Activity of Brainstem Cholinergic Neurons during 22 kHz Ultrasonic Vocalization in Rats

By

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Abstract

Adult rats emit 22 kHz ultrasonic alarm calls in aversive situations. This type of call is a component of defensive behaviour and it functions predominantly to warn conspecifics about predators. Production of these calls is dependent on the central cholinergic system. The laterodorsal tegmental nucleus (LDT) and pedunculopontine tegmental nucleus (PPT) contain largely cholinergic neurons, which create a continuous column in the brainstem. The LDT projects to structures in the forebrain, and it has been implicated in the initiation of 22 kHz alarm calls. It was hypothesized that release of acetylcholine from the ascending LDT terminals in mesencephalic and diencephalic areas initiates 22 kHz alarm vocalization. Therefore, the tegmental cholinergic neurons should be more active during emission of alarm calls. The aim of this study was to demonstrate increased activity of LDT cholinergic neurons during emission of 22 kHz calls induced by air puff stimuli. Immunohistochemical staining of the enzyme choline acetyltransferase identified cell bodies of cholinergic neurons, and c-Fos immunolabeling identified active cells. Double labeled cells were regarded as active cholinergic cells. There were significantly more (p<0.05) c-Fos labeled cells in the LDT of vocalizing animals than in control (non-vocalizing air puffed and naïve non-airpuffed) animals. Although the numbers were low, there were also significantly more (p<0.05) double-labeled neurons in the LDT of vocalizing animals than in the non-vocalizing controls. Such a difference between vocalizing and control animals was not found in the neighbouring PPT nucleus. Results suggest that there are cholinergic and non-cholinergic cells, which are selectively active in the LDT during emission of 22 kHz alarm calls.
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List of Abbreviations

AHPOA: Anterior hypothalamic preoptic area
ANOVA: Analysis of variance
Ch: Cholinergic cell group
ChAT: Choline acetyltransferase
Cy3: Cyanine 3.18
FITC: Fluorescein isothiocyanate
Fos: Protein product of the proto-oncogene c-fos, an IEG
IEG: Immediate early gene
IgG: Immunoglobulin G
LC: Locus coeruleus
LDT: Laterodorsal tegmental nucleus
LH: Lateral hypothalamus
LS: Lateral Septum
LV: Long vocalizing
MPA: Medial preoptic area
NV: Non-vocalizing
PBS: Phosphate buffered saline
PPT: Pedunculopontine tegmental nucleus
REM: Rapid eye movement
SV: Short vocalizing
USV: Ultrasonic vocalization
VTA: Ventral tegmental area
INTRODUCTION

Animal Communication

Communication is a process that allows exchange of information between or among organisms. According to Bradbury and Vehrencamp (1998) communication involves the provision of information by a sender to a receiver, and the receiver decides how to respond based on the information it receives. The signal is the vehicle that provides the information (Bradbury and Vehrencamp, 1998). Smith and Harper (2003) define a signal as an act or structure which alters the behaviour of other organisms (receivers). This alteration in behaviour indicates that the receiving organism has to process the signal and act accordingly for the purpose of survival. According to Brudzynski (2005), all external stimuli arriving to an animal are subjected to a filtering process by specialized sensory and perceptual mechanisms, both at peripheral and central levels. Those stimuli that allow an animal to make a prediction about the environment, and as a result guide their behaviour, are termed biologically significant stimuli or cues (Brudzynski, 2005).

Animals communicate using a variety of signals based on sound and acoustic reception, light and photoreception, chemical substances and chemoreception, or changes in electrical fields and electroreception.

A factor that influences the value of information is signal reliability (McLinn and Stephens, 2006); evidently a signal has more value when the mode of sending the information is reliable. The quality of the signal maybe critical for its guiding effect on the receiver’s behaviour (Brudzynski, 2005). Chemical signals are an important source of information in the life of aquatic animals as visibility in water is often imperfect.
Breithaupt and Eger (2002) found that crayfish release urine as an offensive behaviour because it deters aggression in an opponent, and it also serves to establish dominance as it contains information on the fighting ability of the signaller. In a non-aquatic environment, dance plays a role in the location and profitability of resources to nestmates. Wenner (1968) mentioned that honey bees (Apis mellifera) have a symbolic dance language that is performed by successful foragers, to advertise the location and relative quality and quantity of a food source. This dance recruits other foragers to leave the hive and find this food source with great success.

In dark environments or habitats, sound signals are important as they can be transmitted at any time of day and night and are less affected by environmental conditions (Marler and Hamilton, 1966). Signal reception in this case can occur through barriers and for a considerable distance. For example, a form of alarm signal is the vibratory alarm of young dampwood termites (Zootermopsis angusticollis) (Hill, 2001). These termites bang their heads to produce substrate-borne vibrations to alert nestmates regarding exposure to spores of a fungal pathogen; this induces the nestmates to escape from the source of the stimulus (Hill, 2001). The quality of these signals is quite valuable as they are able to induce responses that are important for survival.

Griffin (1968) mentioned that bats orient themselves by a natural form of sonar; they emit orientation (echolocation) sounds to locate stationary or moving objects at a distance by hearing their echoes and alter their behaviour based upon information conveyed by these echoes. The orientation sounds emitted fall under the ultrasonic range of 20 to 150 kHz (Griffin, 1968). Since bats live in dark caves and visibility is limited, this echolocation behaviour proves to be necessary for orientation. Another reason for this
type of behaviour is to hunt for food and locate insects. Some insects are equipped with an auditory nervous system that responds selectively to these ultrasonic emissions to help evade predatory bats. Bradbury and Vehrencamp (1998) mentioned that in the ear of some noctuids, there are a single pair of sensory cells attached directly to the tympanum, these cells act as pressure detectors and respond to frequencies in the 15 to 125 kHz range. These ear cells detect the ultrasonic echoes and help the insect to determine how much time it has to fly out of the bat’s path or dive for the ground.

Many other species have developed communication in the ultrasonic range of sound frequencies. For example, killer whales (Orcinus orca) emit echolocating clicks that range in frequency from 2 to 35 kHz, to determine spatial location and for the forage of food (Barrett-Lennard et al., 1996). Sperm whales ( Physeter macrocephalus ) (Madsen et al., 2002) and bottlenose dolphins ( Tursiops truncatus ) (Gannon et al., 2005) utilize echolocation as well; in the presence of their prey this form of sonar is used sparingly to minimize any chance of escape. Although some mammals utilize sounds in an ultrasonic frequency range for spatial orientation, ultrasonic vocalization is also a very important form of intraspecies communication. This form of communication has been studied particularly and extensively in rodents since they have distinct call types and certain behaviours associated with the calls.

Ultrasonic Vocalization in Rats

According to Sales and Pye (1974), in 1948, the bank vole ( Clethrionomys glareolus ) was reported to produce high pitched cries that were barely audible to humans, and it was suggested that these cries were used to communicate with members of the same species.
This was one of the first reports about the emission of ultrasonic vocalization (Schleidt, 1948). With the convenience of electronic technology, ultrasonic sounds were easily translated into the range of human hearing, and more reports of ultrasonic vocalization especially in rodents surfaced.

Rats emit ultrasonic vocalization (USV) in a number of behavioural situations. Rat pups are known to emit short calls, with a peak frequency about 40 kHz known as isolation calls, in response to nest, maternal and sibling isolation (Kehoe et al., 2001; Knutson et al., 2002). The isolation calls result in the dam retrieving the pup (Kehoe et al., 2001; Sales and Pye, 1974), indicating that the dam can hear this type of signal and is able to modify her behaviour to include search and retrieval of the pup.

In association with aggressive motivation (Sales and Pye, 1974) and non aversive or potentially rewarding situations, adult and juvenile rats are known to produce high pitched 50 kHz USV. These calls are short in duration and show rapid frequency changes (Knutson et al., 2002; Thompson et al., 2006; Wintink and Brudzynski, 2001). Fifty kHz calls are commonly produced in irregular bursts, and they are also produced in anticipation of a non-agonistic social contact among adult rats (Brudzynski and Pniak, 2002).

On the other hand, in response to aversive stimuli or in stressful situations, juvenile and adult rats are known to emit 22 kHz USV or alarm calls (Brudzynski and Holland, 2005; Brudzynski et al., 1993; Wintink and Brudzynski, 2001); these calls are long and have a lower frequency compared to the USVs emitted by pups or adult rats in a positive affective state.
22 kHz Ultrasonic Alarm Calls

From the biological point of view, 22 kHz alarm vocalization is a component of defensive behaviour directed to members of a colony, to inform them of impending or potential danger such as the presence of a predator (Brudzynski 2001). During the emission of alarm calls, the animal usually exhibits other defensive behaviours such as crouching, freezing, and a general decrease in motor activity (Brudzynski 2001). Twenty-two kHz USVs emitted by adult rats have common acoustic features that are present in all alarm calls. Single call duration ranges from 300 to 3400 ms, call frequency is sustained within a range of 19-32 kHz and the bandwidth of calls is between 1 and 6 kHz (difference between the lowest and highest frequency) (Brudzynski, 2001; Brudzynski 2005). These features are also present in calls that have been induced pharmacologically, as they are indistinguishable from calls that occur naturally (Fu and Brudzynski, 1994; Wintink and Brudzynski, 2001). A striking aspect of alarm calls in adult rats is the duration of the call and the constant frequency maintained around 22 kHz; this potentially has the ability to convey alarming information to other animals in the colony (Brudzynski, 2001). As the acoustic features of alarm calls are not distinguishable when induced pharmacologically or naturally, they are both able to initiate typical defensive behaviour in naïve animals that become exposed to playback of previously recorded 22 kHz calls (Brudzynski and Chiu, 1995).

These alarm calls also serve to carry information about the emitter’s emotional state (Brudzynski 2005; Fu and Brudzynski, 1994; Jelen et al., 2003). Since it is known that 22 kHz USVs are emitted in aversive situations in rodents, it is important to mention that 22 kHz USVs are correlated to anxiety. Jelen et al. (2003) suggested that 22 kHz USV may
be rather a response to general aversive situation than a direct reaction to a stressful stimulus. In general, anxiety is elicited as a result of the presence of potential danger, while fear is elicited in the presence of direct danger (Jelen et al., 2003).

Rats also emit audible sounds, but these sounds are produced when the predator is present, or close to the rat. In laboratory settings the audible sound can be produced when the rat is about to be touched or picked up. According to Litvin et al. (2006), rats emit sonic vocalization as part of a defensive threat response to an oncoming predator, appearing abruptly as the predator approaches to within a meter from the rat. This sonic vocalization is clearly not an alarm call to other conspecifics. Its main function is to discourage further approach from dangerous opponents by threat of defensive attack, while alarm calls are ultrasonic and produce avoidance behaviour in the conspecific recipients (Litvin et al., 2006).

Animals in anxious or negative emotional state do not initiate defensive attacks if the source of danger is some distance away; one pattern of defence in response to a distant or potential threat is risk assessment. Litvin et al. (2006) described risk assessment as a behaviour pattern facilitating assessment of the threat potential of ambiguous threat sources or novel situations. This behaviour is important as it allows the animal to observe its surroundings in a cautious manner, even when a predator is present. During this time other non-defensive activity such as eating and grooming will be minimized to dedicate valuable physiological resources to the risk assessment. A decrease in motor activity is a feature of defensive behaviour, which is the most common. A study by Brudzynski and Chiu (1995), looked at this type of behaviour by investigating the effect of 22 kHz calls (tactile- or carbachol-induced 22 kHz calls) played back to the rats on their general
ergometric activity. Tactile- or carbachol-induced calls consistently and significantly caused a decrease in the general activity of the rats, and the responses to calls were indistinguishable from those in natural situations (Brudzynski and Chiu, 1995).

In a more recent study Burman et al. (2007) examined the effect of playing back conspecific-generated 22 and 50 kHz USVs on an emergence test in rats. The emergence test is a common test of anxiety which measures a rat's behavioural conflict between exploring a novel environment and avoiding an open area (Burman et al., 2007). Previously recorded 22 kHz USVs, induced by applying pressure with a fingertip to the neck of the rat, were played to rats placed in an emergence box in an open arena. Burman et al. (2003) found that significantly fewer rats exposed to 22 kHz USVs emerged from the box into the open arena, and these rats showed a significantly greater latency to emerge than rats exposed to low amplitude background noise. Emergence behaviour was inhibited due to playback of conspecific 22 kHz USV. This type of behavioural response suggests that the animals were in an increased anxious state induced by these calls. Playback of 50 kHz calls did not significantly inhibit emergence behaviour, suggesting no effect on anxiety and a positive or neutral emotional state in the rats. These findings led Burman et al. (2007) to suggest that the low emergence behaviour of rats exposed to 22 kHz USV was simply not a response to the perception of any conspecific-generated USV, but specific to the perception of the 22 kHz USV.

This behavioural role of 22 kHz USV is not acknowledged by some researchers. Blumberg and Alberts (1991) are of the opinion that 22 kHz USVs have no communicatory significance, and that this type of call is a non-adaptive consequence of laryngeal breaking for which we cannot assign a use. The argument relies on the notion
that the larynx is adapted for the control of respiration and that 22 and 40 kHz USV emissions are accidental and reflect the process of enhanced gas-exchange in the lungs with laryngeal breaking as a by-product (Blumberg and Alberts, 1990). The review by Brudzynski (2001) describes 22 kHz calls as a representation of an elaborate process in the production of a specific whistle-like signal with appropriate and prolonged stabilization of the larynx and slow air flow. Another hypothesis put forward by Blumberg (1992) suggests that ultrasonic emission in rodents is the acoustic by-product of physical compression of the thorax during locomotion. If USVs are a by-product of thoracic compression and have no communicatory value, then rats exposed to 22 kHz USVs should not respond with decreased locomotor activity or exhibit low emergence behaviour, and dams should not respond to pup 40 kHz isolation calls by retrieving pups back to the nest. Obviously this has not been the case, as past studies have shown that the three major USVs emitted by rats in various emotional states do have communicatory value, and that animals respond by processing the received information and change their behaviour accordingly.

**Neural Pathways Contributing to Ultrasonic Vocalization**

To understand further the brain control of ultrasonic vocalization, brain structures and neurochemical pathways involved in the production of these calls have to be examined. A study by Fu and Brudzynski (1994) found that intracerebral injections of glutamate and carbachol into the AHPOA of the rat brain resulted in 50 and 22 kHz calls, respectively. The induction of these two types of USVs by the different pharmacological agents showed that these calls have different neurophysiological and neurochemical
mechanisms. Since 50 kHz calls are said to be emitted during potentially rewarding situations, previous research has shown that the neurotransmitter dopamine play a role in the production of these calls. A study by Wintink and Brudzynski (2001) examined the relationship between glutamate and dopamine in the production of 50 kHz calls. Intraperitoneal injections of amphetamine and intracerebral injections of L-glutamate into the anterior hypothalamic-preoptic area (AHPOA) induced 50 kHz calls, although a combination of both drugs did not have an additive effect on vocalization (Wintink and Brudzynski, 2001). A pre-treatment with systemic haloperidol, a dopamine receptor antagonist, strongly decreased glutamate-induced calls to control levels (Wintink and Brudzynski, 2001). This reversal to control levels by haloperidol showed that 50 kHz calls are dependent on dopaminergic activity. Another study by Thompson et al. (2006) demonstrated the role of local dopamine in 50 kHz USV production. In this study, intracerebral injections of D-amphetamine into the nucleus accumbens resulted in a dose-dependent production of 50 kHz calls in adult rats (Thompson et al., 2006). It was also found that the nucleus accumbens shell and ventral core were responsible for the induction of these calls, and that dopamine D₁ and D₂ receptor antagonists given into the same brain site reversed the amphetamine induced increase in 50 kHz calls (Thompson et al., 2006).

According to Burgdorf and Panksepp (2006) the ventral striatal dopamine system is responsible for the production of 50 kHz calls. This ascending dopamine system, the mesolimbic and mesocortical systems that project from the VTA to the ventral striatum and nucleus accumbens, is known to modulate reward-seeking and motivated behaviour (Ikemoto and Panksepp, 1999).
Role of the Cholinergic System in 22 kHz USV

In contrast to the implication of dopamine in the production of 50 kHz calls, the laterodorsal tegmental nucleus (LDT) which has cholinergic projections to basal forebrain areas and septum, was shown to play an important role in the initiation of 22 kHz USV (Bihari, Hrycyshyn and Brudzynski, 2003; Brudzynski, 2001; Brudzynski and Barnabi, 1996). Two major pontomesencephalic areas found in the brainstem that contain cholinergic neurons are the LDT and the pedunculopontine tegmental nucleus (PPT) (Brudzynski and Barnabi, 1996; Losier and Semba, 1993). Most neurons of these nuclei are cholinergic neurons; they synthesize and utilize acetylcholine as a neurotransmitter at the terminals in various brain regions (Butcher, 1995).

Acetylcholine is synthesized in a single step from two precursors: choline and acetyl coenzyme A. This synthesis is catalyzed by the enzyme choline acetyltransferase (ChAT). Since ChAT is present in the cytoplasm of cells, cholinergic cells are easily identified by using ChAT as an immunocytochemical marker (Houser et al., 1983). Previous studies relied upon acetylcholine’s hydrolytic enzyme acetylcholinesterase (AChE) as a histochemical label for cholinergic neurons. A drawback to this method was the lack of specificity; non cholinergic neurons are known to be AChE rich because they can be cholinceptive neurons (Mesulam et al., 1984). As a result, ChAT detection was developed and validated as a histochemical marker, identifying cholinergic neurons that were both ChAT positive and rich in AChE content (Mesulam et al., 1984).

The use of ChAT as an immunolabel marker yielded more information about the detailed distribution of cholinergic neurons in the rat brain. Relying on ChAT histochemistry, Mesulam et al. (1983) identified six main cholinergic cell groups (Ch) in
the rat brain. Each cell group or nucleus consisted of cholinergic neurons that projected to other, sometimes distant regions in the brain. The groups contained neurons within the medial septal nucleus (Ch1), vertical limb of the diagonal band of Broca (Ch2), horizontal limb of the diagonal band of Broca (Ch3), nucleus basalis (Ch4), PPT (Ch5) and LDT (Ch6) (Mesulam et al., 1983).

The targets of these cholinergic cells groups included the hippocampus, which is innervated by Ch1-2, the olfactory bulb which is innervated by Ch3 and the amygdala and neocortex which are both innervated by Ch4 in the rat (Mesulam et al., 1983) and monkey (Mesulam et al., 1984). Ch5 and Ch6 neurons located in the pontomesencephalic reticular formation provide the major source of cholinergic innervation to the thalamus (Mesulam et al., 1983 and Mesulam et al., 1984). Furthermore, Schafer et al. (1998) mentioned cell groups that employ acetylcholine as a neurotransmitter and promote homeostatic functions of the nervous system, and these groups are included in the ascending reticular activating systems of the brainstem, as well as in the forebrain, which play a role in attention, arousal and memory. Also cholinergic projections to cortical and limbic structures are critical for various cognitive abilities and may serve a modulatory function in cognition by optimizing cortical information processing and influencing attention (Katz-Brull et al., 2005). Therefore activities that depend on selective attention, which is a component of conscious awareness, are modulated by the cholinergic system. Finally, there is also the striatal interneuronal system involved in extrapyramidal motor function with the cholinergic neurons located in the dorsal striatum without long-distance connections.
The PPT (Ch5) cholinergic neurons are said to play a crucial role in the genesis of rapid eye movement (REM) sleep. Cholinergic stimulation of the PPT elicits a complete array of REM phenomena including muscle atonia (Honda and Semba, 1995; Verret et al., 2005). The activity of the PPT cholinergic neurons is said to increase their firing rate during synchronization of fast rhythms in waking as well as in REM sleep (Kobayashi et al., 2004). Majority of the PPT neurons projecting to the thalamus, release acetylcholine which is important in controlling behavioural state (Ainge et al., 2004). Lesions in the PPT result in deficits in complex associative processing and in attentional and reaction time performance (Ainge et al., 2004). Within cholinergic projections to the thalamus, the PPT does play a critical role in cortical arousal. In this study, the PPT was chosen as a control structure with numerous cholinergic cells which do not project to the medial cholinoreceptive vocalization strip.

Role of Laterodorsal Tegmental Nucleus in Ultrasonic Vocalization

Previous studies have shown that cholinergic neurons in the LDT (Ch6) have ascending projections to the ventral tegmental area (VTA) (Yeomans and Baptista, 1997), anterior hypothalamic, preoptic and septal regions (Brudzynski, 2001; Brudzynski and Barnabi, 1996), tectum and basal ganglia (Bihari et al., 2003). Oakman et al., (1999) also pointed out that LDT neurons project to structures associated with the limbic system, such as the anterior and midline thalamic nuclei. PPT cholinergic neurons also project to the substantia nigra (SN) and sites involved in sensorimotor function, and they also have descending targets to pontine and medial pontine reticular formation areas and the cranial nerve nuclei (Oakman et al., 1999).
When LDT cholinergic target sites, such as the lateral septum and MPA, are cholinergically stimulated, 22 kHz vocal responses are elicited. Studies have shown that the administration of carbachol, a muscarinic agent, into the hypothalamic/preoptic area (Brudzynski and Bihari, 1990) and septum (Bihari et al., 2003) can initiate 22 kHz calls. The vocal responses from these forebrain targets led to a map of cholinceptive neural structures that stretched from the tegmentum to the preoptic area and septum (Brudzynski, 2001). This stretch of tissue is collectively known as the medial cholinceptive vocalization strip (Figure 1, Brudzynski, 2001). The ascending projections of the LDT and a few from the PPT have been suggested to be involved in limbically driven behaviour, arousal and behavioural activation. In the case of cholinergic stimulation, emotional arousal is manifested by species-typical alarm vocalizations (Brudzynski et al., 1998). An electrophysiological study by Brudzynski et al (1998) showed a decline in the mean firing rate of anterior hypothalamic-medial preoptic region cells as a result of LDT electrical stimulation or direct treatment of hypothalamic-preoptic neurons with the muscarinic agent carbachol. It was hypothesized that as a result of the cellular inhibition in the medial forebrain, defensive behaviours manifest themselves, such as freezing, crouching and the emission of 22 kHz alarm calls.

Another study by Brudzynski and Barnabi (1996) investigated the role of the ascending cholinergic projection from the ponto-mesencephalic cholinergic nuclei to the mediobasal hypothalamic-preoptic region in the production of 22 kHz USVs. L-glutamate, an excitatory amino acid, was injected into the LDT (source of the projection), or a muscarinic agonist, carbachol, was injected into the AHPOA (target site). As a result, comparable 22 kHz alarm calls were induced in both cases (Brudzynski and Barnabi, 1996).
A pre-treatment with scopolamine, a muscarinic antagonist, in the AHPOA attenuated vocalization induced by LDT stimulation with glutamate (Brudzynski and Barnabi, 1996). This result indicates that the LDT does play a role in initiating 22 kHz USVs, as glutamate stimulation of the LDT activates all cells in the LDT, and blocking cholinergic postsynaptic receptors in the AHPOA decreases the glutamate induced USV response from the LDT.

Furthermore, to examine the role of the ascending mesolimbic cholinergic projection from the LDT in production of 22 kHz USV, a study by Bihari et al. (2003) sought to compare vocalization induced by septal carbachol stimulation and by glutamate stimulation of LDT cells. Carbachol injections into the lateral septal area and glutamate given into the LDT both induced 22 kHz USVs (Bihari et al., 2003). Pretreatment of the septum with the muscarinic antagonist scopolamine, antagonized 22 kHz USVs induced by glutamate injections into the LDT (Bihari et al., 2003). Anterograde axonal tracers revealed that the LDT directly projected to the lateral septum. These anatomical results further suggested that the ascending mesolimbic cholinergic system plays a role in the initiation of 22 kHz USVs (Bihari et al., 2003).

Interestingly, it has been suggested that LDT and PPT cholinergic projections to the VTA is important for reward (Yeomans and Baptista, 1997; Rada et al., 2000). This reward pathway is said to arise in the midbrain tegmentum, where dopaminergic cells project to the nucleus accumbens (Rada et al., 2000) and substantia nigra (Yeomans and Baptista, 1997). The study by Rada et al. (2000) investigated the release of acetylcholine in the VTA by examining self-stimulation and ingestive behaviours. Rats in this study were trained to self-stimulate the lateral hypothalamus (LH), and microdialysis probes
inserted in the VTA collected cerebral fluid for acetylcholine analysis (Rada et al., 2000). The results showed that LH self-stimulation and the ingestion of food and water caused a release of acetylcholine in the VTA, and these behaviours were inhibited by the muscarinic antagonist, atropine (Rada et al., 2000). Rada et al. (2000) suggested that acetylcholine release from the midbrain cholinergic cells may stimulate dopamine release in the nucleus accumbens. The outcome of this study suggests the presence of another pathway originating from the LDT that participates in reward. This study highlights the complex circuits of the brain and how emotionally opposite pathways can arise from the same nucleus.

Nonetheless, the cholinergic neurons of the LDT are clearly important in the production of 22 kHz USVs, as demonstrated by many of the previous studies. However, there is no direct evidence about association of their activity with the emission of ultrasonic calls. The current study seeks to verify the activity of the LDT cholinergic neurons during the emission of 22 kHz ultrasonic calls.

**Neuronal Activation Indicated by c-Fos Protein**

A review by Kovacs (1998) mentioned that the expression of c-Fos, a 55 kDa protein, can be induced by a number of factors: neurotrophic factors, neurotransmitters, depolarization and elevation of intracellular/intranuclear calcium. Under basal conditions, c-fos mRNA and protein levels are very low. After an acute challenge, levels of c-fos mRNA are increased within minutes, and these levels peak after 30 minutes (Kovacs, 1998). This rapid induction of Fos protein and its gradual fading from the cell nucleus after a few hours is in agreement with Herrera and Robertson (1996).
Since the *c-fos* gene is located in the nucleus, what mechanism leads to its increase in expression? Stimulated progenitor cell proliferation may serve as an example. Larocca and Almazan (1997) reported that carbachol treatment in oligodendrocytic progenitors resulted in increased progenitor proliferation and activation of *c-fos* gene expression. The mechanism that promotes this type of activity was unclear, and Larocca and Almazan (1997) examined whether mitogen-activated protein kinases (MAPK) (intracellular proteins) were activated as a result of muscarinic receptor stimulation in oligodendrocyte progenitors. Carbachol stimulation resulted in a downstream activation of MAPK p42, also known as extracellular signal regulated kinase 2 (ERK2), in the cell cytoplasm but not in the nucleus (Larocca and Almazan, 1997). ERK2 when phosphorylated serves as an intermediate between the cell nucleus and cytoplasm (Fumagalli et al., 2005). ERK2 upon activation is translocated into the nucleus where it targets transcription factors such as cAMP response element binding protein (CREB) and ets-like gene-1 (Elk-1) and initiates the transcription of immediate early genes such as *c-fos* (Ambrosini et al., 2000). The transcription of the *c-fos* gene in the nucleus results in a protein product that can be used as a quantifiable tool to study neuronal activation.

*c*-Fos protein level is low under basic conditions, but can increase to high levels following stimulation, which is why it has been used as a neuronal marker to identify active neurons in many parts of the brain in response to a variety of stimuli. For example, *c*-Fos was used to locate active neurons in the mesencephalic locomotor region in the rat brain following an intrasubpallidal injection of picrotoxin (Brudzynski and Wang, 1996), various brain regions in rats after exposure to alarm pheromones (Kiyokawa et al., 2005).
and also to identify brain regions that participate in the defence response elicited by exposure to a continuous 20 kHz ultrasound tone (Beckett et al., 1997).

Since c-Fos is a nuclear protein, it allows easy detection of other markers that target the cytoplasm with standard double label techniques, and it also allows the phenotype of the activated neuron to be identified (Hoffman, Smith and Verbalis, 1993). Another advantage with using c-Fos as a tool for studying neural mechanisms is that its expression peaks from 30 minutes to two hours after a stimulus is delivered, and this delay allows the investigator to handle the animal prior to euthanasia without apprehension that such handling will induce non-specific Fos expression (Hoffman, Smith and Verbalis, 1993).

A study by Ainge et al. (2004) mentioned some disadvantages to using c-Fos as a marker for neuronal activity. A major drawback is that c-Fos cannot be used to mark cells with a net inhibitory synaptic or transcriptional drive. Other drawbacks are the varied threshold for c-Fos induction throughout the brain and the induction of c-Fos by a wide variety of factors including handling and circadian rhythms (Ainge et al., 2004). For the present study, these drawbacks will be taken into consideration, and appropriate immunohistochemical controls will be used to avoid non-specific induction of the IEG.

c-Fos is regarded as one of several IEGs that link external cellular signals with phenotypic changes in brain cells (Herrera and Robertson, 1996). Since c-Fos can be activated by a varied group of stimuli and it plays an important role in the study of neuronal activation in different brain regions, it will be applied to the present study. c-Fos will be used as a tool to determine the population of active cholinergic cells in the LDT and overall neural activity of the LDT compared to that of the PPT and control animals.
Rationale

Previous studies have demonstrated that direct stimulation of the LDT (a source of cholinergic cells) with glutamate (Bihari et al., 2003; Brudzynski and Barnabi, 1996) and intracerebral injection of carbachol into the lateral septum (Bihari et al., 2003) and AHPOA (postsynaptic cholinceptive fields) (Brudzynski, 1994; Brudzynski and Barnabi, 1996; Brudzynski and Bihari, 1990) initiated 22 kHz ultrasonic alarm calls in rats. A study by Savoy (2005) induced 22 kHz calls by carbachol injection into the medial AHPOA or by air puff stimulation. As a result, characteristic 22 kHz alarm-type vocalizations were elicited in either case. A subsequent immunohistochemical analysis yielded a significantly higher number of Fos labeled nuclei identified by a c-Fos protein marker and active cholinergic cells identified by a choline acetyltransferase (ChAT) marker in the LDT of vocalizing rats compared to non-vocalizing controls of carbachol treated rats (Savoy, 2005).

Double labeled cells in the LDT were found in the vocalizing rats after intracerebral injection of carbachol but not in the vocalizing animals in response to air puff. In that study, air puff stimuli were relatively weak and induced 22 kHz vocalizations which lasted for a short time. The goal of the present study is to repeat air puff stimulation with higher air pressure and for a longer time, and elicit longer vocal responses. Such an approach is predicted to aid in the visualization of double-labeled neurons in the LDT. In the present study, 22 kHz vocalization was induced by a non-painful and non-invasive air puff procedure. Following the vocalization response, activity of LDT neurons was measured by histochemical detection of c-Fos protein, and the cholinergic nature of the LDT neurons was demonstrated by labelling for choline acetyltransferase (ChAT). Cells
that were double labeled for c-Fos and ChAT are regarded as active cholinergic cells. This approach will ultimately lead to a better understanding of the role of cholinergic LDT cells in the production of 22 kHz ultrasonic alarm calls.

**Hypotheses**

The testable hypothesis is that activity in cholinergic cells in the LDT underlies 22 kHz alarm calls. This leads to the prediction that cholinergic cells of LDT nucleus will be active during emission of 22 kHz vocalization induced by air-puff stimulation. Rats that do vocalize for longer than 20 minutes up to one hour will demonstrate higher levels of cellular activity in the LDT than rats vocalizing for a shorter time and non-vocalizing controls. This will be indicated by a large number of Fos labelling in the LDT. It is also predicted that long vocalizing animals will demonstrate a greater number of active cholinergic (double labeled) cells in the LDT than non-vocalizing controls (air puffed or naïve non-airpuffed controls).
METHODS

Animals

The study was performed on 38 adult male Wistar rats (Charles River, St. Constant, Que., Canada), weighing 260-400 g. After arrival, animals were housed in pairs in clear polycarbonate cages with dust free corn cob bedding (The Andersons Industrial Product Group., Ont., Canada) and dark plastic hiding tubes. Room temperature, air flow and humidity were maintained constant with a 12:12 hour dark/light cycle. Rats had access to standard pelleted chow (Lab Diet, Oakville, Ont., Canada) and water ad libitum. The animal handling and experimental procedures outlined in this study all adhered to the guidelines set out by the Brock University Animal Care and Use Committee, and were in agreement with the guidelines of the Canadian Council for Animal Care.

Experimental Design

Prior to air puff conditions, rats were regularly handled (a minimum of two to three minutes for three to five days) and gradually habituated to human touch. The animals were divided into four groups depending on their response to initial air puff stimulation. The group conditions were: a) non-vocalizing (NV), i.e. air puff followed by no vocalization, b) short vocalizing (SV), i.e. air puff followed by vocalization lasting up to 30 minutes, c) long vocalizing (LV) i.e. a minimum of 40 or more minutes of vocalization induced by air puff stimulation, and d) non-stimulated or control animals.
A. Behavioural Study

Air Puff Stimulation

This type of stimulation is gentle and non-painful, and it is devoid of such harsh stimuli as those associated with aggression, threat or electrical shock (Knapp and Pohorecky, 1995). Their application is, however, not pleasant and unpredictable for rats, and it causes aversive response. Each animal was placed in a plexiglass recording cage (25 cm x 18 cm x 18 cm). The walls and ceiling of the cage were padded with sound-attenuating material, and the inner surface of the cage was lined with absorbent paper. This lining was changed after each animal. Air puff parameters were set according to the study by Knapp and Pohorecky (1995), the air puff pressure was between 50 psi (3.5 kg/cm²) and 55 psi (3.9 kg/cm²), and a distance of approximately 15 cm was kept between the air tube and rat. The rats were stimulated at 15 to 20 s intervals with air puffs (provided by an Airstim Unit, San Diego Instruments Inc, San Diego, CA) directed at the nape until alarm calls were induced or until 15 air puffs had been delivered, whichever came first. An air puff was delivered again when the rat ceased emitting 22 kHz USV, but no more than the application of 15 consecutive air puffs, then the recording session was concluded. The animals were then returned to their home cages. Depending on the animal's response, each session lasted from 4 to 60 minutes.

Recording and Analysis

Alarm calls were recorded by an ultrasonic microphone model SM-1 with a working frequency range of 10 to 180 kHz (Ultrasound Advice, London, UK), which was mounted on the sidewall of the cage, approximately 2 cm above the floor surface. Signals
from the microphone were fed to an S-200 bat detector set at 1/16 frequency division ratio (QMC Instruments, London, UK) and the broadband detection and then from the bat detector to a standard tape recorder (NexxTech CTR-121, Orbyx Electronics, Concord, Ont., Canada). Signals from the tape recorder were subsequently analyzed manually using a sonograph (DSP Sona-Graph signal analysis work station, model 5500, Key Elemetrics Corp., Pine Brook, NJ). Individual alarm calls were analyzed by manipulating sonograph cursors to obtain detailed information about call duration in milliseconds (ms), sound frequency and bandwidth in kHz. Length of individual calls was achieved by reading the time span between two cursors positioned at the beginning and end of each call. Call bandwidths were measured by the frequency difference between two horizontal cursors positioned at the lowest and highest frequency for each vocalization. The peak frequency for a call was regarded as the strongest component expressed in decibels (−dB) measured from the top of the scale of the power spectrum within each call's spectrum. For each sonographic analysis, the first 20 calls in a given session were analyzed.

B. Histochemical Procedure

Tissue Preparation

Ninety minutes after completion of each air puff experiment, each animal was euthanized with an overdose of intraperitoneal sodium pentobarbital (Bimeda-MTC Animal Health Inc, Cambridge, Ontario, Canada). This survival time was determined to be suitable for euthanasia after the preliminary study of maximum Fos protein expression (Savoy, 2005). As soon as breathing stopped, the animals were immediately transcardially perfused with a fixative containing paraformaldehyde (4%) and picric acid
(15%). The brains were extracted following perfusion and post-fixed for two hours at room temperature. For cryoprotection, each brain was immersed in a 10% sucrose solution (made up in a 0.1 M PBS solution) at 4°C overnight, followed by a 30% sucrose solution (in 0.1 M PBS) for 48 hours at 4°C. Cryoprotected brains were rinsed in PBS and bound to a metal disc by mounting medium (Tissue Tek, Miles Scientific, Naperville, IL). Brains were cut into 25 μm sections in a cryotome (Model 2800 Frigocut, E, Reichert-Jung) set to -20°C. Tissue sections from the LDT and PPT were taken from each animal and stored in Eppendorf tubes containing 500 μl of 0.1 M PBS and maintained at 4°C. Sections were processed for choline acetyltransferase (ChAT) and Fos immunohistochemistry.

**Immunohistochemistry**

LDT and PPT sections collected from each animal underwent immunohistochemical procedures designed to detect both Fos protein and ChAT. The sections were rinsed in PBS before being placed in a permeabilization solution containing 4% Triton-X-PBS and 10% normal donkey serum and agitated for 45 minutes. Tissue sections were washed three times with PBS, and incubated in anti-Fos primary antibody (H-125, rabbit anti-c-Fos polyclonal antibody, Santa Cruz Biotechnology, Santa Cruz, CA) at a dilution of 1:100, overnight at 4°C. Sections were washed three times with PBS and incubated in a second primary antibody for ChAT (AP180F, goat anti-ChAT, Chemicon International, Temecula, CA) at a dilution of 1:200 for 24 hours at 4°C.

The sections were incubated with the secondary antibodies for Fos (Cy3 conjugated donkey anti-rabbit IgG, Chemicon International, Temecula, CA) and for ChAT
[fluorescein (FITC) conjugated donkey anti-goat IgG, Chemicon International, Temecula, CA], both at a dilution of 1:200 for 40 hours at 4°C. After this incubation, the sections were washed meticulously and were mounted on poly-L-lysine (Sigma) coated microscope slides. A SlowFade-Light antifade reagent (Cedarlane Laboratories Ltd., Hornby, Ont) was applied directly to the sections, and a cover slip was placed over the sections. The cover slip was sealed to the microscope slide with the application of nail lacquer around the edges of the cover slip, to prevent the tissues from drying out.

**Antibody and Anatomical Specificity**

To control for the occurrence of false positives, where staining appears in instances where there is no specific protein, measures were taken to determine antibody binding specificity for c-Fos and ChAT. According to Butcher (1995), the mesopontine cholinergic complex has the largest collection of cholinergic cells in the brainstem, and it is composed prominently of cholinergic perikarya in the PPT and LDT. Savoy (2005) also showed that LDT of carbachol-induced vocalizing animals demonstrated ChAT labeled cells as well as Fos labeled cells. With this information, sections from the LDT were used to determine histochemical and anatomical specificity. Four separate LDT tissue sections were obtained from a long vocalizing animal (LV 70), and after every section, one of the four antibodies was not applied as a control. Standard protocol was followed for the other three sections. For instance, the first section was incubated with anti-c-Fos, anti-rabbit IgG and anti-goat IgG but not anti-ChAT, the following section was incubated without anti-c-Fos, and the section following lacked anti-rabbit IgG (Table 1 (Savoy, 2005)). The sections were examined with the fluorescent confocal microscope
set to FITC and Cy3 excitation and emission filters to determine antibody specificity, as fluorophores are optically visible with the appropriate filter set.

Since c-Fos has low basal level expression, another structure was employed to determine Fos anatomical and histochemical specificity. The rostral portion of the far lateral hypothalamus is known to have cholinergic neurons (Butcher, 1998). c-Fos expression seen in this region in addition to ChAT expressing cells, made this structure an ideal positive histochemical control. The neighbouring medial hypothalamic areas served as the negative control.

<table>
<thead>
<tr>
<th>Control</th>
<th>Primary Fos Ab</th>
<th>Secondary Fos Ab</th>
<th>Primary ChAT Ab</th>
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**Table 1:** Excluded staining steps to determine histochemical specificity of c-Fos and ChAT antibodies.
**Fluorescent Confocal Microscopy**

The confocal microscope (CI Laser Confocal Microscope, Nikon Inc., Mississauga, ON) was used to examine microscope slides prepared from each experimental animal. The fluorophore-conjugated secondary antibody FITC has an excitation maximum of 496 nm and an emission maximum of 518 nm, while Cy3 has an excitation maximum of 554 nm and emission maximum of 565 nm. These secondary antibodies have to be efficiently excited to yield optimum structural labels. The main dichroic excitation lasers of the confocal microscope are 488 nm (Argon), 543 nm (Helium/Neon) and 633 nm (Helium/Neon) respectively. The settings of the dichroic emission filters was similar to those of Savoy (2005). The first emission filter was set to 530 nm with barrier filters set at 515/530 nm for FITC, and the second dichroic emission filter was set to 625 nm with barrier filters set to 585/540 and 665 LP for Cy3.

Brain areas corresponding to the PPT and LDT (Figure 2) were scanned, and the labelling pattern was noted, as only cholinergic cells were clearly labeled green. Two fields of view of $2.5 \times 10^5 \mu m^2$ were scanned to allow for an accurate representation each of the LDT and PPT nuclei. From an individual 25 $\mu m$ tissue section, an individual scan of 1.08 $\mu m$ was selected for analysis. The selection of scan depth was useful when trying to determine whether Fos labeled nucleus was located in the nuclear space of a cholinergic neuron and not above or below the neuron.
Figure 2: Schematic diagram showing coronal sections of the rat brain. Boxes identify scan areas in the midbrain stereotaxic planes AP=1.00 mm for the pedunculopontine tegmental nucleus (PPT) (a), and AP=0.20 mm for the laterodorsal tegmental nucleus (LDT) (b) according to the atlas by Paxinos and Watson (1986). Abbreviations: Aq: aqueduct, CnF: cuneiform nucleus, DRV: dorsal raphe nucleus, ECIC: external cortex of the inferior colliculus, PnO: pontine reticular nucleus, oral part, py: pyramidal tract, RtTg: reticulotegmental nucleus of the pons, scp: superior cerebellar peduncle, VLPAG: ventrolateral periaqueductal gray. Bar: 1 mm.
**Manual Quantification of c-Fos and ChAT Expression**

Digital images of the LDT and PPT scans were obtained by the EZ-C1 software (version 3.2, Nikon Inc., Mississauga, ON). C-Fos and ChAT labeled cells were manually counted based on the following particular criteria: a) ChAT labeled (green) cells are part of a large population of similarly labeled cells within a nuclear border according to a stereotaxic atlas (Paxinos and Watson, 1986), b) labeled cell colour intensity is brighter than background and c) ChAT labeled cell structure was relatively spherical with a dark space for unlabeled nuclear material close to the cell’s centre. The criteria for Fos labeled cells were: a) Fos labeled nuclei were relatively spherical or elliptic and b) the size of nuclei was proportionally smaller than that of the entire cell. The criteria for a double labeled cell were: a) a ChAT (green) labeled cell with a Fos (red) labeled nucleus within the cell body and b) the Fos labeled nucleus was within the darker nuclear space of the ChAT cell.

**Statistical Analysis**

A Kruskal-Wallis One-Way analysis of variance (ANOVA) was performed to test for statistical significance across experimental groups for c-Fos expression and active cholinergic cells in the LDT and PPT, followed by a Mann-Whitney U analysis to reveal the differences between groups. Where appropriate, a Chi square ($\chi^2$) test was used to determine differences between experimental groups. Results were considered significant if a p value of less than 0.05 was achieved. All data analyses were performed using Instat (version 3.05, Graphpad Software Inc., San Diego, CA).
RESULTS

A. Behavioural Response

Animal Responses to Air Puff

The control group consisted of eight animals, and the air puffed non-vocalizing group was made up of eight animals as well. Depending on response time, nine animals that vocalized for less than 30 minutes were selected for the short vocalizing group (SV, n=9), and 13 animals that vocalized for 40 minutes up to one hour were selected for the long vocalizing group (LV, n=13).

USVs were induced as a result of the air puff stimuli to the rat’s. Animals in the vocalizing category (SV and LV) exhibited characteristic defensive freezing, crouching and an absence of locomotor activity and exploratory behaviour during vocalization. Animal behaviour was similar to that reported by Brudzynski and Holland (2005), Knapp and Pohorecky (1995) and Savoy (2005). Animals in the LV group often did not resume exploratory behaviour or locomotor activity for a long period of time; they found a “home corner” in the recording chamber and remained there in a crouched position. Figure 3 illustrates a typical ultrasonic alarm call induced by air puff stimuli. This particular call lasted for 1034 ms, and the peak frequency was 24.3 kHz.

Acoustic Parameters of Vocalization Induced by Air Puff

All calls emitted by animals in the vocalizing category (SV and LV, n=22) were collected and analyzed. The average acoustic values fit the parameters recognized for 22 kHz USV. The average sound frequency from 22 animals varied between 20.7 and 24.4
kHz (Figure 4a), with an average of 22.76 +/- 1.08 kHz (SEM). The average duration of calls lasted from 386.4 to 1751 ms (Figure 4b), with an average of 988.73 +/- 0.386 ms (SEM). Finally the average bandwidth was between 2.5 and 5.4 kHz, with an overall average of 3.26 +/- 0.22 kHz (SEM) (data not shown).
Figure 3: A sonogram of a 22 kHz ultrasonic alarm call (thick horizontal line) induced by air puff stimulation. Call frequency [kHz] is shown over time [s]. The sound frequency and duration for this call are 24.3 kHz and 1.034 s. The short fragment (line on the left) represents the end of a previous call. Two blank areas represent the background noise between calls. During the call, the noise is electronically suppressed.
Figure 4: Acoustic parameters for USVs induced by air puff. (a) Bars depict average frequency (kHz) of single call per rat and (b) bars depict average duration (ms) of single call per rat. n=22. Bars are arranged in random order based on the sequence of observations.
B. Immunohistochemical Analysis

*Immunohistochemical Controls – Anatomical and Histochemical Specificity*

Antibody specificity was determined with several controls using sections from the LDT of a long vocalizing animal (Table 1). In the c-Fos and ChAT labelling procedure specific steps were excluded from the protocol. Figure 5a shows a confocal image scan of the LDT lacking the primary ChAT antibody. The image, although bright as a result of FITC application, shows that the altered immunolabeling protocol did not label any cells. Figure 5b also has a similar deficit in specific labelling, and it is darker due to the exclusion of FITC, a ChAT conjugated secondary antibody against rat, from the antibody protocol. Figure 5c shows a confocal image of the LDT that underwent the standard labelling protocol with both primary and secondary antibodies for ChAT staining. In this figure, ChAT immunopositive neurons are labeled green, and the cells are almost spherical, with a black space in the centre indicating the nuclear space. In Figure 5d, the primary c-Fos and secondary Cy3-conjugated (Figure 5e) antibodies were omitted from the protocol. Both images show non-specific binding to the antibody present, no cell nuclei and varying levels of brightness. Figure 5f shows a confocal image of the LDT that followed the complete immunohistochemical protocol with several Fos labeled nuclei.

A positive control step was performed with sections of the far LH (Figure 6), where some cholinergic cells were expected, using the standard histochemical protocol. Fluorophores in both ChAT and c-Fos immunopositive cells emitted fluorescence accordingly. These immunohistochemical controls showed that the antibodies labeled some cholinergic cells, as expected. The relevant antibodies consistently labeled ChAT and Fos cells present in the structure examined, and labeled cells were clustered in the
area which corresponds to the anatomical location of the far LH nucleus. There were no ChAT-labeled cells in the neighbouring nuclei. Double labeled cells had red nuclei located in the nuclear space of the ChAT stained cells (Figure 6b, bottom). Cholinergic cells in the far LH project most probably to the cortex, and were also active during vocalization. Due to the control measures taken, the probability of falsely including non-ChAT cells or Fos positive cells was negligible.
Figure 5: Confocal images of the LDT of a vocalizing animal (LV 70) following immunohistochemical controls for anatomical and antibody specificity. Specific steps in antibody protocol were excluded. ChAT primary antibody (a) and FITC-conjugated secondary antibody (b) application were excluded. C-Fos primary antibody (d) and its secondary, conjugated fluorophore, Cy3 (e) application were excluded. Images of standard protocol of ChAT staining (c) and c-Fos (f) label. Bar: 50 μm.
Figure 6: Coronal section of rat brain showing the lateral hypothalamus (LH) according to the atlas by Paxinos and Watson (1986) (a). Confocal images of the LH following unaltered labelling protocol. Cholinergic cells (green) and c-Fos (red) (b). Bottom image is an EX Cl superimposed version of double labelling, indicated by white arrows. Bar: 50 µm.
c-Fos Expression in LDT

Air puff stimuli at 50 to 55 psi caused an increase in the number of c-Fos labeled cells in the LDT of vocalizing rats (Figure 7). To examine whether any difference existed between vocalizing and non-vocalizing animals in general, data from the control and non-vocalizing animals were combined to make a non-vocalizing category, and data from the short vocalizing and long vocalizing animals were combined into the vocalizing category. A Mann-Whitney test was performed to determine if a statistical significance existed between the non-vocalizing and vocalizing experimental groups. The test revealed a statistical significance (U=91, p<0.01); the vocalizing condition had significantly more Fos labeled cells in the LDT than the non-vocalizing experimental condition (Figure 7a).

A Kruskal-Wallis One-Way ANOVA test determined that a significant difference existed across the experimental conditions (KW = 17.1, p<0.001, Figure 7b). A Mann-Whitney U test revealed that the difference between the control and air puffed non-vocalizing groups was not statistically significant (U=13.5, p=0.059), but approaching the level of significance. A Mann-Whitney U test indicated that the long vocalizing group had significantly more (U= 7.0, p<0.0004) Fos labeled LDT cells when compared to the control group. Further Mann-Whitney U tests revealed that the long vocalizing group had significantly more (U= 18.0, p<0.01) Fos labeled cells in the LDT than the non-vocalizing group. When compared to the short vocalizing group, the long vocalizing group also had significantly more (U=14.0, p<0.002) Fos labeled cells in the LDT nucleus. No significant differences existed between the control and short vocalizing groups or the non-vocalizing and short vocalizing groups (Figure 7b). Figure 8 illustrates an example of Fos labeled nuclei in the LDT nucleus. The increase in LDT Fos
expression in long vocalizing and vocalizing animals, but not in the non-vocalizing controls, implies that there is a link between LDT Fos activity and vocalization.

**c-Fos Expression in PPT**

This increase in Fos expression was also present in the PPT of vocalizing animals that received the air puff stimuli (Figure 9). A Mann-Whitney U test was performed to determine whether any significant difference existed between the non-vocalizing and vocalizing categories. There were significantly more Fos labeled cells in the PPT of vocalizing animals than non-vocalizing animals (U=99.5, p<0.02; Figure 9a).

A Kruskal-Wallis ANOVA test revealed a significant difference across experimental conditions of Fos labeled cells in the PPT (KW=14.0, p<0.003). Furthermore, a Mann-Whitney U test was used to determine if any significant difference occurred between groups. There were significantly more Fos labeled cells in the PPT of non-vocalizing animals than in controls (U=12.0, p<0.04). Long vocalizing animals had significantly more Fos labeled PPT cells than control animals (U=6.0, p<0.0003), and more than short vocalizing animals (U=24.5, p<0.03) (Figure 9b). No significant differences existed between the non-vocalizing and long vocalizing animals or between short vocalizing and control animals (Figure 9b). Illustrated in Figure 10 is a confocal image of PPT Fos labeled cells.

Further analysis was performed to determine the difference in Fos expression between the LDT and PPT. A Kruskal-Wallis test revealed significant differences across the experimental conditions in the LDT and PPT (KW=22.2, p<0.0001) (Figure 11). A Mann-Whitney U test revealed that there was a significant difference between the number
of c-Fos labelled cells in the LDT and PPT in the vocalizing category (two dark orange bars, \(U=101.0, p<0.001\)), and no significant differences existed between these nuclei for the non-vocalizing category (two light bars, Figure 11). Thus, the results indicated there is more c-Fos activity in the LDT of vocalizing animals than non-vocalizing animals. These results imply that there is a stronger link between the cholinergic cells of the LDT and vocalization than cholinergic cells of the PPT, and that the PPT might not be critical in the production of vocalization.
Figure 7: Bar graphs illustrating the average number of air puff induced c-Fos labeled cells in the LDT per tissue volume. The tissue volume was calculated by multiplying the scanned area by the thickness of the preparation. (a) c-Fos labeled cells in vocalizing or non-vocalizing categories (vocalizing category included short-and long vocalizing animals) and (b) c-Fos labeled cells in all experimental groups (* p<0.01).
Figure 8: Fluorescent confocal images demonstrating c-Fos (red) and ChAT (green) labeled cells of the LDT. (a) LDT scan of non air-puffed non-vocalizing control, (b) air puffed non-vocalizing animal, (c) air puffed short vocalizing animal and (d) air puffed long vocalizing animal. White arrows indicate c-Fos labeled cells in the LDT. Abbreviations: 4V: fourth ventricle, LC: locus coeruleus. Bar (a-d): 50 μm.
Figure 9: Bar graphs illustrating the mean number of air puff induced c-Fos labeled cells in the PPT per tissue volume. Tissue volume was calculated by multiplying the scanned area by the thickness of the preparation (a) c-Fos labeled cells in vocalizing or non-vocalizing categories (vocalizing category included short- and long vocalizing animals (b) c-Fos labeled cells in all experimental conditions (* p<0.05).
Figure 10: Examples of fluorescent confocal images demonstrating c-Fos (red) and ChAT (green) labeled cells of the PPT. (a) PPT scan of non air-puffed non-vocalizing control, (b) air puffed non-vocalizing animal, (c) air puffed short vocalizing animal and (d) air puffed long vocalizing animal. White arrows indicate c-Fos labeled cells. Bar: 50 μm.
Figure 11: Comparative bar graph illustrating the number of c-Fos labeled cells in the LDT and PPT of the vocalizing and non-vocalizing categories. [(*p<0.0001), n.s.: not significant].
C. Double Labeled Cholinergic Cells

**LDT and PPT Cholinergic Cell Population**

The activity of cholinergic cells was examined to verify their involvement in the production of 22 kHz USV. Double labeled cells were considered active cholinergic cells, and they were counted only when the Fos labeled nucleus (red) was directly located in the nuclear space of ChAT (green) labeled cell. First, double labeled cholinergic cells of the LDT and PPT were combined to determine their overall role in vocalization. The cholinergic cells of the LDT and PPT were counted together, because the distribution of the cholinergic cells did not follow the boundaries of the nuclei.

A Kruskal-Wallis ANOVA test revealed a statistically significant difference across experimental conditions (KW=10.5, p<0.01) (Figure 12). Additional analysis by Mann-Whitney U test showed significantly more active cholinergic cells in long vocalizing animals than controls (U=12.0, p<0.003), and short vocalizing than controls (U=16, p<0.02) (Figure 12). The increasing trend of active cholinergic cells across the experimental groups, with the long vocalizing group as the highest, indicates that the long vocalizing response to air puff stimuli is associated with active cholinergic cells. Figures 13 and 14 illustrate confocal microscope images of double labeled LDT and PPT cholinergic cells, respectively.

**LDT Double Labeled Cholinergic Cells**

Next, the active cholinergic cells (double labeled cells) of the LDT were examined. Due to low counts, large number of zeros, and not normal distribution, a Chi square ($\chi^2$) test was performed. It revealed that there were significantly more active cholinergic
(double labelled) cells in the long vocalizing animals than in short vocalizing animals ($\chi^2=6$, $p<0.01$) or the non-vocalizing group ($\chi^2=4$, $p<0.04$; Figure 15a). The duration of vocal response appears to make a difference, since there are significantly more active cholinergic cells in the long vocalizing group compared to the non- and short vocalizing groups. As the $\chi^2$ test indicates, the high number of active cholinergic cells found in the long vocalizing group is not by chance. The increased number of active cholinergic cells in the vocalizing group suggests that the heightened activity of the cholinergic cells is implicated in the intense production of ultrasonic calls.

**PPT Double Labeled Cholinergic Cells**

Further statistical analysis was performed to examine the double labeled cholinergic cell count in the PPT. A $\chi^2$ test revealed a statistically significant difference between the non-vocalizing and short vocalizing groups $\chi^2=4.2$, $p<0.04$, and non-vocalizing and long vocalizing groups $\chi^2=5.4$, $p<0.02$ (Figure 15b). In general, it is of interest to note that the mean of double labeled cells in all groups combined found in the PPT ($M=0.82$, $SEM=0.354$) was lower when compared to the mean of all groups combined of double labeled cells found in the LDT ($M=1.13$, $SEM=0.415$). A low mean of active cholinergic PPT cells compared to the LDT cells indicates the PPT may not play a critical role in the production of vocalization.

If the cholinergic cells in the PPT are not involved in the production of 22 kHz calls, then the fraction of double labeled active cells in long and short vocalizing animals would be similar. This is illustrated in (Figure 15b) for the PPT, but not in the LDT (Figure
15a). The separation of active cells between short and long vocalizing groups is significantly different in the PPT than the LDT.

In general, a small percent of active (double labeled) LDT cholinergic cells were found. An analysis of the LDT cholinergic cell population (=100%) showed the non-vocalizing group had 0.24% of active cholinergic cells, 0.12% of double labeled cells in the short vocalizing group and the long vocalizing group had a high percentage of 0.43% double labeled cells. The control group had the lowest percentage (0%) of active cholinergic cells compared to other experimental groups (Table 2a). The active non-cholinergic cell population, which is also all the fos labeled cells that were not cholinergic (=100%) was also examined and it was found that the control group had 0% active cholinergic (double labeled) cells and a high percent of active cholinergic cells (0.67%) compared to the short vocalizing group (0.54%) (Figure 2b).
Figure 12: Bar graph demonstrating the average number of active cholinergic cells (double-labeled) per scanned tissue volume in the LDT and PPT collectively. Tissue volume was calculated by multiplying the scanned area by the thickness of the preparation. (*p<0.01)
Figure 13: Fluorescent confocal microscope image of LDT double labeled cholinergic cell in an air puffed long vocalizing animal. c-Fos nuclei (red) sit right in the nuclear space of the cholinergic cells (green) (i, ii). (a, b) are enlarged images containing double labeled cells (white arrows). Bar (i, ii): 50 μm, (a, b): 25 μm.
Figure 14: Fluorescent confocal microscope image of PPT double labeled cholinergic cell in an air puffed long vocalizing animal. c-Fos nucleus (red) sits right in the nuclear space of the cholinergic cell (green) (a). (b) shows enlarged image containing double labeled cell of interest (white arrow). Bar (a): 50 μm, (b): 25 μm.
Figure 15: Average number of active cholinergic cells per tissue volume in the LDT (a) and PPT (b) (*p<0.05).
<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Non-vocalizing</th>
<th>Short Vocalizing</th>
<th>Long Vocalizing</th>
</tr>
</thead>
<tbody>
<tr>
<td>Active Cholinergic</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(double labeled)</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>cells</td>
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<tr>
<td>Non Active Cholinergic</td>
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<td>99.88</td>
<td>99.57</td>
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</table>

**Table 2a:** Percent of double-labeled LDT cells. The table shows the percentage of air puff induced active cholinergic and non active cholinergic cells of the LDT across experimental conditions. 100% represents the total number of ChAT cells in the analyzed LDT area.

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Non-vocalizing</th>
<th>Short Vocalizing</th>
<th>Long Vocalizing</th>
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<tr>
<td>Active Cholinergic</td>
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</tr>
<tr>
<td>cells</td>
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</tr>
<tr>
<td>Active Non Cholinergic</td>
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<td>99.46</td>
<td>99.33</td>
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</table>

**Table 2b:** Percent of double-labeled LDT cells. Table shows percentage of active cholinergic (double labelled) cells and active non cholinergic cells (all Fos labeled cells) of the LDT across experimental conditions. 100% represents total number of c-Fos cells analyzed in the LDT area.
DISCUSSION

Summary of Results

The results indicate that air puff stimuli induced species-typical 22 kHz ultrasonic alarm calls in adult male rats. Immunohistochemical analysis of cells in the LDT and PPT showed an increase in c-Fos expression in vocalizing animals compared to non-vocalizing controls. LDT Fos expression was significantly higher than PPT Fos expression. Increased activity of cholinergic cells was examined by identifying double labeled cholinergic cells. Although low, there were significantly more double labeled active cholinergic cells in the LDT of long vocalizing animals than short vocalizing and control animals. The PPT also showed active cholinergic cells in vocalizing animals compared to control; however the number found was lower than that of the LDT, and there was no difference between the long and short vocalizing groups.

Behavioural Response

Air puff application induced 22 kHz ultrasonic alarm calls within the range of acoustic parameters characteristic for 22 kHz USVs. The frequency for averaged calls was maintained between 20 and 25 kHz, average duration of calls was greater than 386.4 ms, and averaged bandwidth was less than 6 kHz. The calls were similar to those that appear in natural conditions. It was observed that the first two calls in the series had slightly higher initial frequency sweeps. The slight initial increase in frequency was modified in the subsequent calls that followed and the frequency was constant. A possible
explanation is that the rat hears itself emit these calls at a higher frequency and adjusts the frequency so that the call has communicatory significance for nearby conspecifics. There was no difference between the type of calls emitted by animals in the short and long vocalizing groups. Emitted calls were similar, and the difference among groups lay in the total number of vocalizations.

The type of behaviour exhibited by the rats as a result of air puff stimuli was as expected. All exploratory and grooming behaviour ceased, the animal crouched, froze and emitted 22 kHz alarm calls. These actions are components of defensive behaviour exhibited in vocalizing rats (Brudzynski, 2001). The alarm vocalization was a response to a potential threat, as the air puff stimuli had transient tactile and acoustic components that were unpredictable to the animal. The behavioural response to this air puff suggests that the animal is in a negative affective state, which is closely associated with anxiety. Brudzynski and Holland (2005) defined anxiety as a lasting negative affective state present without an obvious threatening stimulus and/or without any indication about its actual appearance and consequences. The results suggest that since emission of 22 kHz USV is a response to an unpredictable air puff stimuli, 22 kHz alarm calls should be correlated with anxiety and not fear.

**Analysis of c-Fos labeled LDT Neurons**

The current study was initiated by the absence of active cholinergic cells (double labeled) in the LDT of air puffed vocalizing animals found in the study by Savoy (2005). The current study used an air pressure of 50 psi (3.5 kg/cm²), which was higher than the 40 psi employed by Savoy (2005). The stimuli were sufficient to induce 22 kHz alarm
calls and other defensive behavioural responses lasting for a longer time. The present results support the hypothesis that cells of the LDT nucleus were active during emission of air-puff-induced 22 kHz USV. Statistical analysis of LDT and PPT Fos labelling revealed a significant increase in Fos expression in vocalizing animals when compared to non-vocalizing controls. The increase in Fos expression was significantly higher in LDT cells than PPT cells, suggesting that there is a strong correlation between 22 kHz USV and the active LDT neurons.

Previous studies have demonstrated that stimulating the LDT with glutamate induced species-typical 22 kHz alarm calls (Bihari et al., 2003; Brudzynski and Barnabi, 1996). As the LDT has cholinergic projections to forebrain structures, pre-treatment with a muscarinic antagonist, scopolamine, in the lateral septum (Bihari et al., 2003) significantly reduced 22 kHz USV response elicited by LDT stimulation. Scopolamine also antagonized the carbachol response induced directly from the AHPOA (Brudzynski and Barnabi, 1996). Results from the current study imply that 22 kHz USV emission is associated with LDT activity. There is a greater involvement of LDT activity than PPT activity as indicated by the number of Fos labeled cells. Since the PPT does not have extensive ascending projections, it is not directly linked with the initiation of the emission of USVs. Future research is required to examine the pattern of c-Fos expression in structures that comprise the cholinceptive vocalization strip in the vocalizing and non-vocalizing animals.

Analysis of Active Cholinergic LDT Neurons
Double labeled cholinergic cells were examined to determine the level of cholinergic cell activity. A low number of double labeled active cholinergic cells was found in the LDT. Long vocalizing animals had significantly more double labeled cells than non-air-puffed non-vocalizing controls. An even lower number of active cholinergic cells was found in the PPT, with no statistical significance across experimental groups. The high number of double labeled cells found in the LDT can be attributed to the emotional component of vocalization; as mentioned earlier, the LDT has cholinergic projections to structures in the limbic system. The results are also consistent with findings in the study by Bihari et al. (2003) where only 10% of ChAT positive cells within the LDT were retrogradely labeled when fluorogold was injected into the lateral septum and medial preoptic area. This suggests that only a few cells are active during vocalization and these cells have direct projections to forebrain structures. A study by Kubota et al. (1992) found that the size of ChAT immunoreactive neurons of the LDT were medium to large. It is possible that due to the structural size of LDT cholinergic neurons, only a few of these large neurons are required to be active and are necessary for the production of 22 kHz USV and regulation of other defensive behaviours.

The smaller number of active cholinergic cells found in the PPT can be attributed to its cholinergic projections. Although cholinergic neurons found in the PPT are similar to those found in the LDT, there is a difference in the target structures to which each nucleus projects. As mentioned earlier, the PPT projects to the VTA, globus pallidus and cerebellar cortex. The results of the present study suggest that active cholinergic neurons of the PPT are not critical for USV production but regulate other functions. The PPT may contribute to vocalization by a different pathway, but not directly through the medial
cholinoceptive vocalization strip. The PPT also has descending projections to the medullary reticular formation which plays a role in regulating REM sleep and consciousness (Oakman et al., 1999). Therefore, the increased c-Fos activity of PPT cholinergic cells has a possible role of promoting arousal in times of potential danger. It is important to mention that the number of double labelled cells found in the PPT was not dependent on the length of vocalization, as were those found in the LDT. There was a significant difference between short and long vocalizing in the number of double labeled neurons in the LDT but not in the PPT nucleus. In general, the activity of LDT cholinergic neurons supports the role of the LDT in the production of 22 kHz calls.

Active Non Cholinergic Cells of the LDT

The LDT has many non-cholinergic neurons, containing neurotransmitters such as GABA, serotonin and noradrenaline. From the present results, a number of non-cholinergic cells in the LDT were found to be active during the emission of 22 kHz USV. It is also possible that some of these non cholinergic cells could be glial cells. Honda and Semba (1995) mentioned that non-cholinergic cells found in the LDT could be serotonergic and noradrenergic neurons. Other non-cholinergic cells may include glutamatergic and GABAergic cells as well (Honda and Semba, 1995). A study by Verret et al. (2005) confirms the presence of GABAergic neurons in the LDT as these neurons were immunoreactive to glutamic acid decarboxylase (GAD). Losier and Semba (1993) found that serotonergic brainstem neurons had branching axons which innervate both the basal forebrain and thalamus. Noradrenergic and histaminergic neurons also project to the basal forebrain and thalamus (Losier and Semba, 1993). Further immunohistochemical
study is required to determine the identity of active non-cholinergic cells in the LDT of vocalizing animals.

Since previous studies have induced 22 kHz USV by pre- and postsynaptic application of pharmacological agents (in relation to the cholinergic synapse in the vocalization strip, see Figure 16), the current study used an air puff stimulus to presynaptically induce similar calls. The proposed model of vocalization (Figure 16) highlights the fact that presynaptic stimulation of the LDT increased LDT Fos expression in cholinergic cells, indicating LDT activity and the release of acetylcholine from terminal ends in the forebrain (cells for example in the MPA, Figure 16). The outcome of this stimulation would cause acetylcholine release in forebrain structures (exemplified by MPA and LS in Figure 16) and stimulate 22 kHz USV (output in Figure 16). Continuous release of acetylcholine in the forebrain areas could involve a feedback loop to the LDT, resulting in more 22 kHz USV emission (dashed line in Figure 16). This feedback loop from forebrain areas to cells in the LDT was found in the study by Savoy (2005). Intracerebral injections of carbachol into the MPA induced 22 kHz alarm calls and also induced cellular c-Fos activity in the LDT (Savoy, 2005).

The present study has demonstrated that the LDT, a structure previously identified by pharmacological and electrophysiological studies as being essential for the production of ultrasonic alarm calls, contained a large number of c-Fos labeled neurons following air puff induced USVs. This finding indicated that the LDT was indeed active during the emission of 22 kHz ultrasonic alarm calls. Results from this study demonstrated that some of the c-Fos labeled cells were active non-cholinergic neurons, and a low percent of the c-Fos labeled cells were cholinergic (double labelled). The results indicate that only a
few active cholinergic cells are involved in the production of these alarm calls. These findings support the hypotheses outlined in the Introduction that 1.) cholinergic cells of the LDT nucleus are active during the emission of 22 kHz alarm calls, 2.) long vocalizing animals have higher levels of cellular activity compared to non-vocalizing controls and 3.) long vocalizing animals demonstrate a greater number of active cholinergic cells in the LDT compared to air puffed or naïve non-vocalizing controls.
Figure 16: Schematic diagram of a proposed model for 22 kHz ultrasonic vocalization. ChAT (green) labeled cell with a Fos nucleus (red), other cells illustrated are active non cholinergic cell (red) and non-active cholinergic cell (green). Abbreviations: Ach: Acetylcholine, LS: Lateral septum, MPA: Medial preoptic area (for explanation see text page 69).
REFERENCES


Griffin DR (1968) Echolocation and its relevance to communication behaviour. In Animal Communication: Techniques of study and results of research. Edited by Thomas A. Sebeok.. Indiana University Press. Bloomington, Indiana


