Immunohistochemical Study of Laterodorsal Tegmental Neurons
Active During 22 kHz Vocalization

by

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Abstract

An ascending cholinergic projection, which originates in the laterodorsal tegmental nucleus (LDT), was implicated in the initiation of ultrasonic vocalization. The goal of this study was to histochemically examine the activity the LDT following ultrasonic calls induced by two methods. It was hypothesized that cholinergic LDT cells would be more active during air puff-induced vocalization than carbachol-induced one.

Choline acetyltransferase (ChAT), and cFos protein were visualized histochemically as markers of cholinergic calls and cellular activity, respectively. Results indicated that animals vocalizing after carbachol, but not after air puff, had a significantly higher number of Fos labeled nuclei within the LDT than non vocalizing controls. A significantly higher number of double-labeled neurons were discovered in the LDT of vocalizing animals (in both groups) as compared to control conditions. Thus, there were significantly more active cholinergic cells in the LDT of vocalizing than non-vocalizing rats for both methods of call induction.
Acknowledgements

"I believe that you cannot go any further than you can think. I certainly believe if you don’t desire a thing, you can never get it."

Charleszetta Waddles

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# Table of Contents

Abstract 2  
Acknowledgments 3  
Table of Contents 4  
List of Abbreviations 6  
List of Tables 7  
List of Figures 8  
Literature Review 9  
  Animal Communication 9  
  Behavioural Context of Ultrasonic Vocalization in Rats 11  
  Neurochemical Basis of Ultrasonic Vocalization 14  
  Cholinergic System in the Production of 22 kHz Ultrasonic Vocalization 15  
  Laterodorsal Tegmental Nucleus and the Cholinergic System 17  
  C-Fos as a Marker for Neuronal Activity 20  
Rationale and Hypotheses 22  
Materials and Methods 25  
  Animals 25  
  Experimental Design 25  
  A) Pharmacological-Behavioural Studies 26  
    Surgical Procedures 26  
    Drugs and Injection Procedures 28  
    Air Puff Stimulation (Non-invasive Call Induction) 29  
    Recording & Analysis of Vocalizations 29  
  B) Histochemical Studies 30  
    Preparation of Tissues 30  
    Peak Expression of Fos Protein 31  
    Immunohistochemistry 32  
    Immunohistochemical Controls 33  
    Antibody Specificity 33  
    Anatomical Specificity 34  
    Fluorescence Confocal Microscopy 36
Quantification of c-Fos and ChAT Expression

Statistical Analysis

Results

Number of Animals
Localization of Injection Sites
Behavioural Response to Carbachol and Air-Puff
Vocalization Induced by Carbachol Stimulation
Vocalization Induced by Air Puff Stimulation
Comparison of Vocalizations Induced by Carbachol versus Air Puff
Peak Fos Expression
Histochemical Controls
Antibody Specificity
Anatomical Specificity
Fos Labeling in the LDT
ChAT Labeling in the LDT
Comparison of Fos and ChAT Labeling in LDT
Double Labeled Cells in LDT
Fos Labeling in the Lateral Septum

Discussion

Overview of Results
Acoustic Parameters
Fos Labeling in the LDT
Double Labeling in the LDT

Literature Cited
**List of Abbreviations**

4V: Fourth ventricle  
Ab: Antibody  
Ach: Acetylcholine  
AchE: Acetylcholinesterase  
AH/POA: Anterior hypothalamic/preoptic area  
ANOVA: Analysis of Variance  
ASM: Followed by number denotes individual animals in the study  
AP & Voc: Animals who received air puff stimulation followed by subsequent vocalization  
AP no Voc: Animals who received air puff stimulation without subsequent vocalization  
c-fos: Proto-oncogene considered to be an IEG  
c-Fos: Protein product of the proto-oncogene c-fos  
Cch: Carbachol  
Cch & Voc: Animals with intracerebral injection of carbachol followed by subsequent vocalization  
Cch no Voc: Animals with intracerebral injection of carbachol without subsequent vocalization  
ChAT: Choline acetyltransferase  
DR: Dorsal raphe nucleus  
DT: Dorsal tegmental nucleus  
FITC: Fluorescein isothiocyanate  
i.e.: Intracerebral injection  
IEGs: Immediate early genes  
IgG: Immunoglobulin G  
LDT: Laterodorsal tegmental nucleus  
LS: Lateral septum  
MPA: Medial preoptic area  
MPO: Medial preoptic nucleus  
MS: Medial septum  
PBS: Phosphate buffered saline  
PHA-L: *Phaseolus vulgaris* leucoagglutinin  
PPG: Fixative containing paraformaldehyde and picric acid  
PPT: Pedunculopontine nucleus  
SN: Substantia nigra  
WGA-HRP: Wheat germ agglutinin-conjugated horseradish peroxidase
List of Tables

<table>
<thead>
<tr>
<th>Table</th>
<th>Title</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Omission of Antibody Steps in Immunohistochemical Procedures</td>
<td>35</td>
</tr>
<tr>
<td>2</td>
<td>Description of Experimental Conditions and Sample Sizes</td>
<td>40</td>
</tr>
</tbody>
</table>
List of Figures

<table>
<thead>
<tr>
<th>Figure</th>
<th>Title</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td><strong>Midsagittal section</strong>: Rat brain demonstrating medial cholinoceptive vocalization strip</td>
<td>16</td>
</tr>
<tr>
<td>2</td>
<td><strong>Schematic diagram</strong>: Cholinergic system involved in the production of ultrasonic vocalization</td>
<td>24</td>
</tr>
<tr>
<td>3</td>
<td><strong>Coronal section</strong>: Rat brain illustrating desired injection site: the medial preoptic area (MPA)</td>
<td>27</td>
</tr>
<tr>
<td>4</td>
<td><strong>Coronal section</strong>: Rat brain illustrating desired scan areas: a) septal region and b) laterodorsal tegmental region</td>
<td>37</td>
</tr>
<tr>
<td>5</td>
<td><strong>Coronal sections</strong>: Localization of injection sites with Carbachol (Cch) and Saline</td>
<td>41</td>
</tr>
<tr>
<td>6</td>
<td><strong>Sonogram</strong>: Ultrasonic vocalization induced by intracerebral injection of Cch into MPA</td>
<td>43</td>
</tr>
<tr>
<td>7</td>
<td><strong>Sonogram</strong>: Ultrasonic vocalization induced by air puff stimulation to laterodorsal head and neck region</td>
<td>43</td>
</tr>
<tr>
<td>8</td>
<td><strong>Bar graph</strong>: Acoustic parameters following either intracerebral injection of Cch or air puff stimulation</td>
<td>45</td>
</tr>
<tr>
<td>9</td>
<td><strong>Bar graph</strong>: Time post injection of Cch into MPA to perfusion (Mean number of Fos labeled cells expressed in the lateral septum (LS))</td>
<td>47</td>
</tr>
<tr>
<td>10</td>
<td><strong>Microphotography</strong>: c-fos expression in lateral LS at 60, 90 and 120 min post injection of Cch into MPA</td>
<td>48</td>
</tr>
<tr>
<td>11</td>
<td><strong>Fluorescence confocal microscopy</strong>: Images of LDT where application of specific antibodies were omitted</td>
<td>51</td>
</tr>
<tr>
<td>12</td>
<td><strong>Fluorescence confocal microscopy</strong>: Images of LDT where application of specific antibodies were omitted</td>
<td>52</td>
</tr>
<tr>
<td>13</td>
<td><strong>Fluorescence confocal microscopy</strong>: Images demonstrating anatomical and cellular specificity of antibodies used for Fos and Chat detection</td>
<td>53</td>
</tr>
<tr>
<td>14</td>
<td><strong>Bar graphs</strong>: Anatomical specificity of ChAT labeling</td>
<td>54</td>
</tr>
<tr>
<td>15</td>
<td><strong>Bar graphs</strong>: Mean number of Fos labeled cells in LDT in all experimental conditions</td>
<td>58</td>
</tr>
<tr>
<td>16</td>
<td><strong>Bar graphs</strong>: Mean number of Fos labeling in LDT of all experimental conditions (left vs. right side)</td>
<td>59</td>
</tr>
<tr>
<td>17</td>
<td><strong>Bar graphs</strong>: Mean number of Fos labeled cells in LDT of all experimental conditions (Scan 1 vs. Scan 2)</td>
<td>60</td>
</tr>
<tr>
<td>18</td>
<td><strong>Fluorescence confocal microscopy</strong>: Fos labeling in the LDT following Cch into MPA</td>
<td>61</td>
</tr>
<tr>
<td>19</td>
<td><strong>Bar graphs</strong>: Mean number of ChAT cells in LDT of all experimental conditions (Scan 1 vs. Scan 2)</td>
<td>63</td>
</tr>
<tr>
<td>20</td>
<td><strong>Fluorescence confocal microscopy</strong>: Overlapping images of ChAT labeling in LDT</td>
<td>64</td>
</tr>
<tr>
<td>21</td>
<td><strong>Fluorescence confocal microscopy</strong>: Positively labeled ChAT and Fos cells in LDT of vocalizing animal following air puff stimulation</td>
<td>66</td>
</tr>
<tr>
<td>22</td>
<td><strong>Fluorescence confocal microscopy</strong>: ChAT and Fos labeled cells in LDT of vocalizing animal following air puff stimulation</td>
<td>67</td>
</tr>
<tr>
<td>23</td>
<td><strong>Bar graphs</strong>: Fos, double and ChAT labeled cells in LDT</td>
<td>68</td>
</tr>
<tr>
<td>24</td>
<td><strong>Bar graphs</strong>: Number of double labeled cells in the LDT of all experimental conditions</td>
<td>72</td>
</tr>
<tr>
<td>25</td>
<td><strong>Fluorescence confocal microscopy</strong>: Double labeled cell in LDT of vocalizing animal</td>
<td>73</td>
</tr>
<tr>
<td>26</td>
<td><strong>Fluorescence confocal microscopy</strong>: Zoomed scan of double labeled cell in LDT of vocalizing animal</td>
<td>74</td>
</tr>
<tr>
<td>27</td>
<td><strong>Bar graphs</strong>: Mean number of Fos labeled cells in lateral septum of all experimental conditions</td>
<td>75</td>
</tr>
<tr>
<td>28</td>
<td><strong>Schematic diagram</strong>: Revised diagram of cholinergic system involved in the production of ultrasonic vocalization.</td>
<td>81</td>
</tr>
</tbody>
</table>
Literature Review

Animal Communication

For most animals, communication with other members of their species is an integral aspect to their survival. Communication is the method by which individuals interact with each other and are able to maintain societal organization according to their status and function (Shorey, 1976). Typical communication has four components: a mechanism for coding the message, a means of emitting the signal, a medium through which the signal is transmitted (e.g. air or water), and a means of receiving the signal (Shorey, 1976). Animals use a variety of modalities to transmit information to conspecifics, which are, for the most part, divided into five major categories. These include visual, tactile, olfactory, acoustic signals, and in the case of some aquatic animals, electrical field signals (Shorey, 1976).

Visible displays of communication (i.e. colour and postural cues) are used by species such as wolves, to defend territories or to show dominance (Smith, 1977). These cues are effective due to their ability to travel over large distances at high speed but can be limited by environmental factors such as darkness.

Olfactory signals (e.g. pheromones) are used by most members of the animal kingdom and in the case of the rodents such as rats and mice, are a means to elicit mating behaviour (Smith, 1977). Relying on wind (air currents) for dispersal, pheromones are effective across large distances, but diffusion of the signal can affect the speed at which the sender is located (Smith, 1977).

Tactile communication among mammals (e.g. non-human primates) is an integral component of social bonding, grooming or mating, with the only disadvantage being a lack of ability to travel across large distances or through physical barriers (Smith, 1977).
The use of electrical fields as a means to transmit signals is limited to very few aquatic species. In the case of the American eel, the emission of electrical pulses at various frequencies is used as a means of showing “presence” and territorial boundaries (Smith, 1977).

Lastly, acoustic signals are used, for instance, to indicate danger, reproductive readiness and species recognition (Smith, 1977). Acoustic signals can present themselves in two forms. The first are audible stimuli (20 Hz-20 kHz) that are used by humans to communicate and to detect changes in the environment. The second type of acoustic signals presents themselves as inaudible (for humans) stimuli in the form of either infrasound (1-20 Hz) used by whales and elephants, or ultrasounds (above 20 kHz) used by rodents and bats (Sales & Pye, 1974). Acoustic signals have the advantage of transmission over considerable distances without many of the limitations mentioned previously.

One of the first documented species to use inaudible frequencies to transmit signals was the bat (Pierce & Griffin, 1938 cited by Cracknell, 1980). Termed echolocation, ultrasounds are used by bats to both navigate their surroundings in darkness and to hunt for food (Cracknell, 1980, Sales & Pye, 1974). Subsequent studies gradually uncovered the ability of other species to both detect and emit ultrasonic vocalizations. Roeder & Treat (1957) noted the ability of moths to detect the ultrasonic vocalizations of their predators (bats). Thorpe (1967) described echolocation in dolphins. Discovery of this method of communication in rodents came slightly earlier in 1964 by Noirot who noted that adult albino mice displayed maternal behaviours readily to newborn pups but their responsiveness to older pups steadily decreased with time. As a result of this finding and subsequent examinations of this behaviour, Noirot proposed that ultrasonic vocalizations were used by pups to elicit maternal behaviours (Noirot, 1966).

The sound characteristics of ultrasonic vocalizations are similar to those of audible sound with the exception that the frequencies of ultrasounds are above the range of human hearing
(Cracknell, 1980, Sales & Pye, 1974). Ultrasonic wavelengths used by animals also propagate in a similar way to other sounds and attenuate with the distance traveled (Sales & Pye, 1974). This occurs according to the inverse square law, which states that the intensity of any sound decreases with the square of the distance traveled (Sales & Pye, 1974). Since ultrasonic vocalizations attenuate in a similar manner to audible sounds, one might question the benefits of this type of communication by rodents.

The benefits of this type of communication can be attributed to two important characteristics of ultrasounds. First, ultrasonic wavelengths are very short which allows for a high directionality of the emitted signals. No sounds can be heard behind the vocalizing animal unless there are obstructions that would reflect the sounds (Wiley & Richards, 1978). Second, ultrasonic signals are easily reflected particularly by smooth surfaces (Sales & Pye, 1974). The advantage of both of these characteristics becomes obvious when one examines the natural habitat of rodents. On a grassy surface, ultrasounds are reflected and scattered so they do not travel very far. Living primarily in underground burrows, the ability to signal members of a colony inside a burrow system without being directly seen or heard by predators outside of the burrow greatly enhances the colonies' chances of survival. In other words, the directionality and reflective properties of ultrasounds allow for a single rodent, facing the entrance of a burrow system, to warn the entire colony of impending danger by emitting ultrasonic vocalizations.

**Behavioural Context of Ultrasonic Vocalization in Rats**

Adult rats in a number of situations emit vocalizations of an ultrasonic nature. These include agonistic interactions such as fighting (Sales & Pye, 1974, Thomas, et al., 1983, Alcock, 1998), defensive behaviours (Sales & Pye, 1974), during, and in anticipation of play behaviours (Knutson, et al., 1998), exposure to foot shock (Kikusui, et al., 2003), following acoustic
stimulation (Koch, 1999), during reproductive encounters (McGinnis & Vakulenko, 2003) and in the presence of a predator (Blanchard, et al., 1991).

Regardless of the situation, rats emit ultrasonic vocalizations in behavioural contexts that require a strong emotional response. Typically, adult rats emit two distinct call patterns: a high frequency call of 35-70 kHz with 3-200 ms call duration, referred to as a short call, and a low frequency call of 20-35 kHz with a duration of at least 300 ms but up to 3000 ms, referred to as a long calls (Sales, 1972, Miczek, et al., 1991). In general, short calls are emitted in non-aversive, social interactions, and long calls appear in aversive, dangerous situations.

Short calls, referred to in the literature as 50 kHz calls, are emitted in situations that reflect positive affective states. These include courtship behaviour (Barfield & Greyer, 1972), in anticipation of play behaviour (Knutson et al., 1998), in the presence of an anaesthetized rat (Blanchard, et al., 1993), olfactory traces of other rats (Brudzynski & Pniak, 2002), in environments previously associated with reward (Knutson, et al., 1999) and also during presentation of non-social, yet rewarding stimuli (Burgdorf, et al., 2000). Long calls, known as alarm calls (Blanchard et al., 1991), or 22 kHz calls, are observed in situations that are perceived as potentially dangerous (Brudzynski & Barnabi, 1996, Brudzynski, 1994, Brudzynski, Ociepa, & Bihari, 1991), or stressful (Jelen, et al., 2003). These include: the presence of a predator (Blanchard et al., 1991), being defeated (Corrigan & Flannelly, 1979), in response to painful stimuli (Thomas & Barfield, 1985) such as application of foot shock (Brudzynski et al., 1991), in response to frightening stimuli like handling and touch (Brudzynski & Ociepa, 1992), or following startling air puffs (Knapp & Pohorecky, 1995). In other words, 22 kHz alarm calls are consistently observed in situations that are of an aversive nature.

The behavioural contexts associated with ultrasonic calls are well supported by the literature. However, there is debate over the functional significance of the calls, and two schools
of thought have emerged. One group of researchers argues that these calls are communicatory signals (Allin & Banks, 1972, Blanchard, et al., 1991, Brudzynski, 1994, Brudzynski & Chiu, 1995, Brudzynski, Bihari, Ociepa & Fu, 1993) and another group argues that 22 kHz calls are merely an acoustic by-product of a physiological process designed to increase gas exchange in the lungs (Blumberg & Alberts, 1990, Blumberg & Stolba, 1996, Blumberg, et al., 2000). This second notion is based on the observation that ultrasonic vocalizations in rat pups (7-14 days old) are directly related to cold exposure (Blumberg & Alberts, 1990, Blumberg & Stolba, 1996). Referred to as laryngeal breaking, these ultrasonic vocalizations are used by newborns to maintain elevated lung volume and are driven by a metabolic need for increased oxygen by heat producing organs within the body (Blumberg & Alberts, 1990, Blumberg & Stolba, 1996, Jourdan, et al., 1997). According to this hypothesis, the ultrasonic vocalizations emitted by pups are merely a by-product of this physiological process and, therefore, carry no communicative value (Blumberg & Stolba, 1996). Conversely, these same ultrasonic vocalizations emitted during cold exposure are used by pups outside of a nest as an effective means to initiate maternal retrieval back to the nest (Allin & Banks, 1972, Blumberg & Alberts, 1990). Since these ultrasonic vocalizations have been shown to successfully instigate a maternal response that is directly related to the pup’s increased rate of survival, one could argue in favour of the first school of thought in that ultrasonic vocalizations do retain communicative value. If these ultrasonic vocalizations did not contain communicatory value, dams within a nest would retrieve non-vocalizing pups from outside of the nest with an equal frequency as vocalizing ones. Research by Ferrell & Alberts (2002) demonstrated that this is not the case. When isolated from the nest, dams retrieve vocalizing pups with a greater frequency than anaesthetized pups from the same litter, even when the dam’s sense of smell is occluded (Ferrell & Alberts, 2002).
Further evidence lending support to the notion that ultrasonic vocalizations retain communicative value is the slight variability in acoustical parameters observed in different situations. Following an earlier observation that hypothermic vocalizations in pups differed in their acoustic properties from isolation calls recorded from the same pups prior to hypothermia (Hofer & Shair, 1992; Brunelli, et al., 1994), determined that following hypothermia, calls emitted by pups were lower in frequency, longer in duration and had longer intervals between calls. As a result, dams responded to hypothermic vocalizations emitted from pups within a maze with greater speed and accuracy (dams had an easier time finding pups within a maze with hypothermic calls than isolation calls) (Brunelli et al., 1994). The notion that pups are able to emit acoustically different calls under different situations, which have direct implications to the pups' rate of warming and recovery and ultimately survival, is another piece of evidence lending support to the idea that ultrasonic vocalizations retain communicative value.

**Neurochemical Basis of Ultrasonic Vocalization**

Since ultrasonic vocalizations differ in acoustic parameters and the behavioural contexts under which they are emitted, it is not surprising that a distinction also extends to the neural mechanisms that underlie the production of 22 kHz and 50 kHz vocalizations. 22 kHz vocalizations have been induced by carbachol, a muscarinic agonist of the cholinergic system (Brudzynski, 1994), which inhibits neurons when intracerebrally injected into the mediobasal hypothalamic area (Brudzynski, et al., 1998; Brudzynski, et al., 1991). On the other hand, 50 kHz vocalizations can be elicited by glutamate, which has an excitatory effect on neurons (Brudzynski et al., 1991) when intracerebrally injected into the same area (Fu & Brudzynski, 1994). Importantly, injection of relevant pharmacological antagonists successfully prevented
the emission of either call type. Injection of MK-801 antagonized the effects of glutamate (Fu & Brudzynski, 1994), and scopolamine antagonized the effects of carbachol (Brudzynski, 1994).

Cholinergic System in the Production of 22 kHz Ultrasonic Vocalization

In the rat brain, a band of cholinergic axons extends from the LDT in the brainstem and innervates several basal and midbrain structures prior to reaching the forebrain region (Brudzynski, 2001, Koyoma, et al., 1994, Satoh & Fibiger, 1986). Intracerebral injection of carbachol (Cch), a predominantly muscarinic agonist, into the anterior hypothalamic/preoptic area (AH/POA) and other medial areas innervated by the LDT has been shown to reliably induce emotional-aversive responses with ultrasonic vocalizations (Brudzynski, 1994, Brudzynski et al., 1991, Fu & Brudzynski, 1994). These vocalization-inducing regions have been termed the medial cholinocceptive vocalization strip (Figure 1) (Brudzynski, 2001, Brudzynski, 1998).

In an effort to gain insight into the role of the cholinergic projections that innervate the mediobasal forebrain area, Brudzynski & Barnabi (1996) investigated the role played by L-glutamate on the induction of ultrasonic vocalizations. Injection of L-glutamate into the LDT resulted in ultrasonic vocalizations, which were comparable by sound frequency and single call duration to those emitted following intracerebral injection of Cch into the AH/POA (Brudzynski & Barnabi, 1996). However pretreatment of the AH/POA with scopolamine, a muscarinic antagonist, prior to injection of L-glutamate into the LDT, resulted in a 70% attenuation of the vocalization responses (Brudzynski & Barnabi, 1996).

In a more recent study designed to examine the role of ascending cholinergic projections from the LDT to the septum in the induction of 22 kHz vocalizations, Bihari, et al., (2003) found similar results. Injection of Cch into the septum resulted in vocalizations that were indistinguishable from those emitted following injection of glutamate into the LDT.
(Bihari et al., 2003). LDT-induced vocalization responses were once again attenuated following pretreatment of the septum with scopolamine (Bihari et al., 2003). A second aspect of this study involved the localization of cholinergic projections terminating in the septum. When fluorogold, a fluorescent retrograde tracer, was pressure injected into the septal sites inducing vocalization, and sections of tissue were immunohistochemically counterstained with ChAT, several double-labeled cells were discovered within the boundaries of the LDT (Bihari et al., 2003). As a result of these direct and indirect examinations of this system, it is now well accepted that cholinergic input, originating in the LDT and innervating mediobasal forebrain and diencephalic regions, contributes to the initiation and production of 22 kHz vocalizations in the rat.

**Laterodorsal Tegmental Nucleus and the Cholinergic System**

The term cholinergic refers to a neuron’s ability to use acetylcholine (Ach) as a neurotransmitter (Kandel, et al., 2001). Early work by Loewy (1921), established Ach as having an inhibitory effect within the peripheral nervous system. Demonstrating Ach’s ability to act as a neurotransmitter within the central nervous system took many more years of research. This work depended on the ability to demonstrate the presence of Ach, its synthetic enzyme choline acetyltransferase (ChAT), the hydrolytic enzyme acetylcholinesterase (AchE) and the presence of postsynaptic receptors able to respond to the application of Ach (Mesulam et al., 1984).

Early studies by Shute & Lewis (1967) and Jacobowitz & Palkovits (1974) relied on AchE as a marker for cholinergic neurons and proposed the source of ascending cholinergic neurons to be the LDT. Attempts to elucidate the specific location (topographic morphology) of cholinergic neurons were hampered by an inability to specifically label them. This, coupled with the discovery of this enzyme in non-cholinergic neurons (Eckenstein & Thoenen, 1982,
Armstrong, et al., 1983), raised concerns about the reliability of early morphological studies of cholinergic pathways in the brain.

Development of monoclonal antibodies specific for ChAT by Levey & Wainer (1982) and Eckenstein & Thoenen (1982) quickly shifted the focus from using AchE as a neuronal marker to using ChAT. The restriction of the ChAT enzyme to cholinergic neurons has made it a reliable and accepted marker for these neurons and, as such, has made it the “marker of choice” over the last two decades. Localization of cholinergic neurons by ChAT immunohistochemistry has been applied to the brains of humans, (Oda & Nakanishi, 2000, Kasashima, et al., 1998), monkeys (Mesulam et al., 1984), cats (Kimura, et al., 1981), and rats (Armstrong et al., 1983, Eckenstein & Thoenen, 1983, Houser, et al., 1983).

Regardless of the species, cholinergic neurons (cell bodies) have been found in several specific locations within the brain. These include the medial septum and the diagonal band of Broca (Kasashima et al., 1998, Mesulam et al., 1984, Houser et al., 1983, Levey et al., 1983, Armstrong et al., 1983), the medial habenular nucleus (Mesulam et al., 1984, Houser et al., 1983), the striatum (Kasashima et al., 1998, Mesulam et al., 1984, Armstrong et al., 1983) the tegmentum (Kasashima et al., 1998, Mesulam et al., 1984, Armstrong et al., 1983), and the oculomotor nucleus (Kasashima et al., 1998, Mesulam et al., 1984, Levey et al., 1983, Kimura et al., 1981).

The first overview of the central pathways in the rat brain using monoclonal antibodies against ChAT was provided by Mesulam et al., (1983). As a result of this study cholinergic cells were found to be aggregated into the following six groups: the medial septum (Ch1), the vertical limb of the diagonal band of Broca (Ch2), the horizontal limb of the diagonal band of Broca (Ch3), the striatum (Ch4), the pedunculopontine nucleus (PPT) (Ch5), and the laterodorsal tegmental nucleus (LDT) (Ch6) (Mesulam, et al., 1983).
Cholinergic innervation of the hippocampus is provided by neurons in the medial septum and the diagonal band of Broca (Ch1-Ch3), and neurons from Ch4 innervate the amygdala (an area of the limbic system) (Mesulam, et al., 1983). Closer examination of Ch5 by Hallanger and Wainer (1988) demonstrated that injection of *Phaseolus vulgaris* leucoagglutinin (PHA-L), an anterograde tracer, into the PPT revealed labeled fibers in the thalamus, the lateral hypothalamus and the amygdala. Confirmation of these results was achieved once wheat germ agglutinin-conjugated horseradish peroxidase (WGA-HRP), a retrograde tracer, injected into these areas revealed ChAT positive neurons within the PPT (Hallanger & Wainer, 1988). Examination of pathways originating from the LDT (Ch6) by Satoh and Fibiger (1986) using injections of PHA-L into the LDT found two ascending pathways. The first traversed through the dorsomedial tegmentum, caudal diencephalon, medial forebrain bundle and diagonal band of Broca. Parts of this band were found to innervate the midbrain, diencephalon (thalamic nuclei) and telencephalon (prefrontal cortex) (Satoh & Fibiger, 1986). The second pathway was shorter and more diffuse having limited innervations with the median raphe, interpeduncular and mammillary nuclei (Satoh & Fibiger, 1986).

The PPT (Ch5) and the LDT (Ch6) form the basis of the tegmental cholinergic neurons. They are distributed in a continuous longitudinal column in the brainstem with the PPT located rostral to the LDT, which is situated more caudal in the brain (Honda & Semba, 1995). Efferents from the LDT and PPT have considerable overlap in their targets within the brain, but they differ in their pathways with the LDT being more mediobasal in location, and the PPT being situated more ventrolateral in the tegmentum (Satoh & Fibiger, 1986).

Ascending projections from the LDT and PPT form a prominent portion of the ascending cholinergic reticular activating system (RAS) (Cornwall, et al., 1990). Inputs from LDT are
found in the frontal cortex, diagonal band and preoptic area (areas associated with limbic system) (Cornwall et al., 1990).

The LDT and PPT have descending pathways to the substantia nigra (SN) (Gould, et al., 1989), the pontine nucleus and the medullary reticular formation (Semba & Fibiger, 1992). These pathways have been implicated in rapid eye movement (REM) sleep (Semba & Fibiger, 1992), the acoustic startle response (Koch, 1999, Jones & Shannon, 1998) and locomotion (Garcia-Rill, et al., 1987).

C-Fos as a Marker for Neuronal Activity

During the cellular response to external stimuli, the first sets of genes activated are those that do not require de novo synthesis of proteins (Herrera & Robertson, 1996). Theses genes are referred to as immediate early genes, or IEGs. IEGs are rapidly activated upon cellular stimulation and their expression is not prevented by protein inhibitors (Sheng & Greenberg, 1990). One of the most extensive IEG studied to date is c-fos. This proto-oncogene is the cellular homologue of the viral oncogene v-fos and encodes a 62 kDa protein that resides in the nucleus of a cell (Herrera & Robertson, 1996).

Outside of the nervous system, c-fos has been localized to developing embryonic tissues. In situ studies of developing mice reveal high levels of c-fos expression in specific regions of the developing skeleton and in the fetal liver (Herrera & Robertson, 1996).

Within the nervous system, basal levels of c-fos protein are low (Dragunow, et al., 1987, Caubet, 1989), but increase in specific brain regions following relatively mild stressors such as tactile stimulation of the whiskers (Mack & Mack, 1992), brief restraint (Chastrette et al., 1991), and also following more severe stimuli such as foot shock (Bullitt, 1990), and generalized seizures (Morgan et al., 1987). Following stimuli, Fos is rapidly (within 15 min) and transiently
activated (Li et al., 1999) and is expressed in response to hormone and neurotransmitter induced elevations in intracellular levels of calcium and c-AMP (Morgan & Curran, 1991, Dragunow & Robertson, 1988). Since activation of c-fos in the brain can be induced by a variety of intrinsic and extrinsic sources, the detection of its protein product, c-Fos, has proven to be an effective indicator of neural activity (Herrera & Robertson, 1996).

Although the expression of c-Fos is not the only indicator of neural activity, assessment of the protein can provide an index of functional responses in behaving animals. Thus, in the present study, the induction of c-Fos expression by specific stimuli was used to identify neural populations in the LDT that may be involved in transmitting vocalization-associated neural signals into the brain.
Rationale and Hypotheses

It has been well documented that intracerebral (i.e.) injection of Cch into the AH/POA, an area of the medial cholinceptive vocalization strip, results in the emission of characteristic 22 kHz alarm-type vocalizations (Brudzynski, 1994, Brudzynski & Barnabi, 1996, Brudzynski et al., 1991, Brudzynski & Bihari, 1990, Fu & Brudzynski, 1994) (Figure 2: “Activation by Cch”). Previous work has also demonstrated that calls emitted following i.e. injection of Cch into the AH/POA do not differ in their acoustical parameters from naturally occurring 22 kHz calls (Brudzynski & Chiu, 1995, Brudzynski et al., 1991). The study of Cch-induced and air-puff induced 22 kHz calls is based on the assumption that 22 kHz calls induced by both methods of stimulation are the same type of calls which should involve the LDT (Figure 2: “VOCALIZATION”). CCh-induced and air-puff induced calls should also have comparable acoustic characteristics.

The LDT has been proposed to be the source of cholinergic neurons that innervate several areas along the medial cholinceptive vocalization strip (Figure 2: “LS” and “MPA”). Since Cch targets postsynaptic neurons in relation to cholinergic terminals, the LDT neurons should not be involved (not active) in the initiation of 22 kHz vocalization following i.e. injection of Cch into the MPA (Figure 2: “Activation by Cch” leading to “OUTPUT” or 22 kHz calls). On the other hand, when initiating 22 kHz calls under natural conditions (i.e. following air puff stimulation), the LDT should be active since the ascending cholinergic vocalization system is activated presynaptically (Figure 2: “INPUT”, leading to “OUTPUT” or 22 kHz calls). Following vocalization, LDT activity will be measured by immunohistochemical localization of the nuclear protein c-Fos and by a counterstain for cholineacetyltransferase (ChAT), a marker of cholinergic neurons.
The experimental paradigms of this study were designed to ultimately lead to a greater understanding of the role of the LDT nucleus in the initiation of 22 kHz vocalizations.

Predictions

Examination of the two main predictions of this study is based on the testable assumption that rats who vocalize after an i.c. injection of Cch into the MPA (Cch & Voc) or after air puff stimulation (AP & Voc) will emit the same 22 kHz alarm-type vocalizations. Provided this assumption holds true, the following predictions will be examined:

**Prediction #1:** Rats who receive air puff stimulation, and subsequently vocalize, will demonstrate higher levels of cellular activity in the LDT than rats who vocalize following direct i.e. injection of Cch into the MPA. This will be indicated by an increase in the number of Fos labeled nuclei within the LDT of vocalizing animals.

**Prediction #2:** Rats in the AP & Voc condition will have a higher number of double-labeled cells (i.e., highly active cholinergic cells) in the LDT than non-vocalizing (control) conditions.
Figure 2: Schematic diagram of cholinergic system involved in the production of ultrasonic vocalization. The medial chilinoceptive vocalization strip is represented here by two structures only: MPA and LS. Cholinergic cell repressed by dotted circle and non-cholinergic cells by empty circles.
Material and Methods

Animals

A total of 65 male Wistar rats were obtained from Charles River (Montreal, Quebec) and were labeled ASM 1-ASM 66. Upon arrival, animals were housed in pairs in translucent polycarbonate cages (19x10x8”), on 1/8” corn cob bedding (Fisco Ent. Bolton, ON) in a temperature-controlled room with a 12:12 hour light: darkness cycle. Animals had access to rodent pelleted chow (LabDiet, Oakville, ON) and tap water ad libitum. All procedures involving the use of rats were approved by Brock University Animal Care & Use Committee and were performed within the procedural guidelines of the Canadian Council on Animal Care.

Experimental Design

The 65 animals used in this study were divided into six groups depending upon: a) the induction method of vocalization (either injection of Cch into the MPA or air puff stimulation) and b) the animal’s response when placed within the recording chamber (pharmacological condition) or plastic cage (air puff condition).

For pharmacological induction of vocalization (n=32), 3 conditions were used: a) intracerebral injection of Cch followed by vocalization (Cch & Voc), b) intracerebral injection of Cch without subsequent vocalization (Cch no Voc) and c) intracerebral injection of saline (Saline).

For the air puff call induction (n=34), 3 conditions were used: a) air puff followed by vocalization (AP & Voc), b) air puff without subsequent vocalization (AP no Voc) and c) a naïve, or un-puffed, control (Naïve) recorded in the same cage as air puffed animals.
A. Pharmacological/Behavioural Studies

Surgical Procedures

Animals weighing between 293 g & 450 g were administered an analgesic, Buprenorphine, (Buprenex, Reckitt & Coleman Pharmaceuticals, Richmond, VA) at 0.01 mg/kg, i.p.) 10 minutes prior to commencement of surgical procedures. Rats were anaesthetized by an intraperitoneal injection of a mixture of ketamine hydrochloride (40 mg/kg, i.p, Ayerst Veterinarian Laboratories, Guelph ON), and xylazine hydrochloride (3-6 mg/kg, i.p. Bayer, Etobicoke, ON). Once a sufficient depth of anaesthesia was reached, as indicated by the absence of motor reflexes, the surgical area (dorsal side of the head) was shaved.

Rats were mounted in a stereotaxic apparatus (D. Kopf Instruments) whose ear bars were coated with lidocaine hydrochloride (20mg/ml, Xylocaine Viscous 2%, Astra Pharma Inc, Mississauga, ON) and whose incisor bar was positioned 3.3 mm below the ear bar plane. Ophthalamic ointment (Lacrilube, Allergan, Irvine, CA) was applied to prevent keratitis, and the surgical area was prepared by application of iodine soap, ethyl alcohol and iodine, respectively. A small surgical incision was made along the midline of the skull, and fascia overlaying the skull was removed. When required, a few drops of Xylocaine (2%) were applied to the surgical area as a local anesthetic.

Calibration of two of the required surgical planes (anterior and lateral) was achieved with use of the apparatus zero reference bar, and a small hole was drilled stereotaxically through the skull at the designated site above the desired target structure of the right medial preoptic area (MPA). The coordinates for the MPA were determined using a stereotaxic atlas (Paxinos & Watson, 1986) and were A=8.0 mm anterior to the interaural plane, L=1.0 mm to the right of the midline, and V=7.2 mm below the surface of the brain. The MPA (Figure 3) was selected as the designated injection site based on previous research by Brudzynski (1994) and Brudzynski &
Figure 3: Coronal section of a rat brain 8.08 mm from the interaural plane demonstrating the desired unilateral injection site (grey dot, right side of brain), within the MPA (medial preoptic area). Abbreviations: CPu-caudate putamen, GP-globus pallidus, BS- Bed nucleus of stria terminalis, MPO-medial preoptic nucleus, MPA-medial preoptic area, LPO- lateral preoptic area, ox-optic chiasm. Scales in mm. From Paxinos & Watson (1986).
Barnabi (1996), which determined that injections of carbachol (Cch), a muscarinic agonist, into this structure could most reliably induce 22 kHz vocalizations.

A cannula of 15 mm (+/- 0.1 mm) in length, fashioned from a 23 G stainless steel needle was lowered into the opening, implanted one millimeter above the injection site, and adhered to the skull and jeweler's screws with dental acrylic (methyl methacrylate resin, Perm Hygenic Corp., St. Catharines, ON). The external opening of the cannula was sealed with a pre-fitted wire plug pin.

When animals were removed from the stereotaxic apparatus, a small amount of topical antibiotic (Surolan, Janssen, North York, ON) was administered around the edge of the wound, and up to 3 ml of warm isotonic saline was injected subcutaneously. Animals were monitored within a heated recovery chamber until a sufficient level of consciousness was reached. Once fully conscious, surgical animals were housed individually for the duration of the recovery period, prior to initiation of injection procedures (6-7 days).

**Drugs and Injection Procedures**

For this study, one of two substances was injected into the MPA of each of the 32 animals. Experimental animals received an injection of 1 µg of carbachol (Cch, carbamylcholine chloride, Sigma, St. Louis, MO) dissolved in 0.2µl sterile isotonic saline, and control animals received an injection of the equivalent volume of isotonic saline (0.2 µl).

Injection procedures began with the removal of the plug pin from the implanted guide cannula. The injecting cannula, 16 mm in length, attached to a microinjector (Hamilton constant rate syringe, CR 700-20, Reno, NA) via polyethylene tubing, was then inserted into the implanted guide cannula, and the appropriate amount of solution was administered at a constant rate of 10 nl/s. Following an additional 30 s the injecting cannula was removed and replaced
with the initial plug pin, and the animal was placed within a recording chamber. The latency of vocalization onset was recorded as time from inserting the plug pin to the emission of the first vocalization. Each animal was subjected to one injection of either carbachol (Cch) or saline into the MPA prior to being placed into the recording chamber. The injection volume and concentration of Cch chosen for this study were based upon results of a previously published dose-response study performed by Brudzynski (1994).

**Air Puff Stimulation (Non-invasive Call Induction)**

At the time of stimulation animals weighing between 375 g and 480 g were subjected to the air puff paradigm. Each animal was placed in a polycarbonate cage (19x10x8") lined with bedding and covered with a wire lid to prevent escape. The air puff apparatus consisted of polyethylene tubing attached to an air puff stimulator (AirStim, San Diego Instruments, San Diego, CA). Air puff stimulation procedures began with the application of an air puff (40 psi) to the rat's head and neck region. An air puff was manually administered to this region approximately every 15s until the rat began to vocalize or until 16 air puffs had been administered, whichever came first. Parameters used for air puff stimulation were based upon previous work by Knapp and Pohorecky (1995).

**Recording and Analysis of Vocalizations**

Following injection of either carbachol or saline, animals were placed within a padded recording chamber, and their vocalizations were recorded for a minimum of 10 minutes. The chamber was equipped with an ultrasonic microphone (SM-1), attached to a frequency divider (Bat Detector, S-200, QMC Laboratories, London, England) set to the frequency ratio division of 1/16. The average distance between the microphone and the rat was approximately 6 inches (15
cm). The signal was then sent to a sonograph (Sona-Graph, Kay Elemetrics digital signal processing station, model 5500-1, Pine Brook, NJ) as well as to a cassette tape recorder where divided vocalizations were recorded on audiotapes.

Recording of vocalizations induced by air puff stimulation involved the same paradigm as above, except that the recordings were taken from within the polycarbonate cage rather than the padded recording chamber. The openings provided by the wire lid covering the top of the cage were required to ensure proper application of air puffs. The distance from the rat to the microphone in this condition was approximately 8 inches (20.5 cm). The difference in quality of recording between these two cages was negligible for the purpose of this study.

Analysis of vocalizations was performed manually using the sonograph (Sona-Graph, Kay Elemetrics digital signal processing station, model 5500-1, Pine Brook, NJ). Signals from the recorded tapes were analyzed to obtain sonograms and power spectra from single calls. Detailed analysis of call duration, bandwidth and sound frequency was achieved by manual manipulation of the sonograph cursors. Length of individual calls was achieved by reading the time span between two cursors positioned at the beginning and the end of each call’s sonogram. Individual call bandwidths were measured by the frequency difference between two horizontal cursors positioned at the lowest frequency and at the highest frequency for each vocalization. The strongest component of the power spectrum within each call’s bandwidth was taken as the peak sound frequency for that call.

B) Histochemical Studies

Preparation of Tissues

Following intracerebral injection of either Cch or saline, or following the air puff paradigm, rats were sacrificed by an overdose of pentobarbital (Somnotol, MTC Pharmaceutical,
Cambridge, ON). Upon cessation of breathing, each animal was transcardially perfused with a fixative containing paraformaldehyde (4%) and picric acid (15%). Following perfusion, the brains were extracted and post-fixed for an additional two hours at room temperature.

At this point each brain underwent cryoprotective steps in the form of immersion in a solution of 10% sucrose/0.1 M PBS solution at 4°C overnight, followed by a 30% sucrose/0.1 M PBS (Na₂HPO₄, NaH₂PO₄, NaCl, dH₂O) solution for 48 hours at 4°C. Cryoprotected tissues were rinsed in PBS and adhered to a metal tissue holder by mounting medium (Tissue Tek, Miles Scientific, Naperville, IL). A cryotome (Model 2800 Frigocut, E, Reichert-Jung), set to -20°C, was utilized to cut the tissues into 25 μm thick coronal sections.

Tissues sections from the septal regions, as well as the laterodorsal tegmental nucleus (LDT), were taken from each experimental animal for immunohistochemical staining. Additionally, for histological verification of injection sites, tissue sections corresponding to the MPA were taken from Cch and saline injected animals. Individual tissue sections were stored in Eppendorf tubes in 500 μl of PBS and maintained at 4°C until required to undergo immunohistochemical procedures.

**Peak Expression of Fos Protein**

A total of 5 animals were utilized to determine the most suitable sacrifice time post injection to achieve the highest number of Fos labeled cells within the brain.

The times from the end of recording to transcardial perfusion were as follows: 0, 30, 60, 90 and 120 min respectively. The first animal was anesthetized immediately after recording and then perfused. Although the perfusion took 5-10 min, this animal was labeled as time zero. Following perfusion, 25 μm thick sections of tissue containing the lateral septum underwent
immunohistochemical staining procedures designed to detect the presence of Fos protein (see subsection “Immunohistochemistry”).

Following immunohistochemistry, slides containing the lateral septum were examined by fluorescent microscopy (Zeiss, West Germany) with the appropriate wavelength (absorption peak of 550 nm) selected to view the slides. Areas within this structure were identified, mapped and photographed using Kodak 400 ASA film. The lateral septum was chosen as a comparative target site due to the large number of c-Fos positive cells in this region following ultrasonic vocalization (Savoy, et al., 2001).

Expression of c-Fos protein for these five time intervals was determined by both the visual analysis of photographs and by cell count performed by digital image analysis taken from photographic negatives. The software used for this application was the Analytical Imaging Station (AIS, Imaging Research Inc, St. Catharines, ON). The criteria used to identify labeled nuclei were: a) morphologic characteristics of the nuclei (i.e. whether the nuclei were reasonably circular in shape) b) the relative brightness compared to background (measured by relative optical density of digital images), c) size of the individual nuclei (approximately 2µm), and d) whether the distribution of the brightly fluorescing cells followed the anatomical boundaries of the lateral septum according to a stereotaxic atlas (Paxinos & Watson, 1986). The most appropriate survival time, post stimulation, was determined to be 90 minutes (see Results section, Figures 9 & 10).

**Immunohistochemistry**

For each animal four septal sections and four sections containing the LDT underwent immunohistochemical procedures designed to detect both, Fos protein and choline acetyltransferase (ChAT). Permeabilization of sections involved 45 minutes of agitation in a
solution of 4% Triton-X-PBS and 10% Normal Donkey Serum. Tissues were washed with PBS, and primary antibody (H-125, rabbit anti-c-Fos polyclonal antibody, Santa Cruz Biotechnology, Santa Cruz, CA) was added at a dilution of 1:100 prior to an overnight incubation at 4°C.

The following day, the tissues were washed (x3) with PBS and a 1:100 dilution of the second primary antibody (AP180F, goat anti ChAT, Chemicon International, Temecula, CA) for ChAT was applied and the tissues were re-incubated at 4°C overnight.

The final incubation step involved the simultaneous application of 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) at a dilution of 1:200 for both antibodies. After 48 hours at 4°C, the sections were washed thoroughly and transferred to poly-L-Lysine (Sigma) coated microscope slide. To reduce the incidence of photobleaching, SlowFade-Light antifade reagents (Cedarlane Laboratories Ltd., Hornby ON) were applied directly to the tissues. A pre-cleaned, autoclaved, cover slip was placed over the tissues and the edges were sealed around by application of nail lacquer.

Immunohistochemical Controls

Antibody Specificity

Prior to data analysis several controls were implemented to provide insight into the specificity of binding for both Fos and ChAT antibodies. These involved the omission of various steps in the incubation process (refer to Table 1 for omitted antibody in each control).

Briefly, four controls were performed on four separate tissue sections from one vocalizing animal (ASM 45). In each control only one of the four applied antibodies was omitted, leaving the remainder of the antibody incubations unchanged. Each section of tissue
containing the LDT (or lateral septum in control #3) was examined by confocal microscopy under individual wavelengths for each of the fluorophores (Cy3 and FITC).

**Anatomical Specificity**

This study also employed several anatomical controls. The first involved the examination of the septal sections for each animal prior to examination of the LDT. Since the lateral septum of Cch-induced vocalizing rats is known to demonstrate high levels of Fos-labeled cells (Savoy et al., 2001), and the neighboring structure of the medial septum/diagonal band demonstrate cholinergic cells (Mesulam, et al., 1983, Zaborszky, et al., 1991, Page & Sofroniew, 1996), these sections of tissue provided unique insight into the level of anatomical specificity of both Fos and ChAT labeling.

A second anatomical control involved the examination of structures directly neighboring the LDT, namely the reticular formation. As this structure does not contain cholinergic cells (Mesulam et al., 1983) it was not expected to contain ChAT labeling and provided an internal negative control.
Table 1: Omission of antibody steps used to determine specificity of Fos and ChAT antibodies.

<table>
<thead>
<tr>
<th>Control Number</th>
<th>Primary Fos Ab (Rabbit anti-c-Fos polyclonal)</th>
<th>Secondary Fos Ab (Cy3 conjugated Donkey anti Rabbit IgG)</th>
<th>Primary ChAT Ab (Goat anti-ChAT)</th>
<th>Secondary ChAT Ab (FITC conjugated Donkey anti Goat IgG)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
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<td>Unaltered</td>
<td>Unaltered</td>
</tr>
<tr>
<td>2</td>
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<td><strong>Omitted</strong></td>
<td>Unaltered</td>
<td>Unaltered</td>
</tr>
<tr>
<td>3</td>
<td>Unaltered</td>
<td>Unaltered</td>
<td><strong>Omitted</strong></td>
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<tr>
<td>4</td>
<td>Unaltered</td>
<td>Unaltered</td>
<td>Unaltered</td>
<td><strong>Omitted</strong></td>
</tr>
</tbody>
</table>
Fluorescence Confocal Microscopy

Microscope slides for each experimental animal underwent critical examination by use of confocal microscopy (Nikon C1 Laser Confocal Microscope, Nikon Inc.). The main dichroic excitation lasers are 488 nm (Argon), 543 nm (He/Ne) and 633 nm (He/Ne) respectively. The first dichroic emission filter was set to 530 nm with barrier filters at 515/530 nm, and the second dichroic emission filter was set to 625 nm with barrier filters set to 585/540 and 665 LP.

First, areas corresponding to the MS and LS were scanned (Figure 4a) and utilized as internal controls for labeling intensity. Second, areas of tissue corresponding to the LDT were examined, the labeling pattern was noted, and subsequently the areas were scanned. The chosen scan areas followed the pattern of cholinergic cells within the LDT so that two individual, overlapping, scans of identical size (4x10^4 μm²) were performed to ensure that an accurate representation of the LDT was achieved (Figure 4b). The first scan area was positioned the most dorsal within the LDT and closer to the midline of the brain. The second scan area of identical size was placed nearby so that it overlapped with the first scan area but still remained within the boundaries of the LDT. During quantification the scan areas were referred to as Scan 1 and Scan 2 respectively.

Quantification of c-Fos and ChAT Expression

Expression of both c-Fos protein and ChAT in the septal regions and in the LDT were determined by use of digital images. These images were acquired by use of EZ-C1 software (Nikon, Inc, Mississauga, ON), which accompanied the confocal microscope. Cell counts were achieved by use of specific criteria set within the quantification software (Simple PCI Software, Compix, Inc).
Figure 4: Coronal section of rat brain 9.2 mm and 0.16mm from interaural zero plane demonstrating scan areas (squares) in the septal region (a) and the laterodorsal tegmental region (b). Inlaid images in both a and b indicate location of coronal section within mid-sagittal plane. Abbreviations: (a) cc-corpus callosum, LS- lateral septum, Cpu- caudate putamen, MS- medial septum, BST- Bed nucleus stria terminalis, VDBV- diagonal band (vertical limb). (b) CI- inferior colliculus, CE- cerebellum, 4V- fourth ventricle, cp-cerebellar penduncle, LDT- laterodorsal tegmental nucleus, DT- dorsal tegmental nucleus, DR- dorsal raphe nucleus, MT- motor trigeminal nucleus, RT- reticular formation. From Paxinos & Watson (1986). Scale in mm.
For both Fos labeled nuclei and ChAT labeled cells the following criteria were used; a) the brightness of targets as compared to background and b) whether the distribution pattern of labeled nuclei and/or cells fell within the boundaries of the septum and the LDT nucleus according to a stereotaxic atlas (Paxinos & Watson, 1986). Fos labeled nuclei had additional detection criteria of a) the relative size of the nuclei (proportionally smaller than the entire cell) and b) the geometric characteristics i.e. whether the nuclei were relatively circular in shape.

The LDT from both the left and right side of each experimental animal contained two overlapping scan areas. Counts of Fos and ChAT labeled cells were taken for each of these scan areas. However, quantification of double labeled cells within the LDT using the scan areas size of 4x10^4μm^2 presented a unique challenge. It was noted during the counting procedure that a number of double labeled neurons were outside of the scanned area but still within the boundaries of the LDT. As a result the area used for quantification was increased to 25x10^4μm^2 in an effort to cover the entire LDT nucleus and to ensure best results for this analysis.

**Statistical Analysis**

Analysis of all data was performed using SPSS software (version 11.1, SPSS Inc, Chicago, IL) or GB STAT (version 6.5, Dynamic Microsystems Inc, Silver Springs, MD). Peak Fos expression, call parameters (call duration, bandwidth, frequency, latency, number of calls per five minutes), and Fos labeling in the LS were examined by use of a univariate analysis of variance (ANOVA). Overall counts for the number of Fos and ChAT labeled cells in the LDT were analyzed independently by a One-Way analysis of variance (ANOVA). Where appropriate, Bonferonni’s correction was applied to post hoc analysis. Double labeling within the LDT was examined by use of a Kruskal-Wallis One-Way ANOVA with further exploration using the Mann-Whitney U test.
Results:

Number of Animals

One animal (ASM 20) was excluded from the entire study due to a lack of successful recording of vocalizations post air puff stimulation. Two animals, ASM 1 and ASM 3, which were used in the preliminary Fos expression study (Time 0 and Time 30), were also excluded from all subsequent analysis for consistency. Therefore, a total of 62 animals were utilized in this study (refer to Table 2 for n values for experimental conditions).

Localization of Injection Sites

Carbachol was unilaterally injected into the right medial preoptic area of 23 animals (Figure 5, filled circles). Of these, 13 (57%) were on target, while the remaining 10 (43%) were in the neighboring areas. All injection sites that induced calls were localized in the medial preoptic area, at the border of the anterior hypothalamic areas, and at the border of the lateral preoptic area.

Injection sites for Cch that did not produce calls were localized near the medial preoptic area (n=10), one site at the border of the lateral preoptic area and one site in the fornix (Figure 5, empty circles). Since spread of the injected substance in a volume of 0.2 μl is approximately 0.5-1 mm from the injection site (Myers, 1974), all of the sites were included in the study. The furthest injection site in the fornix was also retained because of its proximity to the dorsal portion of the median preoptic nucleus (above the anterior commissure).

Isotonic saline was injected into the medial preoptic area of 9 animals. Of these, 4 (44%) were on target, while the remaining 5 (55%) were in the neighboring areas (including 3 sties at the border of the anterior commissure). Injection of saline failed to induce 22 kHz ultrasonic vocalizations from any of the sites (Figure 5, dotted circles). Thus, all injection sites were included in the study.
Table 2: Description of Experimental Conditions and the Sample Size per Condition.

<table>
<thead>
<tr>
<th>Experimental Condition</th>
<th>Description</th>
<th>(n)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pharmacological</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cch &amp; Voc</td>
<td>i.c. injection of carbachol with subsequent vocalization</td>
<td>(11)</td>
</tr>
<tr>
<td>Cch no Voc</td>
<td>i.c. injection of carbachol without subsequent vocalization</td>
<td>(10)</td>
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<tr>
<td>Natural Call Induction</td>
<td></td>
<td></td>
</tr>
<tr>
<td>AP &amp; Voc</td>
<td>air puff stimulation with subsequent vocalization</td>
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</tr>
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</tbody>
</table>
Figure 5: Location of injection sites within the medial preoptic area where carbachol or saline was injected. Each circle represents an injection site for one animal. Alarm calls were induced from 13 sites (filled circles). Injections into remaining 19 sites (empty circles for carbachol and dotted circles for saline) failed to induce 22 kHz calls. Abbreviations: CPU-caudate putamen, ac-anterior commissure, GP-globus pallidus, BS-Bed nucleus of stria terminalis, MPA-medial preoptic area, LPO-lateral preoptic area, MPO-medial preoptic nucleus, MPN-Median preoptic nucleus, ox-optic chiasm. Scale: 1mm.
Behavioural Response to Carbachol and Air-Puff

In an effort to determine whether vocalizations elicited by carbachol or by air puff stimulation were species-typical alarm calls, sonographic analysis was performed. Figure 6 demonstrates a typical call pattern following intracerebral injection of Cch. This sonogram depicts a series of four calls, each with a peak frequency of 23.0 kHz, and with call durations of 0.487s, 0.272s, 0.319s, and 0.322s, respectively. The average duration of the entire response for all animals in this experimental condition was 8.3 min (± 0.73 min).

Figure 7 demonstrates one of the typical calls induced by air puff stimulation to the head and neck region of a rat. The call indicated on this sonogram (excluding a short fragment of the next call to the right) has a peak frequency of 23.7 kHz and a total duration of 1.622s. The average response duration was 11.6 min (±0.97 min) for all AP & Voc animals.

Regardless of whether calls were induced by direct intracerebral injection of Cch or by non-invasive application of air puff, similar behavioural characteristics were observed between experimental conditions. Similarly to the results of Fu & Brudzynski (1994), animals in both vocalizing conditions ceased locomotor activity while emitting calls. Animals assumed a hunched posture and were completely motionless during vocalizations (with the exception of visible deeper breathing patterns which coincided with the calls). Activity and/or exploration of the recording cage would always resume between each series of calls.

The acoustic parameters of all calls measured in these two conditions were within the boundaries of alarm calls (peak sound frequency between 20 and 25 kHz and single call duration more than 200ms).
Figure 6: A sonogram of ultrasonic vocalizations induced by intracerebral injection of 1 µg of carbachol into the medial preoptic area of a rat. (ASM 51)
The peak sound frequency and duration of the emitted calls are: 23.0 kHz and 0.487s (first call), 23.0 kHz and 0.272s (second call), 23.0 kHz and 0.319s (third call) and 23.0 kHz and 0.322s (fourth call), respectively.

Figure 7: A sonogram of ultrasonic vocalization induced by air puff stimulation to the head and neck region of a rat. (ASM 49)
The peak sound frequency and duration of this call are 23.7 kHz and 1.622 s. The short sonogram line on the right hand side is the beginning of the next call.
Vocalization Induced by Carbachol Stimulation

Injection of carbachol into the medial preoptic area (MPA) resulted in the emission of 22 kHz alarm-type vocalizations. The average peak sound frequency was determined to be 23.79 +/- 2.7 kHz (SEM) (Figure 8, left black bar), and the average bandwidth was 5.96 +/- 2.1 kHz (SEM) (Figure 8, left gray bar). The average call duration was 0.62 +/- 0.08 s (SEM, Figure 8, left hatched bar), and the average latency of the onset of vocalization was 1.33 +/- 0.18 min (SEM) (Figure 8, left blank bar). Calls after Cch were emitted at a rate of 235 +/- 28 calls/5min (data not shown).

Vocalizations Induced by Air Puff Stimulation

Air puff stimulation resulted in the emission of characteristic 22 kHz alarm calls. The average peak sound frequency was 25.33 +/- 2.59 kHz (SEM, Figure 8, right black bar), and the average bandwidth was 15.01 +/- 2.03 kHz (SEM) (Figure 8, right gray bar). The average call duration was 1.18 +/- 0.08 s (SEM) (Figure 8, right hatched bar) and the average latency of vocalization was 1.61 +/- 0.17 min (SEM) (Figure 8, right blank bar). Rate of call emission was 116 +/- 27 calls/5min (data not shown).

Comparison of Vocalizations Induced by Carbachol versus Air Puff

Statistical analysis of these parameters (Figure 8) by use of Analysis of Variance (ANOVA) revealed significant differences among the vocalization parameters (F(2,7)=39.07, p<0.0001). Exploration of data using unpaired t-tests revealed no significant difference for latency (t(23)= 1.091, p=.287, ns), and peak sound frequency (t(23)= .410, p=.685, ns). Significant differences were discovered for the average call duration (t(23)= 4.771, p<.0001), the average call bandwidth (t(23)= 3.078, p=.005, and the number of calls emitted per 5 min (t(23)= 3.077, p=.005)
Figure 8: Bar graph demonstrating acoustic parameters for vocalizations following Cch or air puff. The Y axis on the left side of the graph pertains to bars for frequency and bandwidth and the Y axis on the right side of the graph pertains to latency (min) or call duration (ms). Bandwidth and call duration after air puff significantly differed from those after Cch (t-test, * p<0.005, ** p<0.0001).
In short, calls after Cch injection were emitted with faster rate and were shorter in duration but were maintained at the same sound frequency as calls emitted following air puff stimulation.

**Peak Fos Expression**

In an effort to determine the most suitable length of time from stimulation to maximal Fos expression, five animals (ASM 1,3,5,8,9) were sacrificed at 0, 30, 60, 90 and 120 min post injection of Cch into the MPA, and were fixed by perfusion with PPG fixative.

Analysis of cell counts obtained from the lateral septum (LS) of vocalizing animals revealed a significant difference in the number of Fos labeled cells at different times post stimulation (Figure 8, ANOVA, F(4,5)=40.67, p<0.001). Unpaired t-tests revealed significant differences between 30 and 60 min (t(18)=10.03, p<0.0001), 60 and 90 min (t(18)=3.77, p=0.001) and 90 and 120 min (t(18)=2.64, p=0.017). Figures 9 & 10 clearly demonstrate this increase in Fos labeled cells. When compared to the upper and lower most photographs of 60 and 120 min respectively, and to the relative background, a higher number of brightly fluorescing cells can be seen at 90 min post injection of Cch into the MPA.

Based on this analysis the most appropriate time to perfuse animals was determined and, as a result, all subsequent experimental animals were sacrificed 90 min post stimulation.
Figure 9: Time in minutes, from injection of carbachol into the medial preoptic area, to perfusion.
Bar graphs indicate the mean number of Fos labeled cells expressed in the lateral septum of vocalizing animals (1x10^4 μm^2). Vertical lines represent SEM. *p<0.01, **p<0.001, ***p<0.0001.
Figure 10: Microphotographs of coronal sections of the rat lateral septum demonstrating Fos labeled neurons at 60, 90 and 120 minutes post intracerebral injection of Cch into the MPA. The arrow in the leftmost figure indicates the target site in the lateral septum (LS). In the uppermost photograph: 60 min, the middle photograph 90 min and the lowermost photograph 120 min after injection. The insert on the left is taken from the stereotaxic atlas of Paxinos & Watson (1986) 9.2 mm from the interaural plane. Bar: 50μm.
Histochemical Controls

Antibody Specificity

To provide insight into the specificity of antibody labeling, several controls were performed (Table 1). For the first control condition, neighboring sections of LDT from animal ASM 45 underwent identical ChAT incubations with omission of specific steps in the Fos labeling procedure.

As indicated in Figure 11a (top image), incubation with the secondary Fos antibody without the required primary antibody (control #1) resulted in a small background of autofluorescence, but more importantly, a lack of specific Fos fluorescence. As expected, examination of this identical location in the tissue using a wavelength specific for FITC revealed that incubations with unaltered ChAT antibodies resulted in a number of brightly fluorescing neurons, indicated by the presence of bright green cells, as compared to background (Figure 11a, lower image).

Similar results were achieved when the neighboring section of tissue was incubated with the primary Fos antibody without the appropriate Cy3 conjugated secondary antibody (control #2). As indicated in Figure 11b (top image), a small amount of background autofluorescence was noticed, but more importantly, a lack specifically labeled Fos neurons. Similar to the first control, unaltered ChAT incubations resulted in several brightly labeled cholinergic neurons within the LDT (Figure 11b, lower image).

Subsequent controls were designed to shed insight on the specificity of ChAT labeling. Sections of tissues from animal ASM 45 underwent unaltered incubations with Fos antibodies but either the primary or secondary ChAT antibodies were omitted. As demonstrated in Figure 12a (top image), incubation with unaltered Fos antibodies resulted in brightly fluorescing in red
neuronal nuclei, but omission of the primary ChAT antibody (control #3) resulted in a lack of specific cellular labeling for ChAT in the same sections (Figure 12b, lower image).

Similarly, unaltered Fos incubations, followed by application of the primary ChAT antibody without the secondary ChAT antibody (control #4) resulted the presence of specific Fos labeled neurons (Figure 12 b, top image), but a lack of specific ChAT labeled cells (Figure 12b, lower image). As a result of these four controls, the antibodies used in this study were determined to be specific for their intended targets of Fos and ChAT positive neurons.

**Anatomical Specificity**

In addition to antibody controls, this study employed anatomical controls designed to provide the relative amount of fluorescence expected to be observed following immunohistochemical procedures in, and around, cholinergic nuclei. It is known that only the medial septum-vertical limb of diagonal band contains cholinergic cell bodies (Mesulam, et al., 1984; Kasashima, et al., 1998). As indicated in Figure 13, a section of tissue containing the septal regions from an air puff induced vocalizing animal show Fos positive neurons within the LS (a-i), with an absence of ChAT labeling (b-i). On the other hand, the MS, directly neighboring the LS, demonstrated the opposite labeling pattern. As indicated in b-ii, the medial septum contains a dense group of cholinergic cells, with an absence of Fos labeled neurons (a-ii).

A second quantitative anatomical control for ChAT involved the examination of specific areas within tissue sections known to contain cholinergic cells. As mentioned previously, the MS is known to contain cholinergic cells whereas the neighbouring LS does not. Quantification of these areas from vocalizing animals revealed that, as expected, the MS contained cholinergic cells (Figure 14, first bar) and the neighboring structure of the LS did not (Figure 14, second bar).
Figure 11: Confocal images of the LDT of animal ASM 45 (25 μm thick) following immunohistochemical techniques where application of specific antibodies in Fos procedure were omitted.

The area of LDT is indicated by black arrow (top image). In (a) application of primary antibody to Fos protein was omitted, ChAT antibody application steps were unaltered. In (b) application of secondary antibody for Fos protein was omitted, steps involving ChAT antibody application remained unaltered. Bar: 100μm.
Figure 12: Fluorescence Confocal images from animal ASM 45 (25 µm thick) following immunohistochemical techniques where application of specific antibodies in ChAT procedure were omitted.

In (a) application of primary antibody to ChAT was omitted, Fos antibody application steps were unaltered. In (b) application of secondary antibody for ChAT protein was omitted, steps involving Fos antibody application remained unaltered. Location of target structure (black arrow) is indicated in top inlays (a) and (b). Bar: 100µm.
Figure 13: Fluorescent images demonstrating anatomical and cellular specificity of antibodies used for the detection of Fos protein and ChAT.

Abbreviations: row a- images from lateral septum (LS) indicated by arrow in top inlay, row b- images from the medial septum (MS) indicated by arrow in top inlay, column i-Fos labeled cells, column ii-ChAT labeled cells, column iii-overlay of images i and ii. All images taken from animal ASM 45 who received air puff stimulation and subsequently vocalized. Bar: 100μm.
Figure 14: Bar graph demonstrating anatomical specificity of ChAT labeling (in all vocalizing animals). Comparable numbers of ChAT cells were observed in medial septum and laterodorsal tegmental nucleus but not in the neighbouring lateral septum or reticular formation. t-test *p<0.01, **p<0.001.
Examination of the LDT and the neighboring reticular formation revealed similar results to those from the septum. The LDT is known to contain cholinergic cell bodies while the neighbouring reticular formation does not (Mesulam et al., 1983). ChAT-labeled cells were quantified in the LDT and neighbouring reticular formation. As indicated in Figure 14 (right two bars), quantification of ChAT labeling for all vocalizing animals (both pharmacologically and air puff induced), confirmed the presence of cholinergic cells within the LDT (Figure 14, third bar) and the distinct absence of cholinergic cells within the reticular formation (Figure 14, fourth bar). A One-Way ANOVA confirmed significant differences in the distribution of cholinergic cells (F(3,4)=24.76,p<0.0001). As neither the LS nor the reticular formation were expected to demonstrate ChAT labeled cells further exploration of the data set was not performed.

**Fos Labeling in the LDT**

The first analysis performed was to determine whether any significant difference existed in the overall number of Fos labeled cells in the LDT across all experimental conditions. A One-Way ANOVA revealed statistical significance F(5,6)=3.853, p<0.003 (Figure 15). Further analysis of the data using unpaired t-tests revealed that animals in the Cch & Voc condition had a significantly higher number of Fos labeled cells when compared to Cch no Voc (t(43)=3.816, p<0.001), and to Saline (t(39)=3.575, p<0.01) (Figure 15a). No significant difference existed between controls (Cch no Voc and Saline) in the pharmacological conditions (t(37)= 0.125, ns, Figure 15a). When comparing both vocalizing conditions of Cch & Voc and AP & Voc a significantly higher number of Fos labeled cells were found in the pharmacological condition (t(47)=2.214, p< 0.03). Interestingly, no increase in Fos labeled cells was observed across the air puff conditions. Animals in the AP & Voc condition (Figure 15b) demonstrated no significant difference in Fos counts when compared to AP no Voc (t(45)=0.140, ns), and naïve controls.
(t_{43})=0.184, \text{ ns}). No significant difference was found between the control conditions of AP no Voc and Naïve (t_{37})=0.319, \text{ ns}). Since neither of the controls in the pharmacological conditions demonstrated an increase in the number of Fos labeled cells but the vocalizing condition did, the increase in Fos expression in the LDT is specific to vocalization. Unlike the pharmacological conditions, no increase in Fos labeled cells was found in the LDT of any experimental condition in the air puff paradigm.

The second analysis involved a One Way ANOVA to determine whether any difference existed in the labeling pattern of Fos between left vs. right side in the LDT (Figure 16). This was performed to ensure that potential differences in Fos labeling in the pharmacological conditions were not a result of the unilateral injection of Cch or saline into the MPA resulting in a greater number of Fos labeled cells present in the LDT ipsilateral or contralateral side to the injection. Although significance was found (ANOVA F(11,6)=2.862, p<0.001), closer examination revealed that the significance resided across experimental conditions (i.e. the left LDT of Cch & Voc as compared to the left side LDT of Cch no Voc [t_{41}]=4.485, p<0.01] and the left LDT of Cch & Voc as compared to the right LDT of Saline [t_{39}]=4.106, p<0.01]) rather than between the comparable conditions (pairs of bars). Since significance wasn’t found between counts taken from the left and right sides of the LDT, it is safe to assume that a unilateral injection of Cch or Saline into the brain had little to no effect on the laterality of the labeling pattern of Fos in the LDT. In regards to the air puff condition, it is not surprising that significance was not found, as the external stimulus (air puff) was not lateralized.

The third analysis compared Fos labeling in each of two scan areas (Figure 17, Scan 1 vs. Scan 2). Placement of these scan areas followed the pattern of ChAT labeled neurons within the LDT. Fos labeling could occur in close proximity to ChAT cells or further away from them. There were no significant differences observed in the number of Fos labeled cells between Scan
1 and Scan 2 in each condition. However, an overall One-Way ANOVA revealed a significant difference ($F(11,6)=2.325, p<0.01$) which was limited to the difference between Scan 1 in the Cch & Voc condition and Scan 2 in the Cch no Voc condition. Also, regardless of whether the animals were in the pharmacological or natural call paradigms the area labeled as Scan 1 demonstrated a slightly higher number of Fos labeled cells. Examples of c-Fos labeled cells in the LDT are illustrated in Figure 18.
Figure 15: Bar graphs demonstrating number of Fos labeled cells in the LDT in all experimental conditions. (a)- Pharmacological call induction. **Abbreviations:** Cch & Voc- carbachol followed by vocalization, Cch no Voc- carbachol without vocalization, Saline- saline control. (b)- Natural call induction. **Abbreviations:** AP & Voc- air puff followed by vocalization, AP no Voc- air puff without vocalization, Naïve- naïve control. *t*-test, p<0.001 in comparison to Cch no Voc and p<0.01 in comparison to saline.
Figure 16: Bar graphs demonstrating number Fos labeled cells in the LDT of all experimental conditions (left vs. right side). (a)- Pharmacological call induction. **Abbreviations**: Cch & Voc- carbachol followed by vocalization, Cch no Voc- carbachol without vocalization, Saline- saline control. (b)- Natural call induction. **Abbreviations**: AP & Voc- air puff followed by vocalization, AP no Voc- air puff without vocalization, Naïve- naïve control. The differences between bars of each pair were not significant in all conditions.
Figure 17: Bar graphs demonstrating the number of Fos labeled cells in the LDT of all conditions (Scan 1 vs. Scan 2). (a)- Pharmacological call induction. **Abbreviations:** Cch & Voc- carbachol followed by vocalization, Cch no Voc- carbachol without vocalization, Saline- saline control. (b)- Natural call induction. **Abbreviations:** AP & Voc- air puff followed by vocalization, AP no Voc- air puff without vocalization, Naïve- naïve control. Differences between bars of each pair were not significant in all conditions.
Figure 18: Exemplary confocal fluorescence images of labeling pattern of Fos protein in the LDT following intracerebral injection of Cch into the MPA.

Abbreviations: LDT- laterodorsal tegmental nucleus, DT-dorsal tegmental nucleus, 4V-fourth ventricle. Blue squares in the insert indicate location of Scan 1 and 2. Top overlapping images are from right side of LDT and lower overlapping sets of images are from left side of LDT. Bar: 100μm.
ChAT Labeling in the LDT

Placement of the scan areas was performed in such a way that Scan 1 always had a higher number of ChAT-labeled cells than Scan 2. Figure 19 illustrates the number of ChAT labeled cells in the LDT of all experimental conditions. This figure demonstrates that, overall, all experimental conditions had approximately the same number of ChAT labeled cells and always more in Scan 1 as compared to Scan 2. Analysis using a One-Way ANOVA revealed significance in the numbers of cholinergic cells between scan 1 and scan 2. F(11,6)=10.82, p<0.0001. Exploration of the data set using unpaired t-tests revealed significance in the Cch & Voc condition (t(11)=4.021, p<0.01), the AP & Voc condition (t(11)=5.332, P<0.001), the AP no Voc condition (t(11)=5.723, p<0.001), and the naïve condition (t(11)=5.029, p<0.001). The difference did not reach significance in the remaining two conditions.

As shown in Figure 20, Scan 1 (white square) was placed slightly above (in relation to the top of the brain) and aside from Scan 2 (blue square) and resulted in a greater number of ChAT labeled cells than in Scan 1. This figure also clearly demonstrates the variability in the shape and distribution of cholinergic cells. Positively labeled ChAT cells were large, either fusiform or round in shape, and were oriented in several directions. The ChAT labeling was also observed to be uniformly expressed throughout the cells with the exception of the nuclear area which was devoid of the label.
Figure 19: Bar graphs illustrating the number of ChAT labeled cells in Scan 1 vs. Scan 2 in the LDT of all conditions. (a)- Pharmacological call induction. Abbreviations: Cch & Voc- carbachol followed by vocalization, Cch no Voc- carbachol without vocalization, Saline- saline control. (b)- Natural call induction. Abbreviations: AP & Voc- air puff followed by vocalization, AP no Voc- air puff without vocalization, Naïve- naïve control. *t-test, p<0.01, **p<0.001.
Figure 20: Overlapping fluorescence confocal images of the LDT demonstrating ChAT labeling. 
**Abbreviations:** LDT- laterodorsal tegmental nucleus, DT-dorsal tegmental nucleus, 4V-fourth ventricle, DR- dorsal raphe nucleus. The white square indicates the first scan area (Scan 1), and the blue square the second scan area (Scan 2). Left most insert is taken from Paxinos and Watson (1986). Images scanned at 20X magnification. Scale bar: 100μm (top right).
Comparison of Fos and ChAT Labeling in the LDT

Examination of the LDT revealed the presence of numerous single-stained ChAT cells (Figure 21a) or Fos-labeled cells (Figure 21b) in all experimental conditions. In all cases, the pattern was such that Fos labeled nuclei were in close proximity to ChAT labeled neurons (Figure 21c). Closer examination using the “zoom” function in the acquisition software accompanying the confocal microscope revealed Fos labeled nuclei immediately adjacent to ChAT labeled neurons (Figure 22a) and, in some cases, the Fos nuclei were directly overlapping the cholinergic neurons (Figure 22b white arrow) but these ChAT neurons were not double labeled. The relative proportion of double-labeled cells within the LDT as compared to single stained Fos and ChAT-labeled cells can be seen in Figure 23.
Figure 21: Fluorescence confocal microscopy demonstrating positively labeled Fos and ChAT cells within the LDT of an animal who vocalized following air-puff stimulation (ASM 27). Abbreviations: (a)- FITC labeled cholinergic cells (green), (b)- Cy3 labeled Fos nuclei (red), (c)- overlay of images a and b. Scans taken at 20X magnification. Bar: 100µm.
Figure 22: Fluorescence confocal microscopy demonstrating Fos and ChAT labeled cells within the LDT of an animal who received air-puff stimulation and subsequently vocalized (ASM 26). Abbreviations: (a)- 20X scan of LDT demonstrating Fos labeled nuclei (red) and ChAT labeled cells (green) (b)- zoomed scan of image a. White arrow is demonstrating a Fos labeled cell directly overlapping a different ChAT labeled neuron. Scale (a): 100μm (b): 50μm.
Figure 23: Bar graphs demonstrating Fos-, double- and ChAT-labeled cells in the LDT of all experimental conditions. (a)- Pharmacological call induction. Abbreviations: Cch & Voc- carbachol followed by vocalization, Cch no Voc- carbachol without vocalization, Saline- saline control. (b)- Natural call induction. Abbreviations: AP & Voc- air puff followed by vocalization, AP no Voc- air puff without vocalization, Naïve- naïve control.
Double Labeled Cells in the LDT

Double-labeled cells were counted only when the Fos labeled nucleus was directly located within the unstained nuclear space of ChAT neurons. Analysis of the number of double labeled cells in Scan 1 and Scan 2 in the LDT (Figure 24a) by One-Way ANOVA revealed statistical significance \( F(5,6)=15.42, p<0.01 \) Kruskal-Wallis. Further analysis of the data set determined significant differences between Cch & Voc and Cch & no Voc \( p<0.01 \), Mann-Whitney U-test, Figure 24a, black and gray bars) and between Cch & Voc and Saline \( p<0.05 \), Mann-Whitney U-Test, Figure 24a, black bar and clear bar). The Mann-Whitney U-test revealed no significant differences between the pharmacological control conditions of Cch no Voc and Naïve \( p=.543, \) ns, Figure 24a, gray and clear bar).

Also, a U-test performed on the air puff conditions revealed that the AP & Voc condition had a significantly higher number of double labeled cells than the AP no Voc group \( p<0.005 \), Mann-Whitney U-Test, Figure 24a, cross hatched bar and diagonally hatched bar) but not the Naïve condition \( p=.139, \) ns, Figure 24a, cross hatched and vertically hatched bar). No significant difference was determined between the control conditions of AP no Voc and Naïve \( p=.157, \) ns, Figure 24a, diagonally hatched and vertically striped bars). These results suggest that rats that vocalized following either Cch injection or air puff stimulation have a higher number of double labeled cells within the LDT.

During quantification of the LDT labeling, and prior to analysis of the results, a number of double labeled neurons were found to be located immediately outside the two selected areas (Scan 1 and Scan 2) but still within the boundaries of the LDT according to the atlas of Paxinos and Watson (1986). As a result, the size of the quantification area was increased to one large square of \( 25 \times 10^4 \mu \text{m}^2 \) in diameter in an effort to ensure the best representative results of double-
labeled neurons within the LDT. The increased quantification square still remained within the boundaries of the LDT.

The overall number of double-labeled cells in the large scan area is illustrated in Figure 24b. A One-Way ANOVA confirmed earlier significant differences across all experimental conditions (Figure 24b, F(5,6)=29.42, p<0.001, Kruskal-Wallis). A Mann-Whitney U-Test revealed slight variations in significance levels within the conditions. Similar to the previous result, the Cch & Voc condition had a significantly higher number of double labeled neurons when compared to both control conditions of Cch no Voc (p<0.001, Figure 24b, black and gray bars) and Saline (p<0.05, Figure 24b, black and clear bars). No significant difference existed between the Cch no Voc and the Saline condition (p=.346, ns, Figure 23b, gray and clear bars). The vocalizing condition of AP & Voc also had a higher number of double-labeled neurons than the AP no Voc condition (p<0.05, Figure 24b, cross-hatched and diagonally hatched bars) but not higher than the Naïve condition (p=.564, ns, Figure 24b cross hatched and vertically striped bars). However, a significantly higher number of double-labeled neurons were discovered in the Naïve condition when compared to the control condition of AP no Voc (p<0.05, Figure 24b, diagonally and vertically striped bars).

An example of the type of double-labeled cells seen in the LDT is shown in Figure 25. Images (a) and (b) demonstrate ChAT and Fos labeling, respectively, within the LDT of a vocalizing animal following air puff stimulation (ASM 45). An overlay of these images demonstrates a double-labeled cell (image (c), white arrow) with a clearly visible Cy3 labeled Fos nucleus within the nuclear space of a FITC labeled ChAT cell. A double-labeled cell in the LDT of non-vocalizing animal following air puff stimulation (ASM 32) is shown in Figure 26 (a) and (b).
Similar to the previous figure, image (a) demonstrates the presence of both Fos and ChAT labeled cells scanned at 20X magnification. Closer examination of a particular area of this image using the zoom feature within the acquisition software demonstrates a double-labeled cell (Figure 26b, white arrow).

**Fos Labeling in the Lateral Septum**

In order to determine whether increased Fos labeling in the LDT was a unique pattern, Fos-labeled cells in the lateral septum were also counted in all experimental conditions. Fos labeling within the lateral septum (Figure 27) was assessed by a One-Way ANOVA, and a significant difference was present across conditions (F(5)=9.019, p<0.0001). Further examination of the data with unpaired t-tests revealed that Cch & Voc animals had a significantly higher number of Fos labeled nuclei compared to Cch no Voc (t(20)=2.739, p<0.05) and Saline (t(19)=3.092, p<0.05) conditions. No significant difference in the number of Fos labeled cells was found between control conditions of Cch no Voc and Saline (t(18)=0.296, ns). Also, a significant difference was determined between the AP & Voc condition and Naïve (t(21)=5.365, p<0.001), but none was determined between the AP & Voc condition and AP no Voc (t(22)=1.135, ns). Interestingly, a significant difference was present between the control conditions of AP no Voc and Naïve (t(18)=4.142, p<0.01).

Examination of the overall pattern of Fos and ChAT labeling within the septal region using confocal microscopy revealed that cholinergic cells were not present in the LS. Conversely, almost no Fos labeling was evident in the MS. However, among the 62 experimental animals, a total of 2 double-labeled cells were found within the boundaries of the MS. As a result, the double-labeled cells of the MS were not studied further.
Figure 24: Bar graphs of mean number of double labeled cells in the LDT of all experimental conditions. Abbreviations: (a)- number of double labeled cells in smaller scan areas of all experimental conditions, (b)- number of double labeled cells observed in larger scan areas of all experimental conditions. *p<0.05, **p<0.01 (Mann-Whitney U test). Significantly higher counts were compared to one or both control conditions (see text for details).
Figure 25: Exemplary fluorescence confocal images of double labeled cell within the LDT of a vocalizing animal (ASM 45). Abbreviations: (a) FITC labeled cholinergic cells (green) (note the unlabeled nuclear spaces), (b) Cy3 labeled Fos nuclei (red) within the same scanned area, (c) overlapping image of a and b. A double-labeled cell (image c) is indicated by white arrow. Images scanned at 20X. Bar: 100μm.
Figure 26: Exemplary fluorescent confocal image demonstrating a double-labeled cell within the LDT of a non-vocalizing animal (ASM 32).

Abbreviations: (a) confocal image of LDT labeled with FITC (green) and Cy3 (red) scanned at 20X magnification, (b) zoomed area of the same image at the same magnification (blue square) using the zoom feature of the acquisition software clearly demonstrates a double-labeled cell (image (b), white arrow). Bar (a): 100μm, (b): 50μm.
Figure 27: Bar graph demonstrating the mean number of Fos labeled cells within the lateral septum of each experimental condition. *p<0.05, **p<0.01, ***p<0.001 (t-test).
Discussion

Overview of Results

Intracerebral injection of carbachol (Cch), a predominantly muscarinic agonist, into the medial preoptic area (MPA) of rats resulted in the production of characteristic 22 kHz alarm-type vocalizations. Application of an air puff of 40 psi to the head and neck region of rats resulted in the production of 22 kHz vocalizations similar to those elicited following Cch injection. Calls induced by both methods had average sound frequencies of 23-25 kHz and average single call durations of 620-1200 ms. Individual calls following air puff application were found to have a wider bandwidth and were longer in duration than those following Cch injection into the MPA.

Immunohistochemical staining for Fos resulted in a significantly higher number of Fos labeled nuclei in the LDT of rats in the Cch and vocalizing condition than in the non-vocalizing condition. No other increase in Fos labeling was observed across other experimental conditions (air-puff). Interestingly, within the septum, the pattern of Fos labeling was slightly different. A higher number of Fos labeled nuclei was observed in both vocalizing conditions (Cch & Voc and AP & Voc) and in the AP no Voc condition.

Regardless of the size of the scan area (either 4x10^4 µm^2 or 25x10^4 µm^2), examination of the number of double-labeled cells within the boundaries of the LDT revealed that the vocalizing condition of Cch & Voc had a significantly higher number of these cells than did the control conditions of Cch no Voc and Saline. Similarly, the vocalizing condition of AP & Voc also had a significantly higher number of double-labeled cells when compared to the AP no Voc but not the Naïve condition.

Lastly, this study provided insight into a technical issue that the best time to sacrifice rats to achieve the highest number of Fos labeled cells within the brain was 90 minutes post stimulation.
Acoustic Parameters

Prior to examination of the LDT, establishment of whether the emitted ultrasonic calls were comparable between vocalizing conditions was crucial.

The most critical components of the 22 kHz calls were common for both vocalizing conditions (Rationale section: initial assumption). All calls were maintained within the range of alarm frequencies (20-30 kHz), and all single calls were longer in duration than 200 ms. However, analysis of the acoustic parameters of emitted calls (Figure 8) between the vocalizing conditions revealed significant differences between call duration (the length of individual calls) and call bandwidth but no significant differences in call latency or average call frequency. Vocalizations emitted following intracerebral injection of Cch were shorter in duration than those following air puff stimulation and, as a result, the Cch & Voc condition had over twice as many calls per five minute interval.

One possible explanation for the differences in length of calls could be that direct intracerebral injection of Cch into a portion of the cholinergic strip stimulates the vocalization system to such a degree that individual calls are shorter but are emitted at a higher rate so that pharmacological stimulation (or over stimulation) caused stronger activation of the cholinergic vocalization system. The pace of call emission after air puff stimulation was similar to those that appear more frequently in natural conditions (Brudzynski & Ociepa, 1992, Blanchard, et al, 1991).

The significant difference discovered in the bandwidth, or range of call frequencies, between the vocalizing conditions (Figure 8, gray bar) would lend support to the above notion of over stimulation. As indicated in Figure 8, animals that vocalized following air puff stimulation had a wider range of frequencies in their emitted calls than calls after Cch. This is indeed the case when one examines the very first call in a series of calls following air puff stimulation. The
first call always began at a much higher frequency followed by a rapid downward slope in frequency until the desired frequency of 22 kHz was reached (data not shown). Referred to in the literature as frequency fluctuation or modulation (Sales & Pye, 1974), this downward component was never observed in the Cch & Voc condition as rats always began their series of calls at, or very near, the peak frequency of 22 kHz calls. At faster rates of call emission the initial sweep disappeared. Statistical analysis of the average call frequency found no difference between the two conditions (Figure 8).

In short, despite the initial frequency modulation observed in the AP & Voc condition, the mean call peak frequencies between conditions were comparable regardless of whether calls were induced by “postsynaptic” (intracerebral injection of Cch into the MPA), or by “presynaptic” (air puff to the head/neck region) stimulation. Such results are congruent with previous work by Bihari et al., (2003) in which comparable 22 kHz vocalizations were emitted by either “presynaptic” (glutamate into LDT) or “postsynaptic” (Cch into MPA) stimulation.

Lastly, no significant difference was found between the latency of call onset between vocalizing conditions (Figure 8, clear bar) for the dosage of Cch into the MPA (1μg in 0.2μL of saline) and the air puff stimulation at 40 psi used in this study. In other words, regardless of the method of call induction, the length of time from stimulation to the onset of first 22 kHz call was very similar.

The lack of significance between critical acoustic parameters for both vocalizing conditions lends full support to the first assumption of this study. One can therefore conclude that animals in both vocalization paradigms emitted the same 22 kHz alarm-type vocalizations—regardless of whether the calls were induced by pharmacological means or by air puff stimulation.
Fos Labeling in the LDT

Our original model (Figure 2) was based on the notion that the LDT in the Cch & Voc condition would be less active than in the AP & Voc condition since Cch targets the cholinergic vocalization system postsynaptically (Figure 2: “Activation by CCh” leading to “OUTPUT”). As a result of this model, the first prediction examined was that rats in the AP & Voc condition would have a higher amount of activity (as measured by the number of Fos labeled cells) in the LDT than rats after Cch stimulation. Results of this study did not fit this prediction.

Statistical analysis of the overall number of Fos labeled cells within the LDT (Figure 15) revealed two findings. First when compared to pharmacological controls (Cch no Voc and Saline), animals in the Cch & Voc condition had a significantly higher number of Fos labeled cells in the LDT following vocalization. Second, no significant increase in the number of Fos labeled cells was found in the LDT of any animal in the natural call induction condition (AP & Voc, AP no Voc or Naïve). The increase in Fos labeling observed in the Cch & Voc condition is not surprising. There is strong support in the literature demonstrating the involvement of the LDT in the initiation Cch induced 22 kHz alarm-type vocalizations (Jurgens et al., 1996, Brudzynski, 1994, Brudzynski et al., 1991, Fu & Brudzynski, 1994). What is surprising is the lack of increase in Fos labeling in the AP & Voc condition. Initially, based on the lack of significant Fos labeling, one would be tempted to conclude that the LDT is simply not involved in the initiation of 22 kHz vocalizations when they are emitted under “natural” conditions, or that the air puff stimulation is too weak of a stimulus to detect change in LDT activity. However, examination of the level of Fos labeling achieved in the lateral septum of both vocalizing conditions indicates otherwise.
As mentioned previously, the lateral septum is an area innervated by the medial cholinoceptive vocalization strip (Brudzynski, 2001, Brudzynski, 1998), and is known to express high Fos activity following ultrasonic vocalization (Savoy, et al., 2001) (Figure 28: “LS”). As one would expect, following vocalization, both Cch & Voc and AP & Voc demonstrated a significantly higher number of Fos labeled cells in the lateral septum when compared to control conditions. This increase in Fos labeling (in both vocalizing conditions) indicates that at least part of the cholinergic system, and possibly some non-cholinergic cells from the LDT, are involved in the production of 22-kHz vocalizations - regardless of whether the system is targeted postsynaptically (by injection of Cch into the MPA) or by presynaptic means (by application of air puff) (Figure 28). Such a response could happen by mutual connections between the medial preoptic area (MPA) and the LDT (Figure 28: dashed connection back to LDT).

One other interesting finding that warrants’ mentioning is the significant increase in Fos labeling observed in the LS of the AP no Voc condition (Figure 27). This result suggests that the increase in Fos labeling observed in the LS of this condition might be indicative of some aversive emotional state induced by application of the air puff stimulus itself. Since the air puff stimulus is comprised of auditory and tactile components (Knapp & Pohorecky, 1995) it is plausible that one, or both, of these components could lead to an increase in Fos labeling in the LS of animals in this condition. If this were the case, one would expect to observe an increase in Fos labeling in other structures that comprise the limbic system following application of air puff. Since the focus of the present study was to closely examine the LDT nucleus across various experimental conditions, future studies are required to further elucidate which aspect of the air puff stimulus may, or may not, be leading to an increase in the amount of Fos labeling in the LS of the AP no Voc condition.
Figure 28: Revised schematic diagram of cholinergic system involved in the production of ultrasonic vocalization. Cholinergic cell indicated by dotted circle and non cholinergic cells indicated by empty circles.
In general, the increase in Fos labeling observed in the LDT and LS of the Cch & Voc condition indicates that both areas are involved in the production of Cch induced 22 kHz vocalizations. However, the lack of increased Fos labeling observed in the LDT of the AP & Voc condition requires some explanation.

First, one may suggest that the air puff paradigm of 40 psi used to induce 22 kHz alarm-type vocalizations wasn’t a sufficient stimulus to elicit Fos expression within the LDT. Previous work by Knapp & Pohorecky (1995) indicates that a high-intensity air puff of 75 psi can be used to reliably induce 22 kHz vocalizations, but doing so resulted in a large attenuation of elicited vocalizations over the duration of the trial. Also, increasing the pressure of the air puff increased the startle response experienced by the rats (Knapp & Pohorecky, 1995). Since the desired result was for rats to emit a maximal number of 22 kHz vocalizations, without an attenuation of the response, 40 psi was chosen for the current study. The fact that rats in the AP & Voc condition did emit characteristic 22 kHz calls, and demonstrated a significantly higher number of Fos labeled cells in the LS than in naïve animals, indicates that input to the cholinergic system (Figure 28: “Activation by Air Puff”) was sufficient to trigger the output (Figure 28: “VOCALIZATION”) and produce the desired response of 22 kHz alarm-type vocalizations.

A second possible explanation for the lack of increased Fos expression in the LDT of the AP & Voc condition is simply that initiation of the 22 kHz vocal response is not mediated strictly by an overall increase in cellular activity (the number of Fos labeled cells) within this structure. A more important issue to address is whether there is an increase in the number of Fos labeled cells that are cholinergic in nature (as indicated by double labeling).
Double Labeling in the LDT

The second prediction of this study, designed to examine whether rats in the AP & Voc condition had a higher number of active cholinergic cells (as measured by the number of double-labeled cells) in the LDT, was partially supported. It was initially predicted that rats in the AP & Voc condition would demonstrate a higher number of double-labeled cells within the LDT than in the Cch & Voc condition since the vocalization system would be activated presynaptically (Figure 2: “Activation by Air Puff”, leading to “VOCALIZATION”). Examination of the number of double-labeled cells in the LDT (cholinergic cells that are active following emission of 22 kHz vocalizations) revealed that, regardless of the size of the quantification area, both vocalizing conditions (Cch & Voc and AP & Voc) had a higher number of double-labeled cells than the non-vocalizing control conditions (Figure 24, a & b). Though the average number of double-labeled cells observed in the LDT was relatively small, the AP & Voc condition did not demonstrate more double-labeled cells than the AP & Voc condition. The small number of double-labeled cells is consistent with the observation by Bihari et al., (2003) who determined that injection of a retrograde neuroanatomical tracer into the lateral septum resulted in only 5 to 50 retrogradely labeled cells within the boundaries of the LDT.

Although a significant difference in the number of double-labeled cells was observed between the two control conditions of the air puff paradigms (Figure 24 b, AP no Voc and Naive) all other control conditions remained relatively low. Since this increase was not observed in the smaller scan areas (Figure 24 a, AP no Voc and Naive) the increase in the number of double-labeled cells in larger scan area of the Naïve condition may be spurious.

The significant increase in the number of double-labeled cells within the LDT of the vocalizing conditions observed in this study lends support to the notion that initiation of 22 kHz alarm-type vocalizations is mediated, not by an increase in the overall amount of cellular activity
in this nucleus, but rather by a small number of specifically “active” cholinergic cells within this structure. Moreover, these cells are also activated after “postsynaptic” injection of Cch into the MPA, suggesting a mutual connection between the MPA and LDT (Figure 28: dashed line between MPA and LDT). Thus, regardless of the way of activation of the ascending cholinergic vocalization strip, reciprocal connections contribute to the activity of this system, and release of an overt behaviour. Results of this study also indicate that there were about 10-times more active, Fos-labeled cells observed in the LDT of vocalizing rats than double-labeled cells. Since the number of double-labeled cells within the LDT was small and the number of active Fos-labeled cells was high, we cannot exclude the possibility that both cholinergic and non-cholinergic cells in the LDT are active following emission of 22 kHz alarm-type vocalizations.
Literature Cited


