cannula and recovery can be found in section *4.1.a.*

4.2.f: Pharmacological agent and intracerebral injection procedure

The drug was changed from haloperidol to raclopride for intracerebral injection studies because it has been shown previously that intracerebral injection of haloperidol can partially increase the emission of 50-kHz USVs (Thompson et al., 2005). Briefly, 7µg/0.3 µl of raclopride (Sigma-RBI, Oakville, Ont., Canada) was injected into the medial shell of the nucleus accumbens using a constant rate Hamilton® CR 700 micro-syringe (Hamilton Company, Reno, NV) at a rate of ~4.5 nl/s. After injection of raclopride, the injection cannula was left in place for 30 s then withdrawn. The guide cannula was then closed using a sterile plug-in, then the rat was placed in its home cage for 60 s. After 60 s the rat was taken, and 1.0 µg/0.3 µl of carbachol was injected into the LS using a constant rate Hamilton® CR 700 micro-syringe (Hamilton Company, Reno, NV) at the same rate as raclopride. After injection of carbachol, the injection cannula was left in place for an additional 30 s then withdrawn slowly. The guide cannula was closed using a sterile plug-pin, and the rat was then placed into the recording chamber for 10 min.

For the recording of ultrasonic vocalizations, histological analysis of injection sites and statistical analysis of recordings see sections *4.1.c-4.1.d*.

4.3: Results

4.3.a: Pretreatment with systemic haloperidol failed to increase the mean number of carbachol-induced 22-kHz USVs from LS

Injection of the dopamine antagonist haloperidol failed to increase the mean number of recorded 22-kHz USVs compared to veh+carb injection ($\chi^2(3) = 31.6$, $p>0.95$, see Table 1 [tables at end of text] and Figure 24, n=12). Injection of haloperidol also failed to increase the mean

number of recorded 22-kHz USVs when carbachol was injected outside the LS (see Table 1, figure not shown).

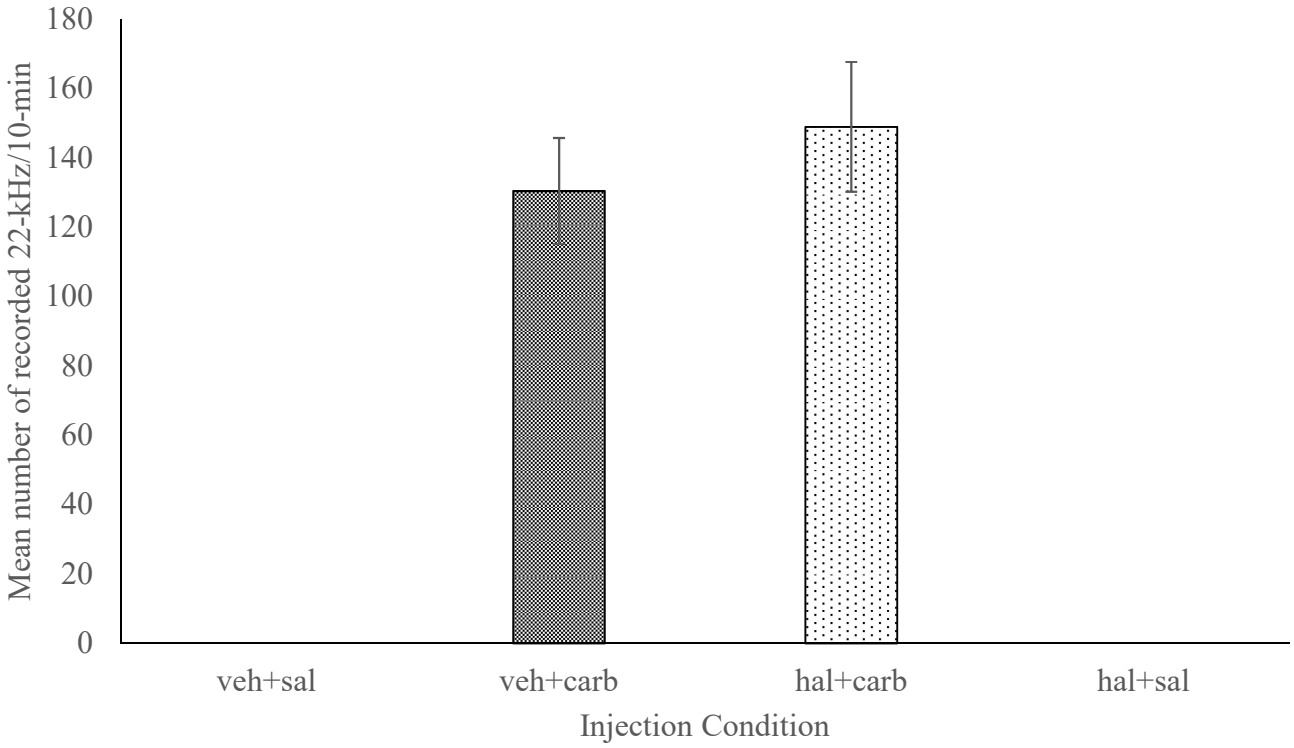
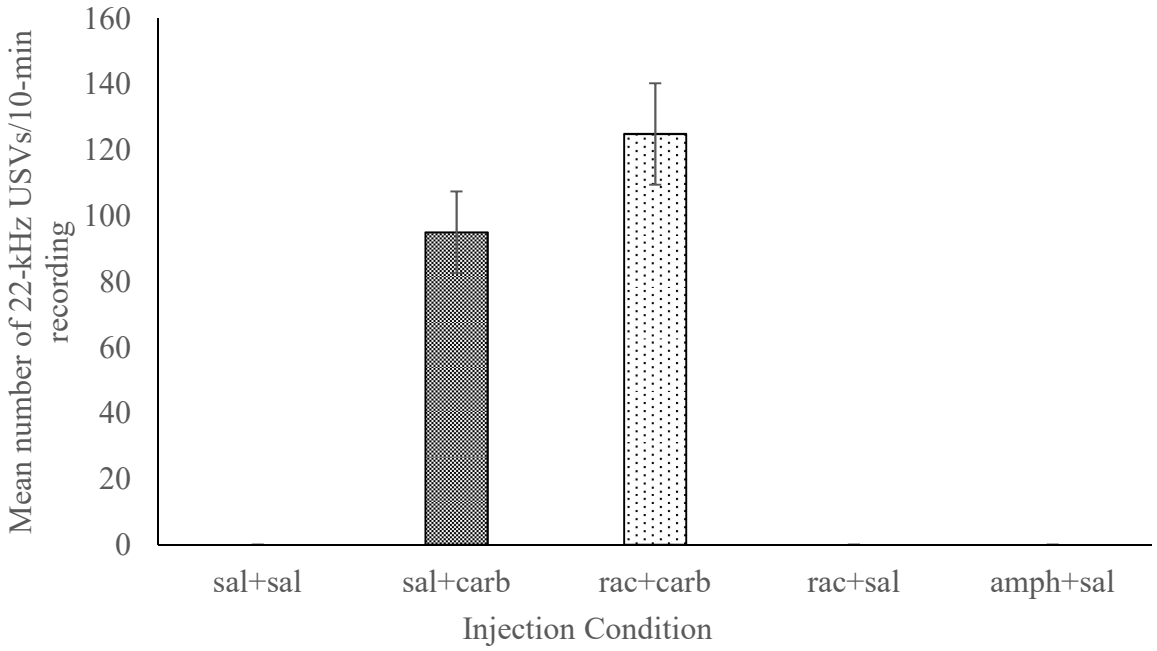


Figure 24: Mean number of recorded 22-kHz USVs during a 10 min recording. There was no statistical difference observed between the number of recorded 22-kHz USVs during veh+carb and hal+carb conditions ($\chi^2[3] = 31.6$, $p > 0.95$, n = 12 rats) *Abbreviations:* veh+sal: vehicle is injected systemically followed by saline injection into the LS 15 min later; veh+carb: vehicle is injected systemically followed by carbachol injection into the LS 15 min later; hal+carb: haloperidol is injected systemically followed by carbachol injection into the LS 15 min later; hal+sal: haloperidol is injected systemically followed by saline injection into the LS 15-minutes later. Veh+sal or hal+sal did not induce any 22-kHz USVs. Localization of injection sites for carbachol is shown in Figure 25C (right side of the brain).

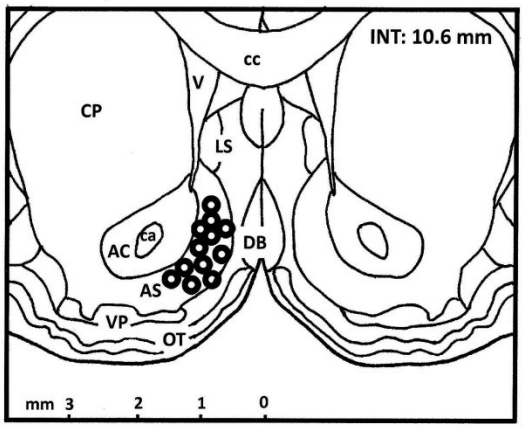
4.3.b: Pretreatment with intracerebral raclopride into the medial shell of the nucleus accumbens failed to increase the mean number of carbachol-induced 22-kHz USVs from the LS

Injection of the dopamine antagonist raclopride into the medial shell of the nucleus accumbens (rac+carb) failed to increase the mean number of recorded 22-kHz USVs compared to veh+carb injection condition ($\chi^2(3) = 45.3$, $p>0.95$, see table 3, figure 25A, for localizations see Figure 25 B and C). Injection of raclopride into the medial shell of the nucleus accumbens followed by injection of carbachol outside the LS failed to increase the number of 22-kHz USVs (see table 6, Figure not shown).

A


Mean number of 22-kHz USVs/10-min recording
160
140
120
100
80
60
40
20
0
sal+sal
sal+carb
rac+carb
rac+sal
amph+sal
Injection Condition

B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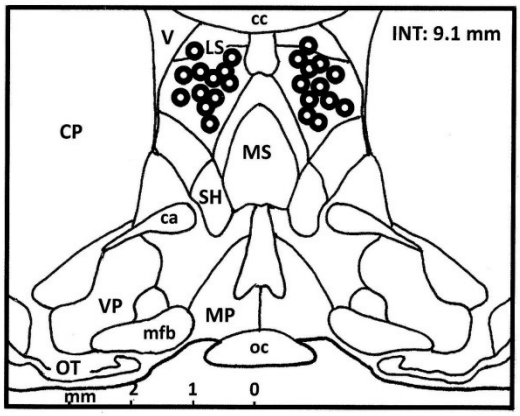
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Figure 25. The mean number of carbachol-induced 22-kHz USVs during a 10 min recording session (**A**). There was no statistical difference between the number of 22-kHz USVs recorded during sal+carb or rac+carb injection conditions ($\chi^2[3] = 45.3$, $p > 0.95$, n = 12 rats). Amphetamine followed by saline in LS (amph+sal) was injected as a negative control. Localization of injection sites in the medial shell of nucleus accumbens is shown in **B** while injection sites in the ipsilateral LS are shown in C (left side of the diagram). The right side of the diagram shows the localization of injection sites for carbachol after systemic haloperidol. *Abbreviations:* sal+sal: saline injected into the medial shell of the nucleus accumbens followed by saline injection into the LS; sal+carb: saline injected into the medial nucleus accumbens shell followed by injection of carbachol into the LS; rac+carb: raclopride injected into the medial shell of the nucleus accumbens followed by carbachol injection into the LS; rac+sal: raclopride injected into the medial shell of the nucleus accumbens followed by saline injection into the LS; amph+sal: amphetamine injected into the medial shell of the nucleus accumbens followed by saline injection into the LS. There were no 22-kHz USVs recorded during sal+sal, rac+sal or amph+sal injection conditions. *Anatomical abbreviations*: AC – core of the nucleus accumbens; AS – shell of the nucleus accumbens; ca- anterior commissure; cc – corpus callosum; CP – caudate-putamen; DB – diagonal band; LS – lateral septum; mfb – medial forebrain bundle; MP – medial preoptic area; MS – medial septum; oc – optic chiasm; OT – olfactory tubercle; SH – striohypothalamic nucleus; V – lateral ventricle; VP – ventral pallidum.

4.3.c: Injection of saline into the medial shell of the nucleus accumbens followed by carbachol injection into the LS induced delayed rebound emission of 50-kHz USVs

Although carbachol injection into LS invariably initiated consistent and long-lasting emission of 22 kHz USVs, we have noticed that this pharmacological response brought about a rebound effect in the form of delayed emission of 50 kHz USVs. These rebound 50 kHz USVs have been termed R-50 kHz calls (see Figure 26). The R-50-kHz calls of both F R-50-kHz USVs and FM R-50-kHz USVs were appearing around the 5th min or later post injection. Time analysis showed the R-50-kHz USVs increased in number with time and reached a maximum after 9-10 min post-injection when the carbachol effects subsided and emission of 22 kHz USVs was waning or was discontinued. Since the R-50-kHz USVs started to appear during the decay of the 22-kHz USV response, and these vocalizations were sensitive to haloperidol and raclopride (see following sections), they were not pharmacologically initiated by carbachol.

An analog rebound phenomenon was not observed after injection of amphetamine into the medial shell of the nucleus accumbens. Amphetamine induced emission of species-typical 50-kHz USVs, and this response gradually subsided. Amphetamine injection condition (amph+sal) was able to significantly increase the mean number of recorded FM 50-kHz USVs when compared to sal+sal injection conditions ($\chi^2[3] = 37.6$, $p = 0.006$, Figure 29). After the emission of 50-kHz USVs subsided, there was no rebound in the form of emission of 22-kHz USV signaling an opposite state to that induced by amphetamine (Figure not shown). Thus, the appearance of the rebound emission of USVs signaling opposite state to that initially induced by the pharmacologic agent was asymmetric. Based on vocalization analysis, the negative emotional state transitioned into a rebound positive emotional state reflected in the number of emitted 50-

kHz USVs. However, the positive emotional state did not transition into a negative emotional state indexed by the number of emitted 22-kHz USVs.

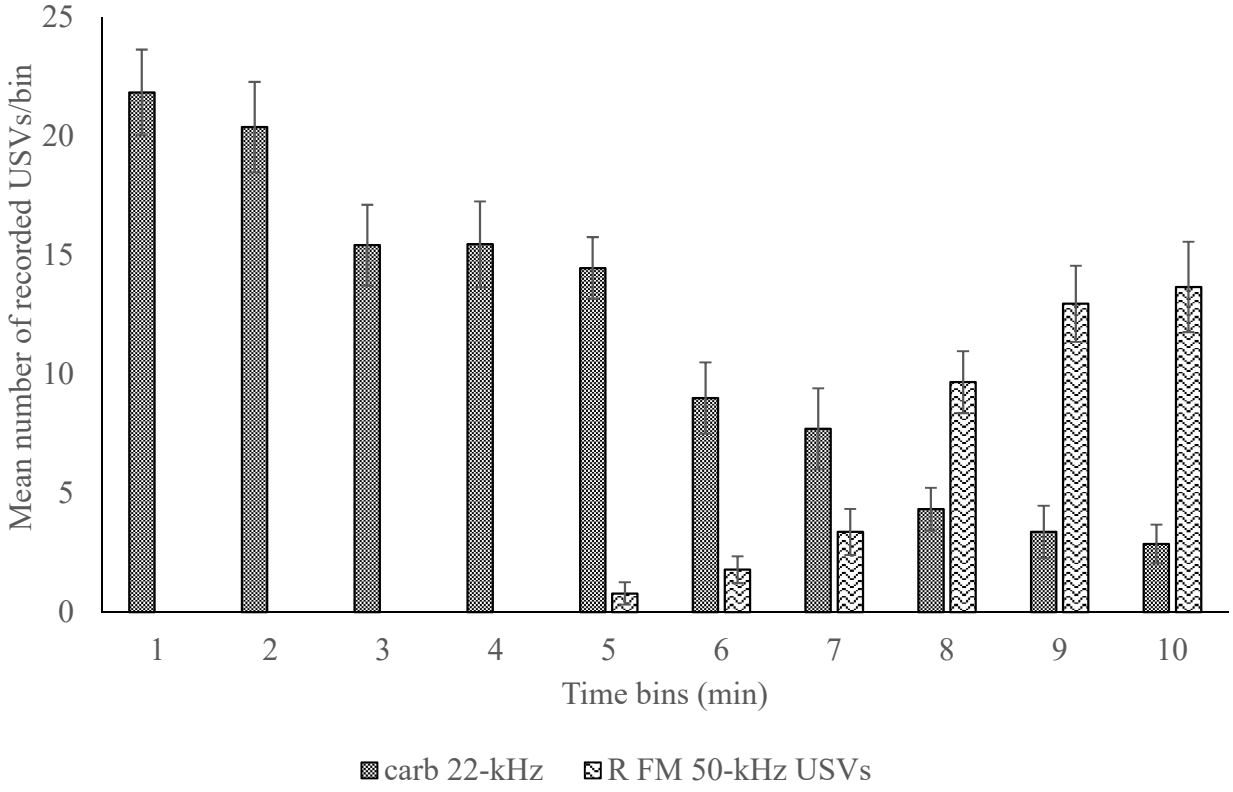

Figure 26. Time bin observations of the mean number of carbachol-induced 22-kHz (dark bars) and R FM 50-kHz USVs (lightly stippled bars) emitted during a 10-minute recording after carbachol. Each bin represents the mean number of recorded 22-kHz USVs, and R FM 50-kHz USVs pooled together from both, veh+carb and sal+carb injection conditions (n = 24, n=12 rats from figure 24 and n=12 rats from figure 25). The maximum response was recorded within the first few minutes, with a trend to a slow decrease to a minimum at the 10 min mark. This trend was opposite when analyzing the rebound FM R-50-kHz USVs. Rebound FM 50-kHz USVs were at a minimum at the 5th min time-bin but reached a peak at the end of the recording where the magnitude of carbachol-induced 22-kHz USVs was at a minimum. Since the 50-kHz USVs

were not initiated by carbachol, but rather began to emerge at the 300 s mark, we have termed these vocalizations *rebound* (R) FM 50-kHz USVs since they are likely to reflect a gradual change to the opposite emotional state of the rat and seem to be initiated by a different neurotransmitter system.

4.3.d: Number of emitted FM R-50-kHz USVs is correlated with the number of carbachol-induced 22-kHz USVs.

After carbachol control injections, i.e., both after sal+carb and the veh+carb injection conditions, when carbachol-induced emission of 22-kHz USVs was subsiding, an increased number of both F and FM R-50-kHz USVs was observed. Thus, we wanted to examine the potential role of the 50-kHz subtypes to determine any relationship between the number of rebound F 50-kHz or FM 50-kHz USVs and the initially emitted 22-kHz USVs.

First, we standardized the number of emitted F or FM R-50-kHz USVs by changing the vocalizations into z-scores. Then the number was plotted against the corresponding number of recorded 22-kHz USVs after carbachol (Figure 27). There was no observed correlation between the number of recorded 22-kHz USVs and the following number of recorded F R-50-kHz USVs ($r_F[22] = 0.11$, $p = 0.59$, dotted correlation line). However, there was a significant correlation between the number of emitted 22-kHz USVs and the number of emitted FM R-50-kHz USVs in the rebound phase of the response ($r_{FM}[22] = 0.56$, $p = 0.005$; Figure 27, dashed correlation line). The positive correlation suggested that with an increasing number of emitted 22-kHz USVs, the magnitude of the emotional rebound state increased, which was reflected in the increased number of R-FM-50-kHz USVs. The FM 50-kHz USVs truly reflected the positive emotional state.

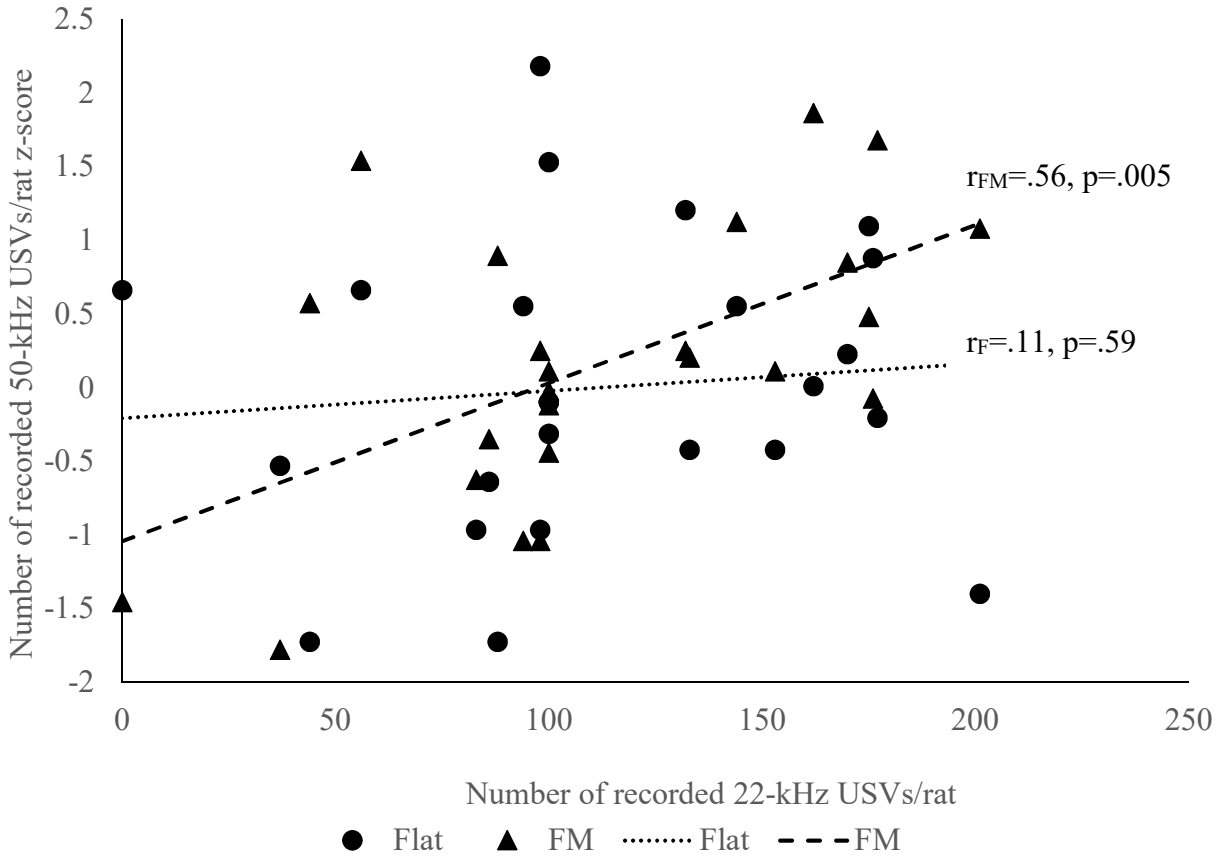

Figure 27: Number of recorded F and FM 50-kHz USVs (standardized) as a function of recorded 22-kHz USVs during veh+carb and sal+carb injection conditions (total n=48). There was a significant correlation between the number of emitted 22-kHz USVs and the number of emitted FM 50-kHz USVs (dashed correlation line, r[22] = 0.56, p = 0.005). There was no correlation between the number of emitted 22-kHz USVs and the number of emitted F 50-kHz USVs (dotted correlation line, r[22] = 0.11, p = 0.59). Note: each point represents the total number of calls emitted by an individual rat. Circles represent total vocalizations emitted/rat, and triangles represent total vocalizations emitted/rat plotted against a number of recorded 22-kHz USVs/rat.

4.3.e: Systemic pre-treatment with haloperidol eliminated the emission of R-50-kHz USVs

In order to investigate the origin of R-50-kHz USVs, we have induced carbachol response from the LS after systemic administration of the dopamine antagonist haloperidol (hal+carb condition).

Intraperitoneal injection of haloperidol followed by carbachol injection into the LS (hal+carb) significantly decreased the mean number of F R-50-kHz USVs compared to veh+carb ($p = 0.027$) and also significantly decreased the number of FM R-50-kHz USVs ($\chi^2[3] = 30.7$, $p = 0.043$, see Figure 28). To understand if these R-50-kHz USVs were a result of carbachol injections into the LS, we analyzed rebound vocalizations when carbachol was injected outside the LS. There was no significant difference in the number of recorded F R-50-kHz USVs ($\chi^2[3] = 15.25$, $p = 0.581$) or FM R-50-kHz USVs ($\chi^2[3] = 9.0$, $p > 0.95$) when carbachol was injected outside of LS compared to veh+carb and veh+sal conditions (Table 7, left part of the table).

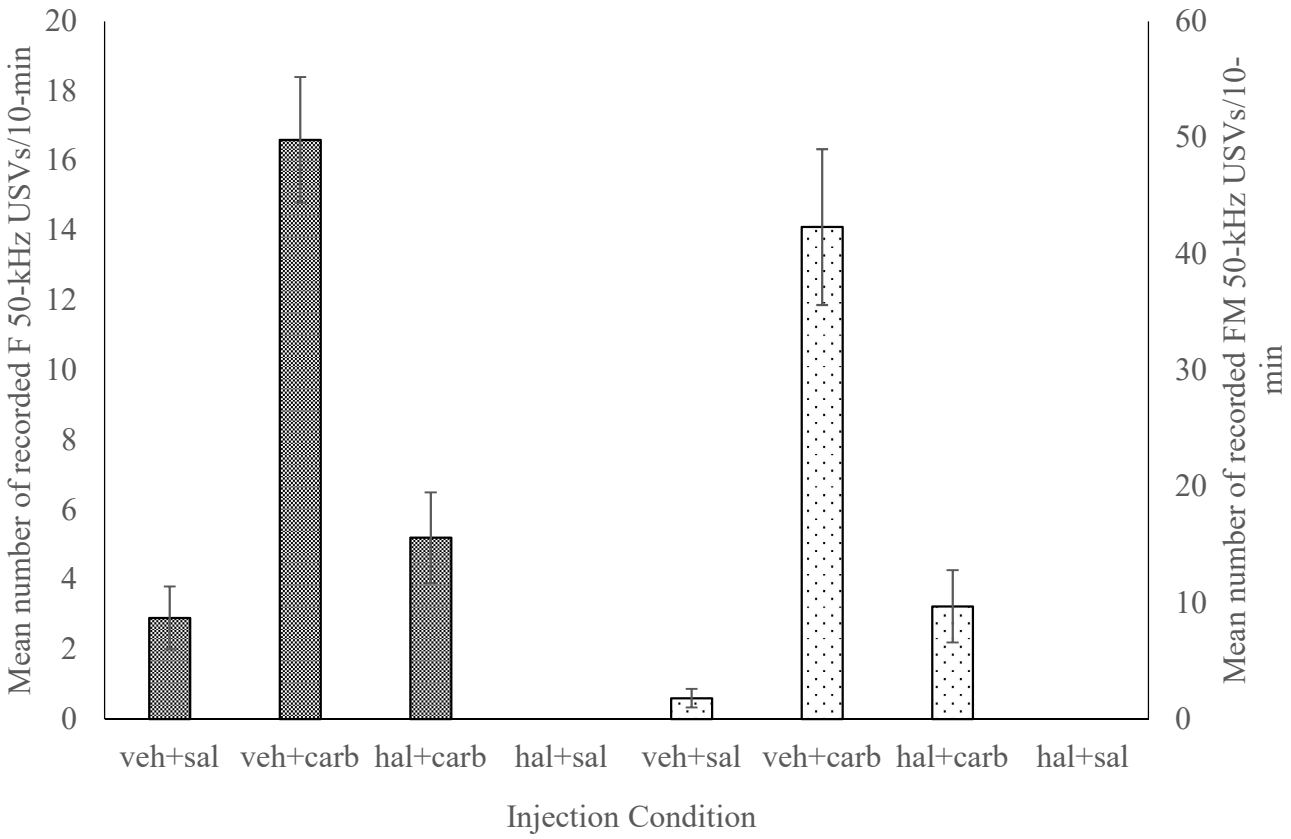

Figure 28: Mean number of recorded R-50 kHz USVs during various injection conditions. Flat USVs (F) are shown by darkly filled bars on the left-hand side. Injection of veh+carb significantly increased the mean number of F 50-kHz USVs compared to both veh+sal condition ($\chi^2[3] = 26.5$, $p < 0.009$, $n = 12$) and hal+sal condition ($p < 0.001$). Pretreatment with haloperidol significantly decreased the F 50 kHz USVs (hal+carb, ($\chi^2[3] = 26.5$, $p = 0.021$). Frequency-modulated (FM) USVs are shown by lightly stippled bars on the right-hand side. In the veh+carb condition, the mean number of recorded FM 50-kHz USVs increased compared to veh+sal ($\chi^2[3] = 30.7$, $p < 0.001$), and hal+sal ($p < 0.001$). Pretreatment with haloperidol significantly decreased the FM 50 kHz USVs (hal+carb, $\chi^2[3] = 30.7$, $p = 0.043$). For a full explanation of injection conditions, see Figures 24 and 25).

4.3.f: Intracerebral pretreatment with raclopride into the medial shell of the nucleus accumbens decreased the emission of R-50-kHz USVs.

In order to provide further evidence that the emissions of both F and FM R-50-kHz USVs were dependent on dopamine transmission in the nucleus accumbens shell, raclopride, a D_2 dopamine antagonist, was intracerebrally injected into the shell of the nucleus accumbens. Injection of raclopride into the medial shell of the nucleus accumbens followed by injection of carbachol into the LS significantly attenuated FM R-50-kHz USVs ($p = 0.016$) and F R-50-kHz USVs ($\chi^2[3] = 23.9$, $p = 0.002$, see Figure 29). To further asses if rebound FM R-50-kHz USVs were induced by carbachol from LS, the number of FM R-50-kHz USVs was recorded when carbachol was injected outside the LS. When carbachol was injected outside the LS, there was no longer a significant difference between the number of recorded FM R-50-kHz USVs between sal+carb and sal+sal conditions ($\chi^2[3] = 37.4$, $p > 0.95$) and F R-50-kHz USVs ($\chi^2[3] = 39.8$, $p > 0.95$, see Table 7).

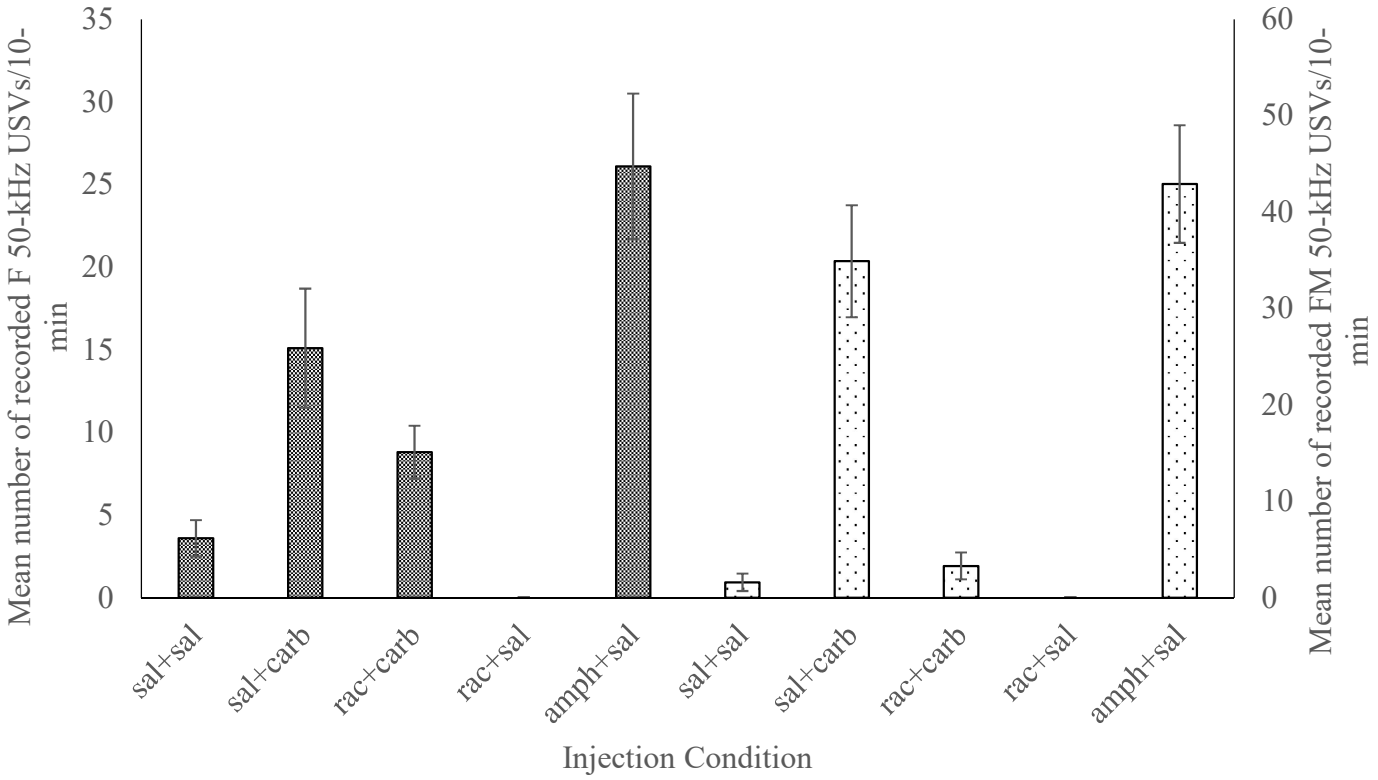

Figure 29: Mean number of recorded 50-kHz and R-50 kHz USVs during various injection conditions. Flat USVs (F) are shown by darkly filled bars on the left-hand side. Injection of sal+carb significantly increased the mean number of recorded F R-50-kHz USVs compared to sal+sal condition ($p = 0.002$). Amph+sal was also able to significantly increase the mean number of F 50-kHz USVs compared to sal+sal condition ($p < 0.001$). Pretreatment with raclopride in the nucleus accumbens has significantly decreased the number of F R-50 kHz USVs (rac+carb, $\chi 2[3] = 23.9$, $p = 0.002$). Frequency-modulated (FM) USVs are shown by lightly stippled bars on the right-hand side. Injection of amph+sal significantly increased the mean number of recorded FM 50-kHz USVs compared to sal+sal condition ($\chi^2[3] = 37.6$, $p = 0.006$), and rac+sal ($p < 0.001$). Injection of sal+carb also significantly increased the mean number of FM R-50-kHz USVs compared to and sal+sal ($p = 0.030$). Pretreatment with raclopride in the nucleus

accumbens (rac+carb) significantly decreased the FM 50 kHz USVs compared to sal+carb injection condition ($\chi^2[3] = 37.6$, $p<.016$) For a full explanation of injection conditions, see Figures 24 and 25).

4.3.g: Intracerebral injection of amphetamine, followed by intracerebral injection of saline into the LS, did not cause emission of 22-kHz USVs.

Initially, the injection of amphetamine was used as a positive control to ensure that raclopride was being injected into neural tissue that was sensitive to producing 50-kHz USVs. Since carbachol was able to induce a rebound of calls representing opposite-valence emotional state, we wanted to determine if amphetamine also caused rebound 22-kHz USVs after the decay of the initial response. Although amphetamine significantly increased both F and FM 50-kHz USVs (Figure 29), there were no recorded 22-kHz USVs during this injection condition upon analysis.

4.3.h: Sonographic features of 22-kHz and 50-kHz USVs recorded during different injection conditions

To determine if there were any significant differences in the acoustic parameters (call duration and peak frequency) of recorded F 50-kHz USVs, FM 50-kHz USVs, and 22-kHz USVs, a one-way repeated measures ANOVA was used. There was no statistical difference in the peak frequency of all recorded 22-kHz USVs ($F[1.1, 25.5] = 0.040$, $p = 0.867$, partial $\eta^2 = 0.044$) or in the call duration ($F[1.8, 43.3] = 0.412$, $p = 0.652$, partial $\eta^2 = 0.018$) between veh+carb and halo+carb conditions (Figure not shown).

There was also no significant difference in the sonographic features of all recorded F and FM R-50-kHz USVs, neither in the call duration ($F[1.9, 62.1] = 2.09$, $p = 0.133$, partial $\eta^2 = 0.061$ and $F[1.18, 27.3] = 2.02$, $p = 0.165$, partial ŋ2 = 0.300), for F and FM USVs respectively, nor in the peak frequency ($F[1.7, 40.3] = 0.563$, $p = 0.552$, partial $\eta^2 = 0.024$ and $F[1.54, 35.5] = .829$. $p = 0.417$, partial ŋ2 = 0.035), respectively (Figure not shown).

We have also analyzed potential acoustic differences across injection conditions. Recording of 22-kHz USVs induced by intracerebral injection of carbachol into the LS preceded by raclopride or saline injection into the medial shell of the nucleus accumbens showed also no significant differences in single call duration ($F[1.5, 35.4] = 1.8$, $p = 0.186$, partial $\eta^2 = 0.073$) or peak frequency ($F[1.4, 33.8] = 1.5$, $p = 0.224$, partial $\eta^2 = 0.064$) (Figure not shown).

Analysis of recorded F 50-kHz USVs did not reveal any significant differences in recorded duration [$F(2.15, 49.6) = 1.1$, $p=.348$, partial $\eta^2=.045$] or peak frequency [$F(2.2, 50.8)=1.9$, $p=.182$, partial $\eta^2=.070$) across injection conditions. There was no significant difference between the duration [$F(2.6, 61.3)=.642$. $p=.567$, partial $\eta^2=.028$] or peak frequency [$F(1.3, 31.8) = 2.0$, $p=.192$, partial $\eta^2=.080$] across injection conditions (Figure not shown).

4.4: Discussion

4.4.a: Systemic injection of haloperidol or intracerebral injection of raclopride into the shell of the nucleus accumbens does not increase carbachol-induced 22-kHz USVs from the LS

The main finding of this experiment was that blocking dopamine receptors via acute systemic injection of haloperidol, or microinjection of the dopamine antagonist raclopride into the medial shell of the nucleus accumbens failed to increase the mean number of carbachol-induced 22-kHz USVs from the LS. Our findings are consistent with previous reports showing

that acute systemic injection of haloperidol did not significantly change the number of recorded 22-kHz USVs in response to anticipation of foot shocks or in other conditioned paradigms (De Vry et al., 1993; Mead et al., 2008; Molewijk et al., 1995; Naito et al., 2003; Sánchez, 2002).

Despite reports showing no changes in the number of emitted 22-kHz USVs in response to acute treatment with haloperidol, there have been reports that show chronic haloperidol treatment can increase anxiety-like behaviours in rats (chronic haloperidol treatment is defined as once per day for at least a seven-day period) (Karl et al., 2006; Rygula et al., 2008). Karl and colleagues (2006) administered haloperidol every day for four weeks and showed high measures of anxiety as compared to controls. The authors in this study measured anxiety using the light/dark box, elevated plus maze, and the open field test. Likewise, Rygula and colleagues (2008) administered haloperidol for three weeks and examined the compounding effects of psychological stress. Consistent with Karl and colleges (2006), chronic haloperidol-treated rats exhibited decreased locomotor activity, rearing, and sniffing behaviours. Rats also displayed a decreased preference for sucrose as well as increased immobility as a result of the forced swim test (Karl et al., 2006). Unfortunately, since there were no recordings of 22-kHz USVs during chronic haloperidol-treated rats in these experiments, it would be interesting to see if chronic haloperidol treatment could change the number of emitted 22-kHz USVs in foot-shock paradigms.

The increase in anxiety after haloperidol in rats has also been reported in human subjects. After six hours of taking 5 mg of haloperidol, human subjects have reported restlessness, dysphoria, agitation, anxiety, and emotional withdrawal (Anderson et al., 1981). The emotional deficits reported by a 5 mg oral dose of haloperidol in humans are paralleled to anxiogenic

experiences reported by human subjects after intravenous injection of 5 mg/kg of haloperidol (Belmaker & Wald, 1977).

The adverse behavioural effects associated with oral or intravenous administration of haloperidol is mimicked by the dopamine depleting drug alphamethylpara tyrosine (AMPTA). Oral administration of 4.5 mg of AMPTA caused, within a six-hour period, feelings of detachment and anhedonia. After a 24-hour period, the subject began to suffer from severe anxiety, restlessness, shame, fear, and depression (de Haan et al., 2005).

The juxtaposition of the different effects of acute haloperidol on the emission of 22-kHz USVs and the increase in anxiety-like behaviours during chronic haloperidol treatment in rats may be attributed to the pharmacokinetic profile of haloperidol. Acute administration of haloperidol, along with other first-generation antipsychotics, increased the firing behaviours of dopamine neuron population within the ventral tegmental area (Grace & Bunny, 1986). However, chronic injections of haloperidol have been shown to decrease the firing pattern of dopamine neurons in the VTA to a largely inhibitory state via a depolarization block mechanism (Chiodo & Grace, 1983; Grace, 1992; Valenti & Grace, 2010; Valenti et al., 2011). The depolarization block leads to decreased levels of dopamine release along target areas of the mesolimbic dopamine system, such as the nucleus accumbens (Blaha & Lane, 1987; Lane & Blaha, 1987; Moore et al., 1998). Thus, the differences in emotional behaviour in rats attributed to acute or chronic haloperidol treatment may be reflected in the pharmacological profile of how haloperidol changes the electrophysiological behaviour of VTA dopamine neurons over time.

Previous results have shown that microinjection of the dopamine agonist apomorphine into the medial shell of the nucleus accumbens was able to decrease carbachol-induced 22-kHz

USVs from the LS. Reports have also shown that acute haloperidol is able to increase the release of dopamine within the nucleus accumbens (Osborne et al., 1994) consistent with findings that acute haloperidol treatment changes the firing pattern of dopamine cells in the VTA from irregular to phasic fast-spiking, yet we did not find any decrease in the number of recorded 22-kHz USVs. A possible mechanism that explains our finding is simply the increased release of dopamine by haloperidol into the nucleus accumbens if off-set by post-synaptic antagonism of D_2 receptors by the antipsychotic (Madras, 2013)

The altered electrophysiological properties of dopamine neurons in the VTA in response to acute haloperidol treatment may not have altered forebrain levels of acetylcholine that promote either expression of behavioural phenotypes associated with anxiety, or the emission of 22-kHz USVs. Despite the lack of evidence of acute haloperidol treatment altering midbrain and forebrain acetylcholine levels, chronic administration of haloperidol has been shown to increase acetylcholine receptor density within the nigrostriatal system (Stock & Kummer, 1981), to increase nucleus accumbens acetylcholine turn-over rate (Bluth & Langnickel, 1985), and to increase *fos* expression within the LS, a condition which can be blocked via application of the muscarinic antagonist scopolamine (Guo et al., 1992). Thus, although acute haloperidol did not alter 22-kHz USVs, chronic treatment may lead to increased sensitivity of the medial cholinoceptive vocalization strip.

4.2: Rebound R-50-kHz USVs were recorded during sal+carb injection condition

An unexpected finding in this experiment was that an injection of carbachol into the LS, which induced expected and lasting emission of 22 kHz USVs, was also able to induce delayed emission of 50-kHz ultrasonic USVs labeled R-50-kHz USVs. The parameters of R-50-kHz

USVs recorded following carbachol injection into the LS did not differ from the parameters of 50-kHz USVs recorded after amph+sal or veh+sal conditions.

We argue that the recorded R-50-kHz USVs are positively emotionally driven 50-kHz USVs that result from the release of dopamine within the nucleus accumbens shell after the response to carbachol fades. The time-bin analysis provides support for this argument since the R-50-kHz USVs observed during the sal+carb injection condition did not appear until the 300 s mark, i.e., after 300 s of uninterrupted emission of 22 kHz USVs. The time bin result combined with the antagonizing effects of dopamine antagonists on R-50-USVs clearly indicate that R-50-kHz USVs were not pharmacologically induced by carbachol but generated a subsequent physiological rebound process initiated by dopaminergic release into the nucleus accumbens.

The recorded R-50-kHz USVs displays characteristics of the opponent-process theory suggested by Solomon and Corbit (Solomon & Corbit, 1974; Solomon, 1980). The opponent-process theory suggests a pattern of emotional dynamics that is described by five different phases. The first phase is the peak of the primary emotional response. After the peak of primary emotional response, the adaptation phase initiates, which transitions into a steady-state, which eventually transitions into the peak of the emotional rebound state. Eventually, the decay of the rebound affective state terminates, and the organism returns to an emotionally neutral state.

The appearance of the R-50-kHz USVs follows the typical patterns that are outlined in the opponent-process model. Initially, carbachol-induced the negative emotional state reflected in the increased number of emitted 22-kHz USVs. However, after the peak response induced by carbachol, the rebound affective state begins to occur with the subsequent emergence of FM R-50-kHz USVs after the initial 300 s. In our recording, the peak rebound affective response

occurred at the 600 s mark where the number of FM R-50-kHz USVs was the highest. Our recording time did not allow us to observe the full reduction and of the emotional rebound and return to the emotionally neutral state.

4.5 Conclusions

The previous research has shown that the initiation of a positive emotional state by apomorphine decreased expression of a subsequent negative emotional state induced by carbachol. The current study examined further the opposite relationship between these emotional states and tested the hypothesis that initial antagonism of dopamine receptors will increase vocal expression of the negative emotional state induced by carbachol measured by emission of 22-kHz USVs.

Our findings showed that antagonism of dopaminergic signaling either via systemic haloperidol or via intracerebral application of the dopamine receptor antagonist raclopride into the shell of the nucleus accumbens did not alter the number of emitted 22-kHz USVs. Our data indicate that inhibition of the mesolimbic dopamine system does not amplify or increase the magnitude of a negative emotional state.

It was also found that prolonged emission of 22-kHz USVs induced by injection of carbachol into the LS brought about delayed rebound effects in the form of emission of R-50-kHz USVs, while prolonged emission of 50-kHz USVs induced by amphetamine did not show such rebound in the form of 22-kHz USVs.

4.6. References

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Table 6. The number of carbachol-induced 22 kHz USVs (± SEM) from LS after systemic antagonism by haloperidol (hal+carb) or intraaccumbens antagonism with raclopride (rac+carb) when carbachol was injected into the LS or outside LS.

	Systemic injection (veh or hal) followed by carb injection in LS (n = 12)		Systemic injection (veh or hal) followed by carb injection outside LS (n = 12)		Microinjection of sal or rac into the shell of the nucleus accum-bens followed by carb into LS (n = 12)		Microinjection of sal or rac into the shell of the nucleus accumbens followed by carb outside LS (n = 12)	
	veh+carb in LS	hal+carb in LS	veh+carb outside LS	hal+carb outside LS	sal+carb in LS	rac+carb in LS	sal+carb outside LS	rac+carb outside LS
22-kHz USVs	130.4 ±15.3	148.9 ±18.7	1.3 ± 1.1	1.2 ± 0.8	95.1 ± 12.4	125.0 ± 15.4	5.5 ± 1.3	1.1 ± 0.6

Note: For abbreviations of injection conditions see figure 24.

Table 7. Number of rebound R-50 kHz USVs and their subtypes (± SEM) that appeared after injection of carbachol into or outside of the LS and their antagonism with systemic haloperidol (hal+carb) or intraacumbens raclopride (rac+carb).

	Carbachol injection inside LS (n = 12)	Carbachol injection outside LS (n = 12)	Carbachol injection inside LS (n = 12)	Carbachol injection outside LS (n = 12)

Call category	veh+carb	hal+carb	veh+carb	hal+carb	sal+carb	rac+carb	sal+carb	rac+carb
F R-50-kHz USV	16.6 ± 1.8	3.3 ± 0.88	1.25 ± 0.6	0	15.1 ± 3.3	8.8 ± 1.8	3.7 ± 1.1	0
FM 50-kHz USV	42.3 ± 6.7	9.75 ± 3.1	0	0	34.9 ± 5.8	2.25 ± 1.4	2.9 ± 1.6	0

For statistical analysis see results.

Chapter 5: General Discussion

5.1: Summary of main findings

The overarching goal of this thesis was to examine the possible antagonistic interactions between the mesolimbic dopamine system and the mesolimbic cholinergic system during the initiation of emotional states. The thesis specifically focused on whether the initiation of a positive emotional state could influence the magnitude of a subsequently expressed negative emotional state. To accomplish this goal, the thesis was divided into three questions, each examining an aspect of the possible antagonistic effect of positive emotionality has on the negative emotionality.

The results obtained in the thesis supported the hypothesis of the thesis. In chapter 2 it was shown that the injection of a dopamine agonist, apomorphine, into the central region of the medial nucleus accumbens shell, decreased the magnitude of carbachol-induced 22-kHz USVs from the main division of the medial cholinoceptive vocalization strip, the AH-MPO. In chapter 3, the injection of apomorphine into the central division of the medial nucleus accumbens shell decreased the magnitude of carbachol-induced 22-kHz USVs from the LS, the most rostral division of the medial cholinoceptive vocalization strip. Since there was a significant decrease in the number of emitted 22-kHz USVs, without changes in spectrographic parameters, it is argued that initiation of a positive emotional state decreased the magnitude of a negative emotional state suggesting an antagonistic relationship between the two ascending mesolimbic systems.

5.2: The lateral habenula has bi-directional connections with both the LTDg and the VTA and may serve as an important nodal point during the regulation of emotional behaviour in rats.

The primary efferent pathway of the lateral habenula (LHb) is the longitudinal fiber bundle the fasciculus retroflexus (fr, Viswanath et al., 2014). This fiber bundle connects the LHb to both the VTA and the LTDg positioning it strategically to influence both tegmental structures that can initiate either 50-kHz USVs or 22-kHz USVs. Conversely, both structures project back to the LHb and can influence LHb function. For example, the VTA sends glutamatergic fibers that co-release GABA and dopamine to the LHb (Mizumori & Baker, 2017). Optogenetic stimulation of these neurons that express dopamine markers but release GABA into the LHb promote conditioned place preference, a measure of positive emotionality (Stamatakis et al., 2013). Whereas optogenetic activation of glutamatergic neurons in the VTA that project to the

LHb promote conditioned place aversion, a measure of negative emotional state (Root et al., 2014)

Conversely, the LHb influences the microcircuitry of the LTDg, and subsequently the expression of anxiogenic behaviours. The LTDg is a heterogeneous nucleus that is composed of glutamatergic, cholinergic, and GABAergic neurons along with the rostral, medial, and caudal divisions. Within the rostral part, the LTDg displays the highest concentration of neurons expressing vesicular glutamate transporter type 2 (VGluT2) mRNA suggesting this subdivision is primarily composed of glutamatergic neurons. The medial division of the LTDg is suggested to contain the highest density of cholinergic neurons since it contains the highest concentration of ChAT-immunoreactivity, while the caudal division has the highest density of GABAergic neurons since this region contains the highest concentration of the glutamic acid decarboxylase isoform 67 (GAD-67) (Wang & Morales, 2009). Yang and colleagues (2016) have fleshed out some of the important circuity connecting glutamatergic neurons from the LHb to GABAergic interneurons within the LTDg in relation to defensive behaviours in mice. The authors showed that optogenetic stimulation of glutamatergic terminals originating from the LHb and terminating inside the LTDg results in freezing behaviour in mice (Yang et al., 2016). Thus, the LHb can inhibit dopaminergic neurons within the VTA by exciting GABAergic neurons within the rostromedial tegmental nucleus (tail of the VTA) and promote the expression of anxiogenic behaviours by exciting GABAergic interneurons within the LTDg. However, vocal expression in mice was not recorded during the optogenetic stimulation of glutamatergic input into the LTDg.

Despite the lack of evidence involving the LHb in the regulation of initiation of 22-kHz USVs, there is indirect support for its role in the initiation of defensive-related behaviours during experimental designs. For example, Brown and Shepard (2013) showed the effects of footshock

and lesion of the fr. Foot shock in rats was able to induce *c-fos* expression in both the lateral habenula as well as the trail of the ventral tegmental area. However, upon lesioning the fr, *c-fos* expression in the tail of the ventral tegmental area is prevented. (Brown & Shepard, 2013). Lateral habenula neurons are also shown to increase *c-fos* expression during exposure to predators. Roseboom and colleagues (2007) showed that, upon exposure to ferret odor, increased Fos positive cells in the lateral habenula within 30 min that remained at a steady level for approx. 120 min. This effect was absent in the medial habenula suggesting a strict role for the lateral habenula in processing anxiety-related information (Roseboom et al., 2007). Although exposure to Ferret odor has not been demonstrated to induce 22-kHz USVs, exposure to the predator odor Fox urine has shown to increase both Fos reactivity in the lateral habenula (Vincenz et al., 2007) and has also been shown to be capable of initiating 22-kHz USVs (Fendt et al., 2018, see Figure 31).

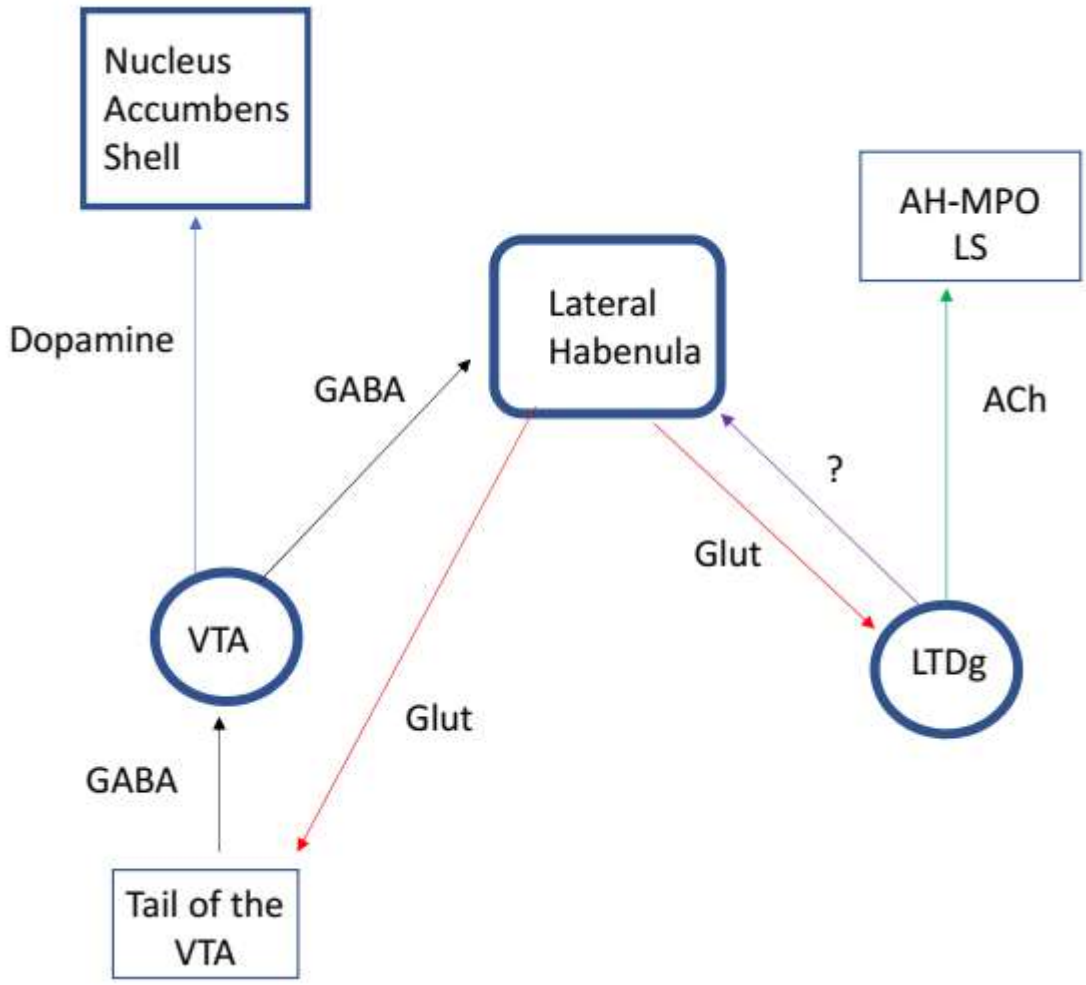

Figure 30: Proposed synaptic connection arguing for the lateral habenula as an important link between the VTA and the LTDg. During aversive conditioned experiments, that can initiate 22-kHz USVs, Fos reactive cells in the tail of the VTA as well as lateral habenula neurons are increased. Fos reactive neurons in the LHb and the tail of the VTA are also increased upon exposing to fox odor, which initiates 22-kHz USVs. Activation of VTA can depolarize a select population of neurons that can inhibit LHb neurons by releasing GABA resulting in conditioned place preference in mice. No study has looked at inhibition or activation of subnuclei within the LHb and 50 kHz or 22-kHz USVs. Data to construct the wiring diagram taken from Brudzynski,

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5.3: Amphetamine did not induce rebound 22-kHz USVs.

The purpose of the fourth chapter was to investigate whether blocking dopamine receptors in the nucleus accumbens, then injecting carbachol into the LS could increase 22-kHz USVs. The results did not support an inverse relationship between the two systems, i.e., that blocking dopamine receptors in the nucleus accumbens did not increase the number of carbachol-induced 22-kHz USVs from the LS.

Interestingly, it was found that 50-kHz USVs were being emitted after carbachol injection roughly 300-400 s after injection. I argued that, since these 50-kHz USVs did not occur *directly* after carbachol injection and were blocked by both systemic and intracerebral application of dopamine antagonists, they represented an emotional rebound state dependent upon dopamine release within the nucleus accumbens. Despite the emotional rebound after carbachol response, there was no 22-kHz USVs observed as a rebound after amphetamine or

apomorphine responses. Despite the lack of rebound 22-kHz USVs, this does not preclude initiation of a rebound negative emotional state after apomorphine or amphetamine microinjections. For example, Ettenberg and colleagues (1999) examined the opponent-process theory by examining the behaviours of rats in a conditioned place preference experimental paradigm after intravenous cocaine administration. They found that the rats that were placed back into the environment 0-min or 5-min after receiving cocaine they returned to the cocaine-paired environment, but rats that were placed back into the apparatus 15-min post cocaine infusion exhibited place aversion response (Ettenberg et al., 1999). Thus, it is possible that the rats would exhibit negative emotionality if given an alternative and more sensitive behavioural tests.

5.4: Alternative hypotheses to 50-kHz USVs that argue against positive emotionally driven vocalizations

Although there is ample evidence to support the role 50-kHz USVs in positive emotions, there are also competing hypotheses that argue 50-kHz USVs are not initiated by positive emotional states. Blumberg & Alberts (1992) makes two separate proposals for the existence of USVs in rats; he proposes that rat pup vocalizations are a by-product of laryngeal breaking used in thermoregulation and adolescent and adult vocalizations are a mechanical by-product of locomotor activity (Blumberg & Alberts, 1991; Blumberg, 1992). Examining Blumberg's hypothesis, Hofer and Shair (1993) examined the effects of removing the laryngeal nerve on thermogenesis in rat pups. The hypothesis was stated that if rats cannot produce USVs, then it should be reflected in their inability to thermoregulate properly. Their results did not fully support Blumberg's thesis. For example, laryngeal-denervated and tracheostomized pups were able to thermoregulate and increase body temperate at a rate comparable to controls. However,

their long-term ability to regulate their temperature waned after the 30-min mark, and they fell behind controls after an hour. Likewise, there is a substantial amount of experimental data that shows mother-dam retrieval is initiated by pup vocalizations suggesting an active signal is contained within the USVs. This suggests that temperature is important for the initiation of USVs in pups, but the USVs are not a by-product of laryngeal activity but rather is a response to external cues that signals separation from conspecifics and expose to dangerous conditions (Hofer et al., 2002).

The second hypothesis put forward by Blumberg was that emission of USVs is a mechanical by-product of locomotion. Analyzing tapes, Blumberg argued that the majority of USVs occur during forepaw compression and thus USVs were a direct product of locomotor activity. However, this hypothesis failed to explain the lack of correlation between locomotion and 50-kHz USVs production as well as the <10% of 50-kHz USVs that occur within 0-0.5 s after forepaw contact with the ground (Knutson et al., 2002; Panksepp & Burgdorf, 2003).

5.5: Conclusions

Our results were sufficient to warrant further investigation into the antagonistic interaction that positive emotional states have on the expression of negative emotional states. However, the thesis did not investigate the possible antagonistic effects a negative emotional state has on the expression of a positive emotional state. Further, the main method to induce either positive or negative emotional states in this thesis was by intracerebral injection of pharmacological compounds. Future research could use more precise control of dopaminergic cells in the VTA and cholinergic cells in the LTDg via optogenetic analysis. Overall, however,

our results to support the hypothesis that positive emotional states antagonize the initiation of a negative emotional state in rats (Brudzynski, 2013).

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Appendix A – Failed Experiment
Initiation of USVs via intracerebral injection of muscarinic and GABAergic agonists into the Lateral Habenula

A. Introduction

Rats can emit ultrasonic vocalizations to signal conspecifics, either positive or negative emotional states (Brudzynski, 2007; 2009; 2013). Negative emotional states can be signaled via the emissions of 22-kHz USVs. These vocalizations have a very long call duration lasting between 300-3000 ms with a low peak frequency (~18-30 kHz) and are emitted in contexts that can cause external physical harm, such as submissive posturing in resident-intruder paradigms (Burgdorf et al., 2008; Kroes et al., 2007). 22-kHz USVs can also be emitted during conditions that cause psychological destabilizations. These conditions include conditioned experiments subjecting rats to air puffs (Naito et al., 2003) or foot-shocks (Prus et al., 2015).

Contrary to the emission of aversively-driven 22-kHz USVs, rats can also emit vocalizations reflecting a positive emotional state. Vocalizations that reflect a positive emotional state are termed 50-kHz USVs. These types of vocalizations have a high peak frequency (~35-80-kHz) and short duration 10-100 ms). The frequency of the vocalizations can be modulated, increasing the peak frequency of the vocalization (Brudzynski, 2013). Rats consistently emit 50-

kHz USVs in response to contexts that promote psychological well-being, such as during rough-and-tumble play (Burgdorf et al., 2008) or expectation of rewarding electrical brain stimulation (Burgdorf & Panksepp, 2000). The non-overlapping acoustic features of 22-kHz and 50-kHz USVs, and the contexts in which they are emitted ensure proper signal fidelity to the receiver.

The anatomical substrates that subserve emission of 50-kHz and 22-kHz USVs are uniquely distinct. The initiation of 50-kHz USVs is in part dependent upon the fidelity of dopamine signaling within the nucleus accumbens shell. Microinjections of dopamine agonists into the shell of the nucleus accumbens unconditionally elicit 50-kHz USVs (Burgdorf et al., 2001; Thompson et al., 2006). The release of dopamine within the nucleus accumbens shell depends upon the activity of dopamine neurons within the VTA. The tonic firing of dopamine VTA neurons leads to an increase in steady-state extracellular dopamine leaves while phasic firing correlates with an increase in the synaptic concentration of dopamine. Under anesthesia, the pattern of activity of VTA neurons presents firing in a highly regular, slow pacemaker pattern. However, in an awake state, afferent influences and GABAergic influences from the RTMg, and local GABAergic interneurons change the behaviour of dopamine neurons that exhibit spontaneous, slow depolarizing membrane currents.

Descending GABAergic control of dopamine neurons in the VTA comes from a variety of different forebrain nuclei that influence rat behaviour. For example, direct GABAergic inputs arise from the VP (Grace, 2016; Root et al., 2015) as well as the lateral hypothalamus (Nieh et al., 2016). Feedforward inhibition of dopamine neurons within the VTA is also provided by the LHb. Glutamatergic fibers of the lateral habenula descend within the dorsal diencephalic conduction system by way of the fasciculus retroflexus to synapse onto GABAergic neurons with the RTMg. Once excited, GABAergic neurons within the RTMg inhibit dopamine neurons

within the VTA (Browne et al., 2018; Graziane et al., 2018; Hong et al., 2011; Jhou et al., 2009; Jhou et al., 2009; Yang et al., 2018).

The activity of glutamatergic projection neurons within the LHb are preferentially excited by stimuli that promote the expression of 22-kHz USVs. For example, reward omission or foot-shock predictive cues conditions, both cause an increase in 22-kHz USVs (Brudzynski, 2007) and excite LHb neurons and inhibit VTA dopaminergic neurons (Jhou et al., 2009). Conversely, a unique population of GABAergic cells within the VTA provides inhibitory input into the LHb.

Optogenetic stimulation of these unique GABA cells inhibits LHb neurons and increases the spontaneous firing rate of VTA neurons *in vivo* (Stamatakis et al., 2003). Despite the evidence involving the LHb in the processing of negative emotional states and its inhibition during positive emotional states, as well as, its a bi-directional connection with both the VTA and the LTDg, the role of the LHb in the USVs initiation has not yet been investigated. Thus, the purpose of the current experiment was to determine if LHb activation or inhibition could initiate either 50-kHz USVs or 22-kHz USVs.

B: Methods and Procedures

B.i: Subjects and Surgery

Twenty-four male adolescent Long-Evans rats (Charles River Laboratories, Saint-Constant, QC Canada) with body weights ranging from 275-300 g at the time of surgery were used in the study. Rats were housed in polycarbonate cages with dimensions of 48 cm x 27 cm x 20 cm at a room temperature-controlled housing facility (23° C ± 2° C) with constant humidity. Rats were on a 12:12 h light dark cycle with *ad libitum* access to standard food pellets and filtered tap water.

After five days of acclimation, rats underwent stereotaxic surgery for unilateral implantation of guide cannula into the LHb. Rats were anesthetized with gaseous isoflurane at a concentration of 5%, then reduced to 3% to maintain the depth of anesthesia. Rats were then placed in a Kopf stereotaxic apparatus (Model 900 David Kopf Instruments, Tujunga, CA). After the rat has been mounted, burr holes were drilled into the skull using an electric drill. Guide cannula (O.D.=650 μm) was implanted into the left LHb. The cannula was constructed from 23 G stainless steel needle (Beckton-Dickinson Canada, Mississauga, ON) and was implanted 1 mm above the intended injection site. Coordinates for the LHb injection were taken from Paxinos & Watson, 2007): A-P: 5.04-6.0 mm; L: 0.6-1.2 mm; D-V: 4.2-4.6 mm below the surface of the dura. The cannula was secured to the rat's skull using jeweler's screws and methyl methacrylate resin (Perm Resin, Hygenic Corporation of Canada Inc., St. Catharines, ON). Rats recovered for a duration of five days after the surgery before they began 72 hours of habituation, and their condition was approved by the staff veterinarian. For further details on stereotaxic surgery, see Fonari et al. (2012). All research protocols were approved by Brock University Animal Care and Use Committee and complied with guidelines and policies set forth by the Canadian Council on Animal Care.

B.ii: Pharmacological agents and intracerebral injection procedure

Muscimol (Tocris, Oakville, ON) , a potent $GABA_A$ receptor agonist, was used to inhibit the LHb neurons. Muscimol was dissolved in sterile physiological saline at a concentration of 29.2 μM, 292 μM, and 2.92 mM solution. Sterile physiological saline was used as an injection control for muscimol injections. Since the LHb receives direct input from the LTDg and contains muscarinic receptors (Vilaró et al., 1990), we also used carbachol to try and initiate 22-kHz USVs from the LHb.

Carbachol was dissolved in sterile physiological saline at a concentration of 18.2 µM, 182 µM, and 1.8 mM. Sterile saline was used as a control for carbachol injections. Injection of drugs and pharmacological controls were used using a constant rate Hamilton® CR 700 micro-syringe (Hamilton Company, Reno, NV) in a volume of 0.3 µl and at a rate of ~4.5 nl/s. The injection cannula was left in place for the 30 s to allow for proper drug diffusion.

B.iii: Recordings of ultrasonic vocalizations

Recording of ultrasonic vocalizations took place in a 25 cm x 18 cm x 18 cm Plexiglas recording chamber. The floor of the recording chamber was lined with a paper towel as corn cobb bedding can influence the emission of USVs (Natusch & Schwarting, 2010). An Avisoft ® CM16/CMPA condenser microphone with a frequency range between 2 kHz – 250 kHz (Avisoft® Bioacoustics, Berlin Germany) was used to record USVs in real time and the data stored in a 16-bit format for later spectrographic analysis using the Avisoft® SASlab program (Avisoft, Germany).

Recorded vocalizations were analyzed off-line by using sonograms. Sonograms were constructed from wave files using a 512 FFT-length with a Hamming window and a 75%-time overlap. Spectrograms were produced with a 488 kHz resolution, and calls were marked manually for their durations, peak frequency, and category of call. Call categories consisted of flat vocalizations, and frequency modulated vocalizations (FM), for a full list of categorized FM calls see Wright et al., 2010.

B.iv: Histology and Localization of injection sites

After injections were finished, rats were anesthetized with an overdose of sodium pentobarbital and received an intracerebral injection of Indian ink (1:100 dilution) for histological determination of injection sites. After injection of India-ink, rats underwent transcardial perfusion with 10% formalin and were stored in formalin for 48 hours. The brains were then coronally sectioned on a freezing microtome (Cryo-Histomat, Hacker Instruments, and Industries, Fairfield, NJ) to a thickness of ~40 μm. Sections were placed on a 1% poly-lysine coated slides then underwent Nissle staining procedure (see Lindroos and Leinonen, 1983 for details). Slides were then cover-slipped, and injection sites (with India ink granules) were histologically verified under projection microscope.

B.v: Statistical Analysis

Results are presented as means with a standard error of the mean (S.E.M). Since the total number of vocalizations was not normally distributed, differences between injection groups were assessed using non-parametric Friedman's ANOVA, followed by Wilcoxon Signed Rank test. Acoustic parameters (peak frequency and duration) were analyzed using an ANOVA. Probability values less than 0.05 were considered significant. Since multiple comparisons were performed, inflation of Type-1 error was controlled using the Bonferroni correction.

C: Results

C.i: Intracerebral injection of various doses of muscimol did not initiate 22-kHz USVs or 50-kHz USVs

The purpose of the current experiment was to investigate if the injection of the potent $GABA_A$ agonist into the LHb could initiate the emission of USVs. A dose-response curve was constructed in order to ascertain the optimal dose of muscimol for the experiment. Injection of

29.2 µM, 292 µM, 2.92 mM, or saline did not induce any 22-kHz USVs. There was also no statistical difference between the number of emitted F 50-kHz USVs ($\chi^2[3] = 0.000$, $p>.95$) or FM 50-kHz USVs ($\chi^2[3] = 8.3$, $p>.95$, see figure 32, and table 8 for values).

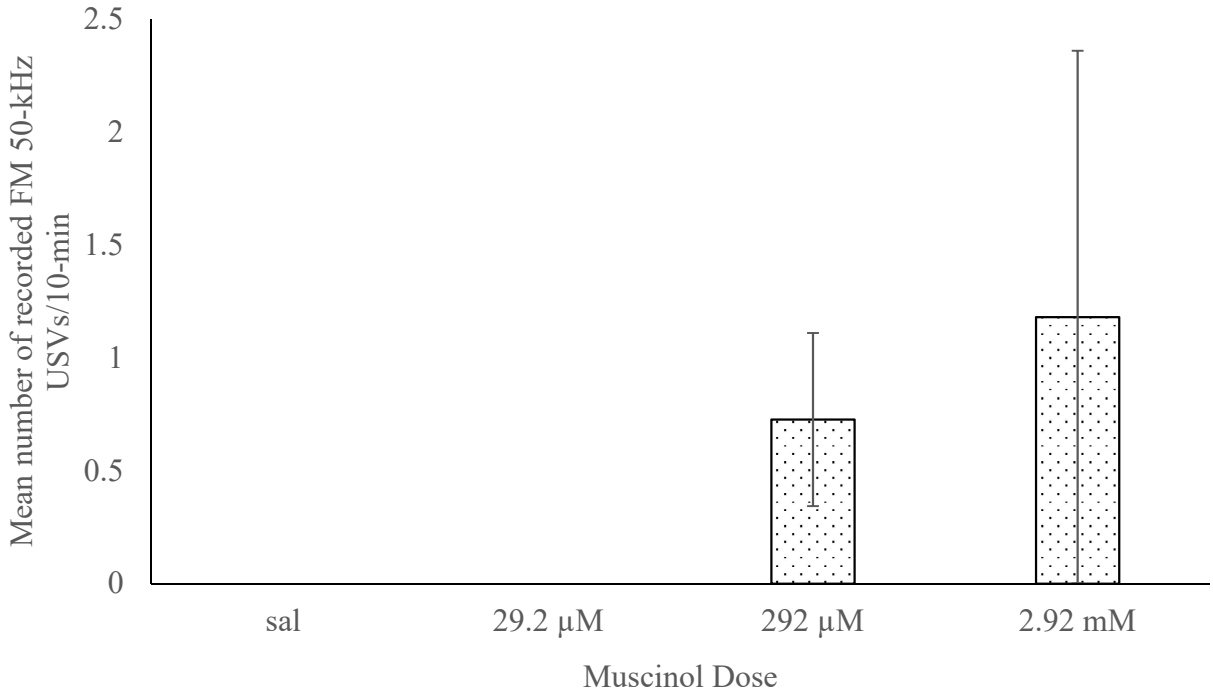

Figure 32: Mean number of recorded FM 50-kHz USVs recorded after Muscimol was injected into the LHb (n = 12). There was no statistical difference in the number of recorded FM 50-kHz USVs across doses of muscimol injected ($\chi^2[3] = 8.3$).

Injection of carbachol was used to assess the ability of broad-spectrum cholinergic stimulation of both nicotine and muscarinic receptors within the LHb. Injection of carbachol did not induce 22-kHz USVs across different doses. Injection of carbachol did not increase the number of F 50-kHz USVs or FM 50-kHz USVs across any doses.

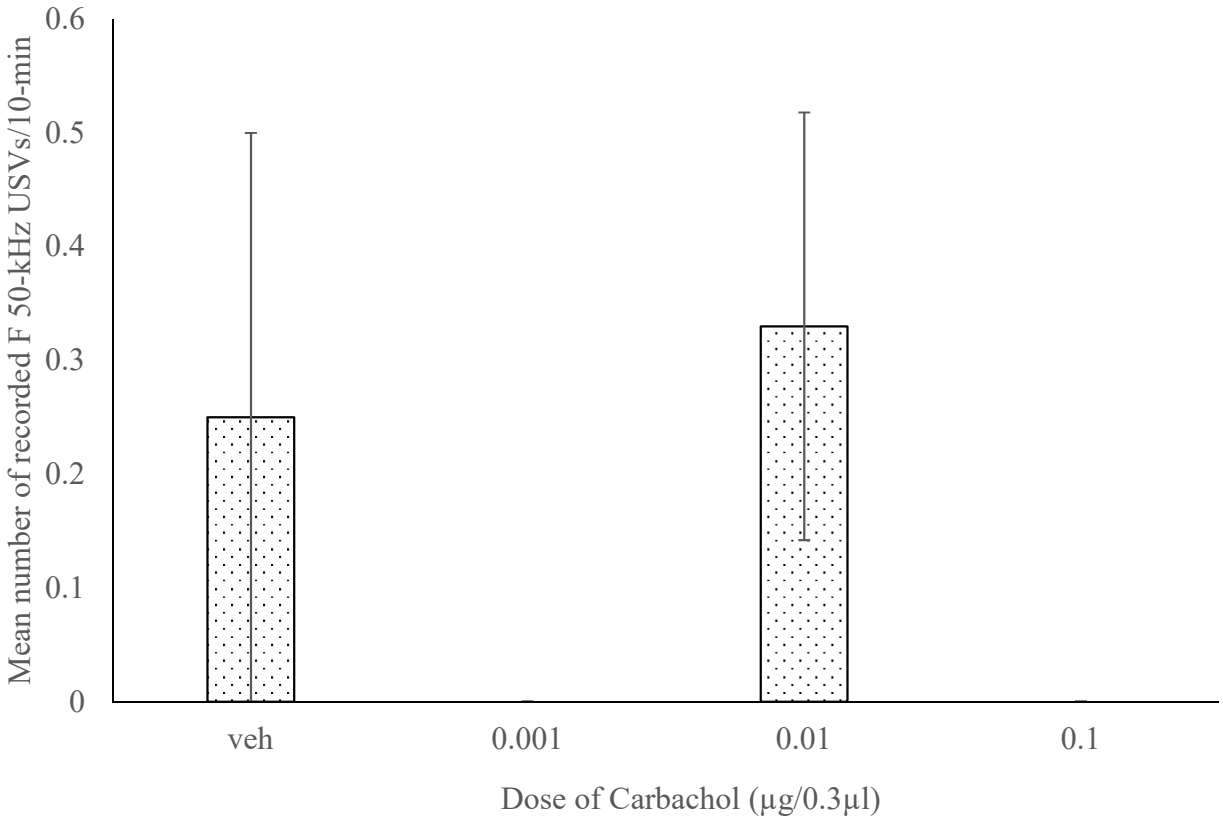

Figure 33: Mean number of recorded F 50-kHz USVs with various doses of carbachol *(n=12)*. There was no statistical difference between the number of recorded F 50-kHz across injection doses.

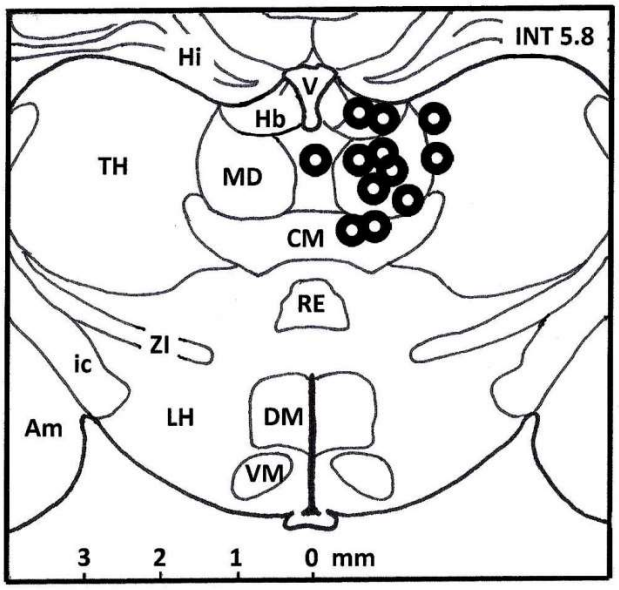

Figure 34: Localization of 12 injection sites (circles) aimed at lateral habenula (left side of Hb). Only one injection site was on target and the second one partially in the Hb. *Abbreviations*: Am – amygdalar complex; CM – centrum medianum; DM – dorsomedial hypothalamic nucleus; Hb – habenula; Hi – hippocampal formation; ic – internal capsule; LH – lateral hypothalamus; MD – dorsomedial thalamic nucleus; RE – nucleus reuniens; TH – other thalamic nuclei; V – cerebral ventricle; VM – ventromedial hypothalamic nucleus; ZI – zona incerta. The coronal stereotaxic section is 5.8 mm from the interaural plane (INT 5.8). Scale in mm.

C.ii: Sonographic features of recorded USVs during carbachol injection

Since no 22-kHz USVs were recorded during the injection conditions, no sonographic analysis of 22-kHz USVs could be accomplished.

C.iii: Sonographic features of recorded USVs during muscimol or carbachol injection conditions.

Since no 22-kHz USVs were recorded, and minimal 50 kHz USVs were recorded during the muscimol and carbachol injection conditions, sonographic analysis was not performed.

D: Discussion

The purpose of the experiment was to determine if intracerebral injection of the cholinergic agonist carbachol, or $GABA_A$ receptor agonist muscimol, could initiate the emission of USVs in rats. The HLb is a very small structure that is smaller than the diameter of the injecting cannula. Histological analysis has revealed that it was a too difficult task to implant properly cannula and inject into the desired nucleus. Such an experiment would require thinner cannulae and a large number of animals, which was not feasible in this thesis. Due to the lack of experimental results, one cannot conclude or exclude the role acetylcholine, or GABA signaling within the LHb plays in the initiation of USVs in rats. Furthermore, without an indication of USVs signaling or proper localizations, we cannot accurately validate the hypothesis. This failed experiment can serve as a preliminary approach that is pointing in the direction of further studies.

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Table 8: Mean (± S.E.M) number of emitted 22-kHz, Flat (F) 50-kHz and Frequency Modulated (FM) 50-kHz USVs recorded during injection conditions.

	Intracerebral injection of Muscimol into the LHb				**Intracerebral Injection of Carbachol into the LHb**			
Category (kHz)	Saline	29.2 µM	292 µM	2.92 mM	Saline	18.2 µM	182 µM	1.8 mM
22 kHz	0	0	0	0	0	0	0	0
50 F	0	0	0	0	.25 ± .25	0	.33 ± .19	0
50 FM	0	0	0.73 ± .38	1.18 ± 1.18	0	0	0	0