The Effects of Quinpirole in Eliciting 50 kHz Calls from the Rat Nucleus Accumbens

by

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Abstract

Fifty kHz rat vocalizations are theorized to reflect a positive affective state, and index the reward value of stimuli (Knutson, Burgdorf & Panksepp, 2002; Panksepp & Burgdorf, 2003; Brudzynski, 2005). Previous studies have identified the neurochemical substrate of this behaviour to be dependent on dopaminergic activity at the nucleus accumbens shell (Burgdorf, Knutson, Panksepp & Ikemoto, 2001; Thompson, Leonard & Brudzynski, 2006). The utilization of d-amphetamine (a non-selective dopamine agonist) in these studies does not address the specific dopamine receptor types involved. The present study aims to identify the role of the D2-like family of receptors in the nucleus accumbens shell in the production of 50 kHz vocalizations in adult rats. Single injections of quinpirole in a saline vehicle were administered to the nucleus accumbens shell of 57 rats, and the number of 50 kHz vocalizations were recorded. An inverted U-shaped relationship was found between quinpirole dose (0.5 μg, 3 μg, 6 μg, 10 μg and 20 μg, all in 0.2μl saline) and the mean number of 50 kHz calls produced. Quinpirole successfully elicited significantly more 50 kHz calls than did a saline control at the 6 μg dose, as did 7 μg/0.2 μl of d-amphetamine injections into the same brain site.

To test whether a selective D2 antagonist could reverse elicited 50 kHz calling, double injections were given that used either saline or raclopride as a pretreatment before quinpirole injections. Saline followed by 6 μg/0.2 μl of quinpirole elicited significantly more 50 kHz vocalizations than did a double injection of saline, while pretreatment with an equimolar dose of raclopride reduced elicited calls to control levels. Raclopride was also used as a pretreatment of 7 μg/0.2 μl d-amphetamine, which elicited significantly fewer 50 kHz vocalizations than saline followed by amphetamine, replicating the finding of Thompson, Leonard & Brudzynski (2006).
Subcutaneous injections of 0.5 mg/kg and 1.5 mg/kg of quinpirole produced a similar number of 50 kHz vocalizations as subcutaneous injection of saline. Wider dose ranges may be explored in future research.

Thus, direct activation of the D₂-like receptors in the nucleus accumbens shell was sufficient to elicit 50 kHz vocalizations in adult rats, an effect which was reversed with selective local antagonism of D₂-like receptors. The D₂-like receptor family also appears necessary for pharmacological activation of 50 kHz calling, as d-amphetamine was no longer able to effectively elicit these vocalizations from the nucleus accumbens shell when the D₂-receptor family was antagonized with raclopride.

The acoustic parameters of elicited vocalizations remained typical of rat 50 kHz calls. Detailed analyses of the acoustic characteristics of elicited calls indicated significant increases in call duration and peak frequency across drug injection groups, particularly among quinpirole dose groups. The implications of these findings are not yet clear, but may represent an important direction for future research into the coding of semiotic content into affective signals in rats.
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Abbreviations

6-OHDA – 6-Hydroxydopamine
ADHD – Attention Deficit-Hyperactivity Disorder
AHPOA – Anterior Hypothalamic Preoptic Area
cAMP – Cyclic adenosine monophosphate
DAT – Dopamine transporter
USV – Ultrasonic vocalization
LDT – Laterodorsal Tegmental Nuclei
NMDA – N-methyl-D-aspartic acid
PAG – Periaqueductal gray
PKA – Protein kinase A
VMAT – Vesicular monoamine transporter
VTA – Ventral Tegmental Area
i.c. – intracannulae
s.c. – subcutaneous
i.p. – intraperitoneal
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*Animal Communication*

The fundamental need for animals to communicate to improve the probability of their survival and reproductive success is well established. Each organism is faced with a variety of challenges to Darwinian fitness which must be overcome if that individual’s genes are to be preserved in successive generations. For animals, particularly social species, transferring relevant information about potential threats, benefits, and social cues between individuals can greatly increase the organism’s ability to avoid danger, and capitalize on available opportunities.

Although the means of communication can be quite diverse, possibly spanning every available sensory system, one of the most ubiquitous forms of intraspecific communication among anurans, avians, and mammals is sound production (Bradbury & Vehrencamp, 1998). Chemical signaling is the phylogenetically oldest method of communication; it is the primary form of communication in single celled organisms, and is conserved in multicellular organisms for intracellular communication (Bradbury & Vehrencamp, 1998). Chemical signals differ from sound or light based communication primarily in the method of transport. Sound or light signals propagate in relatively straight directions away from the sender, while chemical signals must propagate through flow currents in air or water, through passive diffusion, or via direct sender-receiver contact (Bradbury & Vehrencamp, 1998). This method is not only restrictive in the ability to efficiently transmit signals between organisms, but is also slower; the lack of a rapid signal onset and termination also limits the usefulness of temporal pattern encoding in chemical signals (Bradbury & Vehrencamp, 1998). Although chemical
communication is still a valuable sense in many animals, including olfaction and taste, the difficulties in using chemical communication for signaling over a short time span and over longer distances in a predictable direction makes this sensory system less effective in the transmission of certain types of information.

Audible communication is not dependent on passing specific molecules directly to a receiver, or on allowing them to diffuse in a medium and waiting for the molecule to be received. Sound signals also do not require the organism to maintain a line of sight with the communicator as required for visual communication, or direct or indirect contact as required for tactile communication. Although electromagnetic fields have similar properties, electroreception can only be used in water, and is thus generally not practical for terrestrial organisms (Bradbury & Vehrencamp, 1998). Each of these types of signaling is most efficient in some circumstances, and thus in many animals, multiple signal production and reception systems can be found. However, in complex, highly social organisms the communication of complex information is often best accomplished via bioacoustic signaling.

In mammals, a combination of respiratory, laryngeal, and supra-laryngeal control can create a wide variety of acoustic signals (Jürgens & Ploog, 1981). These vocal signals can be within the range of human hearing (approximately 20 Hz to 20 kHz), below 20 Hz, known as the infrasound range, or above 20 kHz, which are known as ultrasonic vocalizations (Garstang, 2004; Sales & Pye, 1974).

**Ultrasonic Communication**

George W. Pierce originally detected ultrasonic signals in the 1930s in bush crickets (Sales & Pye, 1974). Ultrasonic emissions (23-28 kHz) were later detected by
Anderson (1954) in socially isolated rats. Anderson thought these ultrasonic emissions to be a method of echolocation, although the possibility of ultrasonic communication was not dismissed (Sales & Pye, 1974). Experiments by Rosenzweig, Riley and Krech (1955) and Riley and Rosenzweig (1957) later determined that rats likely did not use ultrasonic vocalizations for echolocation, leaving the likely purpose of these sounds as some enigmatic form of communication. The adaptive benefits of communication in the ultrasonic range in not yet entirely clear. Some researchers claim that these sounds are less detectable by predatory species, thus allowing communication between prey animals that is less susceptible to eavesdropping (Wilson & Hare, 2004). While some predators, such as owls, may not be able to detect ultrasonic calls, many predatory species do detect sounds at high frequencies – cats, for instance, can hear well into the 70 kHz range (Barnett, 1963). A competing hypothesis posits that the high frequency nature of these calls limit the range of the signal, such that high frequency sounds are more easily disrupted by environmental interference, particularly in the habitats of rat colonies such as underground burrows or tall grasses (Sewell, 1970; Bradbury & Vehrencamp, 1998).

Rats are a social species, and thus tend to congregate into colonies that maintain a close average proximity (Barnett, 1963). Ultrasonic signals may be audible to nearby rats, but often do not transmit far enough to attract predators and allow them to localize the signaling rat.

The ultrasonic vocalizations (USVs) of rats have thus far been classified into three main types: the infant isolation call, the alarm call, and the social call. The adult call types are physically distinct from each other and are separated by large gaps in physical sound parameters between them, such that these types do not lie on a continuous scale
All three calls are theorized to convey "conceptually distinct messages" and have been demonstrated experimentally to elicit different responses from the recipient, and operate on different anatomical and pharmacological substrates, which shall be discussed in the following sections.

**Infant Rat Vocalizations**

Infant-isolation vocalizations are produced upon the rats separation from its caregiving dam. This condition is simulated experimentally primarily by the physical removal of the pup from its litter and by the administration of ambient cold stimuli that presumably simulate isolation (Okon, 1972; Allin, 1971). The number of these vocalizations peaks six to eight days after birth, gradually declining between days eleven to fifteen, with almost no calls observed after day twenty (Naito & Tonoue, 1987; Kehoe, Callahan, Daigle, Mallinson & Brudzynski, 2001). The physical characteristics of the calls change gradually over this time, likely mediated by physical changes to the pups size, larynx size and muscular power, and possibly due to an overall behavioural development as well (Brudzynski, Kehoe & Callahan, 1999). This type of ultrasonic vocalization is highly variable across all physical characteristics, but can be categorized into several morphological sonographic types, including single and repeated frequency sweeps (Brudzynski, Kehoe & Callahan, 1999). Calls may vary from 20 to 160 ms in duration, 10 to 120 kHz in frequency range, and a bandwidth between 2 to 180 kHz (Brudzynski, Kehoe & Callahan, 1999). It appears likely that multiple physical characteristics of this vocalization type may contribute to the signal content, however the number of frequency sweeps appears to be a particularly meaningful attribute (Brudzynski, 2005). Zippelius & Schleidt (1956) have observed that detection of pup
isolation calls elicits searching and retrieval behaviours in dams, and these calls can act as directional cues to facilitate that search (Brunelli, Shair & Hofer, 1994). These infant-isolation calls match the characteristics needed for a signal to subserve the purpose of aiding detection and localization, including an abrupt beginning and ending, frequency sweeps, and multiple calls in succession (Brudzynski, Kehoe & Callahan, 1999; Marler, 1959; Marler & Hamilton, 1966).

Pharmacological study indicates that these infant-isolation calls can be triggered by noradrenaline agonists independent of environmental cues (Hard, Engel & Lindh, 1988), such as with clonidine, an adrenergic agonist with an anti-hypertensive effect (Blumberg et al, 2000). Infant-isolation vocalizations have been reduced when cholinergic agonists were administered systemically, possibly indicative of an immature acetylcholine system (Kehoe, Callahan, Daigle, Mallinson & Brudzynski, 2001). Infant calls are inhibited by opiates (Carden & Hofer, 1991; Kehoe, Callahan, Daigle, Mallinson & Brudzynski, 1988), benzodiazapines (Gardner & Budham, 1987; Hard & Engel, 1991) and glutamatergic NMDA receptor antagonists (Winslow, Insel, Trullas & Sholnick, 1990) suggesting a connection with analogues of fear and anxiety.

Anatomically, the structures involved in the production of these calls are complex, including such structures as the thalamus, hypothalamus, amygdala, nucleus accumbens, preoptic area, septum, and the anterior cingulate cortex projecting to the periaqueductal grey (PAG; Hofer, 1996). Middlemis-Brown, Johnson and Blumberg (2005) have demonstrated that cortically aspirated and decerebrated pups were capable of responding to cold stimulation with isolation-calls, indicating that this type of vocalization is highly dependent on brainstem activity, although higher functions are
likely involved in regulating the behaviour, particularly calls made in response to social cues.

22 kHz Rat Vocalizations

In adulthood, the ultrasonic vocalizations of rats take on other forms. One adult type is the alarm call, also known as the 22 kHz call, because the vocalization is characterized by a very low bandwidth around a typical peak frequency of approximately 22 kHz. These calls also exhibit a long duration, typically between 300 to 2000 ms (Brudzynski, 2001). These types of vocalizations can be elicited via a sudden and unexpected puff of compressed air that is accompanied by a loud hiss or by exposure to aversive stimuli, including predatory stimuli (Brudzynski & Holland, 2005; Borta, Wohr & Schwarting, 2006; Blanchard, Blanchard, Agullana & Weiss, 1991). Alarm calls are produced much more frequently by pair-reared rats than individually-reared rats, and are elicited more frequently by less socially dominant rats, indicating that these calls are sensitive to complex social contexts (Inagaki, Kuwahara, Tkusui & Tsubone, 2005). Call perception is associated with a defensive freezing behaviour or with fleeing into a protective burrow, as well as with strain-dependent changes in locomotor activity (Blanchard, Blanchard, Agullana & Weiss, 1991; Brudzynski & Chiu, 1995; Commissaris, Palmer, Neophyyou, Graham, Beckett & Marsden, 2000).

Twenty-two kHz calls have been functionally differentiated from audible sounds that are produced by rats in similar antagonistic situations: Twenty-two kHz ultrasonic calls appear to be used as alarm calls that warn conspecifics of danger, whereas sonic vocalizations referred to as “defensive threat cries” are used as threat vocalizations that
warn predators of a potential defensive attack by the vocalizing rat (Litvin, Blanchard & Blanchard, 2007).

Experiments by Endres, Widmann and Fendt (2007) have shown that rats are preferentially able to acquire a learned defensive response to 22 kHz calls relative to comparable alternative sounds, and that these learned responses are very resistant to extinction; this indicates that rats are predisposed to “comprehend” 22 kHz calls as predictive of danger. These data indicate that this vocalization is associated with an anxiety response and signals to colony mates as an alarm call that danger is present. The peak frequency, bandwidth, and morphology exhibit very little variability in which to encode the semiotic value of the signal thus leaving duration a potentially important variable, both in individual calls and in calling bouts, that may provide an indication of affective intensity (Brudzynski, 2001).

Alarm calls can also be induced pharmacologically. Twenty-two kHz vocalizations can be elicited by the direct central injection of a predominantly muscarinic acetylcholine agonist, carbachol, into the mediobasal forebrain (Brudzynski & Bihari, 1990; Brudzynski, 1994; Brudzynski & Barnabi, 1996). Elicited 22 kHz vocalizations were acoustically indistinguishable from naturally produced calls, except that call duration and intensity increased and bandwidth decreased with higher doses of carbachol (Brudzynski, 1994).

Further study has provided an anatomical localization of this pharmacological effect, resulting in what has been termed as the ‘medial cholinceptive vocalization strip’ (Brudzynski, 1994; Dencev, Hrycyshyn & Brudzynski, 1996; Brudzynski, 2007). Cholinergic agonists injected into the medial cholinceptive vocalization strip, that
includes the anterior hypothalamic preoptic area, ventral pallidum, and the lateral septal nucleus, can elicit 22 kHz vocalizations regardless of motivational state of the animal (Brudzynski, 2001; Brudzynski, 1994). Calling elicited by these injections can be reversed with pretreatment with muscarinic cholinergic antagonists such as atropine or scopolamine (Brudzynski & Bihari, 1990; Brudzynski, 1994; Brudzynski & Barnabi, 1996).

This cholinceptive region is innervated by ascending cholinergic pathways that originate at the laterodorsal tegmental nucleus (LDT; Brudzynski, 2001). Injections of L-glutamate directly into the LDT of rats were capable of eliciting 22 kHz calling (Brudzynski & Barnabi, 1996). Glutamate injected into the medial cholinceptive vocalization strip did not initiate 22 kHz vocalizations, indicating that a different neurochemical system than glutamatergic is involved in initiation of vocalization from this region (Fu & Brudzynski, 1994). Cholinergic antagonists injected to the preoptic or anteromedial hypothalamic areas significantly antagonized calling elicited by glutamatergic activation of the LDT. However, the effect was less than complete reversal since the antagonists were only able to diffuse to a small section of the larger medial cholinceptive vocalization strip.

Taken together, these data indicate that the activation of LDT neurons with glutamate promotes acetylcholine release in the medial cholinceptive vocalization strip and functions as the triggering mechanism for activation of 22 kHz vocalizations.

50 kHz Rat Vocalizations

Another form of adult rat ultrasonic vocalization is the 50 kHz call. This call is characterized by a short duration (usually about 30-50 ms), a higher peak frequency range
(35-72 kHz), and a narrow bandwidth (5-7 kHz; Brudzynski, 2005; Portfors, 2007). These calls can exhibit a number of different morphologies of varying complexity. The most readily identifiable characteristic that may encode the intensity of communication is the number of vocalizations emitted over time. The number of calls emitted over time has been shown to vary with the salience of behavioural stimuli and by successive dose ranges of drugs that elicit 50 kHz calls (Brudzynski & Pniak, 2002; Thompson, Leonard & Brudzynski, 2006; Burgdorf, Knutson & Panksepp, 2000).

Most recent hypotheses hold that 50 kHz vocalizations reflect a positive affective state and that they are associated with approach behaviour (Knutson, Burgdorf & Panksepp, 1999; Knutson, Burgdorf & Panksepp, 2002). These calls are often elicited by stimuli that are rewarding to the rat such as anticipated access to preferred food (Burgdorf, Knutson & Panksepp, 2000), and under social stimuli such as contact with a conspecific or stimuli suggesting the presence of conspecifics (Brudzynski & Pniak, 2002), during play fighting (Knutson, Burgdorf & Panksepp, 1998), before and during mating in both sexes (Bialy, Rydz & Kaczmarek, 2000; McGinnes & Vakulenko, 2003), contact with an anesthetized conspecific (Blanchard, Yudko, Blanchard & Taukulis, 1993), and even while being “tickled” by an experimenter (also known as “heterospecific play;” Burgdorf & Panksepp, 2001; Panksepp & Burgdorf, 2000). Anticipation of rewarding drugs or electrical stimulation of the brain in the ventral tegmental area (VTA) or lateral hypothalamus (areas associated with approach and self-stimulation reward) can also elicit 50 kHz calls (Burgdorf, Knutson & Panksepp, 2000).

Early studies of this behaviour elicited 50 kHz calls from glutamatergic activation of the anterior hypothalamic-preoptic area (AHPOA), which was reversed by MK-801,
an N-methyl D-aspartate (NMDA) antagonist (Fu & Brudzynski, 1994). Brudzynski and Pniak (2002) later demonstrated that MK-801 injections to the AHPOA significantly reduced the number of 50 kHz vocalizations in social situations that evoked these calls in controls.

Knutson, Burgdorf and Panksepp (1999) found that high frequency ultrasonic vocalizations could be used in rats as an indication of preference for amphetamine, a dopaminergic agonist with rewarding properties. In 2001, Wintink and Brudzynski found that systemic amphetamine (2 mg/kg) elicited 50 kHz calling in rats and that administration of glutamate to the AHPOA significantly increased the number of calls produced (Wintink & Brudzynski, 2001). This effect was completely reversed, however, by a systemic pretreatment with haloperidol, a non-selective dopamine antagonist (Wintink & Brudzynski, 2001). This illustrates the dependence of AHPOA glutamate elicited 50 kHz calling behaviour on a dopaminergic substrate.

Dopamine

Dopamine is a catecholamine neurotransmitter, involved primarily in movement, arousal, cognition, reward, motivation, and affect (Berridge, 2007; Lee, Pei, Moszczynska, Vukusic, Fletcher & Liu, 2007). Dopamine is produced by cell bodies localized to three areas of the brain: the arcuate nucleus, the substantia nigra, and the ventral tegmental area (VTA; Elsinga, Hatano & Ishiwata, 2006; Mercuri, Saiardi, Bonci, Picetti, Calabresi, Bernardi & Borrelli, 1997). The arcuate nucleus of the hypothalamus has dopaminergic axons projecting to the infundibular region (the tuberoinfundibular pathway) and is involved in prolactin regulation (Kageyama, Takenoya, Hori, Yoshida & Shioda, 2007). The substantia nigra projects to the striatum (the nigrostriatal pathway)
and is involved in the control of movement by the basal ganglia (Mercuri et al., 1997).

The VTA projects to the limbic system (the mesolimbic pathway), particularly the nucleus accumbens, which is involved in reward and motivated behaviour. This pathway also connects to the cortex (the mesocortical pathway) via a partially separate population of dopamine neurons in the VTA, particularly the frontal cortex (Seamans & Yang, 2004). The mesocortical pathway regulates higher motor function execution, motivation, and cognition (Seamans & Yang, 2004).

Knutson, Burgdorf and Panksepp (1997) found that 50 kHz vocalizations could be used as an index of systemic amphetamine preference in rats. The association of 50 kHz calling rates and reward implies that the reward circuits of the midbrain are critical to 50 kHz call function. Behavioural studies found that 50 kHz calls are produced in other naturally rewarding situations (mating, food reward, play, and social contact), which implies that the nucleus accumbens and the larger pathways of reward processing as involved in 50 kHz vocalization processing (e.g., Burgdorf, Knutson & Panksepp, 2000; Brudzynski & Pniak, 2002). These data suggest a key role for nucleus accumbens dopamine in 50 kHz calling behaviour in rats.

The Nucleus Accumbens

The nucleus accumbens is a primary target of the VTA projections in the mesolimbic dopamine pathway. The nucleus accumbens is a limbic structure that is involved in affect, motivation, and reward, and is often characterized as the limbic/motor interface (Mogenson, Jones & Yim, 1980). Drugs of abuse, as well as natural reward conditions such as sucrose intake, reliably elicit dopamine release into the nucleus accumbens, indicating a key role for this site in reward processing (Rada, Avena &
Hoebel, 2005). Thus, this structure is potentially an ideal site for the initiation of 50 kHz vocalizations, which are hypothesized to be associated with a positive affective state (Brudzynski, 2007). VTA activation of dopaminergic cells in the nucleus accumbens, modulated by limbic and cortical inputs, could activate 50 kHz vocalization behaviour. This may be accomplished though efferents to the ventral pallidum and descending connections to brainstem structures that initiate the physical production of vocalization (the PAG and widespread areas within the reticular formation; Dujardin & Jürgens, 2005; Jürgens & Hage, 2007).

Burgdorf, Knutson, Panksepp and Ikemoto (2001) showed that nucleus accumbens dopamine may be critical to the production of 50 kHz vocalizations by successfully eliciting 50 kHz calls with microinjections of amphetamine directly into the nucleus accumbens. The nucleus accumbens, however, is not a homogeneous structure, but rather, consists of two main components that are anatomically and functionally dissociable: the nucleus accumbens shell and core (Sellings & Clarke, 2003; Pecina, Smith & Berridge, 2006). The nucleus accumbens core is associated with motor activity modulation and motivated behaviour, whereas the nucleus accumbens shell is associated with affective modulation and hedonic reward (Cardinal, Parkinson, Hall & Everitt, 2003). A quantitative mapping study by Thompson, Leonard and Brudzynski (2006) identified the nucleus accumbens shell as an important structure for eliciting 50 kHz calling behaviour. In summary, the nucleus accumbens shell has been identified as a key component for the 50 kHz vocalization behaviour in laboratory rats, which is dependent on dopaminergic activation and which may be modulated by various other brain structures and transmitter systems.
Dopamine Receptor Types

Studies in the 1950s and 1960s on the substance R-apomorphine and its interactions with other dopaminergic drugs such as chlorpromazine led researchers in the late 1970s to hypothesize two types of dopamine receptors (Kebabian, Tarazi, Kula & Baldessarini, 1997). Further molecular and genetic studies led to the identification of five dopamine receptor types that have been classified into two receptor families. The D₁-like receptor family includes the D₁ and D₅ receptors, while the D₂-like family includes the D₂, D₃ and D₄ receptors. Current research continues to examine the anatomical and functional characteristics of these receptors, and further differentiate these types into specific isoforms, such as the D₂-short and D₂-long isoforms which show differences in anatomical localization and may have functional differences as well (Kebabian, Tarazi, Kula & Baldessarini, 1997; Lee, Pei, Moszczynska, Vukusic, Fletcher & Liu, 2007; Giordano III, Satpute, Striessnig, Kosofsky & Rajadhyaksha, 2006).

Elsinga, Hatano and Ishiwata (2006) have hypothesized from reviews of current dopamine literature that the “predominant subtypes” (D₁ and D₂ receptors) are primarily involved in the excitation of a behavioural response, while the other, less predominant subtypes (D₃, D₄ and D₅) play mostly inhibitory roles. In some instances, the D₁-like and D₂-like receptor families have demonstrated an antagonistic effect on a specific behaviour (locomotor activity from the ventral pallidum), while in others they have shown complimentary effects (locomotor activity from the nucleus accumbens; Gong, Neill, Lynn & Justice, 1999). In order to identify the effects of a particular receptor type on a specific behaviour at the level of the neural structure of interest, direct microinjections of specific pharmacological agents are used to selectively activate one or
more receptor types. To claim that a substance has a pharmacological effect on a specific biological outcome, the effect of that substance must be significantly greater than that of a vehicle control, the behaviour must be proportional to the dose of the substance, and the effect must be reversible by selective antagonism of that neurochemical system. This method has been used in previous research on the D1-like and D2-like dopamine receptors of the nucleus accumbens in rats, such as in studies of rat locomotor behaviour.

Rat Locomotor Behaviour

It has been well established that amphetamine microinjections to the nucleus accumbens of rats can elicit increased spontaneous locomotor activity (Pijnenburg, Honig, Van der Heyden & van Rossum, 1976; Sellings & Clarke, 2003). Given the expanding knowledge of dissociable dopamine receptor family functions, some researchers turned to examinations of the role of D1 and D2 systems in the initiation of pharmacologically induced spontaneous locomotor activity. Dreher and Jackson (1989) injected selective D1 and D2 agonists (SKF38393 and quinpirole, respectively) to the nucleus accumbens and found that both were capable of eliciting increases in locomotor activity. However, this effect was markedly greater when both drugs were injected concurrently. Essman, McGonigle and Lucki (1993) found that this effect was not uniform throughout the nucleus accumbens core, and localized a significantly increased locomotor response to a D1/D2 combination to the caudal-central portion of the core. Seemingly contradictory to these findings, Mogenson and Wu (1991) found that quinpirole microinjections to the nucleus accumbens attenuated the locomotor eliciting effects of amphetamine. Later studies by Wu, Brudzynski, and Mogenson (1993) found that injections of quinpirole to the nucleus accumbens increased rat locomotor activity.
only when the rat was exhibiting low levels of initial locomotor activity and elicited decreased locomotor activity when initial levels of locomotor activity were high. Injections of SKF38393 increased locomotor behaviour regardless of initial level of activity. Thus, although D₁ and D₂-like receptor systems appear to function in an additive way towards the same behavioural output in some conditions, there still appears to be a complex dissociable function of the two receptor systems depending on the anatomical localization of injection. In the neighbouring ventral pallidum, the D₁ and D₂ receptor systems have been shown to operate in a non-synergistic fashion including possible opposite, antagonistic functions on locomotor activity in rats (Gong, Neill, Lynn & Justice, 1999).

**Quinpirole**

Quinpirole hydrochloride is a selective D₂-like receptor family agonist that has a relatively high affinity for D₂ receptors (Kebabian, Tarazi, Kula & Baldessarini, 1997). D₂ receptors can be found on both the pre-synaptic cell membrane, and the post-synaptic cell. Pre-synaptic D₂ receptors are often hypothesized to be autoreceptors that cause inhibitory hyperpolarization and that functionally modulate DAT activity to reduce dopamine concentrations in the synapse (Perez, White & Hu, 2006; Pothos, Przedborski, Davila, Schmitz & Sulzer, 1998). Post-synaptic D₂ receptors, however, may also be excitatory, depending on the specific population of neurons and their interactions with other neurochemical receptor systems. D₂ receptors were found to have a role in neuronal excitation in striatal cells, including the nucleus accumbens shell, but the full extent of the D₂ receptor type’s role in excitatory processes remains unclear (Perez, White & Hu, 2006). Thonton, Evans and Wickens (1987), and Mogenson and Wu (1991)
null
have found agonistic effects of quinpirole on locomotor behaviour, suggesting that quinpirole can promote overall dopaminergic activity at some sites.

**Purpose of the Present Study**

Following a similar model of pharmacological exploration, the present study has attempted to identify the possible role of the D₂-like dopamine receptor family in the production of 50 kHz rat vocalizations. Quinpirole-hydrochloride was used as a D₂-like dopamine receptor agonist, and raclopride was used as a D₂-like receptor antagonist. Microinjections of a range of quinpirole doses were injected into the nucleus accumbens shell, as this was identified as a key structure in eliciting 50 kHz rat vocalizations by Thompson, Leonard and Brudzynski (2006). Using 57 cannulated male rats, the 50 kHz vocalization response was quantified for various doses of quinpirole and compared to a counterbalanced vehicle control condition (0.2μl saline). D-amphetamine was also used as a positive control to confirm that our experimental procedure was capable of reproducing the 50 kHz vocalization response found in Burgdorf, Knutson, Panksepp and Ikemoto (2001) and Thompson, Leonard and Brudzynski (2006), and that any response elicited by activation of the D₂-like receptor family was consistent with an amphetamine mediated dopaminergic response, which activates all available dopamine receptor types.

The present study also utilized a double injection design to examine antagonism of quinpirole injection elicited 50 kHz vocalization by raclopride pretreatment behaviour. Raclopride was also used to pre-treat injections of 7 μg/0.2 μl of d-amphetamine to replicate the Thompson, Leonard and Brudzynski (2006) finding that antagonism of the D₂-like receptor family is sufficient to reverse 50 kHz vocalizations elicited by overall dopaminergic activation by amphetamine. A parallel group of non-cannulated rats
received systemic injections of quinpirole or saline in a counterbalanced order to examine the potential for peripherally administered quinpirole to elicit 50 kHz calling.

In addition, acoustic parameters were recorded from the first twenty calls detected from each session for analysis, including the mean call duration, peak frequency and bandwidth, in order to confirm that the calls that were detected were of the appropriate type, and to examine any changes in these calls as a result of the pharmacological manipulations.

Hypotheses

Based on the findings of Burgdorf, Knutson, Panksepp and Ikemoto (2001) and Thompson, Leonard and Brudzynski (2006) that microinjections of amphetamine into the nucleus accumbens shell are capable of eliciting significant increases in the number of 50 kHz vocalizations emitted by rats, and the finding by Thompson, Leonard and Brudzynski (2006) that this effect can be antagonized by a selective D2-like receptor antagonist, it is expected that:

1. Microinjections of quinpirole to the nucleus accumbens shell will induce a dose-dependent increase in the rate of species-specific 50 kHz ultrasonic vocalizations. Responses are expected to increase by quinpirole dose until reaching a stable plateau.

2. The quinpirole induced vocalization response will be antagonized by a local pretreatment of an equimolar dose of raclopride, a D2-like dopamine receptor antagonist.

3. A similar increase in 50 kHz vocalizations will be obtained after systemic injection of quinpirole.
4. Pharmacologically elicited responses will possess similar acoustic characteristics to saline induced vocalizations.
Methods

Animals

Eighty-nine male wistar rats were acquired from Charles River, St Constant, Quebec, Canada for this study. Male rats were used to eliminate variability due to female rats’ estrous cycle. Initially, rats were kept in pairs in cages and were separated after surgery into single housing in clear plastic cages, with dust free wood chip bedding and opaque plastic tubes for hiding and environmental enrichment. All cages were maintained in a common room with an ambient temperature of 22±2°C and a 12:12 hour light-dark cycle. Animals were fed pellets of Rodent Lab Diet # 5001 (PMI Nutrition International, Brentwood, MD) and received tap water ad libitum. All rats were friendly and healthy, and were between 240 and 450 g of body weight at the time of surgery.

Cannulae Implantation Surgery

Each rat was weighed and prepared for surgery by the administration of anesthesia through an intraperitoneal injection of 40-60 mg/kg ketamine hydrochloride (Ayerst Veterinarian Laboratories, Guelph, ON) and 4-6 mg/kg xylazine hydrochloride (Bayer, Etobicoke, ON). The depth of anesthesia was tested by monitoring reflex activity with a toe pinch. Once the rat was unresponsive, the surgical area on top of the head was shaved and excess fur was removed with a portable vacuum.

The rat was then mounted on the surgical apparatus (David Kopf Instruments, Tujunga, CA) by securing the two ear bars into two small indentations in the rat’s skull within the external ear canal. The incisor bar was then placed under and behind the rat’s incisor teeth, 3.3 mm below the ear bars horizontal zero plane, and a snout clamp tightened. At this point the rats’ head was secured against movement in any direction.
Once secured, the rat's eyes were treated with a protective gel (artificial tears) to maintain moisture during surgery (Alcon, Mississauga, On) and the surgical area was prepared with betadine soap, followed by isopropanol, followed by a betadine solution. This was intended to protect the surgical area from bacteria that is naturally present in the Wistar rat's fur and inflammation that could be caused by the entry of foreign pathogens into the surgical area. A sterilized protective sheet was placed over the rat's body to further protect the surgical area, and maintain the rat's body temperature.

An incision was made with a scalpel, approximately two cm in length, along the longitudinal suture of the skull from caudal of lambda to rostral of bregma points. The surface of the skull was scraped to remove the periostium, so that the bone was clean and smooth, to facilitate the adhesion of acrylic (Perm, Hygenic Corporation of Canada, St.Catharines, ON). A retractor was placed onto the incision to expose the surface of the skull, and the area was cleaned of any excess fluids. The stereotaxic apparatus zero coordinates were determined using a calibrating arm and the drill attachment. The holes in the skull were localized relative to these zero coordinates. This position was confirmed by verifying that the drill touched the skull at the peak of lambda, and the lateral zero coordinate was in line with the sagittal suture up to bregma. Using the rat stereotaxic atlas by Paxinos and Watson (Paxinos and Watson, 1986), drilling coordinates were recalculated so that the drill penetrated the skull directly above the caudal portion of the nucleus accumbens shell. For the purpose of retaining some injection site variability anterior coordinates ranged from 9.8 mm to 10.2 mm rostral of lambda, with the most common coordinate being 10.0 mm. Lateral coordinates were typically 1.1 mm to either side of the midsagittal suture, but ranged from 1.0 mm to 1.5 mm. Depth was typically
6.0 mm ventral to the surface of the brain, but some cannulae were implanted to depths of 6.5 mm and 7.0 mm.

Bilateral openings were drilled into the skull that were of sufficient size to allow for the implantation of two guide cannulae made from 23 gauge stainless steel needles. The depth of the dura mater was found using the guide cannula and recorded as the horizontal zero surface, and the depth of the nucleus accumbens shell was recalculated from the level of the dura mater. The dura mater was then punctured with a needle, allowing the smooth entry of the cannula into the brain. The cannula was lowered into the desired position so that the tip of the cannula terminated 1 mm above the target structure, allowing the injection cannula to extend past the guide cannula and into the target. Four sterile jewelers screws were then placed in the skull below bregma on an angle to help hold the dental acrylic and the guide cannulae in place. One guide cannula was inserted to the appropriate depth, and while it was held in place by the stereotaxic apparatus, dental acrylic was placed on the skull around the anchoring screws and the cannula. The acrylic was smoothed and allowed to harden so that the cannula implantation arm could be retracted, leaving the cannula implanted in the brain. The process was repeated on the contralateral opening, adding more acrylic to the remaining cannula and overtop of the existing acrylic, forming a smooth dome. Once the acrylic had fully hardened, the incision was sutured shut around the acrylic dome.

Sterile stainless steel plug pins were inserted into the guide cannulae to prevent their blockage and infection. An injection of 3 ml of saline was given to re-hydrate the animal, and the rat was placed in a heat lamp warmed environment to recover. Animals received oral analgesics after surgery (Tylenol). All rats that underwent surgery were
allowed to recover for no less than one week before injections, during which time they were administered oral analgesics as necessary.

**Pharmacological Agents and Microinjections**

Quinpirole hydrochloride (Sigma, St.Louis, MO), a selective D₂-like family dopamine receptor agonist was used intracerebrally (i.c.) at doses ranging from 0.5-20 μl/0.2 μg. d-amphetamine (Sigma), a non-selective dopamine agonist was also used, at 7 μg/0.2μl. Raclopride (Sigma), a selective D₂-like family dopamine receptor antagonist was used at doses equimolar to 6 μg/0.2 μl quinpirole (11.5 μg/0.2 μl), and 7 μg/0.2 μl d-amphetamine (9.4 μg/0.2 μl). Sterile physiological saline was used as the vehicle for all injections, and as a control substance.

All injections were performed with a 30-gauge stainless steel injection cannula connected by PE-10 polyethylene tubing to a Hamilton CR-700 microsyringe filled with mineral oil. The mineral oil from the syringe pushed the drug solution into the brain. The rats were gently restrained during injection, but were fully conscious and active in a restricted range during the injection. After injection, a thirty second delay was maintained before removal of the injection cannula to allow for proper diffusion of the drug away from the injection site. For those rats receiving double injections, a five minute delay was maintained before the procedure was repeated in the same cannula. Once the injection cannula was removed, the plug pin was reinserted, and the rat was transferred immediately to the recording chamber.

**Quinpirole Dose Response**

In experiment 1, thirty rats were given microinjections of quinpirole. The rats were injected in a counterbalanced order, such that half of the rats in a particular series
were injected with a 0.2μl saline control first, and half were administered the test substance first, and the other substance given for the second injection. In some later conditions, two doses of test quinpirole were counterbalanced with each other. No rat received multiple intracannulae injections less than one week apart to minimize the risk of conditioning effects and drug sensitization. The range of doses of quinpirole that were given was 0.5 μg, 3 μg, 6 μg, 10 μg, and 20 μg, all in 0.2 μl. A microinjection of 7 μg of d-AMPH/0.2 μl was also given to the contralateral cannula of each rat as a positive control for induction of 50 kHz vocalizations.

Raclopride-Quinpirole Antagonism

To examine possible pharmacological antagonism of elicited 50 kHz calls, an intracannulae injection of an equimolar solution of a selective-D₂ antagonist, raclopride, was used as a pretreatment of a dose of 6 μg/0.2 μl quinpirole. Twenty-seven rats were injected with either saline or raclopride in counterbalanced order, and recorded for five minutes. The same rat was then injected with the test drug, either 6 μg/0.2 μl quinpirole or saline, and recorded for ten minutes.

Raclopride-Amphetamine Antagonism

Thirteen rats were injected i.c. with either saline or 9.4 μg/0.2 μl raclopride in a counterbalanced order. All pretreatment injections were followed by 7 μg/0.2 μl d-amphetamine. After the d-amphetamine injection the rat was placed in the recording chamber and recorded for ten minutes.

Delayed Response Injection

To examine the possible delayed effects of direct injections of quinpirole on 50 kHz vocalizations, a delayed recording experiment was conducted. Ten rats were
injected i.c. with 10 μg quinpirole or saline in a counterbalanced order, and recorded for five minutes. The animals were then returned to their home cage for forty minutes, then retrieved and recorded for a further ten minutes.

**Systemic Quinpirole Injection**

A series of thirty-two rats were injected subcutaneously (s.c.; on the back, above the spine) with quinpirole, in either a 0.5 mg/kg dose or a 1.5 mg/kg dose, or the same volume of s.c. saline (about 0.2 ml) in a counterbalanced order. Ten minutes after the injection, the rat’s vocalizations were recorded for ten minutes.

**Vocalization Recording**

The recording chamber used was a clear, sterile, translucent polycarbonate cage/enclosure (9 cm x 10 cm) with a microphone mounted directly above the rat’s head. The microphone was connected to an S200 bat detector (QMC Instruments Ltd. London, England), which divided the incoming sound frequency by 16, lowering the pitch of the vocalizations into the human audible range. The output of this device was then recorded on a cassette tape recorder (Optimus CTR-11, Intertan Canada Ltd., Barrie, ON). The signal was simultaneously displayed on a sonograph (DSP Sona-Graph signal analysis workstation model 5500, Kay Elemetrics Corp., Pine Brook, NJ) that displayed the audio signal visually, which facilitated the detection of 50 kHz vocalizations. After recording, the rat was returned to the colony room.

**Acoustic Analysis**

For analysis of all recordings, the cassette tape was replayed on the cassette deck, which fed the signal into the sonograph. The signal was examined visually by the operator for the identification of possible 50 kHz vocalizations. When calls were
null
recognized, the tape and sonograph were paused and a more detailed analysis of the physical properties of the signal were conducted, including the duration of the signal, its bandwidth, peak frequency, and morphology. The beginning and end of the call was marked with time cursors, the duration (the space between the cursors) was recorded, and the power spectrum within that timeframe was computed. The peak frequency of the call was marked on the spectrogram and recorded, and frequency cursors framed the lowest and highest frequency of the signal; the difference between the lowest and highest frequency component of the call was recorded as the bandwidth. If these parameters conformed to the characteristics of a rat 50 kHz vocalization and were of clearly higher intensity than background noise, the call was counted, and its characteristics recorded for statistical analysis. The detailed characteristics were recorded for the first 20 calls, and subsequent calls only counted. For each injection, five minutes of tape was analyzed, although ten minutes may have been recorded.

Histological Procedures

At the conclusion of each experiment, the rats were sacrificed by an overdose of the sodium barbituate (Euthanyl, Vetoquinol Canada Inc., Lavaltrie, Quebec). Rats were perfused with 10% formalin, and their brains were removed. After resting in a formalin solution for a minimum of 24 hours, the perfused brains were sectioned on a freezing microtome (ESBE Scientific, Markham, Ontario), and mounted on polylysinated slides. The slides were then stained with .06% thionin using a Nissl staining procedure. These slides were differentiated in alcohol, cleared in xylene, then cover slipped and examined under a microscope to determine the specific localization of each cannula using the Paxinos and Watson rat brain atlas (Paxinos and Watson, 1986).
Statistical Analyses

For the analysis of single and double injection groups, paired sample t-tests were conducted to identify significant differences in the number of 50 kHz calls produced by rats in relevant groups according to a priori hypotheses. One-way ANOVAs were used to assess differences in the qualitative properties of calls by drug group. Dunnett’s T3 was used to examine group differences in significant omnibus analyses. Alpha level was set at \( p < 0.05 \).
Results

Single Injections of Quinpirole and Amphetamine

Injections of quinpirole produced 50 kHz calls in a dose dependent manner, with the distribution following an inverted U curve (see Figure 1; group mean / 5 minute recording for effective doses: 6 µg quinpirole, M = 33.07, SD = 36.136; 7 µg amphetamine, M = 21.16, SD = 31.53). Microinjections of 6 µg/0.2 µl of quinpirole produced significantly more 50 kHz vocalizations than matched saline controls ($t_{14} = 2.30, p = 0.04$), as did 7 µg/0.2 µl of d-amphetamine ($t_{31} = 2.68, p = 0.01$). The mean number of 50 kHz calls elicited by 10 µg/0.2 µl of quinpirole was relatively high, but did not approach significance ($M = 28.47, SD = 42.07$). The 0.5 µg/0.2 µl dose ($M = 2.89, SD = 4.49$) and the 3 µg/0.2 µl dose ($M = 19.40, SD = 16.81$) did not elicit more 50 kHz vocalizations than saline. The 20 µg/0.2 µl of quinpirole dose also elicited no more calls than saline ($M = 6.67, SD = 7.05$), indicating that at very high doses, quinpirole is no longer effective at inducing 50 kHz vocalizations.

Raclopride Antagonism

Injections of 6 µg/0.2 µl of quinpirole that followed a saline pretreatment produced significantly more 50 kHz vocalizations than double injections of saline ($t_{26} = 2.14, p = 0.04$). When 6 µg/0.2 µl quinpirole followed a pretreatment of an equimolar dose of raclopride (11.5 µg/0.2 µl) the number of calls produced was significantly reduced to control levels ($t_{24} = 2.47, p = 0.02, M = 1.08, SD = 2.47$). Raclopride after saline injections elicited no more 50 kHz calls than did saline alone (see Figure 2).

In a separate group of rats, similar double injections were used to determine the effects of raclopride on d-amphetamine injections to the nucleus accumbens shell.
Experiments by Thompson, Leonard & Brudzynski (2006) found that D₁ or D₂ antagonism could reverse the effects of amphetamine on 50 kHz calling in the rat nucleus accumbens. These findings were replicated in the current study. Amphetamine injected after saline produced significantly more 50 kHz calls than did double injections of saline (t₂₅ = 2.37, p = 0.03). Amphetamine injections that had been pretreated with an equimolar solution of raclopride (9.4 µg/0.2 µl) produced significantly fewer 50 kHz vocalizations than those that had not been pretreated (t₂₅ = 2.16, p = 0.04; see Figure 3).
Figure 1. Mean number of 50 kHz calls produced by single injections of saline, amphetamine, and a range of quinpirole doses (from 0.5 μg/0.2 μl to 20 μg/0.2 μl). A dose-response relationship is observed over successively higher doses of quinpirole. Six μg/0.2 μl of quinpirole produced significantly more 50 kHz vocalizations than saline ($t_{14} = 2.23, p = 0.04$) as did 7 μg/0.2 μl of d-amphetamine ($t_{31} = 2.68, p = 0.01$). The magnitude of response of 7 μg/0.2 μl of amphetamine and that for 6 μg/0.2 μl of quinpirole were similar. $* = p < 0.05$. 

[Graph showing mean number of 50 kHz vocalizations for different doses of quinpirole and amphetamine compared to saline.]
Figure 2. Mean number of 50 kHz calls produced by double injections of saline followed by 6 μg/0.2 μl quinpirole or raclopride followed by 6 μg/0.2 μl quinpirole. Double injections in which quinpirole (6 μg/0.2 μl) followed saline elicited significantly more 50 kHz vocalizations than a double injection of saline (t_{26} = 2.14, p = 0.04). When the same dose of quinpirole was pretreated with an equimolar solution of raclopride (11.5 μg/0.2 μl), the effect was reversed (t_{24} = 2.47, p = 0.02), and the mean number of 50 kHz calls was similar to saline. Saline pretreated with raclopride (11.5 μg/0.2 μl) also produced a response similar to a double injection of saline.

* = p < 0.05.
Figure 3. Mean number of 50 kHz calls produced by double injections of saline followed by amphetamine or raclopride followed by amphetamine (n = 26). Microinjections of amphetamine (7 μg/0.2 μl) into the nucleus accumbens shell elicited significantly more 50 kHz vocalizations (t25 = 2.16, p = 0.04) when following an injection of saline than when following and equimolar solution of raclopride (9.4 μg/0.2 μl). * = p < 0.05.
Delayed Response

To determine if quinpirole could elicit 50 kHz vocalizations in a delayed manner after direct injections to the nucleus accumbens shell, an experiment was conducted in which ten rats were injected with 10μg of quinpirole or saline, then vocalizations were recorded. The animal was then returned to the colony room for forty minutes to its home cage, then retrieved and recorded for another ten minutes. Rather than an increase in vocalizations after the delay period, a non-significant decrease in the mean number of calls was observed for both saline and quinpirole conditions (data not shown). Quinpirole did not elicit more 50 kHz vocalizations than saline prior to or following the delay.

Systemic Injections

Independent-samples t-tests were used to examine differences in 50 kHz calling rates following subcutaneous injections of saline and quinpirole at 0.5 and 1.5mg/kg. There were no significant differences between the number of 50 kHz vocalizations produced by subcutaneous injections of saline and 0.5 mg/kg ($t_{47} = 0.79, p = 0.43, \text{n.s.}$), or 1.5 mg/kg ($t_{47} = 0.83, p = 0.41, \text{n.s.}$) of quinpirole (see Figure 4).

Acoustic Call Characteristics

Data regarding the qualitative acoustic characteristics of 50 kHz calls was also collected from the first 20 vocalizations recorded for all injections, in the form of call bandwidth, duration, peak frequency and rough sonographic morphology. The first three variables were analyzed for group differences by injection type, to determine if the eliciting drug was affecting the physical properties of the calls. The morphology data was retained for future analysis. The physical properties of saline elicited vocalizations
were used as a baseline for statistical comparison in post hoc analysis because these calls were not subject to pharmacological alteration.

The Levene's statistic for these data consistently yielded a significant result, indicating a significant heterogeneity of variance. As a result of this, the Brown-Forsyth robust tests for equality of means are reported here, as this conservative test is less dependent on an assumption of homogeneity of variance. A one-way ANOVA of the bandwidth data by single injection drug groups revealed a significant effect of group on call bandwidth ($F_{6,380} = 6.76, p < 0.001$). Post hoc Dunnett's T3 tests indicated that the 0.5 µg/0.2 µl quinpirole dose elicited calls with significantly smaller bandwidths than saline ($p = 0.01$), and the 20 µg/0.2 µl quinpirole dose elicited calls with significantly larger bandwidths than saline ($p = 0.01$; see Figure 5).

A one-way ANOVA of call duration by injection group also revealed a significant effect of drug injection group ($F_{6,498} = 4.84, p < 0.001$). Post hoc testing with Dunnett's T3 found that the 6 µg/0.2 µl of quinpirole group ($p < 0.001$), and the 10 µg/0.2 µl of quinpirole group ($p = 0.04$) elicited calls with significantly longer call durations than saline (see Figure 6).

A one-way ANOVA of peak frequency by drug injection group again found a significant effect of drug group ($F_{6,313} = 32.57, p < 0.001$). Post hoc Dunnett's T3 revealed that the 0.5 µg/0.2 µl of quinpirole dose elicited calls with significantly lower peak frequencies than saline ($p < 0.001$), while the 6 µg/0.2 µl of quinpirole dose ($p < 0.001$) and the 10 µg/0.2 µl of quinpirole dose ($p < 0.001$) elicited calls with significantly higher peak frequencies than saline (see Figure 7).
Figure 4. Mean number of 50 kHz calls produced by subcutaneous injections of saline, 0.5 mg/kg of quinpirole ($t_9 = 0.676$, $p = 0.52$, n.s.) and 1.5 mg/kg of quinpirole ($t_{31} = 0.830$, $p = 0.41$, n.s.). Neither dose of quinpirole produced significantly more 50 kHz vocalizations than did saline.
Figure 5. Mean bandwidths in kHz for 50 kHz calls recorded following injections of saline, all doses of quinpirole, and amphetamine (n = the number of calls analyzed). The lowest dose of quinpirole (0.5 μg/0.2 μl) elicited calls with significantly reduced bandwidths from saline (Dunnett’s T3, p = 0.01), while the highest dose (20 μg/0.2 μl) elicited calls with significantly increased mean bandwidth (p = .01). * = p < 0.05.
Figure 6. Mean call durations in ms for 50 kHz calls recorded following injections of saline, all doses of quinpirole, and amphetamine. Moderate doses of quinpirole (6 µg/0.2 µl and 10 µg/0.2 µl) elicited calls with significantly longer mean durations than saline (Dunnett’s T3, p < 0.001 and p = 0.04 respectively). * = p < 0.05, # = p < 0.001.
Figure 7. Mean peak frequencies in kHz for 50 kHz calls recorded following injections of saline, all doses of quinpirole, and amphetamine. The lowest dose of quinpirole (0.5 μg/0.2 μl) elicited calls with significantly reduced peak frequencies compared to saline (Dunnett’s T3, p<.001), while moderate doses (6 μg/0.2 μl and 10 μg/0.2 μl) both elicited calls with significantly increased mean peak frequencies from saline (p<.001). * = p < 0.001.
Injection Localization Data

A quantitative mapping of 50 kHz vocalization sites is provided for 6 μg/0.2 μl of quinpirole, 7 μg/0.2 μl amphetamine, saline followed by 6 μg/0.2 μl quinpirole, and raclopride followed by 6 μg/0.2 μl quinpirole (see Figures 8, 9, 10 and 11 respectively). Each injection site is labeled with a circle, which is proportional in size to the magnitude of the vocal response.

The nucleus accumbens shell was divided into quadrants: the ventral-rostral shell, the ventral-caudal shell, the dorsal-rostral shell, and the dorsal-caudal shell (see Figure 12). No injections reached the dorsal-rostral shell, so it was excluded from future analysis. Independent samples t-tests were used to identify differences in the mean number of 50 kHz calls elicited by injections of 6 μg/0.2 μl of quinpirole to various the anatomical subregions described above (equal variances not assumed).

Injections to the nucleus accumbens shell elicited significantly more 50 kHz calls than injections outside the nucleus accumbens (t_{10} = 3.05, p = 0.01) and injections to the nucleus accumbens core (t_{11} = 2.53, p = .03; see figure 12). Of the subregions of the nucleus accumbens shell, only one region elicited significantly more 50 kHz vocalizations from injections of 6 μg/0.2 μl quinpirole than injections outside the accumbens, the ventral-caudal shell (t_{6} = 3.03, p = 0.02). This region, however, was the only region to receive more than three injections that were included in the analysis, and other sites may yet be shown to have the ability to elicit more 50 kHz calls from D2-like activation.

In order in support the validity of the anatomical localizations of function, a paired-samples t-test of only those injections of 6 μg/0.2 μl quinpirole and saline that
were clearly within the boundaries of the nucleus accumbens shell was conducted. In this way, the potential influence of neighboring nuclei was minimized. Injections of 6 µg/0.2 µl quinpirole within the nucleus accumbens shell elicited more 50 kHz vocalizations than counterbalanced saline injections to the same site, (t_{11} = 2.50, p = 0.03; see Figure 13).
Figure 8. A quantitative mapping of 50 kHz vocalizations elicited by 6µg/.2µl quinpirole injection sites (n = 21). Each injection is labeled by one circle proportional in size to the magnitude of the response. The circles on the upper left margin provide the range of the number of calls.
Figure 9. A quantitative mapping of 50 kHz vocalizations elicited by 7μg/.2μl amphetamine injection sites (n = 34). See legend to Figure 8 for other details.
Figure 10. A quantitative mapping of 50 kHz vocalizations elicited by saline followed by 6μg/2μl quinpirole double injection sites (n = 23). See legend to Figure 8 for other details.
Figure 11. A quantitative mapping of 50 kHz vocalizations elicited by raclopride followed by 6μg/2μl quinpirole double injection sites (n = 23). See legend to Figure 8 for other details. Few induced 50 kHz vocalizations are observed.
Figure 12. Localization of 6 μg quinpirole elicited 50 kHz vocalization response to specific accumbal subregions. 6 μg quinpirole injections to the nucleus accumbens shell as a whole elicited significantly more 50 kHz vocalizations than injections to the nucleus accumbens core (t_{11} = 2.53, p = 0.03, #), or injections outside the nucleus accumbens (t_{10} = 3.05, p = 0.01, *). The Ventral-Caudal subregion of the nucleus accumbens shell elicited significantly more calls than injections outside the nucleus accumbens (t_{6} = 3.03, p = 0.02); other areas of the accumbens did not produce vocalizations at a significantly higher rate than controls. * = p < 0.01, # = p < 0.05.
Figure 13. Mean number of 50 kHz calls elicited from nucleus accumbens shell by saline or 6 μg/0.2 μl of quinpirole. Within the boundaries of the nucleus accumbens shell, injections of 6 μg/0.2 μl of quinpirole elicited significantly more 50 kHz vocalizations than saline injections ($t_{11} = 2.50$, $p = 0.03$). * = $p < 0.05$. 
Discussion

Primary Findings

Microinjections of the D_2-like dopamine receptor agonist quinpirole into the nucleus accumbens shell of rats elicited significantly more 50 kHz vocalizations than did saline controls at the 6 µg/0.2 µl dose. Further, the mean number of vocalizations elicited by quinpirole varied with the dose of the drug, following an inverted U shaped curve (see Figure 1). The number of vocalizations was similar to a positive control group that received injections of 7 µg/0.2 µl d-amphetamine. Localizations of the individual injection sites indicate that the nucleus accumbens shell is particularly sensitive to quinpirole in eliciting 50 kHz vocalization relative to the surrounding area (see Figure 8). Comparing only those injections of 6 µg/0.2 µl of quinpirole that were clearly within the boundaries of the nucleus accumbens shell, the drug’s agonistic effect on 50 kHz vocalizations was significant (see Figure 13).

The double injection procedure revealed that saline followed by 6µg/2µl of quinpirole was able to elicit a significantly greater number of 50 kHz calls than was saline followed by saline (see Figure 2). This effect, however, was reversed by the pretreatment of the injection site with raclopride, a selective D_2-like dopamine receptor antagonist. Raclopride alone had no significant effect on 50 kHz vocalization behaviour relative to saline. Thus, activation of the D_2-like receptor type in the nucleus accumbens shell can elicit 50 kHz calling in rats, in a dose dependent and selectively reversible manner.
D₂ Receptor Function

The D₂ receptors on the presynaptic membrane function as autoreceptors. These receptors, primarily consisting of the D₂-short isoform, are functionally linked with the dopamine transporter (DAT) and can modulate cell membrane potential through K⁺ channel activity, and thus quinpirole activation of these receptors should decrease dopamine release from the presynaptic terminals (Perez, White & Hu, 2006; Lee, Pei, Moszczyńska, Vukusic, Fletcher & Liu, 2007). DAT is a membrane bound protein that maintains dopamine concentrations in the synaptic cleft through upregulation or downregulation of dopamine reuptake. DAT is a target of many drugs of abuse including amphetamine and cocaine (Lee, Pei, Moszczyńska, Vukusic, Fletcher & Liu, 2007) and is differentially modulated by D₂ agonists and antagonists depending on site of injection (Kimmel, Joyce, Carroll & Kuhar, 2001). D₂ autoreceptor activity has also been shown to modulate dopamine vesicle quantal size and the frequency of stimulation-evoked quantal release (Pothos, Przedborski, Davila, Schmitz & Sulzer, 1998).

However, D₂ receptors can also be found on the postsynaptic cell membrane, primarily of the D₂-long isoform, where they are hypothesized to be primarily excitatory in nature and cause a net depolarization of the neuron through G-protein mediated second messenger cascades (Lee, Pei, Moszczyńska, Vukusic, Fletcher & Liu, 2007; Piercey, Moon, Sethy, Schreur, Smith, Tang & Von Voigtlander, 1996). The excitatory nature of postsynaptic D₂ receptors are controversial, as they have been shown to inactivate the cAMP/PKA cascade, however, further studies have outlined mechanisms by which the D₂ receptor may facilitate post-synaptic depolarization (Perez, White & Hu, 2006).
Pharmacological Agents – Quinpirole

Quinpirole hydrochloride is a tricyclic ergoline dopamine agonist (Levant 2002) that has a high affinity for D₂ and D₃ receptors and some affinity for D₄ receptors (Kebabian, Tarazi, Kula & Baldessarini, 1997). As quinpirole acts on both pre- and post-synaptic receptors, the action of this drug may be complex. Systemic injections of quinpirole have been shown to exhibit both excitatory and inhibitory effects depending on dose, injection schedule, and behaviour (Wrobel, Zebrowska-Lupina & Wielosz, 2005; Eilam & Szechtman, 1989).

The time course of the inhibitory and excitatory aspects of quinpirole/D₂ function may be dissociable. Subcutaneous injections of quinpirole at various doses have shown to have a biphasic effect on locomotor activity, both across dose and across time, such that low doses only decreased locomotor activity, whereas high doses reduced locomotor behaviour briefly, then later elicited locomotor excitation (Eilam & Szechtman, 1989). The time course of these biphasic responses was shown to be highly dependent on quinpirole dose (Eilam & Szechtman, 1989), and may be further dependent on mode of injection and locale of interest. The authors of that study remarked that a likely site of action for this biphasic effect was the nucleus accumbens, as stimulation of the accumbens produces locomotor excitation, and because neuronal activity there is modulated by presynaptic D₂ receptors, perhaps in a biphasic manner (Eilam & Szechtman, 1989).

This effect was examined in the present study in a discrete group of injections of 10 μg/0.2 μl of quinpirole followed by a five minute initial recording, then another five minute recording after a forty minute delay spent in the animals own single-housed cage.
No increase in the number of 50 kHz vocalizations was found after the delay. The synaptic basis of this finding is not yet clear, however the possibility remains that locomotor and vocalization pathways respond differently to quinpirole stimulation, perhaps with different receptor type densities or behavioural thresholds. Furthermore, in the present study, doses above 10 μg/0.2 μl of quinpirole elicited 50 kHz calls at baseline levels, indicating a potential suppression effect at high doses. The mechanism of such a suppression effect could be similar to the response observed, as the inhibitory properties of quinpirole may be more prevalent at high doses than the excitatory properties.

Pharmacological Agents — Amphetamine

Amphetamine is a compound that produces elevated extracellular release of catecholamines and serotonin, which exerts its effects primarily on the dopamine system, particularly the d-amphetamine form that was used in this study (Sulzer, Sonders, Poulson & Galli, 2005). Amphetamine is lipophilic, and exerts its effects on extracellular mechanisms and after diffusing across the presynaptic plasma membrane. The exact method of action remains unclear, however, it is known that synaptic transmitter levels are modulated by a variety of mechanisms, including stimulating presynaptic vesicle release and reverse transport via DAT into the synaptic cleft and possibly via the vesicular monoamine transporter (VMAT). Amphetamine also produces other subtle effects, such as mild monoamine oxidase (MAO) inhibition, increased dopamine synthesis, and possible activity at trace amine receptors that react to endogenous transmitter substances that resemble amphetamine (β-phenethylamine for example; Sulzer, Sonders, Poulson & Galli, 2005).
Systemic Injections

Another group of rats was given a subcutaneous injection of quinpirole (0.5 mg/kg) or saline, in a counterbalanced order, and recorded ten minutes later to examine the number of 50 kHz calls produced. When no significant change was found, another dose condition was added in which new rats received subcutaneous injections of 1.5 mg/kg of quinpirole counterbalanced with saline. This too produced no significant change in 50 kHz vocalizations, although a slightly higher mean number of calls were observed. Although this finding was null, future studies may attempt to modify this design, by including a wider dose range, and possibly by instituting a delay condition to examine latent effects of the drug when injected systemically.

Some behavioural changes were observed from rats injected systemically with quinpirole, including lethargy, marked increases in water drinking, and yawning. Several studies have found that locomotor activity can be mediated by D₁ and D₂ receptors, particularly in combination (Dreher & Jackson, 1989), and yawning by D₃ receptors (Collins, Witkin, Newman, Svensson, Grundt, Cao & Woods, 2005). A condition known as polydipsia, or non-regulatory compulsive drinking, is a noted symptom of a minority of human patients suffering from chronic psychotic disorders. This condition can be experimentally replicated in rats with chronic administration of quinpirole, and a previous condition reinstated with amphetamine (Amato, Milella, Badiani & Nencini, 2006; Fraioli, Cioli & Nencini, 1997; Cioli, Caricati & Nencini, 2000). Dopamine administration also likely affects a variety of peripheral organs, particularly the kidneys, and can produce behavioural effects including hyperdipsia (Ladines, Zeng, Asico, Sun, Pocchiari, Semeraro, Pisegna, Wank, Yamaguchi, Eisner & Jose, 2001). A series of
papers by Szechtman and others have noted locomotor and stereotypy responses in rats to peripheral administration of chronic and acute quinpirole (Gabrielle, Culver, Sharma, Zhang, Szechtman & Mishra, 2003; Eilam & Szechtman, 1989; Sullivan, Talangbayan, Einat & Szechtman, 1998; Sullivan, Dogaru & Szechtman, 1992). These observations suggest that quinpirole is successfully crossing the blood brain barrier, and is capable of affecting central dopaminergic function, as was noted observationally in the present experiment. However, systemic injections of quinpirole did not have any detectible effect on 50 kHz vocalization rates at the dose level studied.

Acoustic Call Characteristics Findings

In addition to examining changes in the mean number of 50 kHz calls by drug injection, the present study also examined the qualitative characteristics of recorded vocalizations. Vocalizations elicited by saline injections were taken as representative of naturally elicited baseline calling, since isotonic saline is a pharmacologically neutral substance. Statistics for mean call duration, bandwidth, and peak frequency in different experimental conditions were analyzed. No consistent differences were found between groups in bandwidth (see Figure 5); however, significant and consistent differences among groups were found in the duration and peak frequency data. Injections of 6, and 10 μg/0.2 μl quinpirole produced significantly longer call durations than saline. Lower dose injections (0.5 μg) of quinpirole elicited significantly lower mean peak frequency 50 kHz calls, while 6 and 10 μg/0.2 μl of quinpirole elicited calls with a significantly higher mean peak frequency than saline. The communicative value of these effects, if any, is not altogether clear, however differences in the qualitative properties of elicited vocalizations seem to warrant further study. Since these changes appear to be associated with higher
dose levels, it is conceivable that these changes reflect changes in the intensity of the message, or the urgency, as might occur naturally under more salient stimuli. Future studies may seek to identify not only those stimuli that elicit increases in the number of 50 kHz vocalizations, but also correlate aspects of these stimuli to qualitative changes in calls. Specifically, future studies may attempt to differentiate the behavioural conditions that associate with frequency modulated type 50 kHz calls (which are hypothesized to more clearly reflect positive affect and reward, Burgdorf, Wood, Kroes, Moskal & Panksepp, 2001) from flat 50 kHz frequency calls, or whether some behavioural stimuli elicit 50 kHz calls with a higher mean peak frequency than others.

Recent studies have begun to examine qualitative changes in rat vocalizations. Thompson, Leonard and Brudzynski (2006) found no significant changes in 50 kHz call peak frequency, duration or bandwidth across any drug condition, including saline, amphetamine, haloperidol, lactic acid and SKF-83566 (a D1 antagonist). Wintink and Brudzynski (2001) also examined 50 kHz calls elicited by intracerebral injections of saline and glutamate to the anterior hypothalamic preoptic area and intraperitoneal injections of amphetamine. No significant changes in call peak frequency or call duration were found between any conditions. Fendt, Schwienbacher and Schnitzler (2006) presented evidence that agonism of the acetylcholine system of the nucleus accumbens core can produce 50 kHz vocalizations, and noted that these calls were qualitatively similar to those presented in Brudzynski and Pniak (2002) which were elicited through social contact with conspecifics.

Another study examined the effects of widespread dopamine challenges on sexually elicited 50 kHz calls in male rats have been examined for qualitative changes
(Ciucci, Ma, Fox, Kane, Ramig & Schallert, 2007). Vocalizations from rats given haloperidol injections (0.1mg.kg i.p.) or 6-OHDA infusions to the medial forebrain bundle (which caused dopaminergic cell degeneration) were compared to control rats' vocalizations. Both treatments resulted in decreased call bandwidth. Haloperidol injected rats, but not 6-OHDA infused rats produced fewer calls than controls. It should be noted, however, that this study only considered 50 kHz calls of the ‘trill’ morphology, and did not examine peak frequency or call duration as dependent variables. The primary finding of this study, that widespread dopaminergic challenges decrease 50 kHz call bandwidth, may reflect changes in the motor aspect and/or semiotic content, and may be analogous to changes in human vocalization disturbances with dopaminergic dysfunction (such as those found in Parkinson’s disease; Yokochi, 2007). It is unclear as to the nature of this change in content, and a direct modeling of human vocal disturbance has yet to be established.

The findings regarding the qualitative characteristics of calls in the present study do imply some dopaminergic disruption of call bandwidth. Very low doses (0.5 µg/0.2 µl) of quinpirole decreased call bandwidth, and very high doses (20 µg/0.2 µl) of quinpirole increased call bandwidth. Since the effect of quinpirole on synaptic dopamine concentrations has been noted to be biphasic, and the net effect of very high and low central doses has not been established, it is uncertain whether this effect is convergent with the data from Ciucci et al. (2007). Modulation of 50 kHz call duration and peak frequency with moderate doses of quinpirole may indicate that these call properties are disproportionately affected by D₂/D₃ activity. Further research examining the qualitative properties of 50 kHz rat vocalizations may begin to build a more complete profile of the
pharmacological substrates of the physical properties of these calls, and guide research examining the possible communicative value of these properties.

Localization of Injections

Injections of 6 µg/0.2 µl quinpirole to the nucleus accumbens shell elicited significantly more 50 kHz vocalizations than injections outside the nucleus accumbens or injections to the nucleus accumbens core. Injections of amphetamine (7 µg/0.2 µl) to the nucleus accumbens shell also elicited more 50 kHz vocalizations than injections to the core or outside the accumbens. Double injections of saline followed by 6 µg/0.2 µl quinpirole also produced more 50 kHz vocalizations from the accumbens shell than from other localizations. Injections primarily reached the nucleus accumbens shell, with a smaller amount reaching the nucleus accumbens core. Those injections sites that were outside the nucleus accumbens were generally too deep, and reached areas such as the ventral pallidum, horizontal limb of the diagonal band and the olfactory tubercle.

Recent evidence, however, suggests that the nucleus accumbens shell is not an entirely homogeneous region. Although it is not yet entirely clear what functional and anatomical distinctions exist within this region, Reynolds and Berridge (2001) have identified a potential distinction between the rostral and caudal portions of the accumbens shell. Administration of GABAergic agonist muscimol elicited feeding/approach behaviours from the rostral portion, and defensive rearing behaviour from the caudal portion. These data imply an approach-avoidance antagonism based on differing receptor densities within these regions, based on somewhat differing afferent pathways (Reynolds & Berridge, 2001).
Although distinctions noted here appear to function in the reverse direction as data found in the present study, these data were the result of injections of the inhibitory drug muscimol, rather than quinpirole, which may have excitatory activity in this region. If quinpirole is exhibiting some agonistic effect in this region, rather than the inhibitory effects of muscimol, the findings of the current study are supported: that injections to the ventral-caudal region of the nucleus accumbens shell elicit significantly more 50 kHz vocalizations than other regions. The small number of injections for each region and the volume used may obfuscate more specific findings on this topic, and therefore, future mapping studies may examine rostral-caudal distinctions within the nucleus accumbens shell with injections of selective pharmacological agents.

*Mesolimbic Vocalization Pathway*

Much of the dopamine in the brain is produced in two brainstem nuclei, the substantia nigra and the VTA (Elsinga, Hatano, Ishiwata, 2006). While the substantia nigra projects mainly to areas of motor control (Mercuri, Saiardi, Bonci, Picetti, Calabresi, Bernardi & Borrelli, 1997), the VTA projects to limbic areas associated with motivation and reward, including the amygdala, the hippocampus and nucleus accumbens (Giordano III, Satpute, Striessnig, Kosofsky & Rajadhayaksha, 2006). Electrical stimulation of the VTA elicits 50 kHz calls, as does local injection of drugs of abuse (Burgdorf, Knutson & Panksepp, 2000; Burgdorf & Panksepp, 2006). VTA activation produces increases in endogenous dopamine release in the nucleus accumbens, a state consistent with natural reward conditions, including sucrose preference, food rewards, and mating opportunities (Brudzynski, 2007).
Mesolimbic dopamine may be modulated by a large number of other structures that are interconnected with this system. The accumbens communicates reciprocally to the amygdala, hippocampus, subpallidal area, and prefrontal cortex, all of which may modulate the activity of the accumbens (Sullivan, Talangbay, Einat & Szechtman, 1998; White, Whitaker & White, 2006; Wu, Hrycyshyn & Brudzynski, 1996). The amygdala and prefrontal cortex have significant modulatory effects on locomotor excitation elicited from the accumbens (Sullivan, Talangbay, Einat & Szechtman, 1998).

**Potential Role of D₁ Receptors**

The goal of the present study was to identify the role of the D₂-like receptor system of the nucleus accumbens in eliciting 50 kHz vocalizations in rats. However, there is ample evidence that although the D₂-like receptor system is capable of eliciting these calls, the D₁-like system may also be of importance, possibly within the nucleus accumbens shell or core. Interactions between the D₁-like and D₂-like receptor families have been found to have cumulative effects on rat locomotion (Dreher & Jackson, 1989) and synergistic effects on locomotion in the nucleus accumbens (Gong, Neill, Lynn & Justice, 1999). D₁-like and D₂-like receptor families have also exhibited opposite behavioural effects on locomotion when injected to the habenula (Thornton, Evans & Wickens, 1987; Mogenson & Wu, 1991) and ventral pallidum (Gong, Neill, Lynn & Justice, 1999). The relationship of these two systems together in nucleus accumbens mediated activation of 50 kHz vocalization in rats remains to be elucidated in future research.
Potential Role of Other Neurochemical Systems

Many of the studies investigating 50 kHz rat vocalizations have implicated dopamine as the critical chemical system involved, but other neurotransmitters have been investigated in the brain structures relevant to these calls, and their possible roles must also be considered. Dopamine is considered to be the neurochemical basis of the positive affective state in mammals, whereas acetylcholine is involved in the regulation of the negative affective state (Brudzynski, 2007). Paradoxically, Fendt, Schwienbacher and Schnitzler (2006) found that the non-selective acetylcholine agonist carbachol was able to elicit 50 kHz vocalizations from extremely small dose microinjections to the nucleus accumbens core (4 ng/0.5 μl). However, this study administered carbachol in extremely low doses, 375 times smaller than was required to elicit 22 kHz vocalizations from the medial cholinceptive vocalization strip (Brudzynski, 2007). The premise that nucleus accumbens acetylcholine release can potentiate dopaminergically mediated reward responses has yet to be satisfactorily examined. Although some research has indicated that these systems may be positively functionally linked, this appears to be in the context of locomotion and not reward function (Abercrombie & DeBoer, 1997; Zmarowski, Sarter & Bruno, 2005).

Glutamate is another transmitter of interest. In locomotor studies, the locomotor stimulant effects of amphetamine in the nucleus accumbens were blocked by group I, II, and III metabotropic glutamate receptor agonists without affecting basal locomotor activity (David & Abraini, 2003). Glutamatergic activity also modulated the locomotor effects of D₁-like dopamine agonist SKF 38393, and D₂-like dopamine agonist quinpirole (David & Abraini, 2001).
Glucocorticoids have also been shown to reduce the effect of the dopamine agonists amphetamine, amantadine, quinpirole and bromocriptine on locomotion and hyperactivity in the mesolimbic system (Wrobel, Zebrowska-Lupina & Wielosz, 2005).

Corticotrophin releasing factor type 1 receptor antagonist, antalarmin, also affected the dopaminergic system, reversing the D2 receptor up-regulation in the nucleus accumbens and central amygdala exhibited by rats reared in isolation (Djouma, Card, Lodge & Lawrence, 2006). This effect could extend to a stress hormone based modulation of reward and affective expression.

The role of opioid receptors within the striatum is also yet to be fully examined. Studies have also shown that opioid neurotransmission within the nucleus accumbens shell can modulate the hedonic impact of natural rewards, such as foods (Pecina & Berridge, 2000).

The roles, relative importance, and interactions of each of these systems to 50 kHz vocalization behaviour and its motivational substrates remains a key area for future examination.

Relevance to Human Behaviour

The neurochemical and neuroanatomical underpinnings of 50 kHz rat vocalizations can be of relevance to a variety of human conditions. The issue most commonly associated with studies of reward in animals is drug abuse and addictions. Addictions to many drugs of abuse, and other addictions such as gambling, may be by-products of a function of a natural reward system that has become disassociated with its adaptive environment. Inappropriate, maladaptive stimuli can then elicit powerful drives that instead of promoting adaptive fitness become detrimental or dangerous. These
disruptions are theorized to be the result of mesolimbic dopamine, particularly D_1 receptor interactions with NMDA glutamate receptors, in the nucleus accumbens and amygdala (Kelley & Berridge, 2002). All drugs of abuse have been shown to increase VTA activation and subsequent dopamine release in the nucleus accumbens shell, and many drugs of abuse, including amphetamine and cocaine, alter DAT functionality, which is linked to D_2 autoreceptors (Lee, Pei, Mosczynska, Vukusic, Fletcher & Liu, 2007). An understanding of the role of the D_2-like receptor family in reward processing, as it is associated with the social communication of reward in the form of high frequency vocalizations, can be a potentially useful finding, as noted by Panksepp, Knutson and Burgdorf (2002) in reference to rat vocalizations as potential indices of affective states in addiction experiments.

Chronic applications of quinpirole that produce sensitization have induced functional changes in the nucleus accumbens, amygdala, and frontal cortex, and have been implicated as a potential animal model of obsessive-compulsive disorder (Sullivan, Talangbayan, Einat & Szechtman, 1998; Dvorkin, Perrault & Szechtman, 2006). Fifty kHz vocalizations have been proposed to be primitive analogues of human laughter, and clinical implications of this phenomenon as a research tool have been discussed in reference to attention deficit and hyperactivity disorder (ADHD) and depression research as well as drug abuse (Panksepp & Burgdorff, 2003).

The role of the D_2 receptor subtype in schizophrenia, a disorder characterized mainly by wide scale disruption of dopaminergic signaling in the brain (primarily in frontal and limbic regions), is not yet fully understood. Pharmacological dopamine antagonists can induce the D2 receptor subtype into a conformational change, the D2-low
state, leading to decreased affinity for agonists; dopamine agonists induce the D2-high conformational state, which is associated with increased agonist affinity (Piercey, 1996). Seeman and colleagues (2006) have noted that psychotic symptoms may be mediated by D2-high conformational state activity, a premise further supported by the fact that traditional neuroleptics, as well as modern targeted antipsychotics, act selectively on D2 receptors (Lieberman, 2004). The role of the dopaminergic system in affect may also be relevant to studies of schizophrenia, as flat or inappropriate affect expressed in vocal utterances is a diagnostic symptom of the disorder (American Psychiatric Association: Diagnostic and Statistical Manual of Mental Disorders, p.312), a symptom associated with nucleus accumbens function (Mohanty, Herrington, Koven, Fisher, Wenzel, Webb, Heller, Banich & Miller, 2005). Many symptoms of schizophrenia can also be elicited by amphetamine abuse (American Psychiatric Association: Diagnostic and Statistical Manual of Mental Disorders, pp.223-231), and studies of specific dopaminergic function alterations may benefit research regarding both conditions.

Dopamine functioning is also disrupted in Parkinson’s disease, which is disrupted in both the nigro-striatal pathway, which is associated with motor impairments, and the mesolimbic pathway, which is associated with impairments of motivation (Yokochi, 2007). Presently, the D2 agonist Pramipexole is being used to treat Parkinson’s symptoms (Piercey, Hoffmann, Smith & Hyslop, 1996), indicating that further understanding of the potential relationship between dopamine receptor subtypes may bear upon studies of Parkinson’s and other dopaminergic disorders in future research. Persons with Parkinson’s disease also may experience difficulty with vocalization, a symptom
that may be related to disturbances in animal communicative vocalizations (Ciucci, Ma, Fox, Kane, Ramig & Shallert, 2007).

Finally, an understanding of the functional activity of the nucleus accumbens and local D2-like activity may yield a greater understanding of human emotional expression, and interpersonal variability within that characteristic, regardless of its implications to disordered brain function.

**Limitations**

Some aspects of the present study must be interpreted with caution. Due to the degradation of cannulated receptor sites and some health related attrition, it was not possible for all groups to consist of the same animals. Therefore, the design of the experiment was both a between groups design, such that separate groups with separate animals were compared to each other, and a within groups design, such that animals were used in multiple groups and compared to themselves. Rats were still randomly assigned to groups, and comparisons were made in a counterbalanced order, in order to minimize any biasing effects of individual rats that could have potentially skewed the results. Furthermore, all data was inspected to ensure that no outliers were present, and that all rats were exhibiting a typical baseline response.

The use of ANOVAs as the primary means of analysis of the qualitative characteristics of vocalizations may be threatened by the potentially non-normally distributed samples. These samples represent characteristics of animal behaviour, which may be shaped unevenly by the pharmacological agents administered to the brain, and thus samples that are not normally distributed may be expected. Kolmogorov-Smirnov tests of normality are too sensitive when sample size is large, and therefore are not very
useful as an estimate of sample normality. An examination of the individual sample distributions was inconclusive as to the threat to validity posed by the distributions of the qualitative characteristics of 50 kHz calls. As a precaution, non-parametric statistics were conducted on all parameters of call characteristics (call duration, bandwidth, and peak frequency) in addition to the one-way ANOVAs (Kruskal-Wallis one-way analysis of variance). The results of the non-parametric analyses were similar to the results of the ANOVAs, and so the latter was reported and parametric tests used for follow up analyses. The ANOVAs also yielded a significant result in the Levene's test for homogeneity of variance, indicating a significant deviation from homogeneous sample variances between groups. This was corrected by using a robust test of equality of means, the Brown-Forsythe.

Another area of concern for interpretation of the present study is the atypical results of subsequent administrations of saline vehicle injections. Rats responded as expected to the first exposure to saline injections: 50 kHz vocalization responses that were near the five calls per five minute recoding baseline found by other studies (Thompson, Leonard & Brudzynski, 2006). Subsequent injections of saline vehicle, however, elicited rates of 50 kHz calling that were very high, and these rates remained stable for further intra-cannulae injections of saline. The cause of this high rate of calling after multiple saline injections is unclear. This pattern was not found for injections of any other substance, and rates of calling were not uniformly high for substances that followed multiple administrations of saline. There was no significant difference between saline injections that were the first injection given to each rat, and the counterbalanced saline injections that followed another substance. For all statistical analyses, only the
first administration of saline vehicle was used as a baseline, and subsequent saline injections were ignored.

The double injection procedure also yielded puzzling responses. Although the pattern of drug responses was as expected (i.e. quinpirole that followed saline elicited significantly more 50 kHz calls than saline injected twice, and an equimolar solution of raclopride as a pretreatment returned quinpirole induced 50 kHz calling levels back to baseline), the actual number of calls per five minute recording were much lower than those that followed single injections of quinpirole at the same dose (see Figures 1 and 2). This decrease in gross vocalization rates with double injections is consistent with other studies that have utilized the double injection procedure to induce 50 kHz rat vocalizations (Thompson, Leonard & Brudzynski, 2006). The cause of this effect is not known. One possibility is that this may be a result of the duration of the injection procedure causing increased stress in the animal, or the higher total volume of injection and some effect on the brain tissue.

Future Directions

The current study was exploratory in nature, and as such has led to specific ideas for the future research directions. Studies by Szechtman et al. have demonstrated the importance of delayed responses in quinpirole activity, particularly after acute systemic injections (Eilam & Szechtman, 1989). Since the systemic injections yielded null results, a potential course of future research may be to maintain a 40 to 90 minute delay between s.c. injections of quinpirole and vocalization recording. Studies of quinpirole often dissociate between acute and chronic administrations, and if acute injections of quinpirole
fail to elicit 50 kHz vocalization behaviour, it would be advisable to attempt to record them following chronic quinpirole injections.

Another future direction of research would be to expand the use of pharmacological tools to examine the role of other receptor subtypes. The D₁-like receptor family, in particular, would be of great interest, since these two systems have been shown to interact in a variety of ways, and their relationship in the nucleus accumbens activation of 50 kHz calling behaviour has not yet been studied.

Selective D₃ and D₄ agonists have been quite difficult for biochemists to develop (Kebabian et al), and as such the role of these receptor types in the present results are not known. To examine the possible roles of these receptors, the efficacy of quinpirole to elicit 50 kHz calling could be contrasted with pramipexole, a D₂-like dopamine agonists that has a higher D₃ receptor affinity than quinpirole, as an indirect measure of the influence of the D₃ receptor subtype (Piercey, Hoffmann, Smith & Hyslop, 1996). The D₃ receptor is present in relatively high densities in the nucleus accumbens shell, and is therefore a prime candidate for involvement in 50 kHz calling behaviour (Diaz, Pilon, Le Foll, Gros, Triller, Schwartz & Sokoloff, 2000).

Finally, the D₂ receptor itself still has many aspects to investigate. An examination of the pre-synaptic and post-synaptic receptors influence on elicited vocalizations may be of interest, as well as the influence of D₂-high and low affinity conformational states (Seeman, Schwarz, Chen, Szechtmans, McKnight, Roder, Quirion, Boksa, Srivastava, Yanai, Weinshenker & Sumiyoshi, 2006). Examining the effects of the D₂ autoreceptor on synaptic regulation following dopaminergic stimulation in the
nucleus accumbens, particularly after the injection of high doses of dopamine agonists, would also be of value.

**Conclusions**

From the present study, one can conclude that injections of the D₂-like dopamine receptor agonist quinpirole at the 6 μg/0.2 μl of saline dose directly into the nucleus accumbens shell are capable of eliciting 50 kHz ultrasonic vocalizations in rats relative to saline vehicle injections. This response was found to be reversible with pretreatment injections with an equimolar dose of D₂-like dopamine receptor antagonist raclopride. The vocalization response was similar in magnitude to direct injections of 7 μg/0.2 μl d-amphetamine, which was also reversible with pretreatment with an equimolar dose of raclopride. The 50 kHz vocalization response to quinpirole across a range of doses followed an inverted U-shape pattern, and can thus be said to be dose-dependent, reversible, and statistically greater than saline vehicle injections. The present data suggest that the dorsal region of the nucleus accumbens shell is particularly effective at eliciting 50 kHz vocalizations following local quinpirole injections, however this effect requires further, more elaborate mapping techniques to be properly examined and tested. Although no statistically significant effects on 50 kHz vocalizations were observed following subcutaneous injections of quinpirole at 0.5 and 1.5 mg/kg doses, future studies may be required to further examine this possibility using a wider range of quinpirole doses, and additional experimental procedures, including delayed recording and sensitization effects.
References


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Appendix A
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