NEUROBIOLOGY OF BAT VOCAL BEHAVIOR

A Dissertation

by

CHRISTINE PATRICE SCHWARTZ

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

December 2010

Major Subject: Zoology

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Approved by:

Chair of Committee,	Michael Smotherman
Committee Members,	Vincent Cassone
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	Rachel Hull
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ABSTRACT

Neurobiology of Bat Vocal Behavior. (December 2010)

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Vocal plasticity is presumed to be a key element underlying the evolution of human speech and language, but the mechanisms and neuroanatomical basis for this plasticity remain largely unknown. The Mexican free-tailed bat, *Tadarida brasiliensis*, presents a unique opportunity to advance our understanding of the evolution and neurobiology of mammalian vocal communication because this animal displays elements of vocal complexity and plasticity that are more sophisticated than any mammal other than humans, including non-human primates. Current models of vocal control in mammals do not account for the vocal complexity of free-tailed bats. The purpose of this dissertation is to fill that gap in knowledge by identifying a possible neuronal basis for vocal complexity in free-tailed bats. This will be achieved by 1) providing a detailed analysis of the free-tailed bat's vocal behaviors, 2) mapping the distribution of neurotransmitter receptor types suspected of involvement in vocal control, 3) identifying brain regions that exhibit increased neuronal activity during vocalizing, and 4) pharmacologically manipulating putative vocal control regions to confirm and characterize their function in vocalizing.

Analysis of *Tadarida's* vocal behavior indicated that they have a vast vocal repertoire, including many different call types, context-dependent sensory-feedback driven vocal plasticity, and syntactically-organized stereotyped songs. Their vocal behavior changed seasonally, so I mapped the distribution of melatonin binding sites in the brain, finding high densities in the striatum, similar to dopamine receptor distribution. I then used immunohistochemical labeling of the immediate early gene c-fos to map neuronal activation in brains of highly vocal bats to find ROIs activated by vocal production. This technique not only identified all previously known regions of the mammalian vocal motor pathway but also revealed activity in novel brain regions that could potentially account for vocal plasticity, including a localized region of the basal ganglia, the dorsolateral caudate nucleus, and the anterior cingulate region of the frontal cortex. Pharmacological excitation of these regions evoked complex vocal sequences similar to the songs recorded in the field and lab. These results support the hypothesis that the mammalian basal ganglia may play a crucial role in the plasticity and complexity of mammalian vocal behaviors.

DEDICATION

This dissertation is dedicated to my parents, Peter and Sharon Schwartz. Thank you for your support all these years.

ACKNOWLEDGEMENTS

I would like to acknowledge and thank the chair of my doctoral committee, Dr. Michael Smotherman, and the other past and present members of my committee, Dr. Vincent Cassone, Dr. Thierry Lints, Dr. Rachel Hull, and Dr. Heather Bortfeld. I would also like to thank the Department of Biology, Texas A&M University, and in particular the building and maintenance staff at Kyle Field.

NOMENCLATURE

Neuroanatomy

3V	third ventricle
4V	fourth ventricle
ac	anterior commissure
ACg	anterior cingulate cortex
Aq	aqueduct
Au	auditory cortex
BLA	basolateral amygdala
CA3	hippocampus *Cornu Ammonis* 3
Cb	cerebellum
cc	corpus callosum
CdN	caudate nucleus
cp	cerebral peduncle
CPu	caudate putamen
Cx	cortex
ec	external capsule
DG	dentate gyrus
DR	dorsal raphe nucleus
Hp	hippocampus
ic	internal capsule

IC	inferior colliculus
IP	interpeduncular nucleus
LC	locus coeruleus
LDTg	laterodorsal tegmental nucleus
LH	lateral hypothalamus
LHb	lateral habenula
LL	lateral lemniscus
LS	lateral septal nucleus
LV	lateral ventricle
mcp	middle cerebellar peduncle
MD	mediodorsal thalamic nucleus
ME	median eminence
MHb	medial habenula
MnR	medial raphe nucleus
NAcc	nucleus accumbens
PA	paraventricular hypothalamic nucleus
PAG	periaqueductal gray
PB	parabrachial nucleus
Pir	piriform cortex
Pn	pontine nuclei
Pu	putamen
RIP	raphe interpositus nucleus

RLI	rostral linear nucleus
RtTg	reticulotegmental nucleus of the pons
s5	sensory root of trigeminal nerve
SC	superior colliculus
SCN	suprachiasmatic nucleus
scp	superior cerebellar peduncle
SF	sylvian fissure
SNc	substantia nigra pars compacta
SPO	superior paraolivary nucleus
VC	ventral cochlear nucleus
VTA	ventral tegmental area

Call Classification

AH	arrowhead
CF	constant frequency
DFM	descending FM
FM	frequency modulated
LFM	low FM
LL	long ladder
LW	long wave
SL	short ladder
SW	short wave

UFM	upward FM
ULF	upward long flat
V	V call
WAR	warble

Other

FLI	Fos-like immunoreactivity
IHC	immunohistochemistry
ROI	region of interest

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CHAPTER I

INTRODUCTION

The neurological basis of many common speech and language disorders remains elusive, largely because little is known about how complex vocal motor patterns are generated in the mammalian brain. Motor speech production in humans is a complex process requiring precise coordination of multiple muscle groups. Not much is known about the interconnectedness of these motoneuronal pools and how their activities are coordinated to produce syllables. Still even less is understood about how higher brain centers coordinate and link multiple syllables in time to produce meaningful words and phrases. These major deficits in understanding are largely due to the lack of a mammalian model animal exhibiting both a relevant vocal behavior and suitability for neurophysiological studies.

Among mammals, primates, rodents, and bats have been productive non-human models for studying the organization of the vocal motor pathways. But they have not been well suited to the investigation of several of the key issues related to human speech and language disorders, because single syllable production is accounted for by brainstem vocal pattern generators, and there was no clear evidence that these or any other mammals use cortical mechanisms for producing complex vocal sequences. Electrical

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This dissertation follows the style of Neuroscience.

and chemical stimulation of "vocalization regions" in the brains of non-human mammals produced calls identical to recorded natural calls, leaving nothing unaccounted for. I will outline here what is known about the mammalian vocal motor pathways, and delineate how my work investigating the neural basis for a complex vocal behavior by the Mexican free-tailed bat, *Tadarida brasiliensis*, will fill in some of the gaps in the existing knowledge.

The basic vocal motor pathway in mammals is a limbic-based visceromotor triggering of brainstem pattern generators. The limbic system, specifically the anterior cingulate cortex (ACg), amygdala, and hypothalamus, sends inputs to the midbrain periaqueductal gray (PAG), which is responsible for vocal initiation. This limbic input is believed to serve a key role in translating emotional cues and states of arousal into representative species-specific vocalizations (Jürgens and Pratt, 1979, Jürgens, 1982, Jürgens and Lu, 1993, Jürgens et al., 1996). Also, the ACg is thought to provide voluntary control over calling (Sutton et al., 1974). The PAG coordinates respiratory and laryngeal motor neurons of the parabrachial nucleus and nucleus ambiguus necessary for vocalization through the reticular formation (Yajima et al., 1981, Larson and Kistler, 1986, Jürgens, 2002). This non-human vocal motor pathway has been constructed mostly with a variety of lesion studies and electrical stimulations. Electrical and chemical stimulation of the PAG produces vocalizations in all mammals studied, including species of bats, while lesioning this core vocal structure results in mutism (Jürgens and Ploog, 1970, Suga et al., 1973, Yajima et al., 1980, Lu and Jürgens, 1993, Jürgens, 2002). Partial lesions of the PAG abolish some vocalizations while leaving

others intact, suggesting that different regions of the PAG may be important for different vocalization types (Jürgens and Pratt, 1979, Newman and MacLean, 1982). The ACg also produces vocalizations when stimulated, but only when the PAG is intact, indicating that the ACg projects to the PAG (Robinson, 1967, Jürgens and Ploog, 1970, Sutton et al., 1974, Gooler and O'Neill, 1987). Additionally, the PAG receives inputs from other regions of the brain, including medial prefrontal cortex, gyrus rectus, insular cortex, basal ganglia, hypothalamus, amygdala, and thalamus (Dujardin and Jurgens, 2005). This suggests that the PAG integrates information from many sources.

However, the PAG is not involved in all aspects of vocalization. Stimulation of the laryngeal area of the motor cortex produces vocal fold movements even when the PAG is lesioned, indicating that an additional pathway exists that bypasses the PAG entirely, the neocortical pathway (Jürgens and Zwirner, 1996). However, in monkeys, lesions of this area of the motor cortex do not affect production of normal calls (Kirzinger and Jürgens, 1982), and it is suggested that this pathway may be involved in learned or complex vocal behaviors which are not exhibited by the species used in the experiments. Vocal fold movements elicited by the laryngeal motor cortex were blocked by chemical injection into the reticular formation, indicating that this is the site where the neocortical and limbic vocal motor pathways potentially join together (Jürgens and Ehrenreich, 2007). The reticular formation was also found to be a projection area of the laryngeal motor cortex through tract tracing (Simonyan and Jurgens, 2005). Other areas receiving projections include caudate nucleus, putamen, and several thalamic nuclei. Overall, the neocortical pathway for vocal motor control is suggested to be involved in

learned or more complex vocal utterances, but no non-human mammal has provided evidence of this hypothesis.

Humans, on the other hand, exhibit extremely complex vocal behavior. To achieve the remarkable degree of voluntary control and complexity of voice exhibited by humans, it is hypothesized that the human brain evolved a system of direct projections from supplementary motor cortex onto the brainstem motor nuclei controlling laryngeal, articulatory and respiratory musculature not found in other mammals and that these projections are the distinguishing neural feature underlying human speech. Human speech is produced through the activation of specialized regions of premotor cortex, including portions of the supplementary motor area (SMA) and frontal operculum, and subcortical projection areas in the basal ganglia and thalamus (Hardcastle, 1981, Gracco, 1991, Murphy et al., 1997, Schulz et al., 2005, Guenther, 2006). Activity within the SMA is regulated by the basal ganglia as part of a feed-forward loop connecting the striatum, frontal cortex and thalamus. The striatum, a central component of the so-called "motor loop", may play a critical role in regulating the precise timing and sequencing of respiratory, laryngeal and articulatory components of speech. All of the neocortical and subcortical regions identified above as primary elements of voice control in humans have never been identified as part of the species-specific vocal control pathways in other mammals (Jürgens, 2002, Schulz et al., 2005), but the hardware is still present. Cortico-striatal-thalamic loops play a more generalized, well-recognized role in the control of voluntary movements in all mammals and as mentioned previously, there does appear to be a neocortical pathway in non-human mammals based out of the motor cortex

(Jürgens, 2009). This cortical region projects to the striatum and thalamus, so it is possible that this same motor loop vocal pathway exists in non-human mammals. The hypothesized role of the neocortical pathway in non-human mammals is mediating learned and complex vocalizations, an idea that needs an animal model and supporting evidence, which I aim to provide with this dissertation.

In the following sections, I will report on an extensive study of the vocal motor pathway in the *Tadarida brasiliensis*. Chapter II details the materials and methods used for all experiments. In Chapter III, I characterize the free-tailed bat vocal repertoire, providing evidence of complex vocal behavior that provides an excellent candidate system for exploring the neocortical vocal pathway. In Chapter IV, I examine the neural distribution of melatonin and dopamine, two systems potentially vital to the vocal motor pathway. In Chapter V, I use a neural activation marker to pinpoint areas of the brain activated during vocalization in an effort to better characterize the non-human mammalian vocal motor pathway. And finally, in Chapter VI, I provide a conclusion and set the bar for future work.

CHAPTER II

METHODS

Animals

The animals used for all of these experiments were Mexican free-tailed bats, *Tadarida brasiliensis*. The population living in Brazos County, Texas (the location of Texas A&M University) was previously characterized as a non-migratory hybrid population of two subspecies of *Tadarida brasiliensis* (Schmidly et al., 1977) possessing distinguishing morphological characteristic of both the non-migratory *T.b. cynocephala* found in the eastern portion of the state and the migratory *T.b. mexicana* found in the central part of the state. In the evenings of March through October, large numbers of foraging free-tailed bats are easily encountered within the College Station city limits and throughout the surrounding countryside, which is mostly rural. There is also a small subset of animals that remain in the area all year round. The university's football stadium, Kyle Field, appears to be the largest central roosting site for free-tailed bats in Brazos County, although small groups of roosting bats are relatively easy to find in local parking garages, bridges and freeway culverts.

All animal husbandry and experimental protocols were in accordance with NIH guidelines for experiments involving vertebrate animals and were approved by the local IACUC. All experiments for this dissertation were approved and covered under Animal Use Protocol #2007-254.

Field colony

Some vocal behavior data was obtained from wild bats. Echolocation sounds from foraging *Tadarida brasiliensis* were recorded from several different sites in Brazos County, including three small ponds, two large pastures, and on the College Station campus of Texas A&M University. Also, bat communication calls were recorded from three sites in the football stadium over the course of a year to get an illustration of the seasonality of their vocal behavior.

Several experiments required the capture of wild bats. All of the male bats used in autoradiography, western blotting, and immunohistochemistry experiments were obtained from a small, all-male natural roosting site in the football stadium. Females were difficult to find, and were obtained from a separate roost. Females were only used in the vocal behavior studies and the melatonin receptor experiment. Females were not excluded from the other studies, but simply were not found in sufficient numbers to make a meaningful comparison across sexes possible. The bats were obtained from the stadium colony the day before their experimental protocol. They were isolated from the captive colony (see *Captive colony* below) and kept in a soundproof room overnight to help alleviate any stress associated with capture. The bats were then used in experiments the following morning.

Lab colony

A captive colony of *Tadarida brasiliensis* occupy two rooms (4x5x3 m^3) in the Department of Biology vivarium on the College Station campus of Texas A&M

University. These bats were collected from the large colony in the Texas A&M University football stadium, Kyle Field. The captive colony consists of approximately 30 individuals, all males. The rooms have regulated light-dark cycles adjusted with a light timer to mimic the natural external photoperiod. The two rooms are connected with a large sliding door that remains open to provide more room for flight. The rooms are temperature and humidity controlled. Artificial roost sites are available in each room. Bats were trained to feed themselves, and are fed a diet of mealworms supplemented with vitamins and essential fatty acids. This colony of bats was only used for recordings of echolocation calls, buzzes, and songs in the lab and the pharmacology experiments.

Acoustic Recordings

Recording and analysis of calls for vocal behavior experiments

Foraging calls were recorded from March 2005 through October 2006 during the bats' peak periods of nightly foraging activity, which was found to vary seasonally. For ultrasound acquisition I used the UltraSoundGate 116 (Avisoft Bioacoustics, Berlin, Germany: 750 kHz sample rate, 8-bit resolution). The condenser microphone (Ultrasoundgate CM16) had a flat frequency response between 10 and 100 kHz (± 4dB); performance of the microphone was checked with simultaneous recordings made with a calibrated Brüel and Kjær type 4939 ¼"free-field microphone (Brüel and Kjær, Denmark). Sounds were stored and analyzed on a notebook PC computer at a sample rate of 250 kHz at 16 bits/sample. For recordings made at ponds, the microphone was positioned four feet above ground at the edge of the pond and directed upwards at a 45-

degree angle over the pond. The three ponds used in this study varied from 10 to 20 meters in diameter, and thus the average distance between the bats and microphone when the bats emitted buzzes over the pond was roughly 5-10 meters. For recordings made within the city and on campus, the microphone was positioned facing directly upwards. For recordings of communication calls and songs coming from "natural" roosting sites located within the stadium, the microphone was placed 1 m from and directed towards the opening of roost sites, which were thin crevices running along the length of the stadium interior (concrete expansion joints beneath the seats). Weekly recordings were made at the stadium from January-December 2006 to obtain seasonal vocalization data. Sounds were also recorded in the lab under the following conditions: 1) from individual males defending specific roost sites within the bat vivarium, 2) from social groups of 3-5 bats in a small cage in the flight chamber, 3) from individual bats on a feeding platform in either the vivarium or in the flight chamber, and 4) during flight in the flight chamber. The flight chamber was a 6.1m long by 3.0m high by 1.5m wide carpeted flight room, with walls and ceiling lined with acoustic foam (Sonex 1, 3" foam, Acoustical Solutions, Inc., Richmond VA) and temperature maintained within a range of 80 to 90˚ Fahrenheit during recording sessions.

Analysis of echolocation calls and buzzes

Signals were analyzed using both the commercial software program SASLab Pro, version 4.38 (Avisoft Bioacoustics) and Batsound (Pettersson Electronics). Signals were displayed simultaneously as spectrograms and oscillograms. Interpulse intervals (IPIs)

and call durations were measured from the oscillograms and inspected visually for tight correlations with the accompanying spectrogram. For temporal analyses, I used 256-point fast Fourier transforms (FFTs) with 93.75% overlap, providing 976 Hz spectral and .064 ms temporal resolutions. For spectral analyses I used 512-point FFTs, giving 488 Hz spectral and 0.128 ms temporal resolutions. For inspection of finer details of call parameters, the FFT window was adjusted accordingly. Spectral analyses included measures of the minimum frequency (F_{min}), which correlated with the ending frequency of the calls, the maximum frequency (F_{max}), which correlated with starting frequency of the calls, and the frequency of the peak intensity of the call (F_{peak}). Following standards defined in Surlykke and Moss (2000), I took F_{peak} from the power spectrum of the call and defined F_{min} and F_{max} as the frequencies at the lower and upper bandwidths of the spectrum at –15 dB relative to the intensity of the peak frequency. As noted in an earlier study of the bat *Eptesicus fuscus* (Surlykke and Moss, 2000) this method of standardizing spectral measurements proved reliable for search and approach call measures, but is less accurate as signal-to-noise ratios decline, particularly during the buzz phase. For the measures of spectral parameters of faint calls contained within buzzes recorded in the field, only those buzzes in which the standardized measures appeared consistent with measurements taken by hand using the cursor in 1024-point FFTs were included in the final analysis. Declining signal-to-noise ratios were not a significant problem for buzzes recorded from stationary bats in the lab: because of the potential for a differential effect of atmospheric attenuation on the measurements of buzz spectral parameters in the field versus the lab only those buzzes recorded in the field that

included multiple upper-frequency harmonics were included for detailed analysis. However, the measurements presented here for foraging bats are in close agreement with previous published accounts of *Tadarida brasiliensis* echolocation calls under similar conditions (Simmons et al., 1978). These five standardized measures (IPI, Dur, F_{peak}, F_{min}, and F_{max}) are probably insufficient for providing a complete description of the myriad of ways free-tailed bats manipulate their call structure, however these measures do provide an objective and straight-forward foundation for interpreting key observations presented here. I used the statistical software package SigmaStat version 3.1 (Systat Software Inc., Point Richmond CA) for comparison of data sets. All results are presented as means ± standard deviations, and statistically significant differences were tested for using the Mann-Whitney rank sum test or a 1-way ANOVA. P values are provided where appropriate as indicators of the level of significance.

Classification of communication calls

To characterize the *Tadarida* communication repertoire, I developed a classification system based on previously established bat call classification methods (Kanwal et al., 1994, Bohn et al., 2008). I focused my broad classification mostly on two parameters, call duration and frequency range, which resulted in 15 different call types: long waves, short waves, strums, chirps, short ladders, long ladders, trills, low FMs, upward FMs, descending FMs, upward long flats, buzzes, arrowheads, warbles, and V calls (Figure 1).

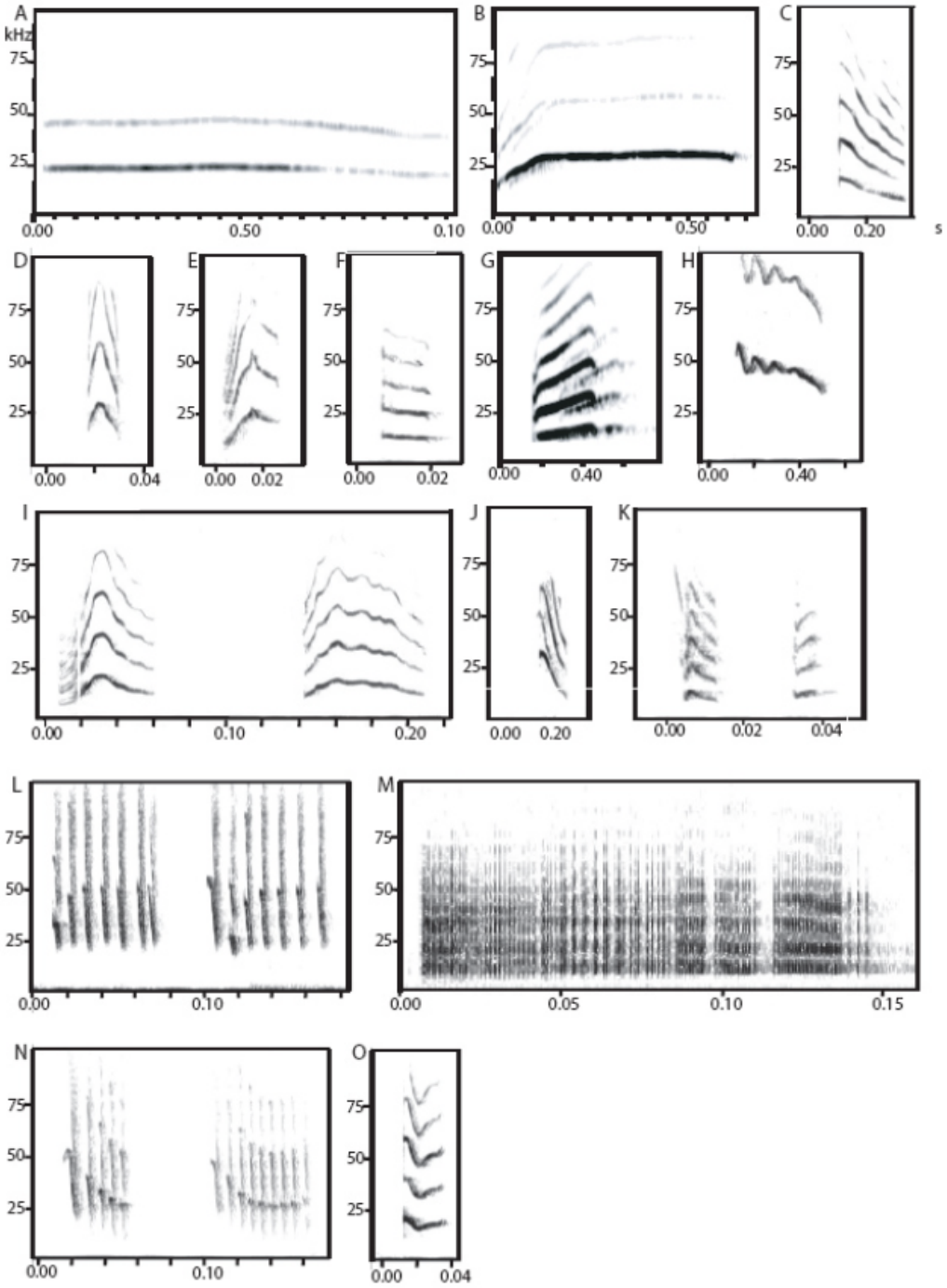

Figure 1. Communication Call Classification for *Tadarida brasiliensis*. An example spectrogram of each call type is provided. A. long ladder, B. upward long flat, C. descending FM, D. arrowhead, E. short wave, F. short ladder, G. upward FM, H. warble, I. long wave, J. low FM, K. chirp, L. trill, M. strum, N. buzz, O. V call.

- Long wave calls start at a low frequency (<20kHz) and rapidly sweep upward to a peak before dropping, arching, or rolling. They are longer than 25 milliseconds. Short wave calls are similar to long waves but shorter, ranging from 10-25 milliseconds. They start out like long waves, but stop at or just after the call peaks, and they do not fall back to the original starting frequency. Wave calls (both long and short) frequently occur in sequences of up to 10 in a row.
- Strums are broadband and appear very noisy on spectrograms. This call type also frequently occurs in sequences. They are one of the longer syllables (>40 milliseconds) and are very low frequency (<20kHz) for the duration of the call. They do not have any significant frequency modulations. Because of their noisy appearance, they are easily recognizable.
- Chirps include a wide range of very short calls, 5-10 milliseconds long. This classification is highly variable and includes both CF and FM calls, both ascending and descending.
- Ladder calls are calls with many prominent harmonics (>4). This classification includes both CF calls and FM calls with a slow frequency incline or decline. They differ from wave calls because they do not come to a peak. Short ladder calls are 10-30 milliseconds, long ladder calls are >30 milliseconds in duration. The lowest harmonic of this call type is usually around 20-30 kHz.

- Trills are sets of 2-7 FM calls that are very short duration (5-7 milliseconds). The intervals between the calls are consistent, usually 3-7 milliseconds. The peak frequency of the calls is also consistent throughout the trill. The frequency range of the trill calls is around 40-20 kHz.
- Buzzes look similar to trills, but they are longer, 5-20 calls within one buzz. Also, the peak frequency, intercall intervals, and call durations all decrease as the buzz progresses.
- Low FM calls look similar to the calls contained within the trills, except that the frequency drops well below 20 kHz. They are short duration (5-10 milliseconds).
- Upward FM calls vary in length from 10-25 millseconds. They sweep up in frequency slowly and smoothly. They are different from the wave calls because they only go up in frequency; they never reach a peak or decline in frequency.
- Descending FM calls are exactly like upward FMs, except that they decline in frequency instead of ascending.
- Upward long flat calls begin at a lower frequency (around 10 kHz) and rise like long wave calls, but they reach a peak and remain at that constant frequency, usually around 25-30 kHz. These are longer duration, around 40 milliseconds.

- Arrowhead calls are 10-25 milliseconds long. They look like an upside down V on a spectrogram and are roughly symmetrical.
- V calls are the exact opposite of arrowhead calls; they look like a V on a spectrogram. Again, this call type is also roughly symmetrical on either side of the center peak.
- Warble calls start at a high frequency (50-60 kHz) and decline in frequency unevenly, usually ending above 25 kHz. They look rippled on a spectrogram. The duration of this call type is around 20-40 milliseconds.

In addition to the 15 call type classifications, I characterized two communication call types seen in isolated bats based on associated behaviors (Figure 2). Protest calls are loud, aggressive short ladder type calls elicited by agitated bats. I recorded many of these calls when handling aggressive singing males or newly captured bats. I was also able to record another call type in isolated bats, the food solicitation call. These calls were produced by tame, hand fed bats begging for food. These calls are lower frequency (<25kHz) and differ by individual.

Characterization of the Tadarida *song*

Songs were recorded both in the field and in the lab as mentioned previously. In addition to audio recordings, video recordings were taken of singing bats in the lab to

establish the behavioral context accompanying the singing behavior. Singing males were examined to establish any physical changes.

The bat song was broken down into components, each of which was characterized. Songs from different individual bats were compared to assess similarities and differences. Natural songs were recorded over the course of a year to determine any seasonal variation.

Recording and analysis of calls for c-fos immunohistochemistry protocol

All acoustic experiments in the lab took place in the flight chamber mentioned above which is lined with acoustic foam which effectively isolated the experiment from all outside noise. All bats were kept in a small wire cage (12x6x6 in) to prevent flying. Vocalizations were monitored by the same Bruel & Kjaer microphone mentioned previously. Incoming signals were digitized with a National Instruments DAQmx, NI PCI-6251 (200 kHz, 16-bit sample rate), and viewed with Avisoft Recorder v3.0. Call rate was determined as the number of calls per second over a thirty minute time period. Movements were tracked with a motion detector (Passive InfraRed Sensor, Parallax, Inc.) attached to the cage and monitored with the DATAPAC 2K2 system. I calculated the movement as the percentage of experimental time the animal spent moving. All auditory stimulations were played through a Sony amplifier (model # STR-DE598) driving a four-speaker array consisting of two Pioneer Ribbon Tweeters (ART-55D/301080) and two Pioneer Rifle Tweeters (ART-59F/301081), arranged to project across the flight room directly in line with the cage. Each speaker provided a flat (±3 dB)

output at a maximum of 85-dB SPL. Echolocation calls were generated using openEX software and the Tucker-Davis Technology (TDT) system III hardware. The auditory stimulus used for these experiments were computer-generated bursts of white noise, which had a center frequency of 33 kHz and -6 dB bandwidth of 16 kHz. These stimuli were generated with RPvdsEx which was set to continuously generate bursts of white noise separated by 200 ms intervals for 30 minutes. This acoustic stimulus was chosen because the parameters match those of natural echolocation calls

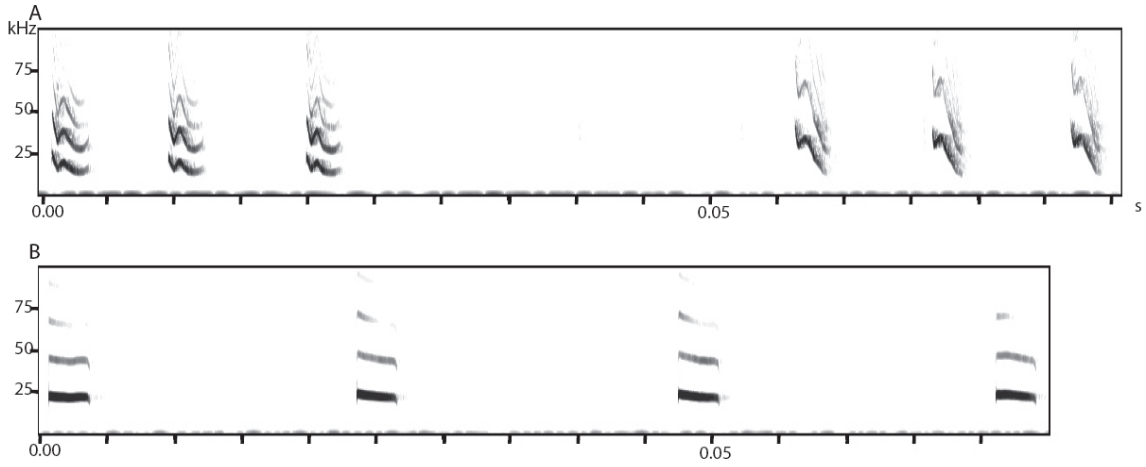

Figure 2. Communication Call Types Classified by Behavior. This figure provides spectrogram examples for two social call types elicited in a specific behavioral context: food solicitation (A) and protesting (B).

Brain Preparation

Brains were extracted for the autoradiography, western blotting, and immunohistochemistry experiments. All animals were killed by injection of sodium pentobarbital (Euthasol) after their experimental protocol. This method of sacrifice is rapid (<5 minutes), so it would not have an effect on gene expression where applicable. The brains were quickly removed, flash frozen in isopentane to minimize cell damage, and stored at -80°C (Sundquist and Nisenbaum, 2005). For the autoradiography and immunohistochemistry experiments, serial coronal sections (16-20 μm) of the entire brain were cryosectioned, thaw mounted on Histobond microscope slides, and stored at -80°C until use. Adjacent brain sections were placed on separate sets of slides so that every fourth slice was in the same set. This provided multiple slide sets from the same brain for control experiments and use of the same animal in more than one experiment. The brains used for western blotting were prepared in a different way (see "**Western Blotting**" section below).

Autoradiography

2-[^{125}I]iodomelatonin

We used slight modifications of previously described protocols for determining the distribution and characterization of melatonin binding sites (Duncan et al., 1986, Dubocovich and Takahashi, 1987, Lu, 1994). The two slide sets were incubated in 0.5M Tris buffer with 0.1% bovine serum albumin at 21°C for one hour. One slide set was then incubated in 100 pM IMEL (PerkinElmer) in Tris buffer and the second set was

incubated in 100 pM IMEL in Tris buffer with 1μM melatonin for two hours at 21°C. For the saturation experiment, the concentrations of IMEL used for this step ranged from 1 pM to 1.2 nM and three different animals were used for each concentration. The slide sets incubated with 1μM melatonin served as controls to determine nonspecific binding. All experimental and control slide sets were then washed in Tris buffer at 4°C for 30 minutes and air dried. The slides used for determining the distribution of melatonin binding sites were dried and apposed to BioMax Maximum Resolution autoradiographic film (Kodak) with a calibrated ^{14}C standard for six weeks at -80°C.

After developing the film, optical density measurements from five sections on the experimental slide set were averaged for each brain area for each animal using NIH Image J (Abramoff et al., 2004). The slides used for saturation studies were dried and apposed to phosphor imaging plates (Fujifilm) with a calibrated ^{14}C standard and incubated for three days at 21°C. The plates were then developed using a phosphorimager (Fujifilm BAS-5000). The optical density of the caudate putamen was measured in five sections from the experimental slide set for each animal and averaged for each concentration in the same manner as above. All sections were subsequently stained with cresyl violet to aid in brain structure identification. Areas exhibiting IMEL binding were also measured on control brains to provide nonspecific binding control data. A standard curve was generated using the optical densities from the calibrated standards. 2-[^{125}I]iodomelatonin binding (nCi/mg protein) from each brain region was calculated using the values from the standard curve. Nonspecific binding was subtracted from total binding to determine specific binding for each brain region. Differences in

IMEL binding between sexes for each brain region were assessed using t-tests. A nonlinear regression was performed on the data collected from the saturation experiment to determine K_d and B_{max}.

Dopamine receptors

To determine the distribution of D1 dopamine receptors, I used slight modifications of a previously described protocol (Kim et al., 2000). All slides were preincubated in assay buffer (50 mM Tris HCl buffer with 120 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, pH 7.4) for 20 minutes. Then, the experimental slide sets were incubated in assay buffer with 1 nM N-methyl-^{3}H SCH 23390 (PerkinElmer) and 5 μM ketanserin (5-HT_2 seratonin receptor antagonist) for 1 hour at 23˚ Celsius. Control slide sets were processed similarly, but also with the addition of 5 μM fluphenazine, a D1/D2 dopamine receptor antagonist. All slide sets were then transferred to ice cold assay buffer for two rinses of 20 seconds each, followed by an ice cold water rinse of 10 seconds. Finally, all slide sets were then dried and apposed to BioMax Maximum Resolution autoradiographic film (Kodak) with a calibrated ^{14}C standard for 17 weeks at -80˚ Celsius.

Similarly, I used slight modifications of a previously described protocol for determining the distribution of D2 dopamine receptors (Lidow et al., 1991). All slides were preincubated in assay buffer for 20 minutes. Then the experimental slide sets were incubated in assay buffer with 1 nM methoxy-^{3}H-raclopride (PerkinElmer) for 45 minutes at 23˚ Celsius. Control slide sets were processed similarly, but with the addition

of 10 μM butaclamol, a dopamine receptor antagonist. All slide sets were then transferred to ice cold assay buffer for two rinses of 5 minutes each, followed by an ice cold water rinse of 5 seconds. Finally, all slide sets were then dried and apposed to BioMax Maximum Resolution autoradiographic film (Kodak) with a calibrated ^{14}C standard for 19 weeks at -80˚ Celsius.

All D1 and D2 films were developed and optical density measurements from five sections on the experimental slide set were measured and averaged for each brain area for each animal using NIH Image J (Abramoff et al., 2004). All sections were subsequently stained with cresyl violet to aid in brain structure identification. Areas exhibiting binding were also measured on control brains to provide nonspecific binding control data. The threshold for binding in the autoradiography experiments was determined by adjusting background level for each image according to the nonspecific binding levels obtained from the control slide sets exposed to melatonin. Any area with binding higher than the nonspecific binding levels was analyzed. A standard curve was generated using the optical densities from the calibrated standards. ^{3}H SCH 23390 and ^{3}H-raclopride binding (nCi/mg protein) from each brain region was calculated using the values from the standard curve. Nonspecific binding was subtracted from total binding to determine specific binding for each brain region.

C-fos Western Blotting

A vocalizing bat was sacrificed and its brain was removed and snap frozen in isopentane (methylbutane). For each 500mg of tissue, I added 300 μl of lysis buffer

(150mM sodium chloride, 1.0% Triton X-100, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate, 50 mM Tris, pH 8.0) and homogenized the brain tissue with an electric homogenizer. I centrifuged the homogenate for three periods of ten minutes, removed the supernatant, and put it in a fresh tube. The samples were stored at -20˚ until use.

To separate the proteins in the sample, I used sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) made with 10% polyacrylamide. The gel was submerged in migration buffer (25 mM Tris base, 190 mM glycine, 0.1% SDS, pH 8.3). The samples and a molecular weight marker were loaded into the gel with Laemmli 2X loading buffer (4% SDS, 10% 2-mercaptoethanol, 20% glycerol, 0.004% bromophenol blue, 0.125 M Tris HCl) and run at 150 V for 1.5 hours.

The proteins that were separated on the gel were now transferred to a membrane for staining. I utilized a wet protein transfer, where the gel and transfer membrane (Millipore Immobilon-P PVDF membrane) were sandwiched between sponges and absorbent paper and clamped tightly together. The sandwich was submerged in transfer buffer (same as above with 20% methanol in place of SDS) and run overnight at 30 mV.

The membrane then underwent a staining procedure similar to the immunohistochemistry protocols in order to determine the specificity of the antibody. The membrane was first blocked in 5% non-fat milk in Tris buffer saline with Tween 20 (TBS-T) for one hour at 20˚C under agitation. Then, the membrane was incubated in c-fos primary antibody with TBS-T and 3% BSA (Abcam, ab7963, 1:2000) for two hours at 20˚C under agitation. The membrane was then washed in TBS-T several times before incubation with the HRP-conjugated secondary antibody (1:2000) in TBS-T for one hour

at 20˚C under agitation. The membrane was washed again and then developed with SuperSignal West Pico Chemiluminescent Substrate. The membrane was then drained, covered in plastic wrap, and exposed to X-ray film for 5 minutes. The film was then developed and analyzed.

Blocking peptide

In order to more conclusively determine whether or not the antibody is specific, I used a blocking peptide to highlight any nonspecific binding. After the protein transfer, I split the membrane into two pieces for the staining step. The buffer with primary antibody was prepared and divided into two volumes. C-Fos peptide (Abcam, ab7997, 1:200) was added to one volume of buffer, and the other received an equivalent amount of buffer as a control. The membranes were incubated in these buffers for two hours at 20˚C under agitation as above. The remaining steps of the staining section were performed the same as stated above, but the two membrane pieces were kept separate.

C-fos Immunohistochemistry

The primary antibody for c-fos, corresponding to N terminal amino acids 1-14 of human c-Fos, was obtained from Abcam (ab7963). The secondary antibody was part of a kit from Vector Labs (Vectastain Elite ABC Kit). Brain sections on slides were traced with a PAP pen (RPI #195506), fixed in ice cold methanol, incubated in a 0.3% H_2O_2 solution, and then rinsed. The slices were then blocked in normal serum and incubated with the primary antibody (1:5000) overnight at 4˚ Celsius. The next day, the slides

were rinsed and then incubated with the biotinylated secondary antibody solution. The slices were then rinsed again, incubated in Vectastain Elite ABC reagent, rinsed, and then finally incubated with diaminobenzidine (DAB) solution with nickel. They were then rinsed, dehydrated, cleared and coverslipped. In addition to c-fos, I also used this same immunohistochemistry protocol with a primary antibody to tyrosine hydroxylase (ab112, Abcam), the first enzyme in the dopamine synthesis pathway, to aid in determining the locations of regions in the striatum and substantia nigra.

Pictures of the extent of each brain area were taken using an Infinity 2 microscope camera connected to a computer running Infinity Capture application software (version 3.7.5, Lumenera Corporation). Consecutive sections from both the right and left sides for each region of interest (ROI) were analyzed for each animal. The fos-like immunoreactivity (5-40 pixels in size) was counted using NIH Image J (Abramoff et al., 2004). Cells were counted in random 0.012 x 0.012 mm square areas over each ROI (Beckett et al., 1997, Neophytou et al., 2000, Sadananda et al., 2008). Data from the right and left sides for bilateral structures were combined and the mean for each ROI for each animal was calculated. The animals were then divided into their respective treatment groups and cell counts were compared between groups of bats using an analysis of variance (ANOVA) with treatment as the factor (echolocating, listening, or silent). The comparisons of interest were echolocating vs. listening, echolocating vs. silent, and listening vs. silent. All comparisons were analyzed using the Holm-Sidak method.

Tyrosine Hydroxylase and Dopamine-beta-hydroxylase Immunohistochemistry

The same protocol used for c-fos immunohistochemistry was used for tyrosine hydroxylase and dopamine beta hydroxylase immunohistochemistry. Both primary antibodies were obtained from Abcam (ab112, ab43868) and the same secondary kit was used. The analysis was also the same, except that all the cells in the entire structure were analyzed rather than counting random squares.

Pharmacology

Bats from the captive lab colony were used for these experiments. All surgical tools were sterilized in an autoclave and further cleaned using a dry glass bead sterilizer (Steriguard). Tools were also rinsed in a 2% chlorohexidine gluconate solution throughout the surgical process. Preparatory surgical procedures are similar to methods published previously (Smotherman et al., 2003, Smotherman et al., 2006). All bats underwent an initial surgery to drill a hole in the skull. Inhalational anesthesia (2.5% isoflurane vaporized in air) was administered using a tube fitted over the bat's face. The bat was then placed in a custom-built stereotaxic apparatus for surgery. Fur was trimmed from the top of the bat's head and the bat was given an injection of lidocaine, a topical anesthetic, prior to the first incision and throughout the surgery as needed. The skin on the top of the skull was cut, exposing the underlying muscles. These muscles were moved aside and minimally cut to expose the skull. The *Tadarida* skull has several landmarks to help navigate the brain below, particularly important were the sagittal

suture and the ridged region running perpendicular to this suture that lies directly over the division between the inferior and superior colliculi. Using these anatomical landmarks and the lab brain atlas sections, I was able to calculate the locations of the ACg, dlCdN and PAG. The ACg was approximately 3 mm forward of the SC/IC ridge and 1.2 mm deep, directly next to the midline. The dlCdN was approximately 2 mm forward of the SC/IC ridge, 2 mm off the midline, and about 1.4 mm deep. The PAG is 250-500 microns lateral to midline and directly beneath the superior colliculus, which is visible at the surface of the brain. Using these coordinates, I used the 3-axis piezoelectric microdrive (Siskiyou Inc., Grants Pass OR) stereotaxic equipment to find the area of the skull overlaying these ROIs. Using a drill fitted with 0.8 mm bits, I drilled a hole in the skull corresponding to the X and Y coordinates of the region of interest. I then covered the hole and exposed brain with bone wax, folded the skin and muscle tissue back over the skull, gave the bat an injection of buprenorphine, and placed it in a cage to monitor its recovery. After the initial surgery, the bat was given three days to recover before receiving an injection. The bats received additional follow-up buprenorphine injections as needed.

On the day of the drug injection, the bat was again anesthetized with isoflurane gas and placed in the stereotaxic apparatus. The bone wax was removed from the hole in the skull, exposing the brain. Glass micropipettes were made with a pipette puller, the tip diameter ranged from 2-5 micron, verified with a dissecting microscope. Kainic acid and bicuculline (5 mM concentration) were used for injection into the brain. Once the micropipette was loaded with drug, it was lowered into the brain to the depth calculated

previously. The drug was pneumatically administered and the pipette was kept in the brain for 30 seconds after the injection. The pipette was then removed, the isoflurane was shut off, and the bat was returned to a plastic cage with a microphone mounted overhead running UltraSoundGate 116 (Avisoft Bioacoustics, see "**Acoustic Recordings**" for more details). Under normal circumstances a bat regains consciousness from the isoflurane and begins calling within 2 minutes of cessation. All vocalizations were recorded for one hour after injection. Controls included saline injections in the same brain regions as well as drug injections in anatomical control sites.

CHAPTER III

VOCAL BEHAVIOR OF *Tadarida brasiliensis*[*]

Introduction

A central question in neuroscience is how complex motor patterns are coordinated in the brain. Bats use several vocal behaviors that exhibit complexity and plasticity that can be advantageous in addressing this question, including echolocation calls, communication calls, and more complex patterns and sequences of both call types. Bats use echolocation to navigate and explore their surroundings both in flight and on the ground. *Tadarida brasiliensis's* diverse echolocation call repertoire spans a range of sonar signal designs usually reserved for comparisons across whole families of bats (Simmons et al., 1978), indicating that they are capable of vast vocal variability. It is well established that echolocation calls are highly sensitive to acoustic cues derived from preceding echoes. However, this sensitivity has not been extended to calls contained within the feeding buzz, since the intervals between calls within a buzz, typically on the order of a few milliseconds, are presumed to be too short to allow for auditory feedback control of call parameters. The buzzes uttered by echolocating bats as they converge upon their fluttering prey represent a unique example of a precisely timed complex vocal motor pattern in a mammal other than humans, and yet little is known about the extent to which bats regulate the temporal and spectral components of their buzzes. Instead,

[*] Part of the data in this chapter is reprinted with permission from "The Tiny Difference Between Foraging and Communication Buzzes Uttered by the Mexican Free-tailed Bat, *Tadarida brasiliensis*" by Christine Schwartz, Jedediah Tressler, Halli Keller, Marc Vanzant, Sarah Ezell, and Michael Smotherman, 2007. Journal of Comparative Physiology A, 193, 853-863, Copyright 2007 by Springer Science + Business Media

feeding buzzes more likely reflect a fixed vocal motor pattern operating independently of sensory feedback (Schuller and Radtke-Schuller, 1990, Dusterhoft et al., 2000, Jürgens, 2000, Fenzl and Schuller, 2002, 2005, Hage and Jurgens, 2006). Thus the feeding buzz may represent a special opportunity to study the hierarchical organization of complex vocal motor patterns.

Several studies have reported that feeding buzzes uttered by foraging bats showed significant variations in duration (Griffin, 1958, Schnitzler et al., 1987, Kalko, 1989, Surlykke et al., 1993, Schnitzler et al., 1994, Kalko, 1995b, Surlykke and Moss, 2000), but most of these studies also reported a generally high degree of consistency in the basic spectral and temporal patterns of calls within feeding buzzes. One recent study demonstrated that big brown bats truncated buzz durations to compensate for shorter distances between prey and background clutter (Moss et al., 2006). Yet no reports have provided convincing evidence that sensory feedback modulates the fine structure of calls within a feeding buzz.

Free-tailed bats also have the ability to control their echolocation vocalizations to avoid periods of noise. Bats given broadband noise jamming their normal echolocation frequency modified their calls across several parameters to compensate for the disturbance, a change that was made within seconds of stimulus onset (Ulanovsky et al., 2004, Gillam et al., 2007, Tressler and Smotherman, 2009). Free-tailed bats were also shown to shift the timing of their echolocation calls to avoid noisy stimuli (Jarvis et al., 2010). This demonstrates plasticity in the vocal behavior of *Tadarida brasiliensis* and also indicates that sensory feedback pertaining to the bat's environment is involved in

the vocal motor pathway generating these calls. This also provides an excellent opportunity to study the mammalian vocal motor pathway.

In addition to echolocation calls, *Tadarida brasiliensis* also possesses a broad and dynamic repertoire of communication sounds including both isolated buzzes and buzzes incorporated into territorial songs (Ma et al., 2004). While the echolocation spectrum of this species is impressive, the communication repertoire is even more vast; a previous report characterized 14 different communication calls with specific behaviors in the Mexican free-tailed bat (Bohn et al., 2008). Communication calls are abundant in groups of bats, both in the field and in the lab, but these types of calls are generally not seen in solitary bats. Several communication calls, including the buzz, are incorporated into songs associated with territorial and courtship behavior. At first approximation, the buzzes seen in communication look strikingly similar to *Tadarida's* foraging buzzes used in a different context.

We provide here an analysis of *Tadarida brasiliensis* vocal behavior, including characterization of both echolocation calls and communication calls. I provide a detailed analysis of the spectral and temporal dynamics of *Tadarida's* foraging and communication buzzes, to assess whether or not these buzzes were identical vocal motor patterns being used in different behavioral contexts. I hypothesized that if the feeding buzz was a fixed motor pattern, then the communication buzzes should closely resemble foraging buzzes in every detail. Alternatively, I hypothesized that if auditory feedback played a significant role in shaping the spectral and temporal dynamics of calls within the buzz during flight, then a very predictable set of differences should appear in the

stationary communication buzzes relative to foraging buzzes recorded during flight. In flight, call durations and inter-call intervals progressively shorten as bats approach a target, thus if similar changes observed within foraging buzzes are also driven by auditory feedback cues, then buzzes uttered by stationary bats should not exhibit progressive reductions in call durations or intervals. Therefore, this analysis offered the opportunity to directly address whether or not auditory feedback influenced call parameters within foraging buzzes. In addition to the detailed assessment of buzzes in different contexts, I also developed a classification system to characterize the communication repertoire of *Tadarida brasiliensis*, which I used to determine any sex-related or seasonal differences in syllable use. I also examine the structure and behavioral context of the territorial/courtship song.

Results

Echolocation calls used during foraging.

A previous study (Simmons et al., 1978) provided a detailed description of the spectral and temporal parameters of echolocation calls used by free-tailed bats in transit but not while foraging. Here I will provide a basic description of the variability of echolocation call types used by the Brazos Valley population of free-tailed bats studied here while foraging, but the focus of my presentation will address details of feeding buzz temporal and spectral parameters. Figure 3 offers a representative example of the typical series of changes in call structure that were observed to occur through a complete foraging sequence, including transitions from search to approach to buzz phases, and

these having been well documented previously in a wide range of bat species (Griffin, 1958, Schnitzler et al., 1987, Kalko, 1989, Surlykke et al., 1993, Kalko, 1995a, Obrist, 1995, Surlykke and Moss, 2000, Schnitzler and Kalko, 2001), will only be touched upon briefly for comparative purposes.

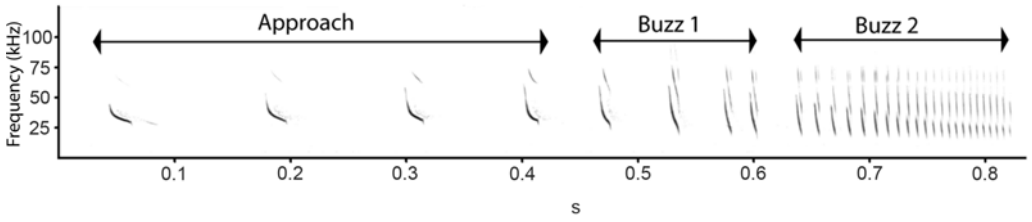

Figure 3. Echolocation Sequence of a Foraging Bat. Sequence of echolocation calls concluding with a feeding buzz emitted by a free-tailed bat foraging over a small pond. Approach phase ended with the transition from an increasing to a decreasing F_{min}, or ending call frequency. Buzz 1 and Buzz 2 are separated by an interval ≥ 20 ms.

As noted previously (Simmons et al., 1978), *Tadarida* use relatively long, constant-frequency (CF) or very shallow frequency-modulated (FM) "search" calls when flying in open space (Figure 4). I analyzed 100 series of 10 sequential search calls taken randomly from approximately 80 hours of recordings at six different sites; in each case I visually identified sequences in which the temporal and spectral parameters of the calls indicated that they were emitted by a single bat passing overhead. I attempted to avoid

pseudoreplication by allowing at least 10 minutes between sequences selected for analysis, by subjective analysis of the records wherein it was often possible to recognize the continued activity of a single bat, and by choosing data sets from a combined total of 28 different recording dates and locations. Mean call frequencies (F_{peak}), call durations, and interpulse intervals (IPIs) were calculated for each sequence, and the resulting 100 means were pooled to generate the graphs shown in Figure 4 B-D. Overall, the mean search call duration was 14.6 ± 1.4 ms. The mean inter-pulse interval was 274 ± 71 ms, however the distribution of IPIs was multimodal (Figure 4B), which is consistent with a conclusion that these bats typically emitted calls on alternating wing beats, which would appear to have been roughly 125 ms apart (the mode at 250 ms is presumed to reflect the time between 2 wing beats). Since this estimate is in close agreement with previous estimates of wingbeat rates of other similarly sized bats (Holderied and Helversen, 2003), I did not analyze this parameter further. The mean search call frequency was 26.4 ± 1.6 kHz, and only included one prominent harmonic. To provide a standardized measure of call bandwidth, the Q_{10}dB value, I measured the –10dB bandwidth of 100 individual search calls taken from 100 different sequences; the mean search call bandwidth was 1.2 ± 0.6kHz, with a mean Q_{10}dB of 23.2 ± 4.9 (Q_{10}dB =F_{peak} divided by the bandwidth of the call at 10 dB below the peak intensity). A power spectrum for a representative search call is shown in Figure 5.

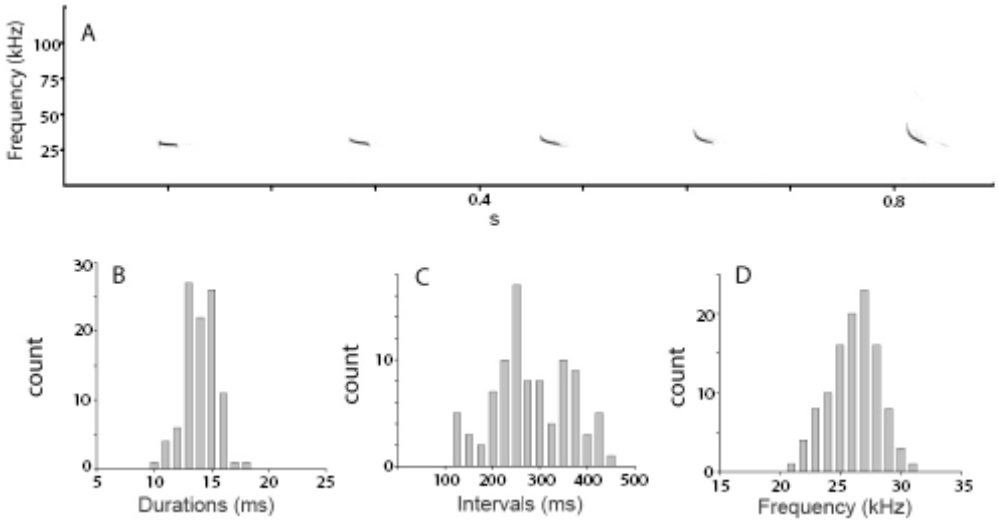

Figure 4. A Series of Search Phase Echolocation Calls. The last call in (A) immediately preceded the first call appearing in figure 3. B, C and D illustrate population-level variability in search call durations (B), inter-call intervals (C), and call frequencies, F_{peak} (D). Measurements of search phase calls were pooled from the means of 100 different sequences of 10 consecutive calls collected from a total of 28 different recording dates and locations in Brazos County, Texas.

Search calls were replaced by a series of progressively steeper linearly frequency-modulated FM "approach" calls as the bat rapidly approaches a target. Approach calls were characterized by progressively shorter durations, a large increase in the starting frequency (F_{max}), an overall increase in bandwidth, and a small rise in the ending frequency (F_{min}) of the call (Figures 3 and 5). For approach phase calls recorded in the field, the F_{peak} was typically located at or within approximately 2 kHz of F_{min} (Figure 5). F_{min} increased up to an average maximum value of 29.5 ± 1.3 kHz for the last approach call emitted before entering the buzz phase (n=100 calls preceding 100

buzzes). For the same data set, the mean –15 dB bandwidth was 8.4 ± 2.0 kHz, although this may underestimate the actual bandwidth due to variability in the recorded F_{max}. In the best recordings, non-overlapping second and third harmonic components were generally present in approach calls. As the bats transitioned from the approach to the "buzz" phase, both F_{max} and F_{min} were lowered (Figure 5).

Previous studies have separated feeding buzzes into two phases: the first phase (Buzz 1) was characterized by the transition from increasing to decreasing F_{min} values, thereby defining the boundary between the end of approach phase and the beginning of Buzz 1, (Schnitzler et al., 1987, Surlykke et al., 1993, Surlykke and Moss, 2000). The transition from Buzz 1 to Buzz 2 is more variable in its definition across species, but in the big brown bat, *Eptesicus fuscus*, whose echolocation calls share strong similarities with *Tadarida*, this transition was characterized by an abrupt decrease in IPI and a slower decrease in F_{min} (Surlykke and Moss, 2000). The above definition of the transition from approach to buzz 1 derived from other bats appears appropriate for *Tadarida brasiliensis* (see Figures 3 and 5), however, the definition of the transition from buzz 1 to buzz 2 is different for *Tadarida brasiliensis* because the temporal patterns of their calls within the buzz are different from *Eptesicus*. I found that the transition from a buzz 1 to a buzz 2 was correlated with the appearance of a single IPI greater than 20 ms that appeared in every buzz (Figure 3), which was followed by a sudden drop in IPI and from there on a smooth sequential decrease in IPIs, durations, and F_{min}. I suggest, based upon an analysis of 100 feeding buzzes, that Buzz 1 always ends with a doublet, characterized by a single short IPI (mean 12.7 ± 2.5 ms, n=100),

which was followed a 20-24 ms IPI (mean of 22.4 ± 0.6 ms, n=100) that preceded buzz 2. I hypothesize that this 20-24 ms IPI separating buzz 1 and buzz 2 is used for a rapid inspiration immediately prior to the main portion of the buzz, but at this time I have no way of testing this. Although respiratory temporal patterns are surely different in flight versus stationary conditions, I presume that the IPIs contained within buzz 2, which began at 10.9 ± 2.5 ms, are too short to allow for intervening inspirations, but the limited evidence available (Smotherman et al., 2006) suggests that vocalizing bats are capable of taking a breath within intervals as short as 20-25 ms, which is similar in duration to the one separating buzz 1 and buzz 2. Based on this, I adopted a physiological definition of Buzz 2 that characterizes it as a single complex vocal motor pattern constrained by respiratory temporal dynamics. Like *Eptesicus fuscus* (Surlykke and Moss, 2000), *Tadarida* were occasionally observed to extend their buzzes by emitting a series of two or three buzzes conjoined by brief 20-30 ms IPIs, but for the purpose of the analyses presented here I treated these as separate, sequential buzzes rather than a single protracted buzz. Only the first buzz in any series of multiple buzzes was included in the analysis. For my analysis of buzz variability and for the purpose of comparing foraging and communication buzzes, I herewith define the foraging buzz as a single buzz 2, beginning and terminating with any IPI greater than 20 ms. This distinction is essential because although communication buzzes closely resemble the buzz 2 phase, they do not follow a buzz 1 phase.

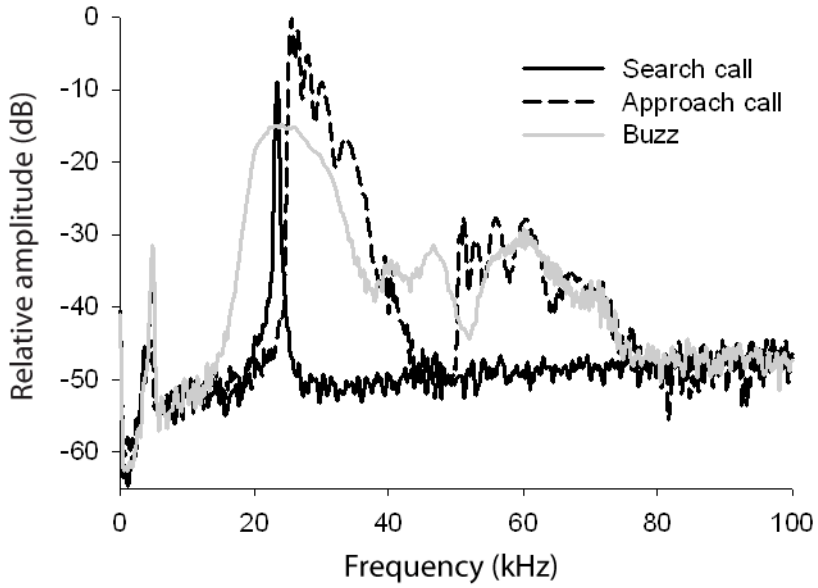

Figure 5. Power Spectra Comparison of Foraging Echolocation Calls. A comparison of the power spectra for a representative search phase call, the last approach phase call, and the first call in buzz phase 2. All three calls were taken from a single series of foraging calls spanning a period of 1.5 seconds. The bat was hunting over a pond. Intensities are given relative to the peak intensity of the loudest of the three calls, the approach call.

The foraging buzz

From a much larger data set, I selected 100 feeding buzzes from foraging free-tailed bats that had good signal-to-noise ratios and in which at least two harmonic components were visible. I initially separated the data into solitary and group foraging conditions, but a statistical analysis failed to identify any significant differences in the temporal or spectral dynamics of buzzes emitted alone versus those emitted in the

presence of other foraging bats. Therefore the data were pooled for subsequent analyses and presentation. Figure 6 illustrates the observed variability in buzz durations. Foraging buzzes lasted an average of 153 ± 44 ms, and included an average of 19 ± 6 calls (Figure 6): the longest was 300 ms and contained 45 individual calls. There was a predictably linear relationship between number of calls and overall buzz duration (Figure 6A), and the slope of this relationship (6.7 ms per additional call) was just above the fastest rate at which the bats were able to emit calls within a buzz (approximately 6.4 ms between call onsets, or 156 Hz). The measured slope in Figure 6A is higher than the maximum call rate because the first call durations and IPIs within the buzz (3.8 ± 0.9 ms and 10.9 ± 2.5 ms, respectively) were roughly twice as long as the mean final durations and IPIs in the buzz (1.8 ± 0.6 ms and 4.6 ± 0.6 ms, respectively), and it took approximately 10 calls to approach the maximum call rate. Figure 7 (A, B) offers two more examples of foraging buzzes differing in duration and number of calls. I looked for but found no consistent indication that buzz durations varied significantly with recording site, while alternatively I found ample evidence of a broad range of buzz durations at every recording site.

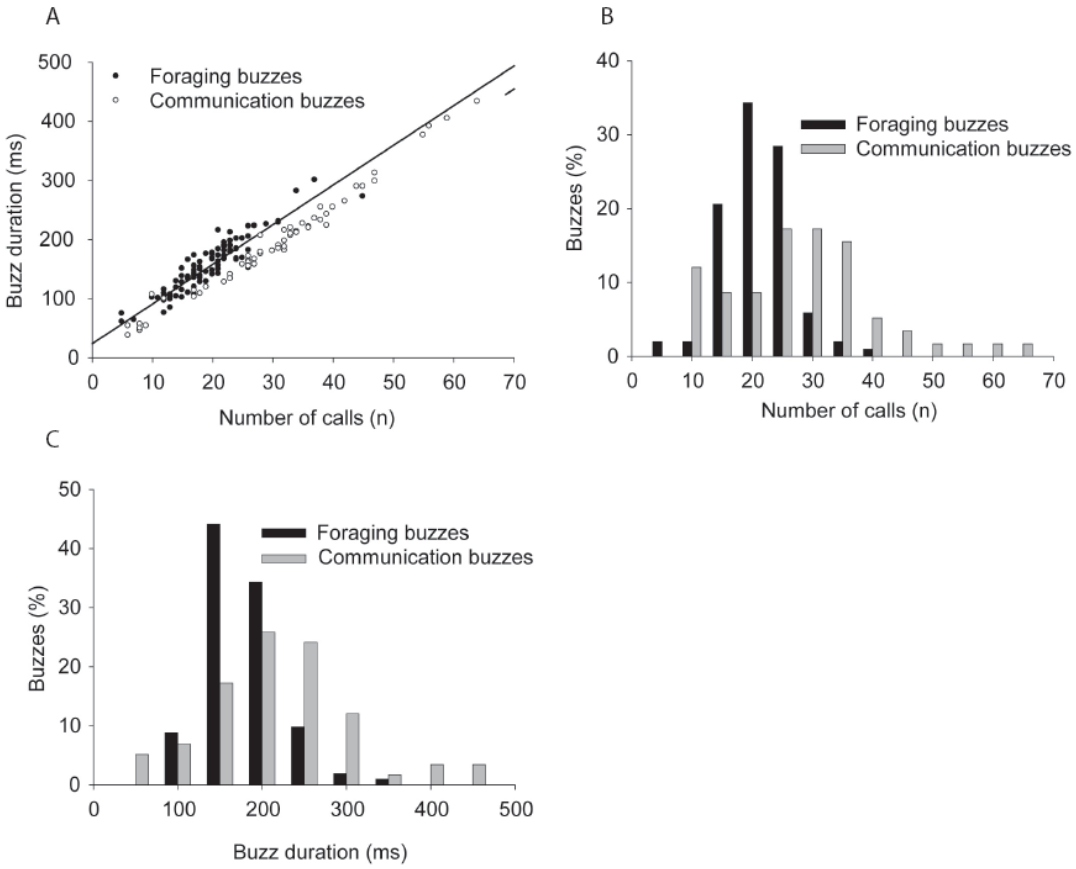

Figure 6. Relationship Between Buzz Length and Number of Calls. This figure shows a comparison of the relationship between the number of calls within a buzz and the resulting buzz duration (A). B illustrates the relative distributions of foraging and communication buzzes composed of different call numbers, while C compares the relative distribution of foraging and communication buzzes of differing durations. For the foraging buzz data in A, m=6.7 ms/call, r2 = 0.85, and for the communication buzz data, m= 6.6 ms/call, r2=0.97. For B, mean and median numbers of calls were 19 ± 6 and 19 calls (foraging) and 30 ± 13 and 31 calls (communication). For C, mean and median durations were 153 ± 44 ms and 150 ms (foraging) and 191 ± 88 ms and 185 ms (communication). N=100 foraging and 58 communication buzzes.

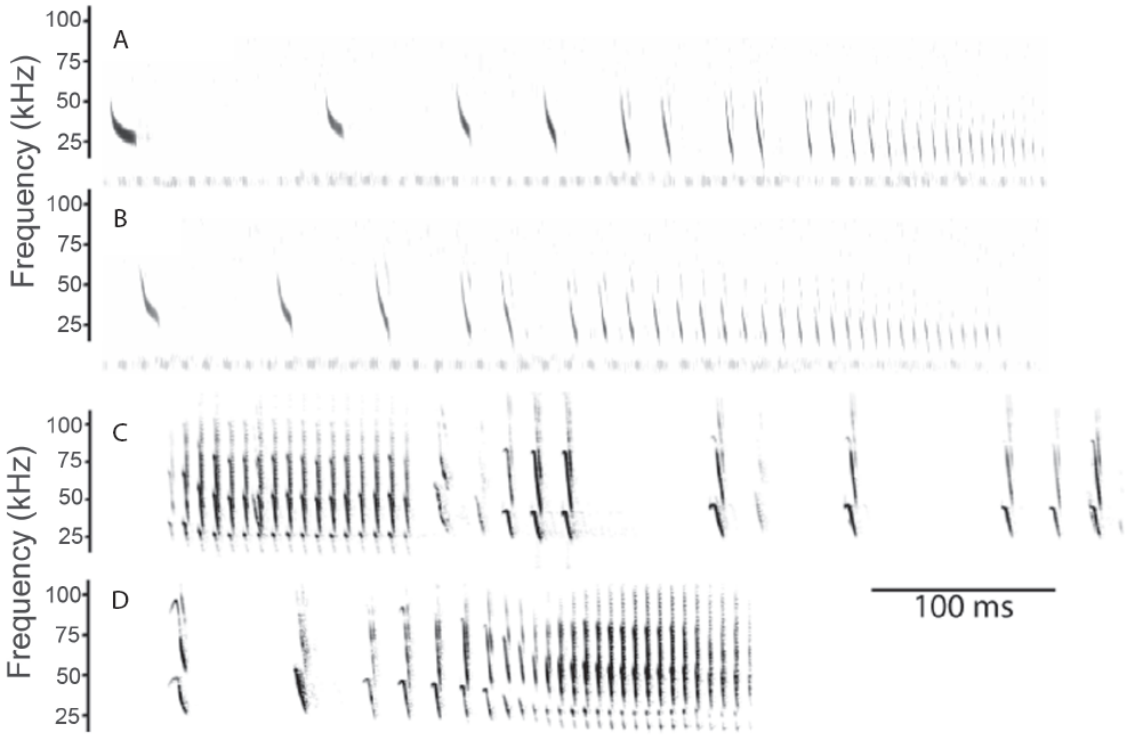

Figure 7. Examples of Foraging and Communication Buzzes. A and B are foraging buzzes, while C and D are communication buzzes. C is a buzz taken from the end of a territorial song. D is an example of a communication buzz uttered alone. Both c and d include examples of echolocation calls before and after the buzz, illustrating the CF-FM call structure used in cluttered spaces.

The communication buzz

Communication buzzes were recorded emanating from *Tadarida*'s natural roost sites in Kyle Field and from captive bats living in the vivarium (Figure 7 C,D). Both isolated communication buzzes and buzzes uttered as part of the *Tadarida brasiliensis* territorial song (Ma et al., 2004) were recorded and analyzed. During routine behavioral observations of self-feeding *Tadarida brasiliensis* within the vivarium, I observed

individual bats emitting buzzes on the feeding station when other bats approached the food dish. These buzzes were often coupled with aggressive physical confrontations. Similarly, when hand-feeding bats on the feeding platform, some bats would emit buzzes as they attempted to chase other bats off the feeding platform. These observations indicated to us that the buzz was used in a communicative rather than an echolocation context. Characterization of the complete song as "territorial" was based upon personal communications with Ms. Barbara French (Bat Conservation International, Austin TX) and Dr. George Pollak (University of Texas, Austin) and upon video recordings that reflect the vigorous defensive behavior that accompanies song production by solitary male bats defending a roost site. When the communication buzz is included in the territorial song, it always appears as the terminating sequence of the song, very similar to the way the "terminal" buzz also represents the conclusion of the foraging echolocation sequence. A total of 58 communication buzzes were analyzed in detail, including 36 isolated buzzes and 22 buzzes taken from the ends of territorial songs. Separate analyses of isolated communication buzzes versus buzzes included in the territorial song revealed no significant differences in the temporal or spectral parameters of the buzzes or calls contained within these subsets, therefore these two data sets were pooled into a common set of "communication buzzes". The vocal repertoire of *Tadarida brasiliensis* also includes a trill, which I have recorded from both flying and stationary bats. These trills are easily distinguished from short buzzes because they typically consist of 5 to 7 syllables separated by longer (12-15 ms), constant-duration IPIs, rather than the rapidly shortening IPIs seen in buzzes. Trills could also be distinguished from buzzes based on

differences in other spectral and temporal measurements, but these details will be presented in a separate report. Trills were not included in the communication buzz data set.

Communication buzzes were significantly longer than foraging buzzes (Figure 6), averaging 191 ± 88 ms ($P < 0.001$), and included significantly more calls (30 ± 13, $P < 0.002$). The slope of the relationship between number of calls and overall buzz duration (Figure 6A) was statistically indistinguishable (6.5 ms per additional call) from foraging buzzes, but the entire relationship appears offset relative to foraging buzzes (Figure 6A) because communication buzzes typically began with shorter IPIs and usually reached the maximum call by the 5^{th} call, rather than the 10^{th} call in foraging buzzes (Figure 8B). Figure 8 presents the results of a detailed measurement of the call durations and IPIs taken from the first 20 calls of all the buzzes that contained 20 or more elements (79 foraging buzzes and 37 communication buzzes). Figure 9 presents the spectral analyses of F_{peak}, F_{max} and F_{min} for the same data set. The first IPI in communication buzzes was 7.5 ± 3.2 ms, and the first 10 IPIs in communication buzzes were all statistically shorter than their counterpart IPIs in foraging buzzes ($P < 0.001$). The maximum call rate within communication buzzes, 6.5 ms per call or 154 Hz, was the same as for foraging buzzes (Figure 8B). In contrast to the obvious difference in IPIs between buzz types, call durations began, progressed, and ended with statistically indistinguishable values for both foraging and communication buzzes (Figure 8A). Call durations began at 3.8 ± 0.9 ms and 4.4 ± 1.4 ms for foraging and communication buzzes respectively, ($P > 0.05$) and

decreased continuously throughout the first 20 calls in both cases to minimum values of 1.8 ± 0.6 and 1.8 ± 0.4 ms, respectively.

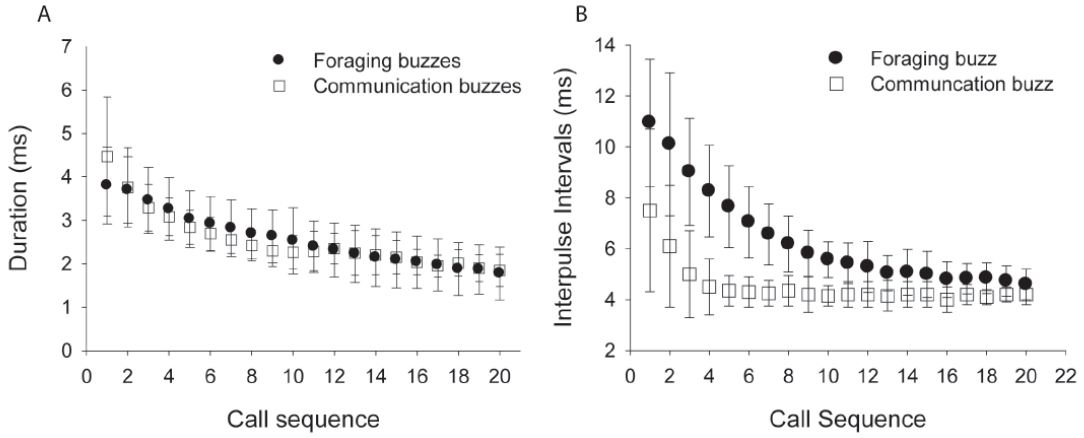

Figure 8. The Sequences of Changes in Call Durations and Inter-pulse Intervals in Foraging and Communication Buzzes. A represents call durations and B represents interpulse intervals. Data shown are the mean ± SD of the first 20 calls/intervals for all buzzes consisting of at least 20 calls (n=79 foraging and 37 communication buzzes). There was no significant difference in call durations in foraging versus communication buzzes, however IPIs were significantly different (P<.001) from the first to the 12th IPI.

Comparing spectral parameters of foraging and communication buzzes

A previous report (Simmons et al., 1978) noted that *Tadarida brasiliensis* uses different call structures when echolocating in open space versus in their roosts or other cluttered spaces. In their natural roosts, in the bat vivarium and in our flight chamber,

Tadarida switched away from the roughly linearly-modulated FM sweeps emitted while foraging outside (Figure 7 A,B) to emitting echolocation calls that contain a short constant-frequency (CF) component followed by an brief FM sweep (Figure 7 C,D and Figure 9A). In some cases (Figure 7) the CF component was preceded by an initial upward FM component, although the shape and duration of the CF component appeared to vary among bats. The CF component of the call is typically the loudest part of the call. Figure 10B compares the power spectra obtained from an outside "approach" call with one obtained from echolocation call emitted in the lab (Figure 10A). Besides the distinct peak of the initial CF component present in the call recorded in confined spaces, one can glean from this comparison that in fact the overall bandwidth of the two call types at approximately –50 dB relative to F_{peak} are not very different (Figure 10B). Rather than making a change in call frequency, my interpretation is that when echolocating in cluttered and confined spaces, *Tadarida* shift the maximum energy of their calls to the uppermost portions of their normal bandwidth and extend the duration of the calls initial segment to further enhance resolution in a cluttered environment.

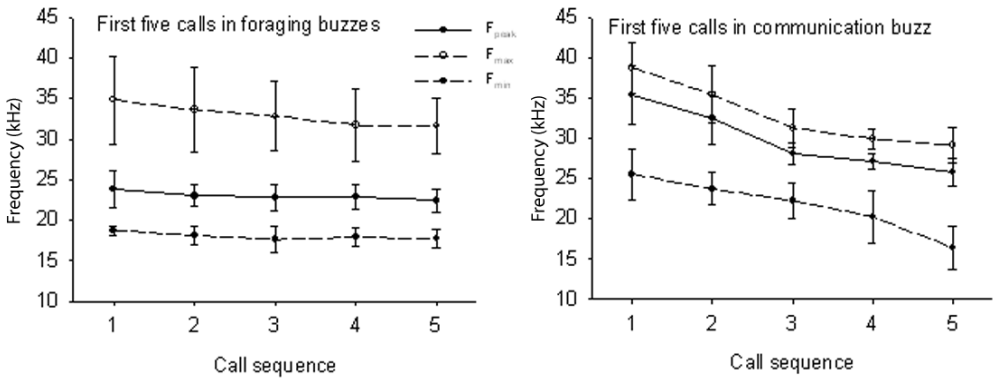

Figure 9. A Comparison of Mean Spectral Parameters of the First Five Calls in Foraging and Communication Buzzes. This uses the same data set as Figure 8. F_{min} and F_{max} are the lower and upper frequencies, respectively, at –15dB relative to F_{peak}.

It also appeared to be the case that when echolocating in cluttered spaces these bats put more energy into several upper harmonics (Figure 7), which dramatically widened the overall bandwidth of the calls in a manner, similar to an adaptation observed across different European FM bats that forage at different distances to clutter (Siemers and Schnitzler, 2004); the closer to clutter they hunt, the wider the call bandwidth. However, since in my experiments the distance from the microphone to the bats was much greater for buzzes recorded in the field compared to those recorded in our vivarium, I cannot unequivocally establish that the absence of multiple higher harmonics in calls recorded in the field was not simply due to atmospheric attenuation.

For echolocation calls recorded in the lab the mean call duration was 4.5 ± 0.1 ms (n=500 calls, data pooled from 100 calls each from five bats). The mean F_{peak}, F_{max}

and F_{min} were 39.2 ± 11.8 kHz, 45.8 ± 7.3 kHz, and 29.5 ± 11.8 kHz respectively. In contrast to recordings of approach phase calls recorded in the field, in the lab F_{peak} was always more closely associated with F_{max} than F_{min}, which reflects the change to a CF-FM call structure. Furthermore, F_{max} rather than F_{min} was the least variable call frequency measurement in the lab recordings. The –15 dB bandwidth was greater in the lab recordings, probably because the microphone was positioned only a meter away from the bat, but the measured F_{min} was similar to the field recorded approach calls. These results are consistent with the previously reported changes in call structure (Simmons et al., 1978) and their conclusion that *Tadarida brasiliensis* modify their echolocation call structure from an FM to a CF-FM call upon entering their roost.

In the communication buzzes shown in Figure 7 C and D, it is evident that calls contained within the communication buzz are of the CF-FM type, and do not exhibit the same spectro-temporal structure of calls contained within foraging buzzes shown in Figures 3 and 7A,B. To provide a more objective comparison of the spectral parameters of calls within foraging and communication buzzes, Figure 9 depicts measurements of F_{peak}, F_{max} and F_{min} for the first five calls of foraging buzzes (7A) and communication buzzes (7B). As was the case in foraging approach calls, the F_{peak} was always closer to the F_{min} in foraging buzzes, whereas in communication buzzes the F_{peak} was always closer to the F_{max}. For the first call in the buzz, measurements of F_{peak}, F_{max} and F_{min} were all significantly higher in the communication buzzes compared to foraging buzzes, however all three values dropped much more steeply over the first five calls in communication versus foraging buzzes. By the fifth call there was no significant

difference ($P< 0.05$) in the F_{max} and F_{min} of foraging and communication buzzes, but the F_{peak} remained significantly higher. Figure 10C compares the power spectra of the first call in a foraging buzz with the first call in a communication buzz. These data illustrate that the change in call structure from an FM to a CF-FM call that accommodates the transition to echolocation in cluttered spaces appears to be maintained in the structure of calls comprising the communication buzzes.

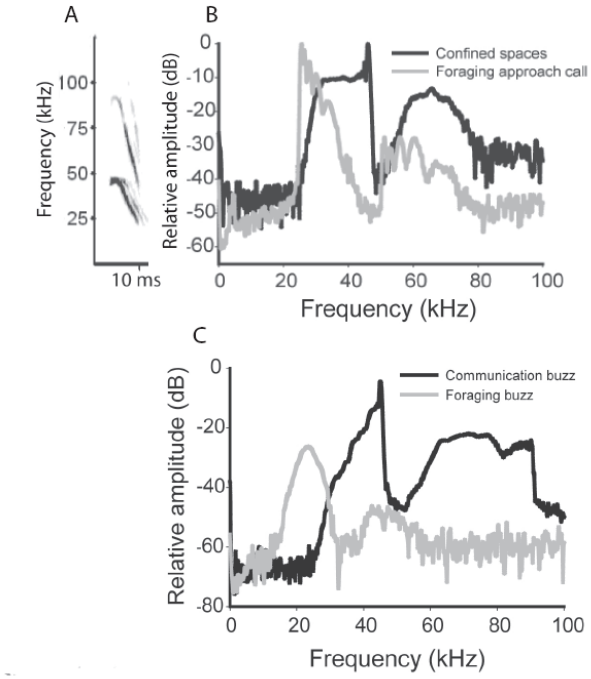

Figure 10. Echolocation Calls in Confined Space. (A) An example of the CF-FM call structure utilized by free-tailed bats echolocating in their roost. B compares the power spectrum of an approach call recorded from a bat foraging over a pond with the spectrum of an echolocation call emitted in the roost (call shown in A). C compares the power spectra of the first calls in a foraging buzz and a communication buzz.

In-flight buzzes uttered in cluttered spaces

Finally, I sought to characterize what foraging buzzes looked like when emitted by bats flying within the cluttered confines of our vivarium, within which the acoustic environment would be identical to the conditions under which the communication buzzes were recorded. I was unsuccessful in training our free-tailed bats to seek and catch either flying insects (waxmoths) or tethered insects within our vivarium as has been done with the big brown bat (*Eptesicus fuscus*) (Griffin, 1958, Moss et al., 2006), however I found that I could provoke buzzes from flying bats in the vivarium by randomly tossing obstacles (small foam balls) in the air across their flight path. Using this simple method I recorded 18 in-flight buzzes from three different bats under conditions in which only one bat was present in the room at a time. Since the bats were not catching prey items, I will refer to these as orientation buzzes. These were bats from which I had previously recorded territorial songs and communication buzzes. Under these conditions, the mean orientation buzz duration was 104 ± 18 ms, and consisted of an average of 12 ± 3 calls per buzz. The duration of the first call within the orientation buzzes was 3.5 ± 0.7 ms, and subsequent calls followed a pattern of change that was statistically indistinguishable from both foraging and communication buzzes. The first IPI of the orientation buzzes was 9.7 ± 2.2 ms, which was significantly longer than the first IPIs of the communication buzzes (7.5 ± 3.2 ms; $P< 0.05$) but also not quite as long as the first IPIs of the foraging buzzes (10.9± 2.5 ms; $P> 0.05$) although this difference was not significant. The calls within the orientation buzzes were of the CF-FM type; the mean F_{pk} was 36.3 ± 3.2 kHz, which was closer to the mean F_{max} (39.1±2.4 kHz) than

the mean F_{min} (24.5±3.8 kHz). Thus, buzzes uttered in flight within a cluttered space exhibited spectral parameters basically identical to those found in communication buzzes, yet the initial temporal patterns of call emissions within the buzz were more similar to those of foraging buzzes uttered by flying bats.

Communication call analysis

We analyzed 15 different call types in both males and females: long waves, short waves, strums, chirps, short ladders, long ladders, trills, low FMs, upward FMs, descending FMs, upward long flats, buzzes, arrowheads, warbles, and V calls (Figure 1). I found that both sexes used all 15 call types and the relative proportion of call types used was not different between males and females (Figure 11). Wave calls were the most predominantly used calls, followed by strums and chirps. Wave calls and strums were often seen repeated in long chains by the same bat. The only sex difference in communication behavior was the fact that the males incorporated several call types into a stereotyped sequence (see *Song Analysis*). Overall, the proportion of calls was the same, but males orchestrated some syllables into a song.

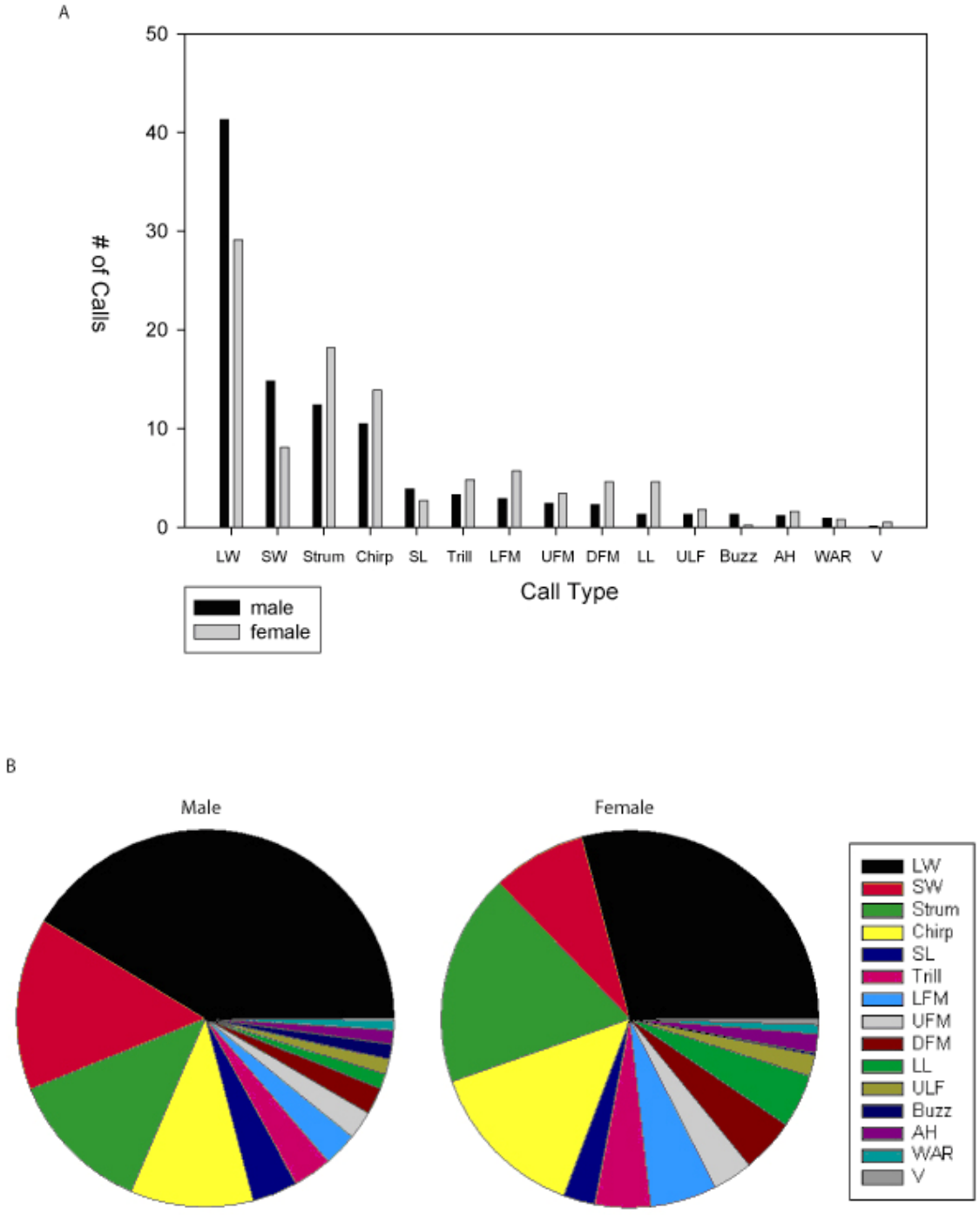

Figure 11. Distribution of Call Types Across Sex. A comparison of the 15 classified call types between males and females reveals that both sexes use the same call types with about the same frequency. The graph in A provides a direct comparison of the calls used by sex and B provides an individual sex distribution.

We also noted two call types associated with specific behaviors that occurred when single bats were isolated from the colony, protest calls and food solicitation calls (Figure 2). Protest calls were produced by agitated bats when handled, while food solicitation calls were produced by begging bats that were trained to take food from a caretaker. These calls were emitted by both males and females. The protest calls could be classified as short ladders, but the food solicitation calls were more difficult to classify based on my system. These calls had different structures between individuals and look very similar in structure to *Tadarida* pup isolation calls (Gelfand and McCracken, 1986).

We explored the seasonality of vocal behavior by looking at seasonal variation in the field in a few individual syllables: trills and communication buzzes, which are part of the courtship song (see *Song Analysis* below), along with a syllable seen in the winter, the strum (Figure 12). Strums were prevalent during the winter, comprising about 20% of all calls used compared to less than 1% of calls used during the summer (Figure 13). Interestingly, buzzes and trills showed the same trend, but opposite of strums. During the summer, buzzes and trills comprised approximately 20% of all calls used and during the winter less than 1%. However temperature appears to also play a small role. On one recording day in late March there was a big drop in temperature (55 degrees Fahrenheit) similar to the temperatures the bats encounter over the winter (Figure 14). No songs were recorded on that day, along with no buzzes or trills. However, strums were recorded and I hypothesized that this call type is potentially correlated with torpor, although these calls are recorded at a very small percentage during the summer as well.

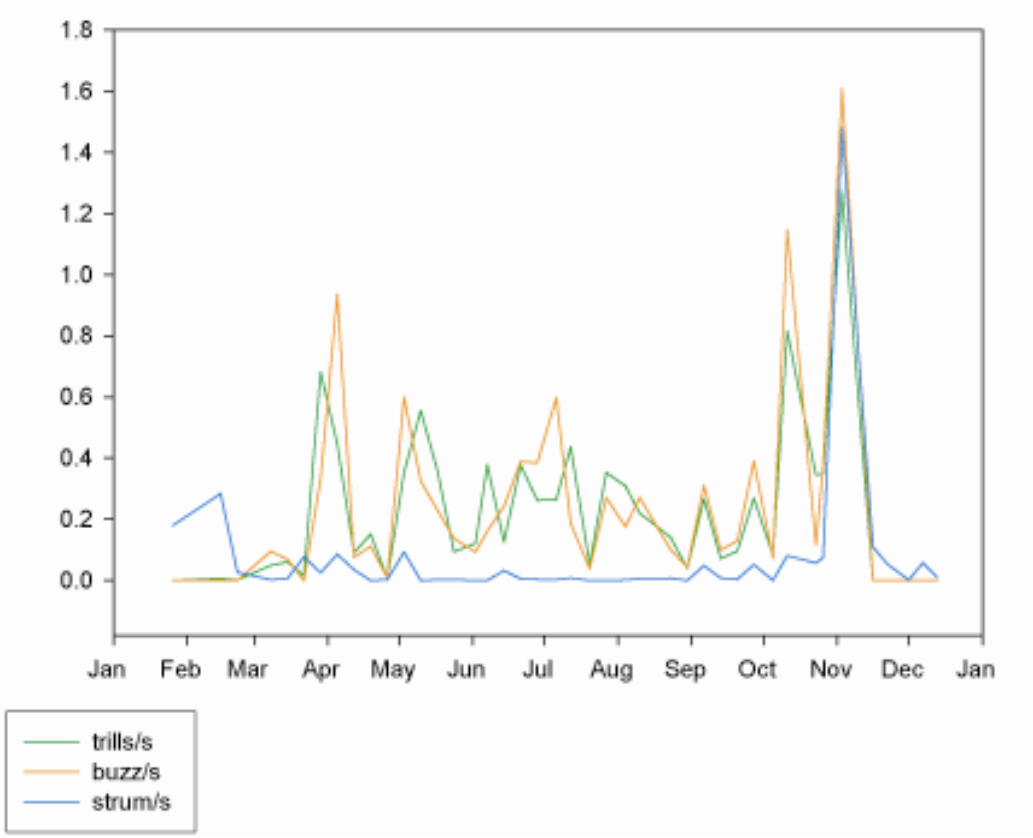

Figure 12. Seasonal Syllable Distribution. The seasonal distribution of trills (green), buzzes (orange), and strums (blue) shows that song syllables (trills and buzzes) are seen in the spring and summer, while strums are used more frequently in the winter, indicating that the vocal repertoire of *Tadarida brasiliensis* changes seasonally. Syllable frequency was calculated as the number of a syllable type divided by the total recorded time.

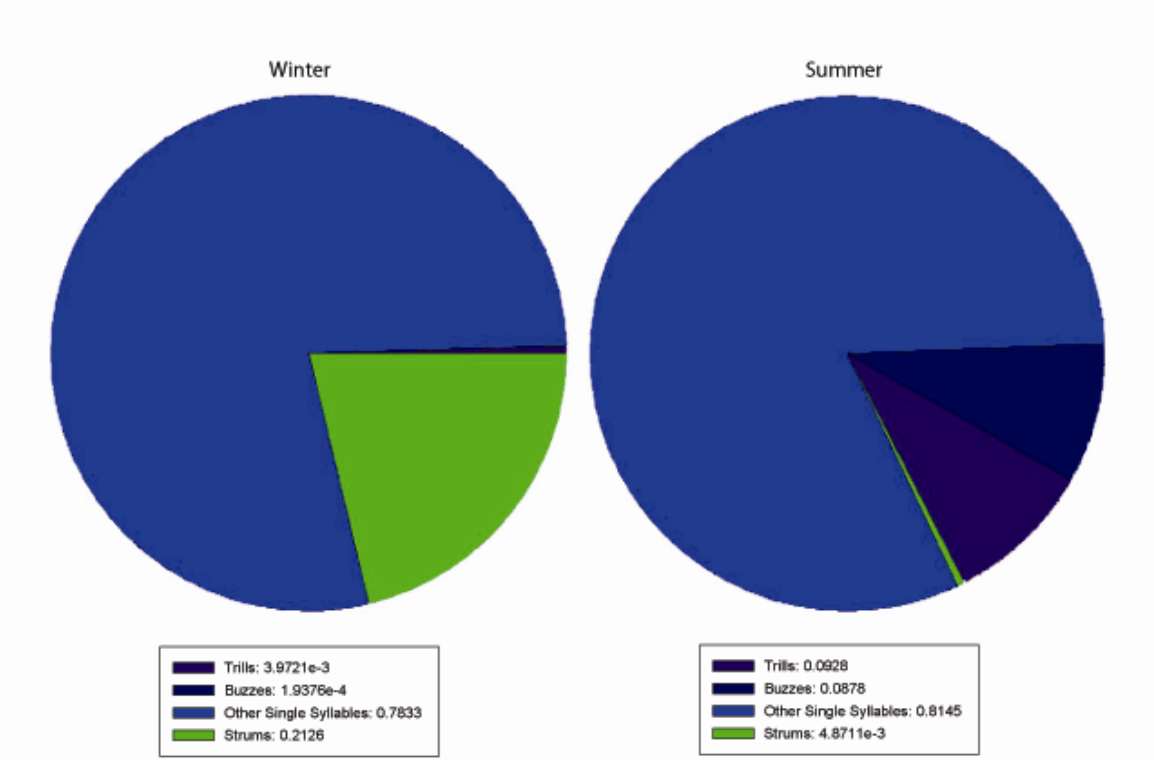

Figure 13. Summer Versus Winter Syllable Preference. This figure shows the relative syllable usage of a population of bats for winter (January and February) and summer (May and June). Syllable frequency was calculated as the total number of a syllable type divided by the total number of calls for the two month period. Strums occupy about 25 percent of the repertoire in the winter, while buzzes and trills fill that portion during the summer.

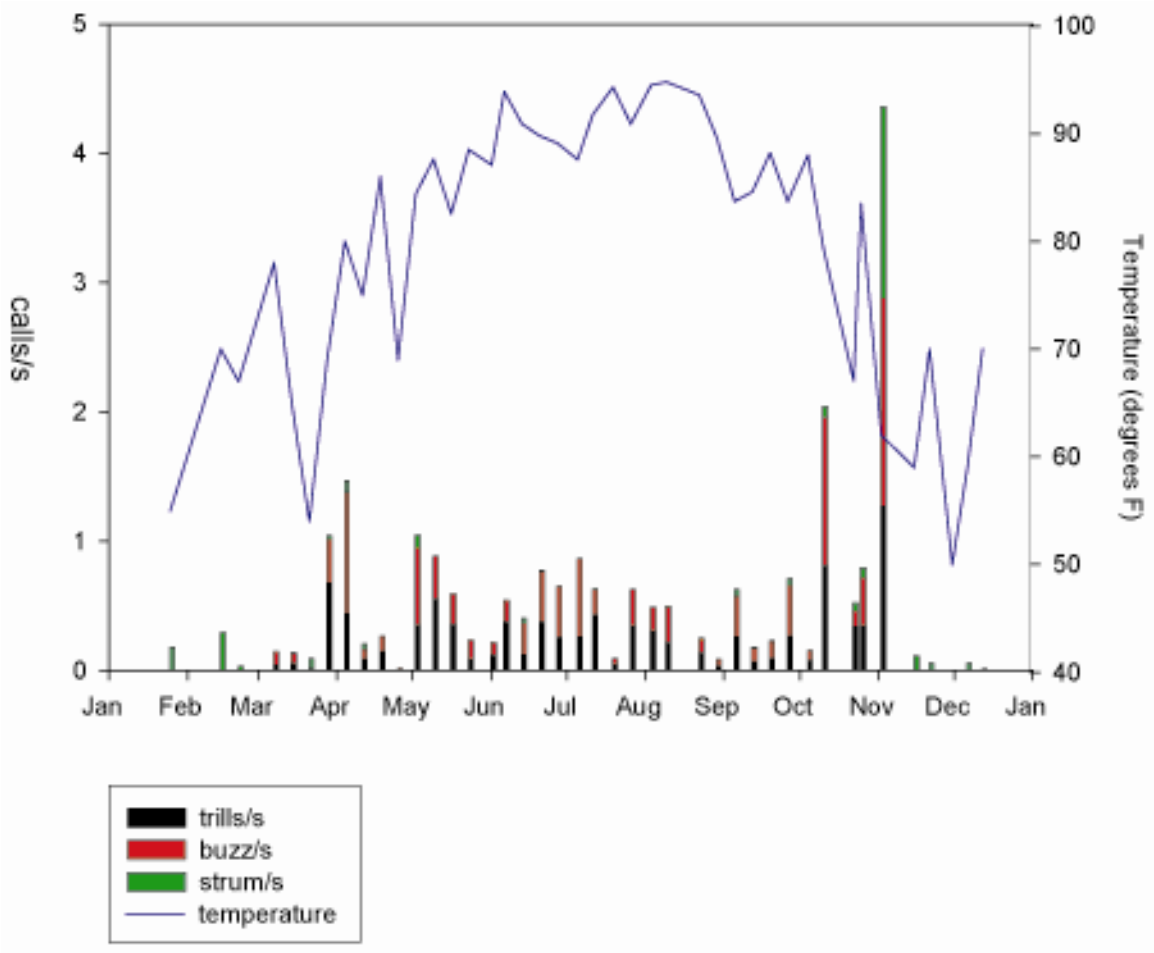

Figure 14. Temperature Effect on Syllable Use. This graph shows the relative distribution of trills (black), buzzes (red), and strums (green) per total number of calls on each recording day over the course of a year. Anytime the emperature (blue) dropped below 60°F, no buzzes or trills were used, only strums. Additionally, in general the total number of calls decreased in lower temperatures.

Song analysis

We recorded vocalizations from bats in the football stadium over the course of a year in an effort to characterize *Tadarida brasiliensis* vocal behavior and to assess whether any seasonal changes in call types used occurred. In early spring, I saw the appearance of vocal patterns emerging that I also was able to record in the lab. This very distinct compilation consists of three types of syllables combined together in a specific order to form a 10-15 syllable long song with a duration of approximately 1-2 seconds (Figure 15). The three syllables are trills, buzzes, and a signature syllable that is unique for each individual bat. A fourth syllable, an introductory syllable, is not always included. Trills consist of 1-4 short duration (3-7 ms) downward FM calls. Buzzes are discussed in detail above (see "*The communication buzz*"). The signature syllables appear to be the individual male's method of making his song distinctive and unique. The signature syllables are variable in all parameters, but they tend to be longer (14-20 ms) than the calls contained in the trill. In the song, the bat begins with 1-3 introductory syllables, although this introduction is not always included, then alternates between trills and signature syllables for several repetitions before terminating the song with a buzz. This structure, however, is just a rough outline. The bats vary every aspect of the song both individually and between individuals (Figure 15). The duration of the entire song is variable due to changes in the different components. The introduction varies in length and is often absent. The alternation between signature syllables and trills is not always 1:1 and the number of each syllable type changes as well. The song can end with more than one buzz. The number of calls within the trills and buzzes can vary greatly and

even the signature syllable can have some modifications. There is a lot of room for variation in this song, while still maintaining a basic structure. This basic song structure is highly consistent among male bats and is not produced by females. However, females do use both trills and buzzes, as mentioned in the previous section.

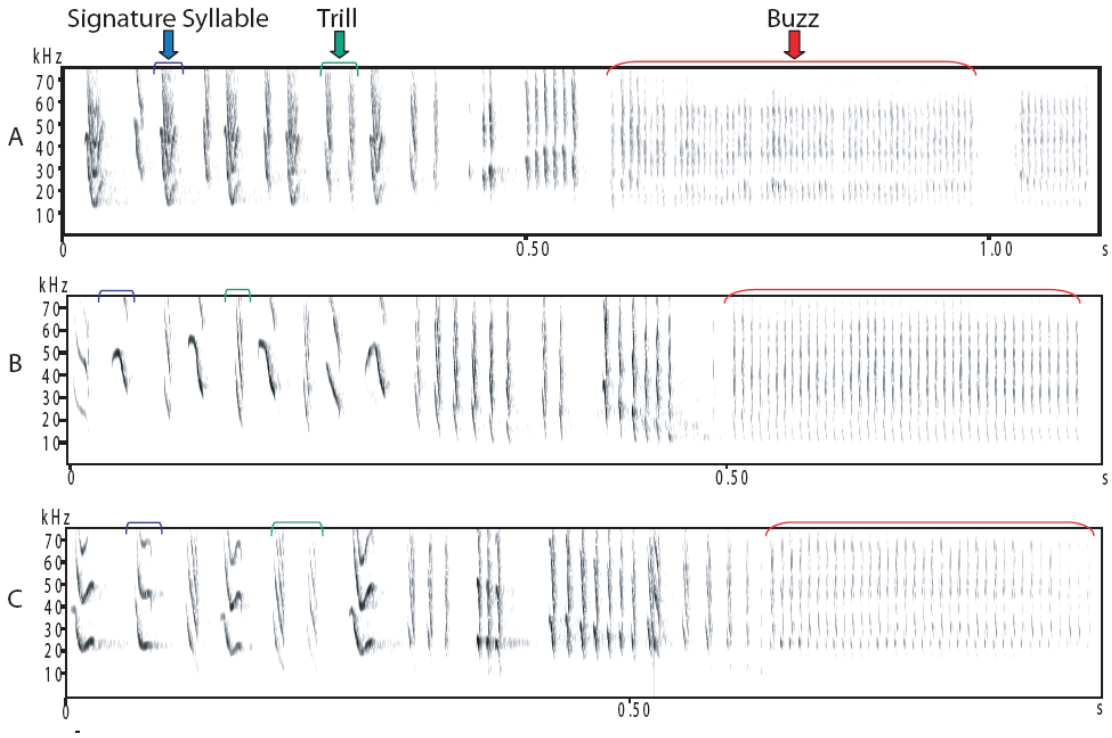

Figure 15. *Tadarida brasiliensis* Courtship/Territorial Song Structure. A-C display songs from three different male bats. The three main song components, trills (green), buzzes (red), and signature syllables (blue) are labeled on each song. This figure illustrates that the *Tadarida* song displays the same basic structure, but exhibits a lot of variability as well.

In addition to audio recordings, I was also able to record videos of singing bats in the lab. Singing males established territories in the available hanging tubes in the vivarium, which they aggressively defended against other males. If another male attempted to enter the tube, the singing male sang loudly and repeatedly, while rubbing his chest and penis on the walls of the tube, behaviors previously observed in males during the mating period (Keeley and Keeley, 2004). If the male did not fly away immediately, the singing male would run down the tube and force the trespasser out. However, singing males allowed approaching females to enter and reside in the territory. Physical examination of singing males showed that these bats had enlarged testes and an enlarged pit on their ventral surface identified as a gular gland. This gland is a specialized sebaceous gland that produces and secretes an oily substance with a musky odor used for scent marking (Gutierrez and Aoki, 1973). While I did not observe any mating, the behaviors exhibited by the males matched previous reports of *Tadarida* mating behavior (French and Lollar, 1998, Keeley and Keeley, 2004). Thus, my observations indicate that the *Tadarida* song is associated with courtship and territoriality.

Because the song is associated with courtship and territoriality, I wanted to determine whether song production was seasonal. In the field, I first recorded songs in early March and consistently saw singing behavior through the end of October (Figure 16). This timing corresponds with the beginning of the *Tadarida* mating season in the field (Keeley and Keeley, 2004). The bats in the lab colony followed a similar schedule; I began to record songs in March. The bats began using song components, trills and

buzzes, to a small degree in the weeks prior to the emergence of the song and there was also a big surge of trills and buzzes in early November, right after songs disappear from recordings (Figure 12).

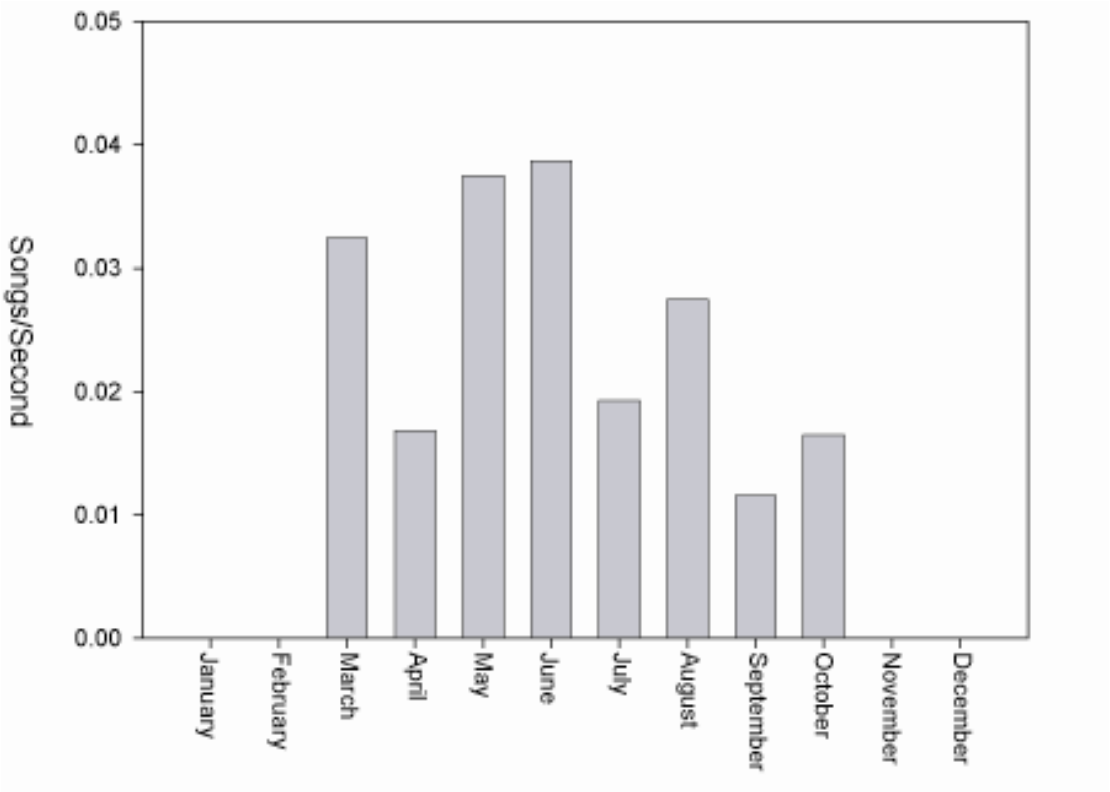

Figure 16. Seasonality of Song. This figure shows that the *Tadarida* song is seasonal, showing up in March and disappearing by November. Song frequency was calculated as the number of songs divided by the total recorded time.

Discussion

Echolocation

The echolocation calls of Tadarida brasiliensis can vary greatly depending on the environment. I found that when echolocating in open spaces, they use CF or very shallow FM search calls. Upon approaching a target, the bats emit a series of progressively steeper linearly frequency-modulated FM "approach" calls which leads up to a buzz. However, in confined spaces, *Tadarida* does not use the same FM approach calls as in the field, but switches to echolocation calls that contain a short constant-frequency (CF) component followed by a brief FM sweep. My interpretation of this change is that when echolocating in cluttered and confined spaces, *Tadarida* shift the maximum energy of their calls to the uppermost portions of their normal bandwidth and extend the duration of the call's initial segment to further enhance resolution in a cluttered environment. This ability to modify the structure of their echolocation calls is interesting because it shows that the bats are modulating their calls in a context-dependent way, using feedback from the environment and making changes. This is valuable to the study of vocal behavior because it shows that echolocation is plastic and complex. Also it is a reliable and robust behavior that bats will do even when alone, which is important for gene expression studies, where it is important to eliminate any unnecessary stimulations that could cloud and clutter gene expression in response to vocalization.

Communication

In my communication call analysis, I classified *Tadarida* social group vocalizations into 15 different call types. What was particularly striking about the communication call analysis, aside from the number of call types this species is capable of producing and the variability within the classifications, was the fact that the proportion of call types used by male and female bats was not really different, even though males produce complex courtship songs and females do not. A characterization of mustached bat vocal behavior also does not report any sex differences (Kanwal et al., 1994), but this species is not known to sing. The sac-winged bat is reported to have a male courtship song, but the report does not indicate whether females use the same syllables (Behr and von Helversen, 2004). This suggests that whatever neural substrate controlling singing behavior in male bats could exist in females as well, but that they are lacking the trigger to activate it. In songbirds, males are always reported as the singers, but there is a growing body of evidence reporting that females also sing, less frequently than males, and only at specific times, such as periods of competition or when caring for their young (Langmore, 1998). One view is that female birdsong is an anomaly and only a byproduct of high androgen levels, but Langmore believes that female song is an adaptive and possibly sexually selected trait. Without debating the function of female birdsong, one definite conclusion that can be drawn from this is that females could produce these complex vocal behaviors with the correct stimulation, whether it is high androgen levels, periods of intense competition or something else. Females and males use the same syllables, but only males produce song, most likely because males have

elevated testosterone levels during mating season that activate the neural substrate necessary for song production. It is possible that given the same factors, females could incorporate those basic syllables into a sequence.

In addition to my classification system of 15 different call types, I also recorded and classified two call types associated with specific behaviors, protest and food solicitation calls. These two call types are important because they are social calls that the bats produce even when they are separated from other bats. Just as with echolocation, these calls could be very valuable to the study of vocal control because the bat can produce these calls alone. Also, this could provide an interesting comparison of echolocation and communication.

Three of the classified call types were analyzed from weekly recordings for an entire year to assess seasonality of vocal behavior. The strum was one of the dominant syllables in the subdued winter repertoire. It was also found at a very small percentage of total calls the rest of the year. I hypothesize that this syllable is associated with cold temperatures and torpor, because it is predominantly seen in the winter and on colder days. Also, I rarely record this syllable type in the vivarium, which is maintained at a higher temperature. The two song syllables, trills and buzzes, exhibited about the same seasonal trend as the courtship song, although they showed up in the recordings a week prior to the first recording of songs and there was a surge of buzzes and trills the week after songs disappeared from the recordings in early November. These are only single data points, but it is possible that the neural mechanism facilitating the song development and production seasonally builds up and breaks down gradually. In many

singing birds, neural attributes of song control nuclei undergo changes seasonally, including changes in size, neuron number and density, and synaptic and dendritic morphology (Smith et al., 1997). In canaries, song syllable repertoire changes seasonally, and they use syllables that are sexually attractive to females during the mating season (Leitner et al., 2001). The emergence of these attractive syllables correlates with increased testosterone levels. Other singing birds have seasonal changes in their repertoires as well, along with changes in testosterone (Smith et al., 1997). It is likely that testosterone plays a role in *Tadarida* singing behavior, especially considering the accompanying phenotype of aggressive behavior, enlarged testes and overactive gular gland. However, testosterone could not be the only factor. The fact that singing behavior is seasonal suggests that melatonin could be involved. Nightly melatonin durations gradually decrease in the spring and early summer and gradually increase in late summer and fall. It is possible that seasonal information from melatonin is the driving force behind the seasonal production of the free-tailed bat courtship song. In songbirds, melatonin receptors are present in the song control system, so localizing these receptors in the free-tailed bat could answer some of the questions about how courtship song is controlled.

Buzzes

Foraging and communication buzzes uttered by the Mexican free-tailed bat *Tadarida brasiliensis* are similar in several key ways: They are both distinguished by the rapid procession of many short calls culminating with a common maximum call rate

of approximately 150 Hz. Within each buzz type call durations are identical at the beginning and ending of the buzz and follow identical patterns of change within the buzz. IPIs began longer and shortened rapidly in both buzz types, and in both cases the call frequency and bandwidth were progressively lowered over the time-course of the buzz. *Tadarida's* foraging and communication buzzes are different in that foraging buzzes began with significantly longer IPIs, in that the communication buzzes were typically 25% longer than foraging buzzes, and that calls contained within the communication buzzes were of the CF-FM type rather than the FM type used by foraging bats. The observed similarities in foraging and communication buzzes are sufficient for us to conclude that these two buzz types are the result of a common vocal motor template being used in different behavioral contexts. I hypothesize that both spectral and temporal differences may be attributable to differences in acoustic conditions, the demands of coordinating feeding with flying, and significant differences in respiratory mechanics of flying versus stationary bats.

The most revealing aspect of the comparison between foraging and communication buzzes is that the calls comprising the communication buzz exhibit spectral parameters consistent with an adaptation for echolocating in cluttered space. Behavioral observations leave little doubt that these buzzes are being used for communicative, not echolocative, purposes: I have observed and recorded buzzes being emitted by bats grappling for possession of a mealworm. If the buzzes were not being used for echolocation, then presumably there would have been no benefit in altering call structures to enhance acoustic resolution in cluttered or confined spaces. It's also

unlikely that the switch to CF-FM calls is relevant to the intended receiver since the receiver is not in a position to use the sounds to better resolve the acoustic scene, which in most cases would be a charging bat. Instead, it appears likely that the incorporation of CF-FM calls into communication buzzes reflects the reflexive nature of the auditory feedback pathway that drives the switch from FM to CF-FM calls when the bats enter the roost (Simmons et al., 1978). This interpretation leads to the conclusion that this auditory feedback pathway operates independent of the neural substrate that coordinates buzzes, which is consistent with experimental results with other species of bats indicating a role for midbrain, or at least sub-cortical, feedback influences on syllable structure (Metzner, 1989, Gaioni et al., 1990, Smotherman et al., 2003, Smotherman and Metzner, 2005).

The observation that communication buzzes were typically longer than foraging buzzes may reflect several influences. Firstly, I hypothesize that there is a functional relationship between respiratory capacity and buzz duration, in which case the duration of a communication buzz would have the potential to reflect either the size or health of the emitter, thereby creating a selective pressure favoring longer communication buzzes. Alternatively, foraging buzzes may have appeared shorter in field recordings because their durations were precisely coordinated with foraging conditions (Moss et al., 2006), and the conditions in which I recorded foraging bats simply favored shorter buzzes. I recorded foraging buzzes in the field that were longer than the average communication buzz, so there seems to be no functional limitation to foraging buzzes that would otherwise explain the difference in mean buzz durations. On the other hand, the

observation that orienting buzzes emitted in the vivarium were significantly shorter than foraging buzzes in open spaces is consistent with previous conclusions that buzz durations are closely matched to the distance of approaching background clutter.

The buzzes uttered by echolocating bats represent a unique example of a mammalian complex vocal motor pattern. Echolocation buzzes by any species of bat are comprised of many individual calls that exhibit precisely defined spectral and temporal properties typically found only within the buzz. Single calls but not buzzes can be elicited by electrical or chemical microstimulation of the midbrain periaqueductal gray (PAG) and the neighboring paralemniscal region (Suga et al., 1973, Schuller, 1986, Schuller and Radtke-Schuller, 1986, Suga and Horikawa, 1986, Schuller and Radtke-Schuller, 1990, Fenzl and Schuller, 2002, 2005). Auditory-vocal feedback pathways appear to pass from the midbrain auditory nuclei through the paralemniscal region (Metzner, 1989) and parabrachial nucleus (Smotherman and Metzner, 2003, Smotherman et al., 2006) before being integrated with the descending vocal motor pathways at or below the level of the PAG (Moss and Sinha, 2003). Somewhere in the bat brain above the level of the PAG is a buzz motor template, and in *Tadarida brasiliensis* this template can be accessed in support of two very different behaviors. Evidence presented here indicates that free-tailed bats can modulate the expression of this buzz template. This is significant because a similar neural template responsible for orchestrating complex multi-syllabic vocal sequences has so far only been identified in one other mammal, namely humans. Thus the differences between foraging and communication buzzes uttered by Mexican free-tailed bats may be small, but therein may lie the keys to understanding how the

mammalian brain orchestrates single syllables into phrases, and ultimately phrases into songs and even speech.

Song

Tadarida brasiliensis courtship/territorial song is a complex sequence of three syllable types that are also found separately in the free-tailed bat vocal repertoire. The song occurs seasonally, emerging at the beginning of mating season. I found that the song had a distinct structure, but there was considerable variability in that structure, both intra- and inter-individually. The song exhibits a fixed pattern which was shown to have syntax (Bohn et al., 2009), which suggests that the neural substrate controlling this complex behavior includes more than the visceromotor pathway. This would be an excellent candidate for studying vocal control and exploring the role of the neocortical pathway, but the song is unreliable in the lab and the colony environment is not suitable for gene expression studies. Singing males will not sing if separated from the colony, which makes sense behaviorally, since the song is triggered by approaching bats and courtship opportunities. The ideal situation for maximal gene expression is to have a solitary bat in a controlled environment, which is not feasible with singing bats at this time.

Conclusion

Overall, I conclude that *Tadarida brasiliensis* has uniquely sophisticated vocal abilities, including the production of a wide vocal repertoire of single syllables, the

ability to modulate calls based on context and environment, and the capacity to create stereotyped yet variable vocal sequences. These capabilities make the Mexican free-tailed bat an excellent non-human mammalian model for vocal behavior. However, due to ambiguities associated with the environment of the experiment in using singing bats, I have chosen to focus my proposed gene expression studies on a more reliable and controllable, yet still complex vocal behavior: echolocation.

CHAPTER IV

MELATONIN AND DOPAMINE DISTRIBUTION IN THE BRAIN OF MEXICAN FREE-TAILED BATS*

Melatonin Introduction

Melatonin, an indoleamine neurohormone produced and released by the pineal gland, is an important regulator of vertebrate circadian rhythms and seasonal processes (Cassone and Menaker, 1984, Wiechmann, 1986, Lincoln, 2006). Melatonin levels are low during the day and high at night, making it an effective conveyer of both time of day and time of year, exerting its circadian and circannual actions through discrete populations of melatonin receptors. In many seasonally breeding mammals, melatonin durations relay information about photic cues to other parts of the brain and body to ensure that reproduction and associated behaviors occur at the optimal time. Reproductive behaviors can be induced in pinealectomized hamsters if given melatonin infusions mimicking longer days of spring (Maywood et al., 1990, Goldman, 1991). Similarly, pinealectomized sheep, short day breeders, can be reproductively induced if given melatonin infusions mimicking shorter days (Karsch et al., 1988). Lesions of melatonin binding sites in the mediobasal hypothalamus of male Syrian hamsters blocks normal testis regression and the associated drops in gonadotropic hormone levels that

* Part of the data reported in this chapter is reprinted with permission from "Distribution of 2-[^{125}I]iodomelatonin Binding in the Brain of Mexican Free-tailed Bats (Tadarida brasiliensis)" by Christine Schwartz, Paul Bartell, Vincent Cassone, and Michael Smotherman, 2009. Brain, Behavior, and Evolution, 73, 16-25, Copyright 2009 S. Karger AG Basel.

normally occur with exposure to short days, suggesting that melatonin acts on the reproductive axis through this region (Maywood and Hastings, 1995). Melatonin also appears to be involved in songbird seasonal reproduction, playing a role in the seasonal regulation of song nuclei volume. In starlings, melatonin implants attenuated the long day increase in the volume of the song nucleus HVC and reduced the volume of another song nucleus, Area X (Bentley et al., 1999). Thus, melatonin appears to act through receptors in discrete brain areas to control seasonal behaviors. When examining seasonal behaviors where the neuroanatomy is not understood, mapping of melatonin receptor distributions could provide useful insight into the neural networks underlying these behaviors.

2-[^{125}I]iodomelatonin (IMEL) binding assays (Vakkuri et al., 1984) are a reliable method for determining the distribution of melatonin receptors in many vertebrates. In mammals, melatonin-binding sites are found in the hypothalamus and pituitary, specifically the median eminence (ME) and pars tuberalis (PT), regions known to directly regulate reproduction, and also in the suprachiasmatic nuclei (SCN), the site of the mammalian circadian clock and recipient of the monosynaptic tract providing light input from the retina, important for environmental entrainment (Cassone, 1990). Additionally, melatonin binding sites are found relatively infrequently in a species-specific manner in other brain regions, including parts of the cortex, thalamus, and hypothalamus (Duncan et al., 1986, Weaver et al., 1989, Stankov et al., 1991b). In birds, melatonin-binding sites are found in the visual system (including visual SCN) and in the song control systems of passerines (Rivkees et al., 1989, Gahr and Kosar, 1996,

Whitfield-Rucker and Cassone, 1996, Bentley and Ball, 2000). A description of melatonin receptor distribution in the brain areas of *Tadarida* could help to identify areas involved in the seasonal processes of breeding and song control as they do in other species.

Mexican free-tailed bats, *Tadarida brasiliensis*, provide a unique opportunity to comparatively study the role of melatonin because these animals display a variety of seasonal behaviors normally found separately in either mammals or birds, such as migration (Fleming and Eby, 2003) and courtship singing behavior (Bohn et al., 2008). In this study, I use *in vitro* autoradiography to localize and characterize melatonin-binding sites in the Mexican free-tailed bat brain. I hypothesize that IMEL binding sites will be found in the ME/PT and also in the SCN, as sites in these regions are found in most mammals. Furthermore, I anticipate that if other IMEL binding sites were present in the bat brain, these sites could be directly involved in migration or seasonal reproductive behaviors, including courtship song production. I also look for sexual dimorphisms in IMEL binding in this study, due to sexual dimorphisms present in reproductive behavior.

Results

Distribution of IMEL-binding sites

We used a custom brain atlas developed in our lab for *Tadarida brasiliensis* to identify the brain areas reported in this paper. The basal ganglia are large, easily

recognizable structures located beneath the external capsule, anterior to the thalamus. The caudate nucleus and putamen, hereafter referred to collectively as the caudate putamen, are one structure in rodents and look very similar in bats, except that the internal capsule more pronouncedly bisects the two structures, similar to the pattern in primates. The positions of the globus pallidus, ventral pallidum, and nucleus accumbens were inferred by cytoarchitectonic features as determined using cresyl violet and cytochrome oxidase stains, and the positions of these structures were similar to those described in rats (Paxinos and Watson, 1998). Similarly, I identified the SCN, ME, and PT based on their relative sizes and positions in the brain. The hippocampus, including dentate gyrus, was also identified based on location and cytoarchitectonics of the structures. A comprehensive review of the available neuroanatomical resources from other bat species also helped identify the major brain regions reported in this paper (Prasada Rao and Kanwal, 2004, Maseko and Manger, 2007).

The entire *Tadarida* brain, from the most rostral tip of the olfactory bulbs to the caudal-most extension of the brainstem nucleus ambiguus, was evaluated to determine where IMEL binding sites were present. The threshold for binding was determined by adjusting background level for each image according to the nonspecific binding levels obtained from the control slide sets exposed to melatonin. Any area with binding higher than the nonspecific binding levels was analyzed. I found binding sites, in order from highest to lowest amount of binding, in the ME/PT, SCN, caudate putamen, nucleus

accumbens, and the granular dentate gyrus of the hippocampus in all bats studied (Figure 17). The nucleus accumbens was the first region with binding to appear in the rostral to caudal direction, followed by the caudate putamen. Binding in these areas persisted for the entire duration of the structures and was relatively uniform throughout each structure. The highest receptor densities were found in the hypothalamus, in the ME and SCN. I include the PT of the pituitary along with the ME as one structure for my analysis because while it is likely that IMEL binding sites are present there due to their prevalence in many other mammals (Stankov et al., 1992, Nonno et al., 1995a, Williams et al., 1995, Williams et al., 1996), these small structures are located so close together and the free-tailed bat is such a small animal that I was unable to differentiate between them on the autoradiographic film, an issue seen in many of the small mammals examined (Weaver et al., 1989, Weaver et al., 1990). In the hippocampus, I quantified the most caudal portion of the granular dentate gyrus. The levels of binding in the rostral portions of the granular dentate gyrus were low and quite variable or even absent in some of the bats surveyed. Binding in the caudal regions was more consistent and dense, so I chose to focus my analysis there.

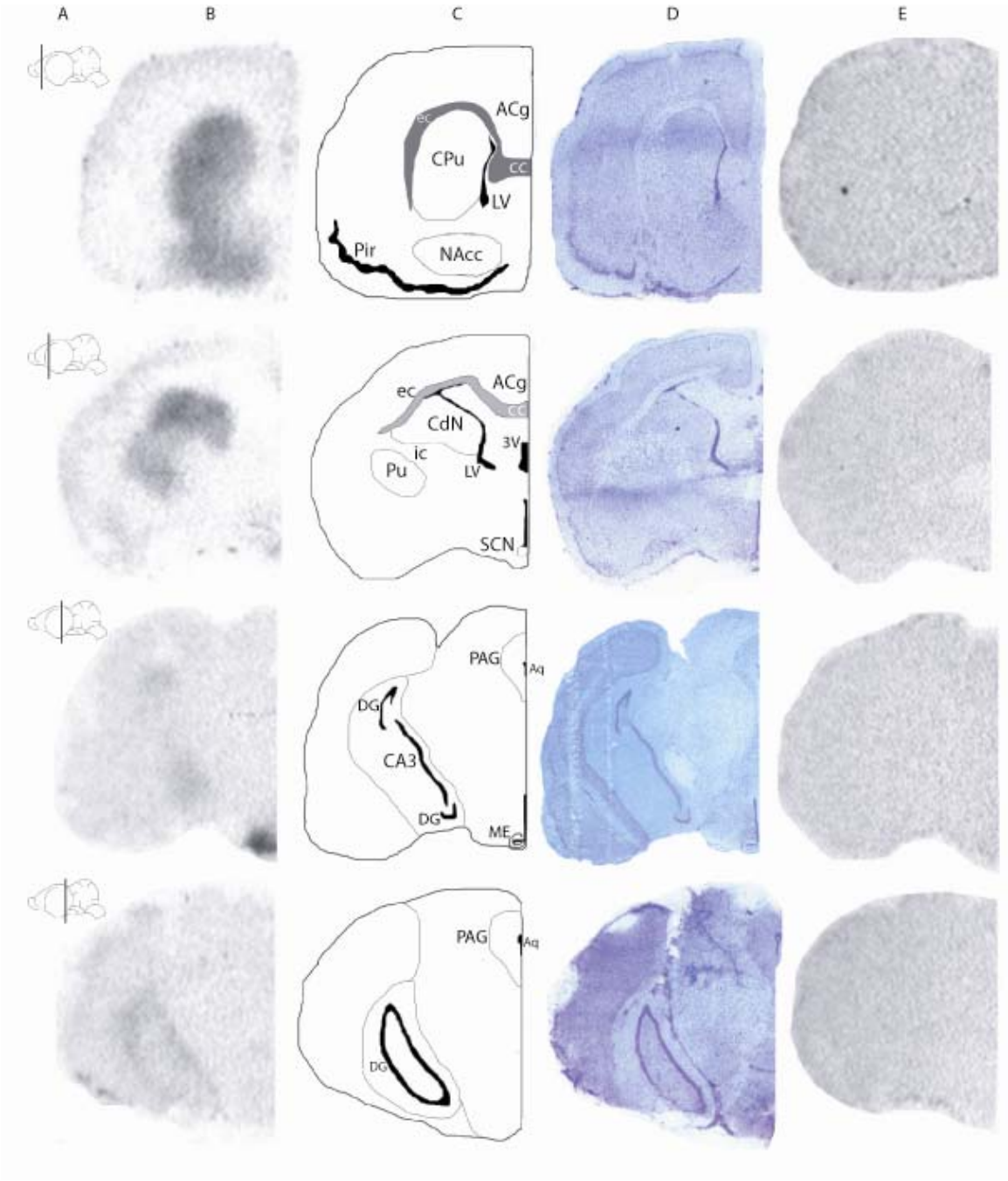

Figure 17. Presence of Melatonin Receptors in the Singing *Tadarida brasiliensis* Brain. Row A indicates the plane of the coronal slice, B shows a representative IMEL autoradiograph, C provides the accompanying brain atlas, D shows the cresyl violet stained sections, and E shows nonspecific binding in control brains.

In my final analysis, I quantified the relative amount of binding in each brain region for both males and females (Table 1). Two of the seven females and one of the seven males only provided data for some of the brain regions because of cryosectioning differences that resulted in the loss of some brain sections. The brains of males and females were analyzed separately to assess any sexual dimorphism in IMEL binding, however, where no significant differences were found, the brain regions are presented together. No significant differences between sexes were found for the ME/PT region (0.136 ± 0.0780 μCi/g, n=13), but binding in the SCN of males (0.0619 ± 0.0247 μCi/g, n=7) was significantly higher ($p=0.048$, $t=2.206$ with 12 d.f) than in females (0.0383 ± 0.0136 μCi/g, n=7). IMEL binding in the caudate putamen and nucleus accumbens of the basal ganglia, (0.0382 ± 0.0237 and 0.0280 ± 0.0174 μCi/g respectively, n=13) and in the dentate gyrus of the hippocampus (0.0173 ± 0.00771 μCi/g, n=11) were not significantly different between sexes. In addition, in one animal, I observed binding in the frontal cortex, including the anterior cingulate cortex. While I can only report actual binding values for this one individual (0.0193 μCi/g), it is possible that IMEL binding sites occur here in the other animals as well, but in levels too low to quantify with autoradiographic techniques.

In addition, six male bats taken out of torpor were also analyzed to determine if there were any seasonal changes in melatonin receptor distribution or density. 2-[^{125}I]iodomelatonin binding sites were found in the exact same areas of the brain in these torpid bats and there was no significant difference in IMEL binding density between the male bats collected in the summer and the torpid male bats collected in the winter.

Characterization of IMEL-binding sites

Saturation experiments were performed to characterize the binding affinity and maximum number of melatonin receptors found in the basal ganglia. The caudate putamen was chosen because its relatively large size allowed for analysis of several sections for each animal. Nonlinear regression analysis on data from my saturation experiments revealed a high affinity binding site (equilibrium dissociation constant (K_d) is 22.3 ± 15.1 pM) in the caudate putamen of *Tadarida brasiliensis* (Figure 18). Because the value obtained was in the picomolar range, the binding sites can be characterized as high affinity. Specific binding plateaued at approximately 200 pM, demonstrating that binding is saturable and the total number of binding sites (B_{max}) was 0.1992 ± 0.0285 fmol/mg protein (Figure 18).

Table 1. 2-[^{125}I]iodomelatonin Binding in Male Versus Female *Tadarida brasiliensis* Brains. (means ± SD).

Brain Region	**Males**	**Females**	**p**	**t (d.f)**
Nucleus Accumbens (NAc)	0.0213 ± 0.0100 (n = 7)	0.0358 ± 0.0222 (n = 6)	0.147	-1.562 (11)
Caudate Putamen (CPu)	0.0297 ± 0.0176 (n = 7)	0.0481 ± 0.0281 (n = 6)	0.179	1.435 (11)
Suprachiasmatic nuclei (SCN)	0.0619 ± 0.0247 (n = 7)	0.0383 ± 0.0136 (n = 7)	0.048	2.206 (12)
Median eminence/Pars tuberalis (ME/PT)	0.187 ± 0.0909 (n = 7)	0.0932± 0.0701 (n = 6)	0.181	1.724 (11)
Dentate gyrus (DG)	0.0148 ± 0.00336 (n = 6)	0.0204 ± 0.0106 (n = 5)	0.253	-1.222 (9)

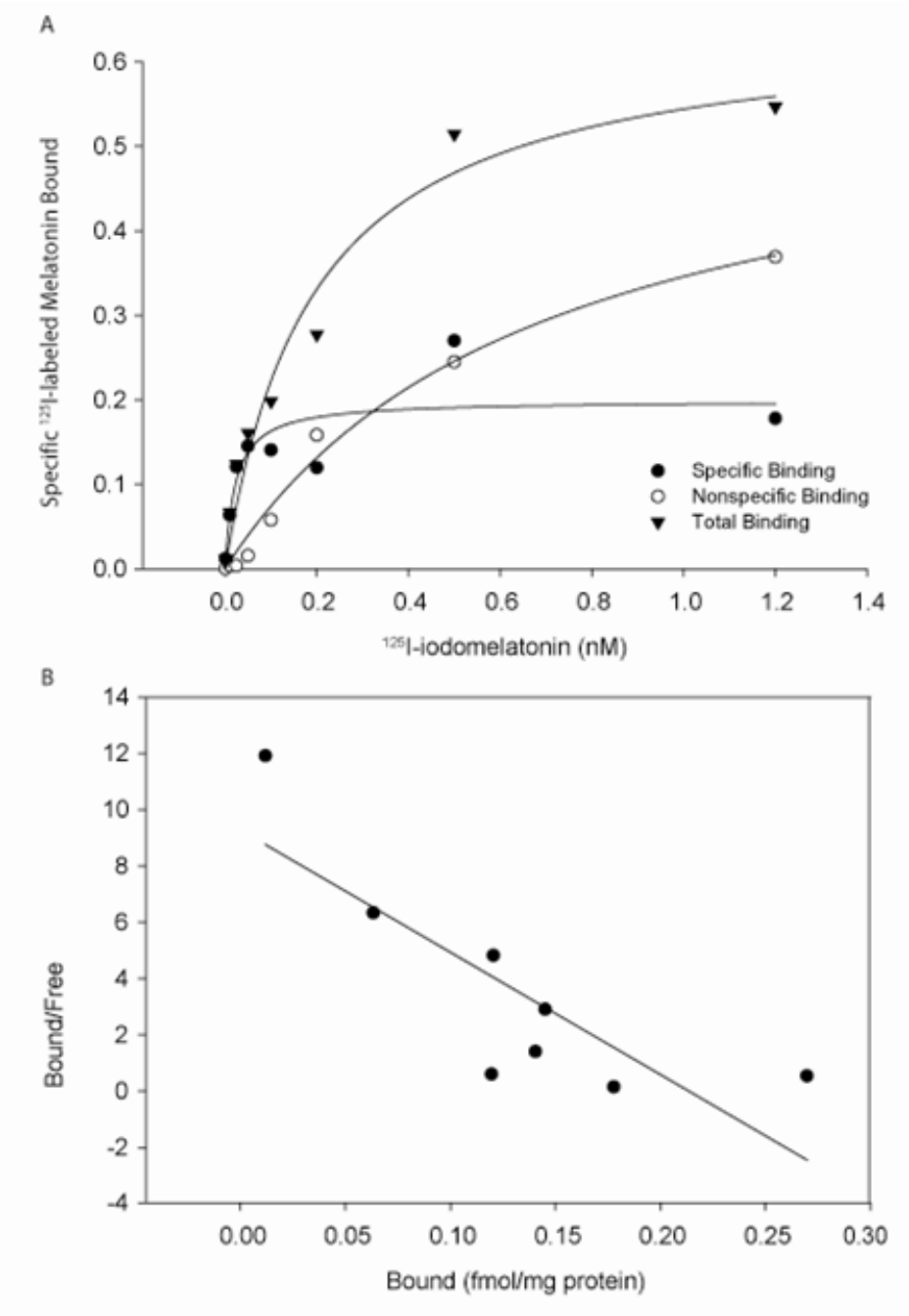

Figure 18. Saturation of IMEL Binding to *Tadarida brasiliensis* Caudate Putamen. The saturation curve is shown in A. Specific binding was calculated by subtracting nonspecific binding from total binding. Each point represents three different animals. B shows a Scatchard plot illustrating the results of a nonlinear regression analysis indicating a high affinity binding site with $K_d = 22.3 \pm 15.1$ pM and $B_{max} = 0.1992 \pm 0.0285$ fmol/mg protein.

Discussion

This study provides a report of melatonin receptor localization and characterization in the Mexican free-tailed bat, *Tadarida brasiliensis*. 2-[^{125}I]iodomelatonin binding sites were found in the caudate putamen, nucleus accumbens, ME/PT, SCN, and hippocampus (Figure 17, Table 1). Saturation experiments revealed a saturable high affinity binding site (Figure 18).

Melatonin binding sites in typical mammalian regions

2-[^{125}I]iodomelatonin binding sites were found in the bat brain in areas previously reported in other mammals: the SCN and ME/PT. The mammalian SCN is the circadian pacemaker and receives monosynaptic input from the retina, allowing the animal to entrain to the photic environment (Cassone and Menaker, 1984, Cassone, 1990). Melatonin production by the pineal is controlled by the SCN via a polysynaptic pathway and the presence of melatonin receptors in the SCN provide the mechanism for melatonin to feedback to the SCN and inhibit its activity. Melatonin receptors in the ME/PT region mediate the effects of melatonin on reproductive behavior and physiology (Cassone, 1990, 1998). Lesions of melatonin binding sites in the mediobasal hypothalamus of male Syrian hamsters blocked the normal testis regression and gonadotropic hormone level drop associated with exposure to short days, suggesting that melatonin acts on the reproductive axis through these sites (Maywood and Hastings, 1995). In the same study, secretion of the reproductive hormone prolactin was unaffected by the mediobasal hypothalamic lesions, but it was speculated that prolactin

levels are instead controlled by melatonin binding sites in the pituitary, specifically pars tuberalis. The presence of melatonin receptors in the bat SCN and ME/PT appears to be consistent with the assumption that these areas control reproductive behaviors in bats via mechanisms common to most mammals.

Sexual dimorphism in 2-[^{125}I]iodomelatonin binding

The SCN was the only area to show sexual dimorphism in IMEL binding in *Tadarida*, with higher binding observed in males. My findings represent the first case of sexual dimorphism in IMEL binding in a mammalian brain region. The only other reported instances of sexual dimorphism in IMEL binding are in the song control system of birds and parts of the visual system and preoptic area in quail, which all displayed higher binding in males (Gahr and Kosar, 1996, Whitfield-Rucker and Cassone, 1996, Aste et al., 2001). The reasons for these differences are unclear, but could potentially be the result of sexually dimorphic neurotransmitters, specifically GABA, which, when given at physiological doses, increases IMEL binding in several brain regions of the quail, including the preoptic area (Canonaco et al., 1994). In addition, sexually dimorphic GABA-like immunoreactivity was found in the song control system of zebra finches (Grisham and Arnold, 1994). While both of these examples are from the bird literature, it seems reasonable that something similar could be occurring in bats. The sexual dimorphism in IMEL binding in the bat SCN might contribute to differences seen in male and female reproductive behaviors, but it must be presumed that changes in SCN

physiology would generally affect all circadian behaviors, due to its role as pacemaker of the mammalian circadian system.

2-[^{125}I]iodomelatonin binding in the telencephalon

The hippocampus is important for short-term memory and spatial navigation. 2-[^{125}I]iodomelatonin binding in the hippocampus was previously reported in other mammals, including primates, rabbits, cattle, and red deer (Stankov et al., 1991a, Stankov et al., 1993, Nonno et al., 1995b, Williams et al., 1996). In bats the hippocampus could be important for migration, as seasonally migrating bats are consistent in their routes and final destinations (Fleming and Eby, 2003). Several species of birds exhibit seasonal changes in hippocampal volume associated with behaviors requiring enhanced spatial memory, such as food hoarding and brood parasitism (Smulders et al., 1995, Clayton et al., 1997, Smulders et al., 2000). However, melatonin receptors have not been found in the bird hippocampus (Cassone et al., 1995), and evidence suggests that photoperiod is only indirectly responsible for changes in hippocampal volume (MacDougall-Shackleton et al., 2003). Seasonal changes in hippocampal volume have not been found in mammals with similar food hoarding behaviors, such as the food-caching gray squirrel, even though the behavior is seasonal (Lavenex et al., 2000). There is no evidence of seasonal or circadian changes in memory capacity or hippocampal functioning that might illustrate a potential role for melatonin in the mammalian hippocampus. It is possible that in mammals certain seasonal behaviors like migration and reproduction might be supported by melatonin receptors in

the hippocampus facilitating increased memory capacity without concurrent changes in hippocampal volume. I found no difference in IMEL binding between males and females, indicating that the role of melatonin receptors in the free-tailed bat hippocampus is most likely unrelated to sexually dimorphic reproductive behaviors.

Three areas of the basal ganglia of the Mexican free-tailed bat brain were found to contain putative melatonin receptors: the caudate nucleus, putamen, and nucleus accumbens. 2-[^{125}I]iodomelatonin binding in the mammalian putamen was previously reported only in red deer (Williams et al., 1996). 2-[^{125}I]iodomelatonin binding in the nucleus accumbens, a ventral component of the basal ganglia, was previously observed in mice (Weaver et al., 1990). Melatonin injections into the nucleus accumbens of rats changed their locomotor activity (Gaffori and Van Ree, 1985, Paredes et al., 1999), but the presence of melatonin receptors in the nucleus accumbens has not been confirmed in the rat. What is particularly striking about the melatonin receptor profile in the bat basal ganglia is that putative melatonin receptors were observed throughout the three regions of the basal ganglia at relatively high densities, which has not been observed in other mammals. This distribution is more reminiscent of the high densities of IMEL binding observed in homologous regions of the songbird brain (Gahr and Kosar, 1996, Whitfield-Rucker and Cassone, 1996, Bentley et al., 1999), which may be correlated with similarities in the seasonal migratory, foraging, and reproductive behaviors displayed by birds and bats. It is also more reminiscent of what the distribution of dopamine receptors would look like, which presents some interesting questions about

melatonin's potential interactions with dopamine (Gehlert et al., 1992, Levant et al., 1993).

2-[^{125}I]iodomelatonin binding in the bat basal ganglia is interesting because the basal ganglia are generally involved in the coordination of complex behaviors, including the orchestration of complex motor sequences (Aldridge and Berridge, 1998, Graybiel, 2000, 2001, Berridge et al., 2005, Grillner et al., 2005). The caudate nucleus is credited with planning complex behaviors, and the putamen is believed to act as a substrate for the integration of multisensory, memory and contextual cues for the ongoing control of behaviors. This control is achieved by a striatal-thalamo-cortical feedback loop through the basal ganglia that selectively activates or modulates subsequent motor patterns (Graybiel, 2000, Hikosaka et al., 2000, Alm, 2004). Extrapyramidal inputs from the motor cortex converge with sensory and memory cues in the basal ganglia to regulate the activity of excitatory thalamic neurons projecting back onto the motor cortex via inhibitory outputs to the globus pallidus. I did not find IMEL binding in the primary output centers of the basal ganglia, the globus pallidus and the substantia nigra (Graybiel, 2000, Grillner et al., 2005). Disruption of basal ganglia circuits seriously disrupts motor coordination in mammals, but less dramatic pharmacological manipulations of synaptic activity can profoundly influence the initiation and drive to complete specialized complex behaviors (Berridge and Aldridge, 2000a, b, Berridge et al., 2005). Melatonin receptors in the *Tadarida* basal ganglia could contribute to the regulation of seasonally specific behaviors such as migration, foraging patterns, prey preferences, and reproductive behaviors, including separation of the sexes, the formation

of maternity roosts, and male courtship behaviors such as singing and territorial displays (Altringham, 1996).

In songbirds, IMEL binding is found in Area X, the avian homologue of the basal ganglia (Gahr and Kosar, 1996, Whitfield-Rucker and Cassone, 1996, Bentley and Ball, 2000). Area X is the part of the song control system involved in learning and development of song (Brenowitz et al., 1997). In a manner analogous to its general function in mammals, the avian basal ganglia is believed to contribute to the development of the characteristic sequence stereotypy of oscine courtship songs (Doupe et al., 2004, Doupe et al., 2005). Through the anterior forebrain pathway, the bird basal ganglia contribute to both the variability of the song during development (Brainard and Doupe, 2000, Kao and Brainard, 2006) and its stereotypy (Scharff and Nottebohm, 1991). I do not know if the basal ganglia in the free-tailed bat are involved in the production of the courtship song, but current models of how non-human mammalian vocalizations are generated in the brain do not include the basal ganglia. Humans however, do rely upon basal ganglia functions for normal speech production (Alm, 2004).

In addition to the areas containing IMEL binding sites in all bats, I also found binding in the frontal cortex, including the anterior cingulate cortex, of one male animal in quantifiable levels. It is possible that binding sites were present here in the other animals, but in levels too low to quantify with autoradiographic techniques. Frontal cortex binding was previously reported in primates and rabbits (Stankov et al., 1991a, Stankov et al., 1993). Similar to these previous findings, the IMEL binding sites of the

Tadarida frontal cortex were not restricted to a specific cortical layer, but rather appeared to be relatively uniform through all layers. The significance of the IMEL binding in the frontal cortex is unclear.

Characterization of 2-[^{125}I]iodomelatonin binding sites

The K_d and B_{max} values determined for the IMEL binding sites in the caudate putamen of the free-tailed bat (22.3 ± 15.1 pM and 0.1992 ± 0.0285 fmol/mg protein, respectively) were within the same range as reported for rabbit, horse, sheep, and human (Stankov et al., 1991a, Stankov et al., 1991b, Yuan et al., 1991). These values indicate that binding sites in the caudate putamen are most likely to be the Mel_{1a} receptor, which has a K_d of 20-40 pM (Reppert et al., 1996). Researchers have identified three different melatonin receptors, Mel_{1a}, Mel_{1b}, and Mel_{1c}, and all but Mel_{1c} have been found in the mammalian brain (Dubocovich, 1995, Reppert et al., 1996, Reppert, 1997). It is unlikely that the receptors found in the caudate putamen are Mel_{1b} receptors because the K_d range for this receptor is around 160 pM. The melatonin receptors have been cloned and belong to a family of guanine nucleotide binding protein (G protein)-coupled receptors (Ebisawa et al., 1994, Reppert and Weaver, 1995). This was supported prior to the actual cloning of the genes by showing that IMEL binding could be inhibited by the presence of a non-hydrolyzable GTP analog (GTPγS), which was subsequently demonstrated in several species (Stankov et al., 1992, Stankov et al., 1993, Wiechmann and Wirsig-Wiechmann, 1994, Williams et al., 1995, Williams et al., 1996). Further

investigations identifying which melatonin receptors are located in each bat brain region are needed.

Absence of seasonal effects on 2-[^{125}I]iodomelatonin binding

It is presumed that seasonal changes in nightly melatonin durations drive seasonal changes in behavior, but it is possible that seasonal changes in receptor numbers may also contribute. In ground squirrels and hamsters it was found that IMEL binding in the PT was shown to decrease when the animals were in hibernation (Stanton et al., 1991, Skene et al., 1993). However, I found no significant difference in IMEL binding between bats collected in the summer and the torpid bats collected in the winter. Ground squirrels and hamsters are true hibernators, while the free-tailed bats only go through periods of daily torpor. Torpor periods are shorter and the animals wake and raise their body temperature to normal levels more frequently. Because free-tailed bats, which migrate to avoid long periods of cold temperatures, do not go through the lengthy depression of body temperature that these other mammals endure might explain why no differences in IMEL binding density were found to be associated with torpor. Torpor can be induced in bats at any time of year by exposure to cold temperatures regardless of photoperiodic conditions, which suggests that torpor is not regulated by the same seasonal and neuroendocrine cues that initiate hibernation. Seasonal differences in IMEL binding in the PT was also shown in mink, but this was not reported to be shown specifically in hibernating animals (Messager et al., 1997).

Mexican free-tailed bats have many other seasonal behaviors in addition to torpor, including migration and courtship behaviors, which could potentially be associated with seasonal changes in IMEL binding. For example, in songbirds, there are significant differences in IMEL binding associated with different seasons in the song control system (Whitfield-Rucker and Cassone, 1996, Bentley and Ball, 2000). However, while *Tadarida brasiliensis* exhibits prominent seasonal behaviors, in particular, a courtship singing behavior similar to birds, no significant seasonal differences in IMEL binding associated with season were found in any brain region. This suggests that the seasonal control of behaviors in this species is probably not occurring through changes in melatonin receptor numbers, but perhaps through some other mechanism.

Conclusion

The Mexican free-tailed bat, *Tadarida brasilensis*, is a unique mammalian model for the comparative study of melatonin and melatonin receptors. The bat brain showed IMEL distribution patterns consistent with that observed in other mammals, but also with high densities of receptors distributed in the basal ganglia. The function of melatonin receptors in the mammalian basal ganglia is unknown, but their comparable localization to the song control nuclei in songbirds may provide a clue about their function in bats. Since the free-tailed bats exhibit a suite of behaviors similar to birds, like flying, singing, and long-distance seasonal migration patterns, my results may shed light on how melatonin regulates seasonal behaviors in the vertebrate brain.

Additionally, since the melatonin binding sites were distributed in the striatum, it is of interest to determine whether this distribution matches the distribution of the dopamine receptors, which are found in this same area in other mammals.

Dopamine Introduction

Dopamine is a monoamine neurotransmitter synthesized in the body from the amino acid tyrosine. It is produced in the brain in the substantia nigra pars compacta and ventral tegmental area (SNc-VTA) of the midbrain. Tyrosine is modified by tyrosine hydroxylase (TH) into 3,4-dihydroxyphenylalanine (L-DOPA), which is then converted by aromatic L-amino acid decarboxylase into dopamine. There are five types of dopamine receptors, D1, D2, D3, D4, and D5, but D1 and D2 receptors the most commonly found types. Dopamine has a variety of functions in the brain, including roles in motivation, mood, sleep, movement, and learning, among others. Disruptions in the dopamine system are at the heart of many pathological conditions, including Huntington's disease, Parkinson's disease, Tourette's syndrome, and schizophrenia, which have helped identify some of dopamine's many functions through their associated symptoms.

Dopamine is the main modulatory neurotransmitter in the basal ganglia, a network of interconnected subcortical nuclei centrally located in the brain that interact with many different areas (Alm, 2004). The input nucleus of this network is the striatum, composed of the caudate nucleus, putamen, and nucleus accumbens (Hikosaka et al., 2000, Alm, 2004). The striatum receives input from most of the cerebral cortex.

The main output nuclei are the interior part of the globus pallidus and the substantia nigra pars reticulata, which project back to the cortex by way of the thalamus and also to the brain stem. The basal ganglia also include some internal modulators, the subthalamic nucleus, the external portion of the globus pallidus, and the SNc/VTA, which specifically modulates the activity of the striatum with dopamine.

A variety of successful radiolabeled dopamine receptor agonists and antagonists have been used to study the distribution of D1 and D2 receptors in mammals (Urwyler and Coward, 1987, Filloux et al., 1988, Lidow et al., 1991, Gehlert et al., 1992, Kirouac and Ganguly, 1993, Levant et al., 1993, Hall et al., 1994, Kim et al., 2000). Both D1 and D2 binding sites were found mainly in the striatum (caudate nucleus, putamen, and nucleus accumbens), but also scattered in some areas of the cortex and olfactory bulbs. In addition, sites of dopamine synthesis in the brain have been identified using antibodies against TH (Jones and Beaudet, 1987, Lavoie et al., 1989). These sites have been found in the SNc/VTA of the midbrain.

Determining the distribution of dopamine receptors is of particular interest in the Mexican free-tailed bat, *Tadarida brasiliensis,* because a previous report from our lab showed the distribution of melatonin binding sites throughout the striatum where it would be hypothesized that dopamine binding sites should be found (Schwartz et al., 2009). Because of this interesting result, I wanted to explore the distribution of the common dopamine receptor types in the free-tailed bat brain to see if the melatonin binding site distribution closely resembles the distribution of either one or both dopamine receptor types. In this report, I use autoradiography to identify D1 and D2

dopamine binding sites in the *Tadarida brasiliensis* brain. I also use TH immunohistochemistry to find the areas of the brain involved in dopamine synthesis and dopamine β-hydroxylase (DβH) immunohistochemistry, which converts dopamine to norephinephrine, to further characterize my TH results.

Results

We established the positions of the regions of the striatum in the *Tadarida brasiliensis* brain in a previous paper (Schwartz et al., 2009). The positions of the SNc/VTA and parabrachial nucleus were inferred by cytoarchitectonic features as determined using cresyl violet and cytochrome oxidase stains, and the positions of these structures were similar to those described in rats (Paxinos and Watson, 1998). Several other neuroanatomical resources from other bat species also helped identify the brain regions reported in this paper (Prasada Rao and Kanwal, 2004, Maseko and Manger, 2007). The entire *Tadarida* brain, from the most rostral tip of the olfactory bulbs to the caudal-most extension of the brainstem nucleus ambiguus, was evaluated to determine where dopamine binding sites and TH and DβH IHC were present.

D1 and D2 receptors were found in the same areas of the *Tadarida* brain, the nucleus accumbens and the caudate putamen of the basal ganglia (Figure 19). Binding was higher in the caudate putamen (D1: 0.00131 ± 0.000193 μCi/g, n = 5; D2: 0.00159 ± 0.000249, n = 4) than in the nucleus accumbens, (D1: 0.000984 ± 0.000141 μCi/g, n = 5; D2: 0.00118 ± 0.000381, n = 4).

TH was found in the substantia nigra, ventral tegmental area, and parabrachial nucleus/locus coerulus (Figure 20). In the SNc I found 70.221 ± 18.119 cells/mm², in the VTA 151.415 ± 32.077 cells/mm², and in the PB/LC 447.954 ± 70.897 cells/mm².

DβH was found only in the parabrachial nucleus/locus coerulus, 641.842 ± 109.434 cells/mm² (Figure 21).

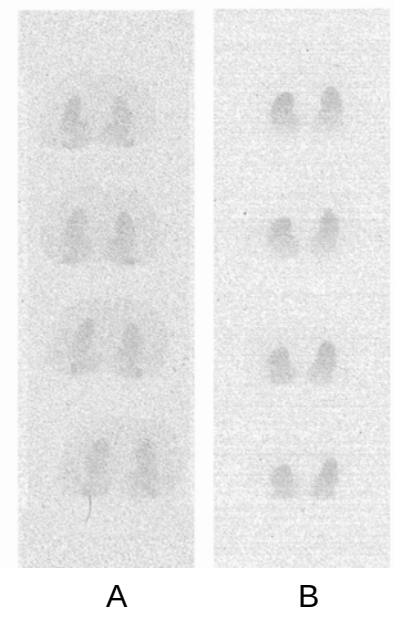

Figure 19. Autoradiographic Localization of D1 and D2 Dopamine Receptors in the Free-tailed Bat Brain. A shows a representative autoradiograph showing binding in the striatum in four adjacent brain sections incubated with ^{3}H SCH 23390, a D1 receptor antagonist. Likewise, B shows a representative autoradiograph showing binding in the striatum in four adjacent brain sections incubated with ^{3}H raclopride, a D2 receptor antagonist.

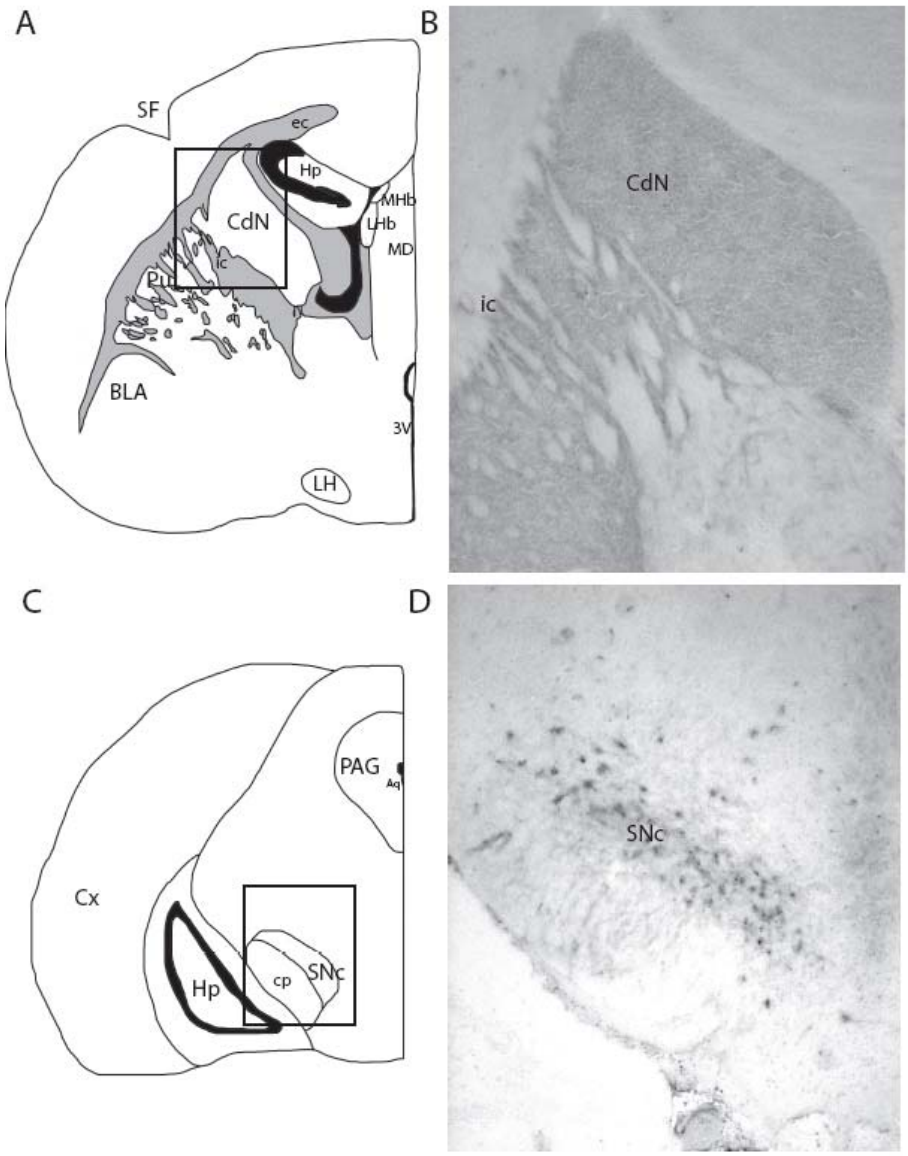

Figure 20. Tyrosine Hydroxylase Binding in the *Tadarida brasiliensis* Brain. This figure shows the results from tyrosine hydroxylase immunohistochemistry. A, C, E, and H are brain atlas drawings showing the location of the pictures directly following. B shows part of the striatum, where there are no cell bodies present with TH, but there are synapse terminals present which show up light gray. D shows TH binding in the substantia nigra pars compacta. Cell bodies with TH show up as black dots. F shows binding in the ventral tegmental area. H shows binding in the parabrachial nucleus and locus coeruleus. J provides a brain guide illustrating the positions of A, C, E, and H.
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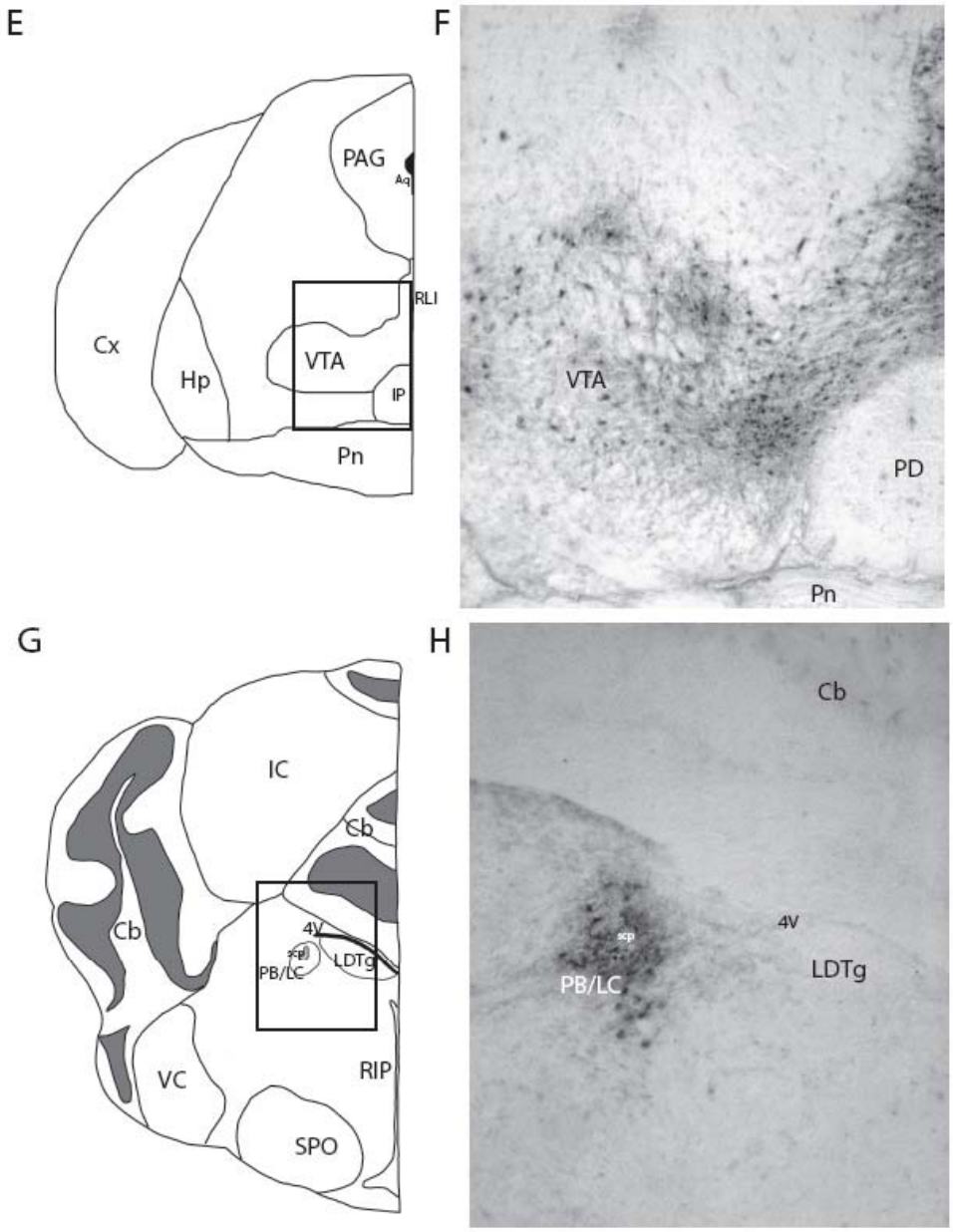

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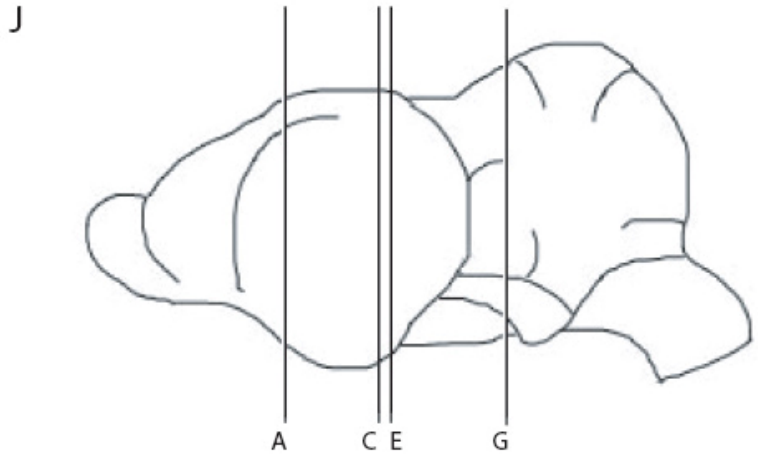

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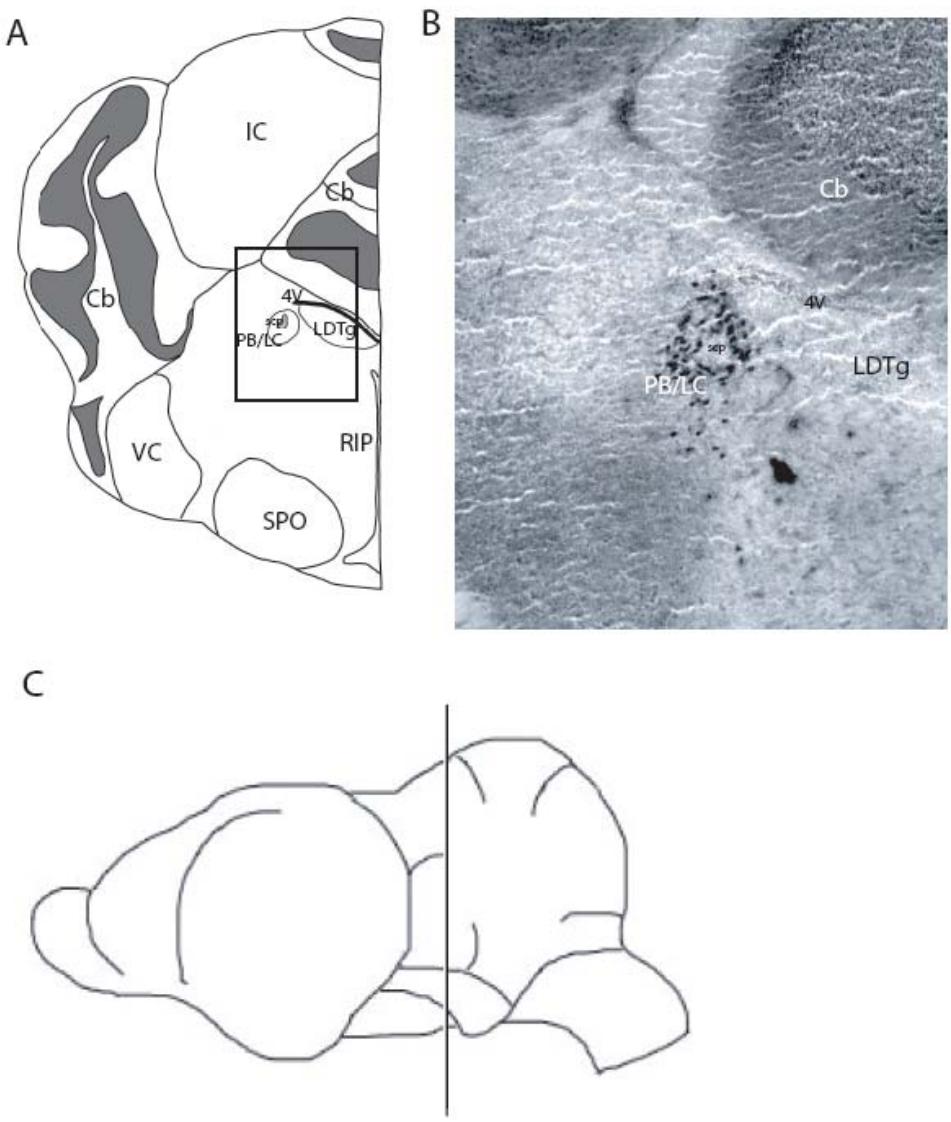

Figure 21. Dopamine-beta-hydroxylase Binding in the *Tadarida brasiliensis* Brain. This figure shows the results from dopamine-beta-hydroxylase immunohistochemistry. A gives a brain atlas drawing of the position of the picture in B, which shows DβH binding in the parabrachial nucleus and locus coeruleus. C provides a brain guide illustrating the position of A.

Discussion

In this study, both D1 and D2 receptors were found in the caudate putamen and nucleus accumbens, showing the exact same distribution as the melatonin binding sites in the previous section. This receptor distribution is also similar to those found in other species. Additionally, TH immunohistochemistry revealed immunoreactivity in the SNc/VTA, locus coerulus and parabrachial nucleus, while DβH immunohistochemistry was found in the locus coerulus and parabrachial nucleus, also similar to other species.

Localization of dopamine receptor sites

In the rat, dopamine receptors were found throughout the forebrain with autoradiography, with the highest levels in the caudate putamen, nucleus accumbens, and olfactory tubercle (Boyson et al., 1986). Lower levels were detected in areas of the cortex, amygdala, globus pallidus, and hippocampus, among others. There could be D1 and D2 receptors in other areas of the free-tailed bat brain which were too low to find with this technique. D1 receptor density was at least 3 fold higher than D2 receptor density in all areas in the rat, while in the bat, the two receptor types were approximately the same. Humans, monkeys, and cats have a similar distribution and D1/D2 ratio to that seen in the rat (Richfield et al., 1987, Camps et al., 1989, Lidow et al., 1991). It is suggested that this higher ratio was due to the fact that an antagonist was used for the D1 receptor type while an agonist was used for the D2 receptor type since dopamine antagonists bind both high and low affinity states of receptors while agonists only label the high affinity states (Camps et al., 1990). I used antagonists for both receptor types in

my experiments which could explain the nearly 1:1 ratio that I see in my data. Also, another analysis in humans using SCH-23390 and raclopride showed a similar ratio to the bat, suggesting that the radioligands used might be contributing to the resulting receptor densities (Hall et al., 1988).

Sites of dopamine synthesis

TH immunoreactivity was previously found in the same areas I found in the bat, the SNc and VTA in the rat and cat, known regions of dopamine synthesis (Jones and Beaudet, 1987, Lavoie et al., 1989). TH was also reported in the locus coerulus and parabrachial nucleus of the cat (Jones and Beaudet, 1987). My findings in the DβH IHC suggest that these hindbrain regions are not dopaminergic but catecholaminergic, but a double labeling study in cattle suggests that there are some dopaminergic cells in this region as well (Berod et al., 1982).

Interaction of dopamine and melatonin

Overall, both the D1 and D2 receptor distribution in the striatum matches the distribution of melatonin binding sites I found previously. This does not indicate that there is a colocalization of these receptors, but it does provide an interesting correlation suggesting some interaction between the two. Melatonin does appear to play a role in the striatum, particularly in relation to dopamine. Ionotophoretic injection of melatonin into the striatum attenuated excitatory responses of striatal neurons to somatosensory cortex stimulation (Escames et al., 1996). Similarly, melatonin application inhibited

spontaneously active caudate putamen neurons (Castillo-Romero et al., 1993). This is thought to occur by increasing the affinity of the D2 receptor (Hamdi, 1998). Melatonin is known to play a similar modulatory role in the retina and hypothalamus, inhibiting dopamine release (Dubocovich, 1983, Zisapel and Laudon, 1983). It is possible that melatonin also plays a role in the dopamine synthesis pathway. Melatonin injection in late afternoon increased TH activity in the caudate nucleus of hamsters, possibly due to a change in affinity of the enzyme (Alexiuk and Vriend, 2007). The injections also increased serotonin concentrations, suggesting that dopamine, melatonin, and serotonin are all interconnected. In addition to having a role as a modulator, melatonin has some protective properties, acting as an antioxidant. Melatonin prevented degeneration of neurons in the SNc in mice treated with 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) and rats treated with rotenone, both models of Parkinson's disease (Lin et al., 2008, Ma et al., 2009). This has led to the recommended use of melatonin as a therapeutic agent in treatment of Parkinson's disease symptoms (Mayo et al., 2005).

Conclusion

In conclusion, this characterization of the *Tadarida brasiliensis* dopamine system indicated that the free-tailed bats are not dissimilar from other mammals. However, this does raise some interesting questions about the interactions between dopamine and melatonin in the striatum, whether or not they also interact with serotonin, melatonin's role as an antioxidant, and the physiological significance of melatonin in the striatum. It is still unclear whether melatonin in the striatum or any of the other brain regions is

related to the seasonality of *Tadarida brasiliensis* vocal behavior, but this remains a distinct possibility. Melatonin binding sites are found in the bird song system (Gahr and Kosar, 1996, Whitfield-Rucker and Cassone, 1996, Bentley and Ball, 2000), so while I cannot concretely conclude anything from this study, it provides us with some target areas for the mammalian vocal motor pathway to explore further.

CHAPTER V

ECHOLOCATION-INDUCED NEURONAL ACTIVATION IN THE MEXICAN FREE-TAILED BAT BRAIN

Introduction

Despite a broad diversity in mammalian vocal behaviors, a limited number of brain structures are suspected of involvement in the production of innate mammalian vocalizations (Newman, 2010). Vocal production in mammals is initiated by activity in forebrain limbic structures, including the amygdala, hypothalamic nuclei and the anterior cingulate cortex, which in turn act through a midbrain gating mechanism in the periaqueductal gray (PAG) to coordinate activity in laryngeal and respiratory motoneuronal pools in the brainstem (Jürgens, 2002). Importantly however, this pathway (Figure 22) has been constructed almost entirely from lesion studies coupled with electrical and chemical microstimulation of the brain regions from which normal sounding vocalizations can be elicited. Few studies have described neuronal activity from the vocal control pathways of spontaneously vocalizing mammals (Larson and Kistler, 1986, Zealear and Larson, 1988, Farley et al., 1992a, Farley et al., 1992b, Dusterhoft et al., 2004), which leaves lingering questions about whether the neural circuits revealed by these methods accurately reflect the neural basis of spontaneous vocal communication in mammals.

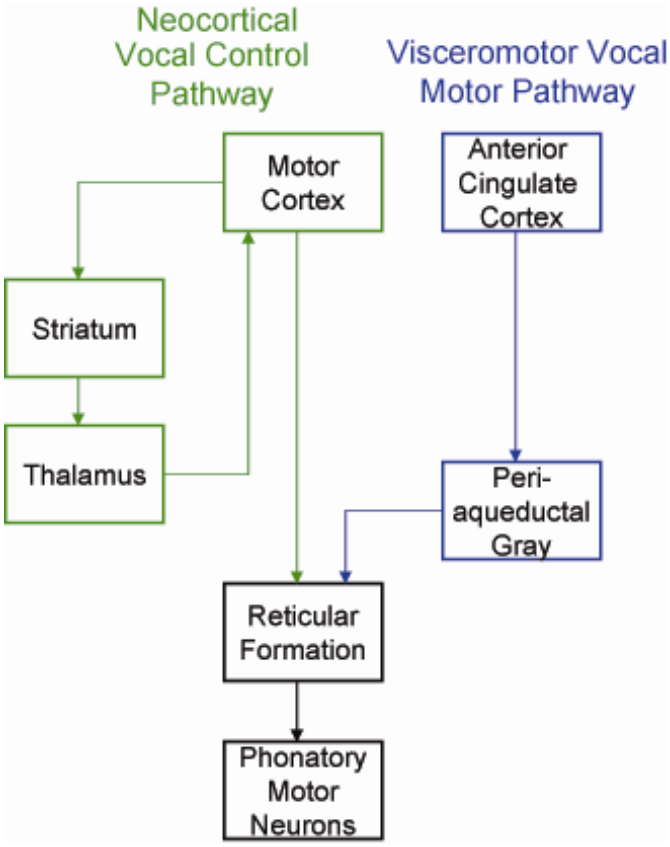

Figure 22. Mammalian Vocal Motor Pathway Model.

Compared to other mammals, humans appear to have a very different neural substrate for speech production, and while this may be justified by the extraordinary complexity of speech, current models of speech motor control are largely based on non-invasive functional imaging techniques in naturally behaving humans (Fox et al., 1996, Guenther, 2006, Tourville et al., 2008). Humans exhibit behavioral traits not common among mammals, including vocal learning, plasticity, and the use of syntax in vocal

communication. These vocal behaviors are dependent upon forebrain brain circuits that exist outside of the limbic-based visceromotor pathway that drives innate vocalizations, and the paucity of evidence for these types of vocal behaviors in non-human mammals may account for their perceived functional impotence in mammalian vocal control.

However, recent studies have revealed that mammalian vocal behaviors may be more complex than previously appreciated. Many mammals, including primates, cetaceans and bats, are now known to make sensory- and context-specific changes in the acoustic structure of their vocalizations (Foote et al., 2004, Madsen et al., 2005, Scheifele et al., 2005, Egnor and Hauser, 2006, Egnor et al., 2007, Smotherman, 2007, Versace et al., 2008), are capable of vocal learning (Weilgart and Whitehead, 1997, Boughman, 1998, Tyack and Clark, 2000, Knörnschild et al., 2006, Knornschild et al., 2010), and can incorporate multiple syllables into complex syntactical vocal patterns (Payne and McVay, 1971, Mitani and Marler, 1989, Weilgart and Whitehead, 1997, Behr and von Helversen, 2004, Rendell and Whitehead, 2005, Behr et al., 2006, Bohn et al., 2008, Bohn et al., 2009). These vocal behaviors would be supported by a model that incorporates forebrain components of the human speech model as modulatory elements in the vocal motor pathway, as predicted by Jürgens (2009). Such a shift in thinking would be promoted by any evidence of neuronal activity in neocortical or basal ganglia structures analogous to those associated with vocal production in humans in a non-human mammal model exhibiting one or more of the sophisticated vocal behaviors listed above.

Echolocating bats possess an extraordinary ability to precisely modulate the sound of their voice to accommodate changes in environmental acoustic conditions and behavioral context (Kalko and Schnitzler, 1998, Ulanovsky et al., 2004, Gillam et al., 2007, Schwartz et al., 2007, Smotherman, 2007, Tressler and Smotherman, 2009, Jarvis et al., 2010). To test the hypothesis that there are other areas of the brain involved in spontaneous vocal behaviors that were not exposed by microstimulation methods, I compared the expression levels of the immediate early gene c-fos in 15 neuroanatomical regions of interest (ROIs) in spontaneously vocalizing versus silent and listening free-tailed bats (*Tadarida brasiliensis*). The cellular expression of c-fos has been shown to be a reliable indicator of neuronal activation (Chaudhuri, 1997, Chaudhuri et al., 2000, Sundquist and Nisenbaum, 2005). Previous studies have used c-fos immunohistochemistry to map the vocal centers in electrically-stimulated tamarins (Jürgens et al., 1996), and a similar study used fluorodeoxyglucose autoradiography to achieve similar results in electrically-stimulated vocalizing rats (Gonzalez-Lima, 2010). To date however, no studies have mapped c-fos expression in a spontaneously vocalizing mammal. My results are constrained by the fact that the animals had to be allowed a modest amount of freedom to move or they would not vocalize, but the results presented here provide clear answers to two key questions: firstly, are all the brain areas identified as vocal control centers by microstimulation studies actually active during spontaneous vocalizing, and secondly, are there any additional brain regions active in spontaneously vocalizing animals that might account for the vocal behaviors not explained by the

current model of mammalian vocal motor control. If so, these brain regions would become important targets of future studies of vocal control in mammals.

Experimental Groups and Data Analysis

Behavior

Three different behavioral groups were used for this experiment: 1) bats that were actively echolocating in a quiet environment, 2) silent bats in a quiet environment, and 3) bats listening to artificial acoustic stimuli while remaining silent. All bats underwent the same handling procedures leading up to the experimental trial; however previous studies have shown that there were significant differences in behavior between bats and within the same bat across trials. Rather than try to force bats to either vocalize or remain silent, bats were placed in the test cage and positioned in the center of the recording chamber and their vocal behavior was quantified in real time for the 30 minute trial. An actively echolocating free-tailed bat may emit up to 5 or 6 calls per second, averaging up to 300 calls per minute for 10 to 15 minutes at a time. It is unusual for a stationary bat to maintain this call rate continuously for 30 minutes, but it is not unusual for an actively calling bat to discontinuously emit between 3000 and 6000 total calls within a 30 minute experimental trial. If at the end of 30 minutes a bat had emitted more than 3000 echolocation calls it was classified as an “echolocating bat”. Alternatively, some bats sit quietly in the test cage for the 30 minute trial. These bats may only emit calls sporadically at rates averaging less than 10 per minute. If at the end of the 30 minute trial a bat had emitted less than 300 total calls, the bat was classified as a “silent

bat". Consequently, all of the bats in the silent group emitted no calls during the 30 minute trial. If a bat emitted between 300 and 3000 calls, it wasn't classified as either silent or echolocating and the bat was returned to the colony. At the end of each 30 minute experimental trial the bat's echolocation performance was assessed and a decision was made whether or not to sacrifice the bat for immunohistochemistry.

Despite individual difference in vocal behavior, there were a few simple things I could do to either encourage or inhibit vocalizing. For example, bats placed in a novel environment will echolocate in order to explore their surroundings. For the echolocation group, I took advantage of this in the following way: The cage containing the echolocating bat was placed in the center of the recording chamber and then surrounded by a tightly-fitting isolation box lined with acoustic foam two hours before the experiment. This greatly reduced the number of echolocation pulses uttered to near zero during the 2-hour time period preceding the actual experimental trial. Keeping the bat in a quiet, isolated environment limits outside stimulation to ensure maximal gene expression during the experiment (Chaudhuri, 1997). At the beginning of the experiment, the isolation box was removed, which effectively encouraged some bats to echolocate and explore the suddenly larger environment beyond their cage during the experiment trial period. The recording chamber was a novel environment to the bats, which also helped to encourage echolocation. Repeated exposure to the recording chamber greatly diminished subsequent call rates in this species. For the echolocating bats included in this study, mean call rates ranged from 0.686 calls/second to 4.5 calls/second and the time spent moving ranged from 31.8% to 49%. The bats in the

echolocation group exclusively used echolocation calls; any data from a bat that emitted social calls was not used. All the bat's calls were recorded, and the files were manually checked for any calls that were not echolocation calls (Jarvis et al., 2010). The animals called for 30 minutes and were kept an additional 30 minutes in isolation before being sacrificed. Subjects differed in call rate, temporal patterning of calls, and other parameters, but I only used animals that called continuously over the 30 minute period (no silent gaps >5 minutes). C-fos protein is detectable as early as 30 minutes after the onset of stimulation, in this case, the point when the bat started to call (Chaudhuri et al., 2000).

Alternatively, to increase the probability of a bat remaining silent for the trial period, bats were placed in the center of the recording chamber but not in an isolation box, and left alone for at least two hours before the experimental trial: once the bats became acclimated to the recording chamber their call rates were greatly reduced simply from the boredom of being in the recording chamber. All vocalizations were recorded during the two hour period preceding the trial to be sure that the bats were sufficiently quiet to ensure maximal gene expression, and video monitoring in real time assured that the silent bats hadn't fallen asleep, but were awake and aware of their surroundings. In general, head and ear movements as well as conspicuous grooming behaviors were taken as evidence that the bats were awake while silent. This group received no stimulus and did not call and provided the baseline data for the experiments.

The listening group contained silent bats that listened to computer-generated echolocation calls without calling themselves. I used the same approach as for the silent

bats, by allowing them to become accustomed to (or bored with) their environment so that they called less during the experiments. None of the bats vocalized in response to a playback, so this was not an issue. After the 30 minute listening phase, the animals were kept for another 30 minutes in silence before being sacrificed. All vocalizations were monitored for the entire experiment. Any animal that called was not used.

Data analysis

We came up with 15 regions of interest (ROIs) to analyze for FLI in the bat brain, based on current and hypothesized models of non-human vocal motor control and accompanying mammalian vocalization literature (Figure 22, Table 2). Additionally, I analyzed one auditory region. The anterior cingulate cortex (ACg), PAG, parabrachial nucleus (PB), amygdala, and the hypothalamic nuclei were chosen because electrical stimulation of these regions results in vocalization (Jürgens and Ploog, 1970, Gooler and O'Neill, 1987). Also, the ACg, PAG, and PB are part of the current vocal motor pathway. The amygdala, ACg, hypothalamic nuclei, lateral habenula, PAG, and raphe nuclei were chosen because they resulted in increased FLI from electrically stimulated vocalizing monkeys (Jürgens et al., 1996). Nucleus accumbens, ACg, amygdala, the hypothalamic nuclei, substantia nigra pars compacta, and raphe nuclei were shown to be afferents of vocal regions of the PAG (Dujardin and Jurgens, 2005). The ACg, caudate nucleus, putamen, mediodorsal thalamic nucleus, and PB are all recipients of projections from the larynx area of the motor cortex (Simonyan and Jurgens, 2002, 2003). Additionally, the basal ganglia and thalamus are important components of the human

speech and birdsong vocal models and form the basis for the hypothesized motor cortical vocal control pathway (Jürgens, 2009).

Locations of the caudate nucleus, putamen, nucleus accumbens, substantia nigra and parabrachial nucleus were found using the resulting sections from the TH antibody protocol (Figure 20). The ACg was considered to be the portion of the cingulate cortex rostral to the anterior commissure (Gooler and O'Neill, 1987). The remaining structures (Table 2) were found using our lab generated brain atlas and published bat brain resources from other labs (Prasada Rao and Kanwal, 2004, Maseko and Manger, 2007, Schwartz et al., 2009). They were also verified with a comparison to mice and rats (Paxinos and Watson, 1998, Franklin and Paxinos, 2008). The bat brain is very similar in structure and size to the mouse brain.

Pictures of the extent of each brain area were taken using an Infinity 2 microscope camera connected to a computer running Infinity Capture application software (version 3.7.5, Lumenera Corporation). Consecutive sections from both the right and left sides for each ROI were analyzed for each animal. The FLI (5-40 pixels in size) was counted using NIH Image J (Abramoff et al., 2004). Cells were counted in random 0.012 x 0.012 mm square areas over each ROI (Beckett et al., 1997, Neophytou et al., 2000, Sadananda et al., 2008). Data from the right and left sides for bilateral structures were quantified separately at first to assess any lateralized activation, but I did not find any ROI with differences in activation on one side, so the right and left sides were combined and the mean for each ROI for each animal was calculated. The animals were then divided into their respective treatment groups and cell counts were compared

between groups of bats using an analysis of variance (ANOVA) with treatment as the factor (echolocating, listening, or silent). The comparisons of interest were echolocating vs. listening, echolocating vs. silent, and listening vs. silent. All comparisons were analyzed using the Holm-Sidak method.

In order to determine if movement had an effect on the FLI in echolocating bats, I performed a linear regression analysis on the FLI for each brain region versus time spent moving. I also looked at the effect of call rate using the same method. Additionally, I also performed a linear regression analysis on a larger group of bats (N=21) to ascertain the relationship between movement and call rate.

Results

C-fos western blotting experiment

The results from the c-fos western blot showed a band at approximately 42 kDa that was blocked with the c-fos peptide (Figure 23). This result is consistent with the predicted molecular weight (Abcam).

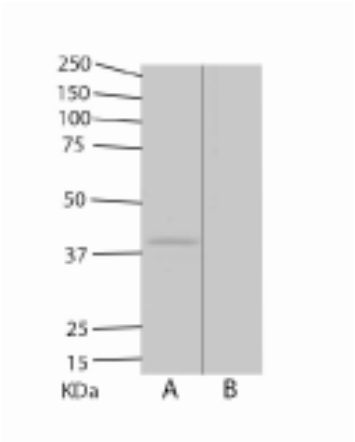

Figure 23. C-fos Western Blot. This picture shows the results of the c-fos western blotting protocol. In A, a band corresponding to 42 kDa indicating that the antibody used is binding to the correct protein. In B, a c-fos blocking peptide was used to block specific binding, so no band is present.

C-fos immunohistochemistry on echolocating, listening, and silent bats

We analyzed 15 ROIs in the forebrain, midbrain, and hindbrain of *Tadarida brasiliensis* (Table 2).

In the forebrain, I analyzed the anterior cingulate cortex (ACg), basolateral amygdala (BLA), striatum of the basal ganglia, paraventricular (PA) and lateral (LH) hypothalamic nuclei, lateral habenula (LHb), and mediodorsal thalamic nucleus (MD). The striatum was divided into five ROIs, the nucleus accumbens (Acb), caudate putamen (CPu), dorsolateral caudate nucleus (dlCdN), ventromedial caudate nucleus (vmCdN), and putamen (Pu) (Figure 24). The CPu was considered to be the rostral joined portion of the CdN and the Pu prior to its bisection by the internal capsule. The CdN, categorized as the caudal portion of the former CPu dorsal to the internal capsule, was

divided into two equal parts based on location, the dlCdN and the vmCdN. The Pu, the caudal portion of the former CPu ventral to the internal capsule, was analyzed as one structure. The ACg, BLA, PA, LH, LHb, and MD were also not divided any further.

In the midbrain, I analyzed the substantia nigra pars compacta (SNc) of the basal ganglia and PAG. The SNc was analyzed as one structure. The PAG was divided into four areas: dorsal (dPAG), ventral (vPAG), left central (lPAG), and right central (rPAG). Each of these four PAG areas was then separated on the rostral-caudal axis into four bins for separate analysis.

In the hindbrain, I analyzed the median (MRn) and dorsal raphe (DR) nuclei, and PB for c-fos immunoreactivity. None of the hindbrain areas were divided into substructures.

In my analysis, I quantified the cell counts of each ROI for each of the three experimental groups (Table 2). One echolocating bat and one silent bat only provided data for some of the brain regions because of cryosectioning errors that resulted in the loss of some brain sections. Overall, I found significantly higher cell counts in the ACg, dlCdN, PA, LH, LHb, SNc, all PAG regions, MRn, DR, and PB in echolocating bats when compared to listening and silent bats (Table 2, Figures 24-27). Cell counts in the MD were significantly higher in both echolocating and listening bats when compared to silent bats. No significant differences between groups were found in Acb, CPu, vmCdN, Pu, or BLA. An atlas representation of the cell locations for each ROI is provided in Figures 24, 25, 26 and 27, showing in particular that the cells in both the dlCdN and SNc were found in very discrete areas of these structures.

Table 2. FLI Counts for Echolocating, Listening, and Silent Bats. Values are ± SD (n). The counts provided for each region are the average number of stained cells per analyzed area (0.12 x 0.12 mm²). The letters below the FLI values indicate significance, if a letter is different from another letter in the same row, those values are significantly different (A is significantly different from B). P values are given in the last column.

	Echolocating	Listening	Silent	P
Acg	4.354 ± 0.887 (8) A	0.840 ± 0.456 (5) B	0.300 ± 0.151 (8) B	<0.001
BLA	0.250 ± 0.177 (8) A	0.160 ± 0.055 (5) A	0.138 ± 0.074 (8) A	0.164
Acb	0.338 ± 0.292 (8) A	0.387 ± 0.189 (5) A	0.186 ± 0.090 (7) A	0.250
CPu	0.487 ± 0.285 (8) A	0.380 ± 0.164 (5) A	0.214 ± 0.157 (7) A	0.083
dlCdN	8.677 ± 3.246 (8) A	1.183 ± 0.346 (5) A	1.240 ± 0.283 (8) A	<0.001
vmCdN	0.912 ± 1.147 (8) A	0.020 ± 0.045 (5) A	0.488 ± 0.304 (8) A	0.133
Pu	0.138 ± 0.130 (8) A	0.124 ± 0.089 (5) A	0.139 ± 0.118 (8) A	0.974
PA	7.417 ± 3.607 (8) A	2.567 ± 1.610 (5) B	3.083 ± 3.150 (8) B	0.015
LH	3.979 ± 0.998 (8) A	0.967 ± 0.380 (5) B	1.000 ± 0.408 (8) B	<0.001
LHb	7.438 ± 2.527 (8) A	1.540 ± 1.004 (5) B	2.275 ± 1.094 (8) B	<0.001
MD	8.188 ± 2.302 (8) A	6.280 ± 1.431 (5) A	3.612 ± 1.336 (8) B	<0.001
SNc	7.571 ± 2.290 (7) A	1.740 ± 0.573 (5) B	1.163 ± 0.550 (8) B	<0.001

Table 2 cont.

	Echolocating	Listening	Silent	P
dPAG	10.332 ± 2.593 (8) A	3.026 ± 1.352 (5) B	2.245 ± 0.921 (8) B	<0.001
1	7.425 ± 3.986 A	2.160 ± 1.941 B	1.875 ± 1.666 B	<0.001
2	10.479 ± 3.572 A	3.667 ± 1.312 B	2.271 ± 1.188 B	<0.001
3	10.771 ± 2.577 A	2.633 ± 1.325 B	2.250 ± 1.330 B	<0.001
4	12.167 ± 3.234 A	3.500 ± 1.532 B	2.521 ± 1.364 B	<0.001
lPAG	6.451 ± 2.113 (8) A	1.904 ± 0.528 (5) B	1.158 ± 0.354 (8) B	<0.001
1	8.275 ± 3.061 A	2.040 ± 1.187 B	1.900 ± 0.793 B	<0.001
2	5.813 ± 2.498 A	1.700 ± 0.681 B	0.646 ± 0.663 B	<0.001
3	6.583 ± 2.136 A	1.733 ± 0.279 B	0.833 ± 0.684 B	<0.001
4	5.438 ± 2.121 A	2.167 ± 0.645 B	1.375 ± 0.635 B	<0.001
rPAG	6.924 ± 1.943 (8) A	2.122 ± 1.010 (5) B	1.477 ± 0.896 (8) B	<0.001
1	9.325 ± 2.496 A	2.360 ± 1.322 B	2.275 ± 1.126 B	<0.001
2	5.771 ± 2.870 A	1.867 ± 0.877 B	0.616 ± 0.322 B	<0.001
3	7.229 ± 3.094 A	2.000 ± 1.505 B	0.875 ± 0.659 B	<0.001
4	5.771 ± 2.415 A	2.300 ± 1.181 B	1.458 ± 0.689 B	<0.001
vPAG	4.479 ± 1.986 (8) A	1.367 ± 1.008 (5) B	0.323 ± 0.269 (8) B	<0.001
1	2.225 ± 1.202 A	0.800 ± 0.548 B	0.125 ± 0.149 B	<0.001
2	5.979 ± 2.837 A	1.733 ± 1.593 B	0.396 ± 0.408 B	<0.001
DR	8.091 ± 2.569 (8) A	2.363 ± 0.553 (5) B	1.511 ± 0.937 (8) B	<0.001
MnR	7.250 ± 1.900 (8) A	1.280 ± 0.179 (5) B	1.543 ± 1.176 (7) B	<0.001

Table 2 cont.

	Echolocating	Listening	Silent	P
PB m l	7.119 ± 1.904 (7) A 5.690 ± 3.056(7) A 3.286 ± 2.277 (7) B	1.833 ± 1.381 (4) B	0.500 ± 0.385 (7) B	<0.001
LL	6.333 ± 2.594 (8) A	5.867 ± 1.609 (5) A	1.542 ± 0.434 (8) B	<0.001

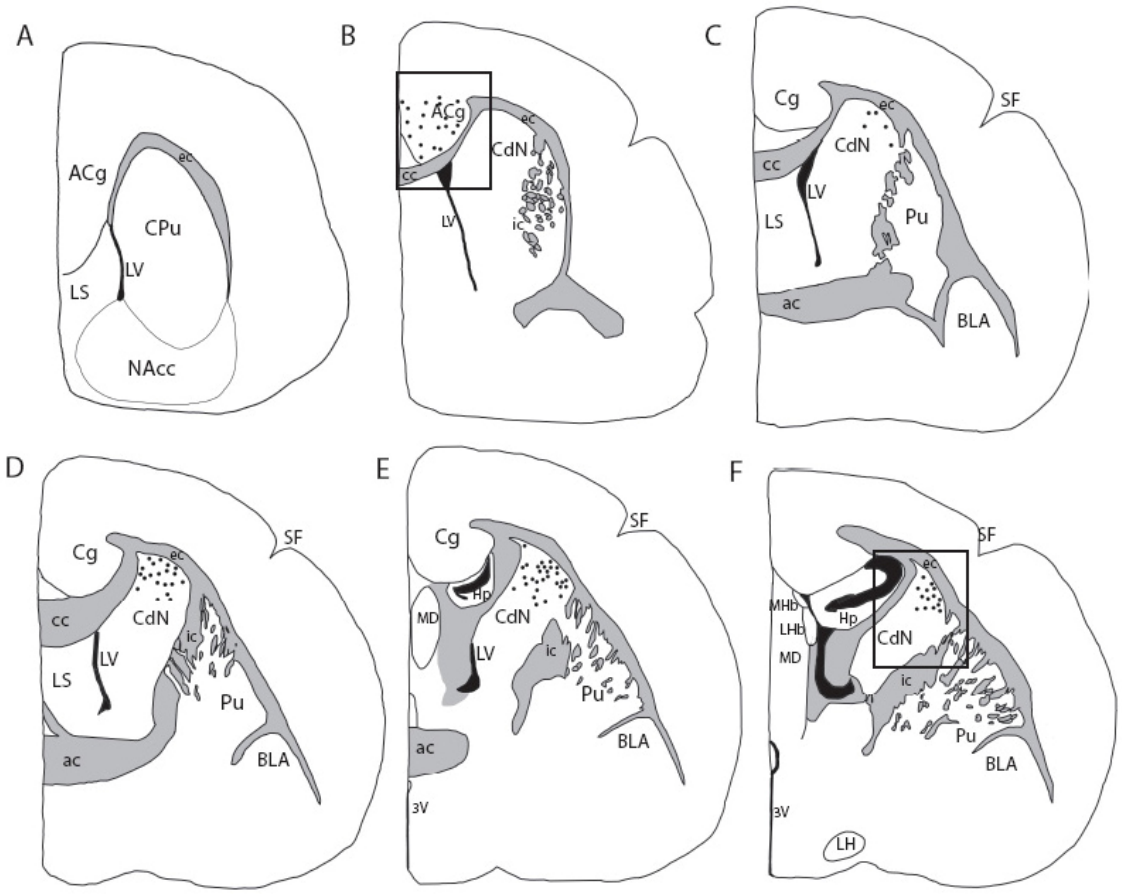

Figure 24. C-fos Binding in Frontal Cortex and Basal Ganglia. A-G are brain atlas drawings showing the positions of the different parts of the cortex and striatum that were analyzed. In addition, B-G show the progression of c-fos binding in the dorsolateral caudate nucleus. H provides a brain guide illustrating the location of the brain atlas drawings. I is a picture of c-fos binding in the anterior cingulate cortex (position shown by the box in B). J is a picture of c-fos binding in the dorsolateral caudate nucleus (position shown by the box in F).

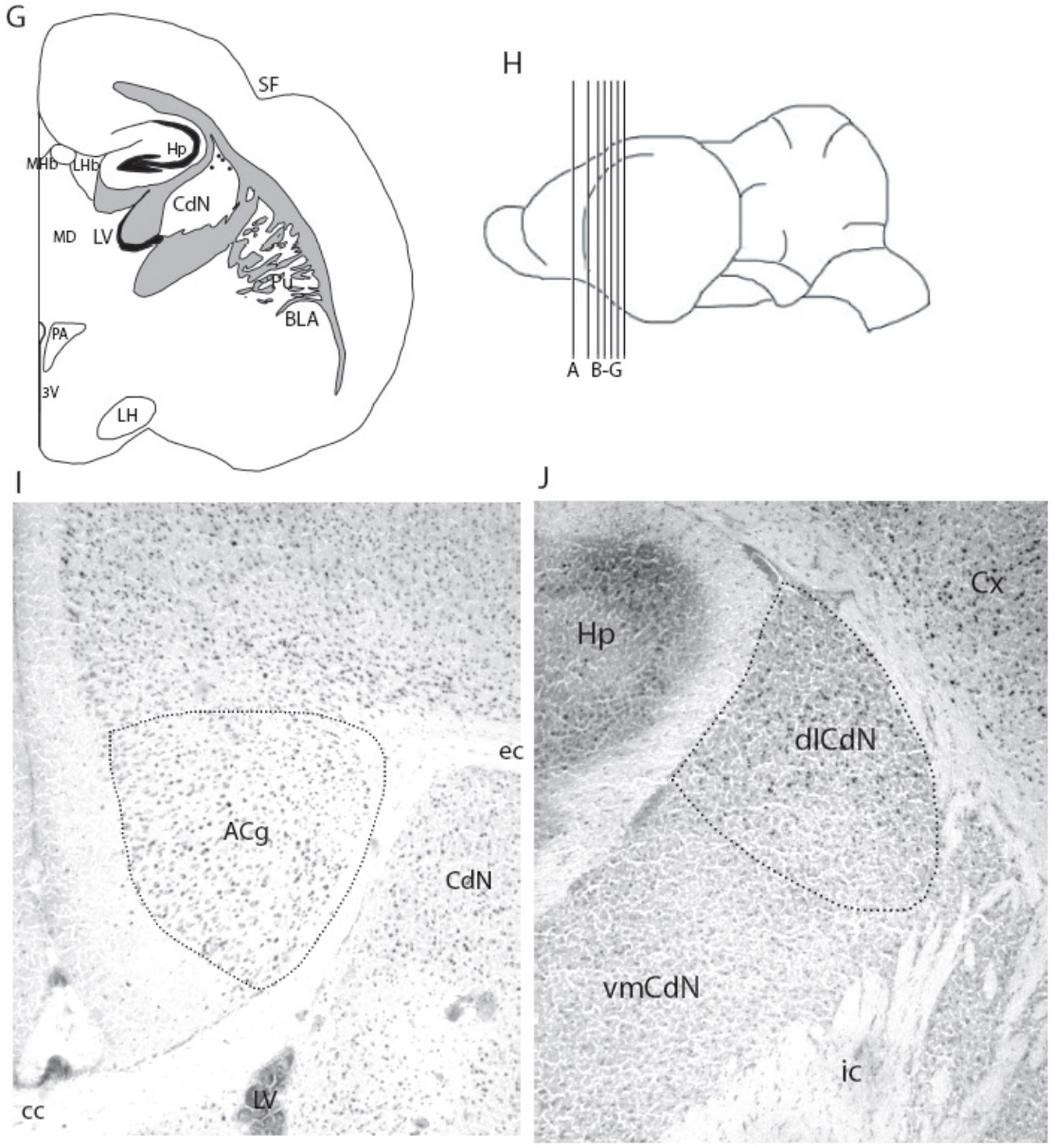

Figure 24 cont.

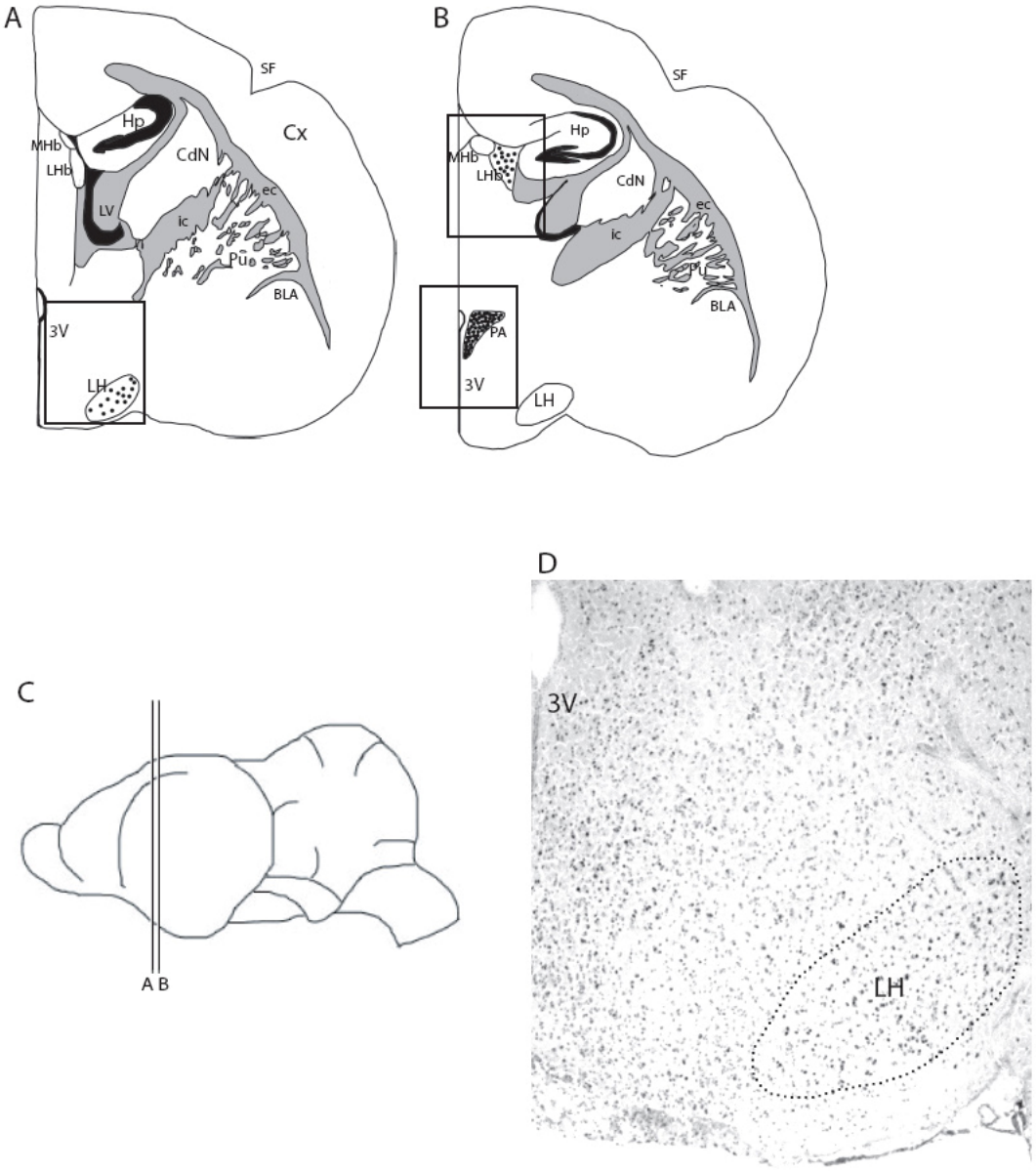

Figure 25. C-fos Binding in Epithalamus and Hypothalamus. A and B are brain atlas drawings showing the positions of the pictures of c-fos binding in D-F. C provides a brain guide illustrating the locations of A and B. D shows c-fos binding in the lateral hypothalamus (position shown by the box in A). E shows c-fos binding in the lateral habenula (position shown by upper box in B). F shows c-fos binding in the paraventricular hypothalamic nucleus (position shown by the lower box in B).

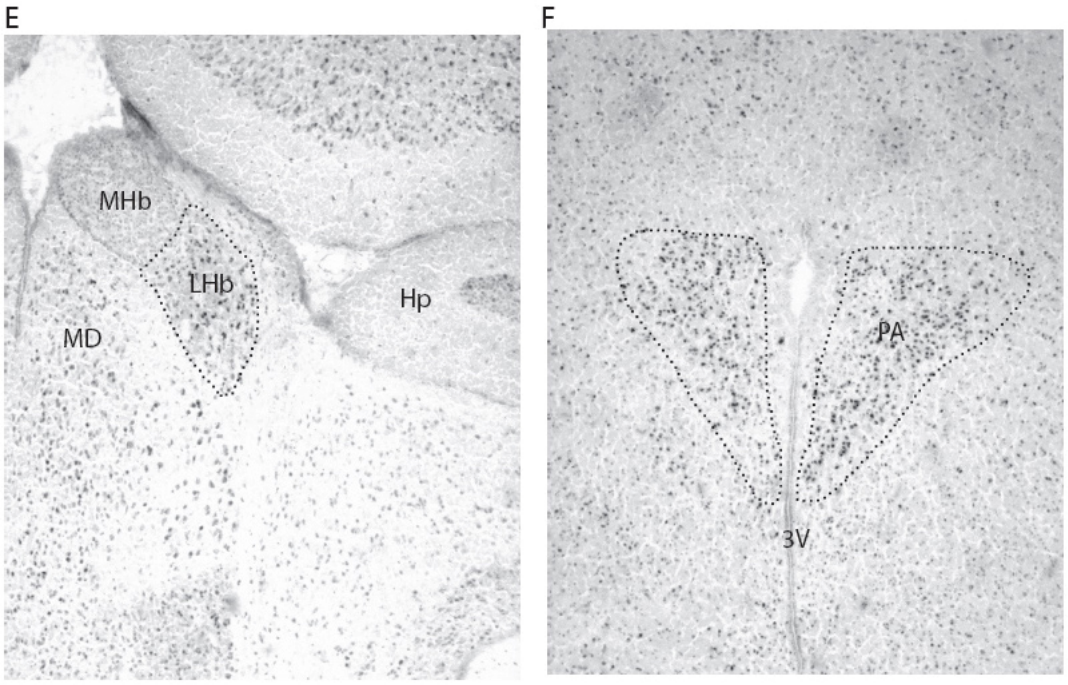

Figure 25 cont.

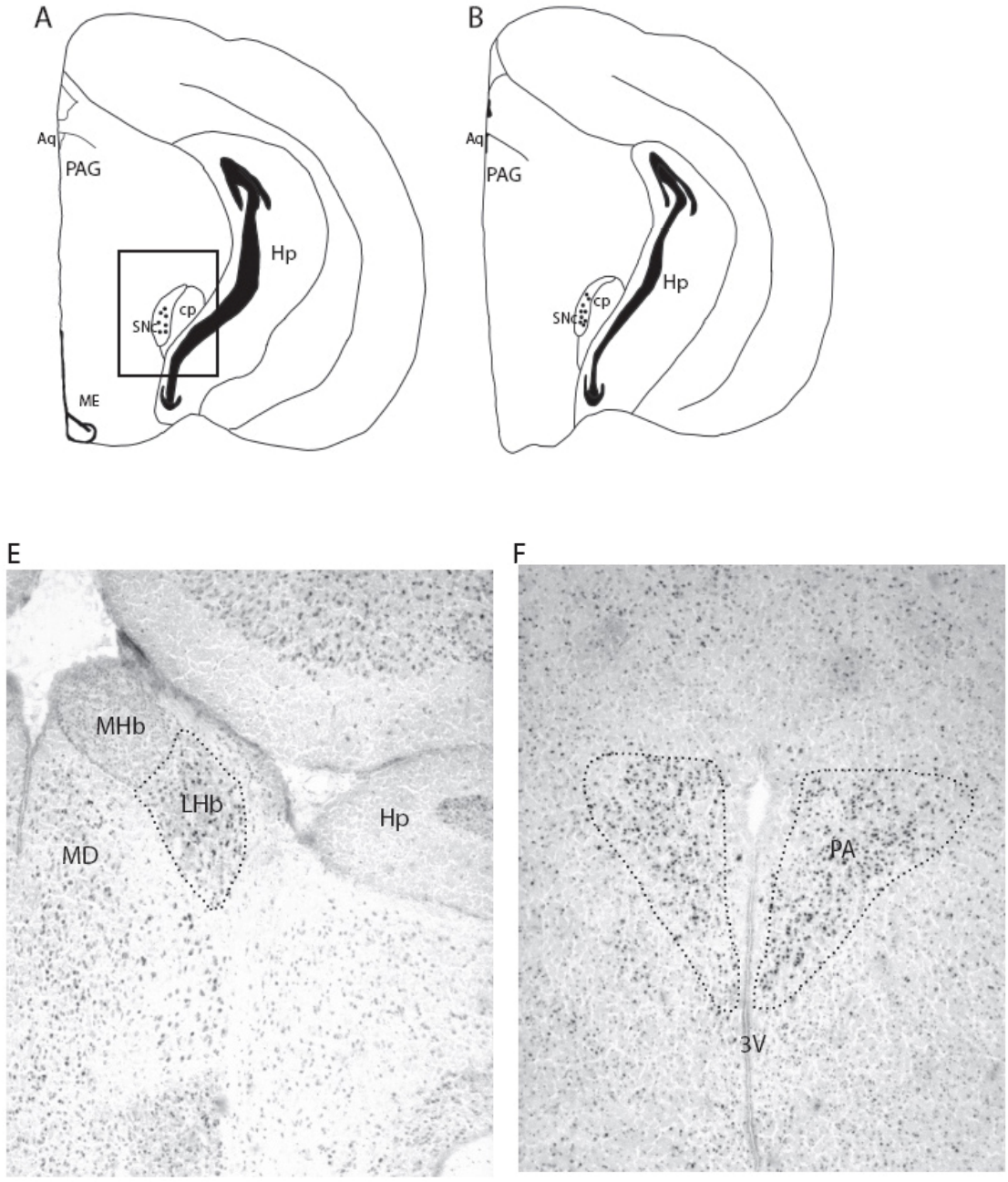

Figure 26. C-fos Binding in the Midbrain. A-C are brain atlas drawings showing the positions of the pictures of c-fos binding in D-E. Additionally A and B show the progression of c-fos binding in the substantia nigra pars compacta. D provides a brain guide illustrating the locations of A-C.A picture of the binding in the SNc is shown in E (the position is shown by the box in A). F shows c-fos binding in the PAG (position shown by the box in C).

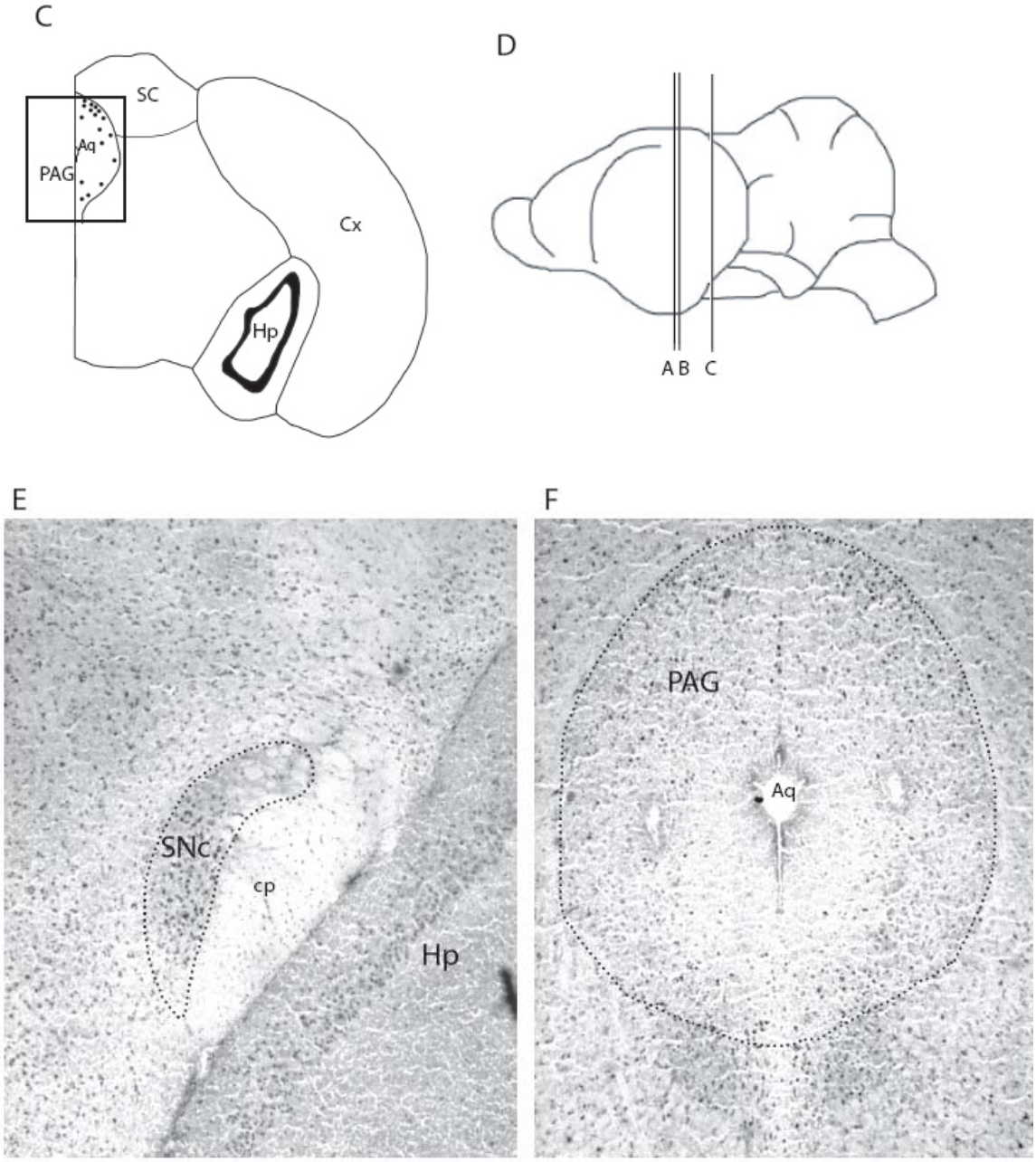

Figure 26 cont.

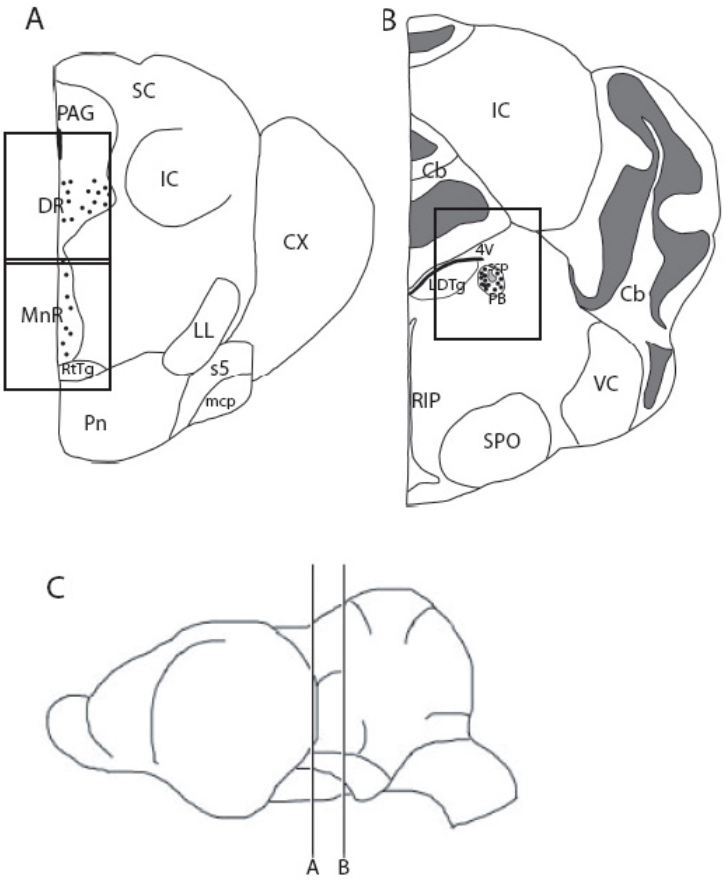

Figure 27. C-fos Binding in the Hindbrain. A and B are brain atlas drawings showing the positions of the pictures of c-fos binding in D-F. C provides a brain guide illustrating the locations of A and B. D is a picture of c-fos binding in the dorsal raphe nucleus (the position is shown by the upper box in A). E shows c-fos binding in the median raphe nucleus (position shown by lower box in A). F shows c-fos binding in the parabrachial nucleus (position shown by the box in B).

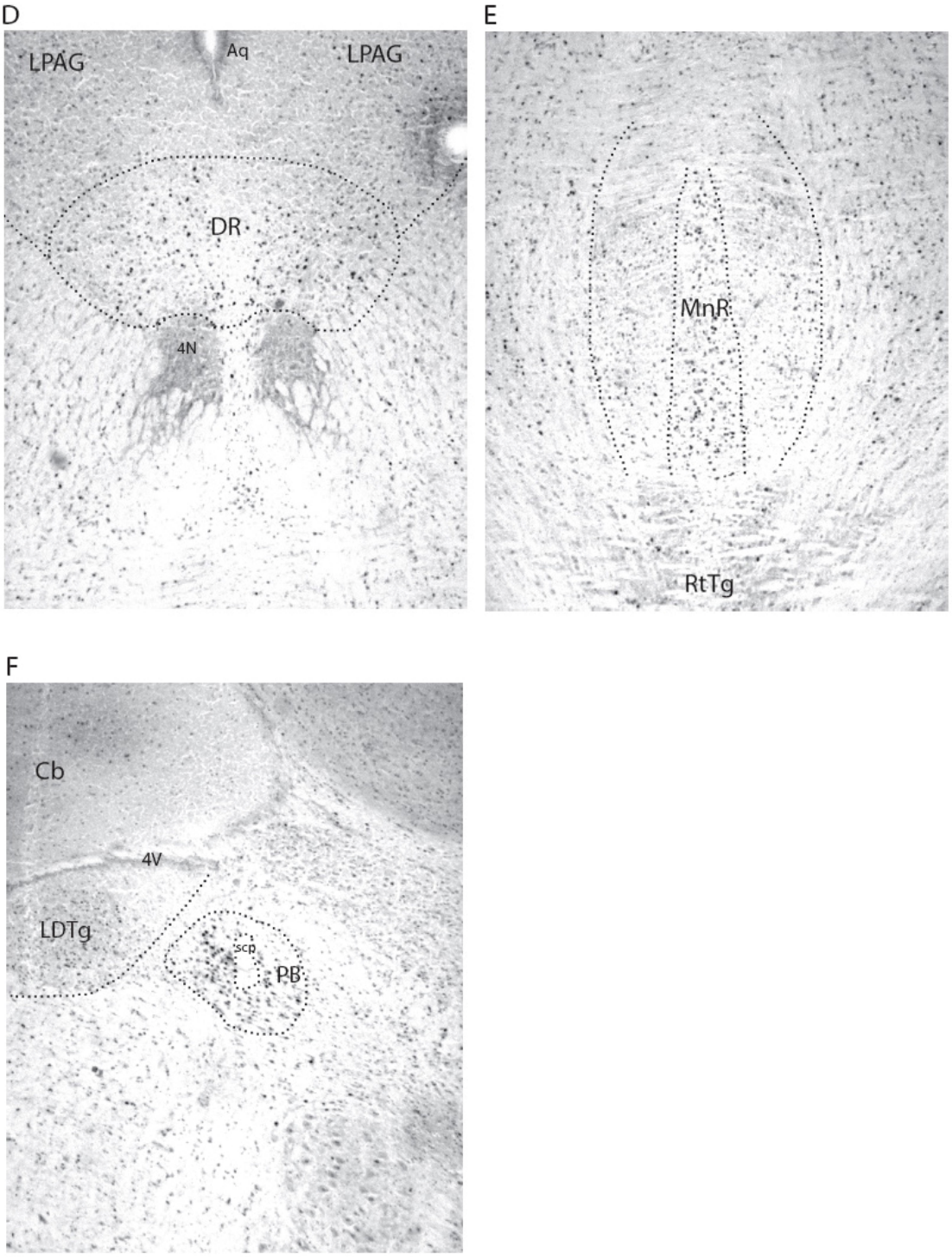

Figure 27 cont.

In the PAG, I attempted to pinpoint any areas with particularly high FLI by separating this relatively large structure into pieces on both the dorsal-ventral axis and the rostral-caudal axis. However, all of these sections of the PAG in the echolocating group had significantly higher FLI than both the listening and silent bats (Table 2, Figure 26). But there were some insignificant differences in the number of cells between some of the PAG regions. The areas of the PAG that had the highest cell counts were dPAG bins 4, 3, and 2 and both lPAG and rPAG bin 1. The collective dPAG had the highest FLI out of all ROIs analyzed, while the collective vPAG had one of the lowest.

Similarly, in the PB, since FLI in echolocating bats was significantly higher than the silent and listening bats (Table 2, Figure 27), I analyzed the medial and lateral PB separately to see if one region had more FLI. The medial PB had significantly higher FLI than the lateral PB. The FLI counts of the lateral PB by itself were not significantly different from those of the listening bats. Some of this PB data was published previously (Smotherman et al., 2009).

In the hypothalamus, I analyzed PA and LH (Table 2, Figure 25). In the PA, there was a lot of variation between groups. Although the echolocating group ended up having significantly higher FLI than the other two groups, there were animals in the listening and silent groups that had cell counts in the range found in the echolocating group, suggesting that activity in this area might not be attributable to echolocation only. However, this is not the case for the LH. This region had the lowest FLI count for all ROIs analyzed, but all bats were very consistent according to their treatment.

Two ROIs in the basal ganglia resulted in higher FLI in echolocating bats. The FLI in the forebrain dlCdN shows up in a very discrete area of the caudate nucleus (Figure 24). In general, the caudate nucleus area is encompassed by the external capsule on the dorsal side, internal capsule laterally, and corpus callosum medially. The dlCdN is the upper half of this structure. Where the FLI begins, the cells are scattered throughout the dlCdN, but more concentrated medially, where the structure comes to a point between the external capsule and the corpus callosum. As the structure progresses back caudally, the hippocampus appears on the medial side, the dlCdN gradually diminishes in size, and the FLI becomes even more concentrated in that medial point. FLI in the dlCdN was observed bilaterally. Interestingly, FLI was not significant in the vmCdN.

The second basal ganglia region with higher FLI in echolocating bats is the midbrain SNc. The SNc is located on the medial side of the substantia nigra pars reticulata, which is just medial to the hippocampus. Again, FLI in this ROI shows up in a discrete area (Figure 26). FLI is absent in the dorsal tip of this structure and is more concentrated in the center and ventral portions.

The thalamic MD was the only ROI significantly different from the silent group in both echolocating and listening bats. This area is directly dorsal and medial to the LHb.

The remaining ROIs with significant FLI, ACg, LHb, MRn, and DR, all had a consistent distribution of FLI throughout the entire structure.

In addition to the established 15 ROIs potentially involved in vocalization, I also analyzed one auditory structure, the lateral lemniscus (LL), as a control. I found that FLI in the LL was significantly higher in both vocalizing and listening bats when compared to silent bats.

Effect of call rate and movement on cell counts

We also analyzed call rate (calls/second) and time spent moving (%) in relation to cell counts within the ROIs that had significantly higher FLI only in echolocating bats to determine if higher call rates or more movement produced higher cell counts. I found no positive correlation between call rate or time spent moving and cell counts. However, only animals in the echolocating group had cell counts significantly higher in these regions than silent and listening bats, so there is clearly an effect associated with this group. This indicates that the cell counts were most likely correlated with some other parameter, including but not limited to temporal pattern of calls, frequency, or call variability. To determine the effect of movement on call rate, I used data from a larger pool of bats (n=21) and found that movement and call rate were weakly correlated (R^2=0.3788), meaning that a bat is more likely to call while it is moving (Figure 28). However, this weak relationship still does not account for the majority of vocalization, it only illustrates that in echolocation vocalization and movement are loosely linked.

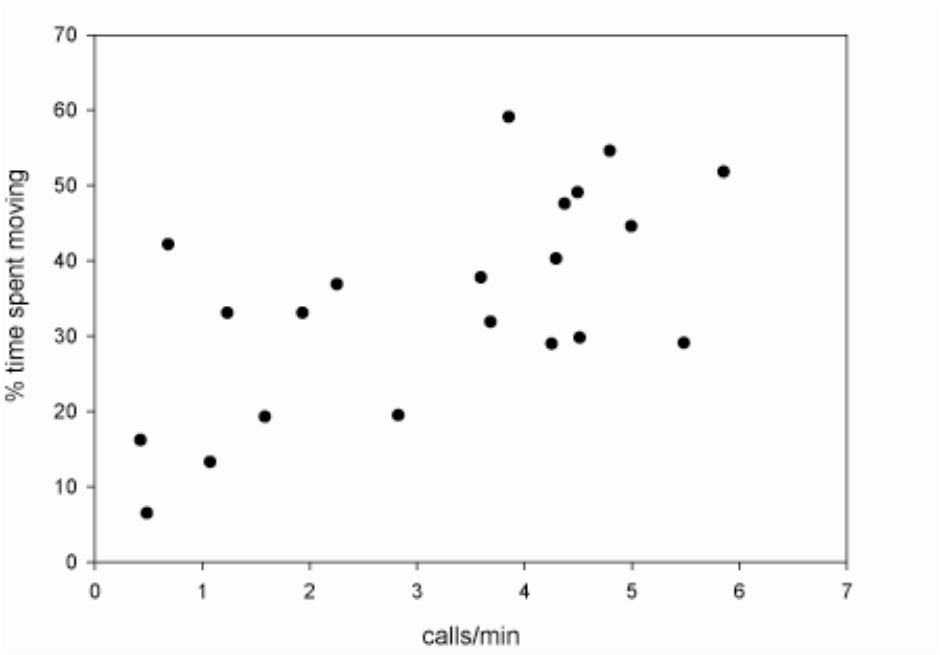

Figure 28. Movement Versus Call Rate. This graph plots the relationship between movement and call rate for 21 echolocating bats, showing that they are weakly correlated (R^2=0.3788). However, this graph also shows that there is a great deal of variability in this relationship.

Pharmacology of vocal motor pathway regions of interest

To further explore some of the regions activated by echolocation, I injected the glutamate agonists kainic acid and bicuculline into the dlCdN, ACg, and PAG, all of which resulted in vocalization (Figure 29). The calls resulting from injections into the dlCdN were song-like sequences with several syllable types including buzzes. From the ACg, both normal looking echolocation calls and song-like sequences similar to the ones seen in the dlCdN experiments were recorded. From the PAG, I got single syllable echolocation-like (downward FM sweeps) calls.

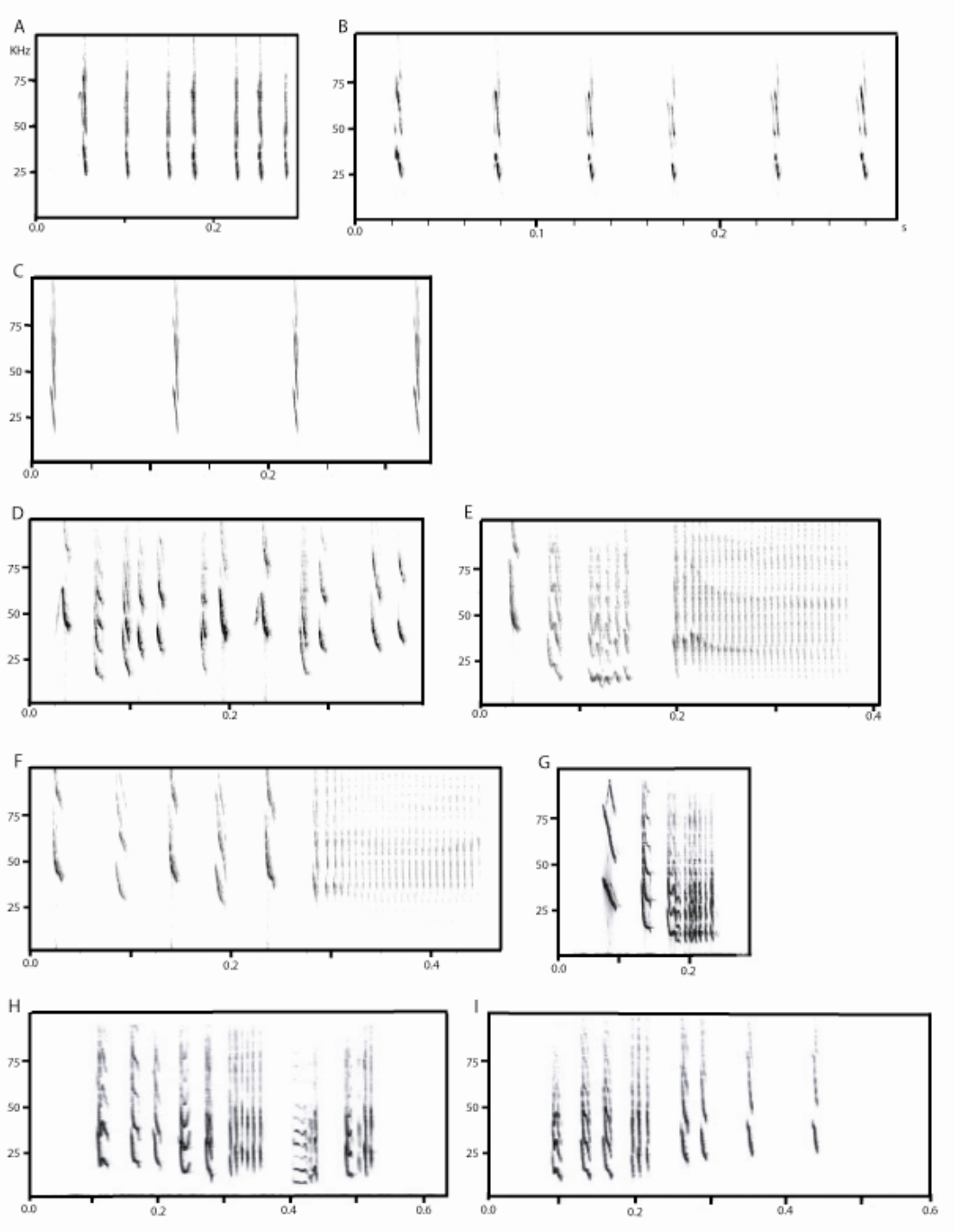

Figure 29. Pharmacological Excitation of Three Vocal Regions of Interest. This figure shows the vocalizations elicited after injection of a glutamate agonist into the PAG (A-B), ACg (C-F), and dlCdN (G-I). Each spectrogram is from a different individual bat.

Discussion

This study reports on vocalization induced c-fos immunoreactivity in the brains of Mexican free-tailed bats, *Tadarida brasiliensis*. Neuronal activation was found in the anterior cingulate cortex, dorsomedial caudate nucleus, paraventricular and lateral hypothalamic nuclei, lateral habenula, substantia nigra pars compacta, PAG, median and dorsal raphe nuclei, and parabrachial nucleus of echolocating animals, but not listening or silent animals (Figure 22, Table 2).

Vocalization induced c-fos expression in known vocal structures

The basic vocal motor pathway in mammals is a limbic-based visceromotor triggering of brainstem pattern generators (Figure 22). The limbic system, specifically the anterior cingulate cortex (ACg), provides voluntary control over calling, sending inputs to the midbrain periaqueductal gray (PAG), responsible for vocal initiation. The PAG coordinates respiratory and laryngeal motor neurons of the parabrachial nucleus (PB) and nucleus ambiguus (NA) necessary for vocalization (Yajima et al., 1981, Larson and Kistler, 1986, Jürgens, 2002). Electrical stimulation of the PAG produces vocalizations in all mammals studied, including species of bats, while lesioning this core vocal structure results in mutism (Jürgens and Ploog, 1970, Suga et al., 1973, Yajima et al., 1980, Jürgens, 2002). The ACg produces PAG-mediated vocalizations when stimulated (Robinson, 1967, Jürgens and Ploog, 1970, Sutton et al., 1974, Gooler and O'Neill, 1987). Additionally, the PAG receives inputs from other regions of the brain, including medial prefrontal cortex, gyrus rectus, insular cortex, basal ganglia,

hypothalamus, amygdala, and thalamus, found through retrograde tract tracing (Dujardin and Jurgens, 2005).

It was expected that I would find echolocation-induced c-fos expression in basic core vocal structures like the ACg, PAG and PB. These serve as an indicator of the reliability of this gene and the technique used for this application. However, to gain further understanding of these regions, I broke the PAG and PB down into segments and analyzed these separately to ascertain whether there were any areas that were specifically active or inactive. I divided the PAG into sections both on the rostral-caudal axis and on the dorsal-ventral axis, but all areas analyzed had significantly higher cell counts than matching regions in the listening and silent animals (Table 2, Figure 26). There were some regions with insignificantly higher FLI counts, in particular the caudal most sections of the dorsal PAG and the rostral lateral PAG. Accounts of electrical stimulation in the PAG reported that stimulation in the dorsolateral PAG resulted in vocalization in both the macaque and the bat (Suga et al., 1973, Larson, 1985). Particularly in the bat stimulation study, the electrode site appears to be in the caudal dorsolateral PAG, judging from the neighboring structures, including the inferior colliculus and dorsal raphe nucleus (Suga et al., 1973). My highest FLI count was found in the dorsal PAG around the same area, suggesting that this section of the PAG might be particularly important for the vocal motor pathway.

We also broke the PB down into two sections, the medial and lateral PB. I found that FLI in the medial PB was significantly higher than in the lateral PB and that when they were separated; FLI in the lateral PB was no longer significantly different from the

listening bats. It is not surprising that these two subregions of the PB would have different activation. The medial PB stimulates laryngeal afferents, while the lateral PB stimulates pulmonary afferents (Smotherman et al., 2009). Since the echolocating animals were the only group vocalizing, and therefore using the larynx, this group should be the only group with activation in the medial PB.

The ACg was not broken down any further. This area was analyzed based on the results from previous stimulation studies (Gooler and O'Neill, 1987). Vocalizations were elicited in this study from sites in the anterior cingulate cortex rostral to the anterior commissure. Similar results were found in the squirrel monkey (Jürgens and Ploog, 1970). In addition to its known role in the vocal initiation pathway, there is a body of evidence that suggests that the ACg is important for audio-vocal integration. This ROI receives extensive input from auditory regions in the superior temporal gyrus (STG), including auditory association cortex, in non-human primates (Paus, 2001). Also, a subset of neurons in the STG reacted to both auditory stimuli and electrical stimulation of vocal regions of the ACg (Müller-Preuss et al., 1980). In experienced human singers, fMRI reveals that the ACg is activated during compensation when auditory feedback to the subject was manipulated to indicate a shift in pitch (Zarate and Zatorre, 2005, 2008). This suggests that the ACg is important for vocal modulation in addition to vocal initiation.

Potential hypothalamic vocal areas

The paraventricular nucleus of the hypothalamus is a central component of the mammal stress response, coordinating both the endocrine system and autonomic nervous system (Engelmann et al., 2004, Benarroch, 2005). There is little evidence to support the involvement of this region in vocalization, aside from a stimulation study where electrical stimulation of this area resulted in aggressive vocalizations and displays, which could be categorized as a stress response (Jürgens et al., 1967). I did find significantly higher FLI in echolocating bats in my experiment, but there were some bats in each experimental group with similarly high levels of FLI. Due to the extensive evidence in the literature that this area is involved in the stress response, I attribute high FLI in the PVN of some bats to be in response to the stress associated with participation in the experiments.

Similarly, it is unclear what role the lateral hypothalamus plays in vocal behavior. The LH receives projections via the medial forebrain bundle, which collects inputs from the olfactory regions, striatum, prefrontal cortex, and limbic regions, among others (Nieuwenhuys et al., 1982). The LH is also interconnected with several of my designated ROIs, including the ACg, LHb, PVN, raphe nuclei, PAG, and PB (Saper et al., 1979, Berk and Finkelstein, 1982). These connections suggest that the LH could provide an integrative role, using sensory input provided through the medial forebrain bundle to influence vocal behavior through the vocal initiation pathway or the serotonin system.

Vocalization induced c-fos expression in the basal ganglia: Evidence for the motor cortical vocal control pathway

It has been recently proposed that another pathway controls plasticity and patterning, the motor cortical vocal control pathway (Figure 22), a cortico-basal ganglia-thalamic loop which originates in the larynx area of the motor cortex, a model based on the vocal motor systems of humans and songbirds (Jürgens, 2009). Stimulation of this cortical area in the squirrel monkey causes vocal fold adduction, even with inactivation of the PAG (Jürgens and Zwirner, 1996). Anterograde tracer injected into the larynx area of the motor cortex indicates that there is a direct connection to the reticular formation which then projects to the NA, completely bypassing the PAG (Simonyan and Jurgens, 2003, Jürgens and Ehrenreich, 2007). This motor cortical vocal control pathway hypothesis attempts to explain the key aspects of mammalian vocalization that have been virtually neglected in past mammalian studies: vocal plasticity and patterning.

In relation to this hypothesis, probably the most interesting areas discovered in these experiments were the dorsolateral caudate nucleus and the substantia nigra pars compacta of the basal ganglia. The basal ganglia are a network of interconnected subcortical nuclei with a central location in the brain important for interaction with many different areas (Alm, 2004). The input nucleus of this network is the striatum, composed of the caudate nucleus, putamen, and nucleus accumbens (Hikosaka et al., 2000, Alm, 2004). The striatum receives input from most of the cerebral cortex. The main output nuclei are the interior part of the globus pallidus and the substantia nigra pars reticulata, which project back to the cortex by way of the thalamus and also to the brain stem. The

basal ganglia also include some internal modulators, the subthalamic nucleus, the external portion of the globus pallidus, and the substantia nigra pars compacta, which specifically modulates the activity of the striatum. Interestingly, no areas of the basal ganglia were induced by electrically-stimulated vocalizations (Jürgens et al., 1996), but nucleus accumbens and globus pallidus were reported as afferents to the PAG in squirrel monkeys (Dujardin and Jurgens, 2005).

FLI in the basal ganglia supports Jürgens' hypothesis about a separate motor cortical pathway for patterning and modulation, and also, as Jürgens' hypothesis is based on the human and birdsong models of vocalization, this also supports the idea that the vocal motor pathway in non-human mammals is more closely related to what is known in these groups that was previously thought. Although Jürgens' pathway is centered around the putamen instead of the caudate nucleus, the evidence presented for the hypothesized pathway would also support the caudate nucleus being the striatal portion of the loop, as this region also receives a direct projection from the larynx area of the motor cortex (Simonyan and Jurgens, 2003, Jürgens, 2009). Additionally, the dorsolateral striatum, including dorsolateral caudate nucleus and putamen, is considered the sensorimotor portion of the striatum which receives inputs from motor, premotor, and sensory cortical areas (Groenewegen, 2003). This motor loop hypothesis is not exclusive to vocal motor behavior. This is the central idea behind general mammalian motor skill learning and production (Hikosaka et al., 2002, Doyon et al., 2003).

The basal ganglia generally seem to play a role in sequence ordering and timing. In rodents, the dorsolateral striatum and the substantia nigra are involved in syntactic

grooming behaviors, shown by increased firing of cells in these areas during movements within the grooming sequence but not during the same movements performed singly (Aldridge et al., 1993, Aldridge et al., 2004). A key area in the bird song system for song development, variability, and sequence maintenance is Area X, an area homologous to the mammalian striatum (Jarvis et al., 1998, Liu and Nottebohm, 2005).

The basal ganglia's role in order and timing is particularly noted in several pathologies. Patients with obsessive-compulsive disorder and Tourette's syndrome, both disorders involving uncontrolled repetitions, show abnormalities in the striatum when observed with positron emission tomography (PET) and fMRI (Graybiel, 2000). Similarly, in autism, where in addition to social and communicative problems, a key symptom is stereotyped and repetitive behaviors, patients exhibit an enlarged caudate nucleus (Langen et al., 2007). Basal ganglia abnormalities are also a likely candidate for the issue at the heart of stuttering (Alm, 2004). Huntington's and Parkinson's diseases are caused by basal ganglia lesions, particularly depletion of dopaminergic cells in the substantia nigra pars compacta, and both present with motor and speech problems (Graybiel, 2000). These pathologies of the basal ganglia have a common underlying feature in that all these patients are unable to control the under or over production of motor function and this has a profound effect on a person's ability to function at a normal capacity.

C-fos expression in the dorsolateral striatum and the substantia nigra pars compacta in echolocating bats falls directly in line with the role these areas are thought to play in sequencing and timing. Echolocation calls occur in long trains at regular

intervals and provide the bat with a spatial map of its environment. The returning echoes dictate future calls, which are modified to accommodate close obstacles or to pinpoint prey items. As mentioned previously, *Tadarida brasiliensis* possesses the ability to rapidly alter its echolocation frequencies to avoid acoustic jamming, a method used by some insect species to avoid predation (Miller and Surlykke, 2001, Tressler and Smotherman, 2009). These bats can also alter the timing of their calls to take advantage of quiet periods within noise (Jarvis et al., 2010). These quick modifications and vocal plasticity would require that the bat possess a sophisticated system of vocal control allowing for vocal modulation and rapid processing of auditory feedback. Additionally, the males of this species produce a complex courtship song which is both species stereotyped and individually adapted which would seemingly require at the very least auditory feedback and possibly a neural mechanism for vocal learning (Bohn et al., 2008, Bohn et al., 2009). The hypothesized candidate system for vocal modulation, incorporating a cortico-striatal-thalamic motor loop (Figure 22), is supported by my finding of vocalization-induced FLI in the dorsolateral striatum.

Dopamine and serotonin in the vocal motor pathway

The sites of synthesis and release of two major neurotransmitter systems, dopamine and serotonin were found to have vocalization-induced FLI in free-tailed bats. In the dopaminergic system, dopamine is produced and released by neurons in the substantia nigra pars compacta. These dopaminergic neurons project to the striatum, the site of both D1 and D2 dopamine receptors, where I also found FLI in a small restricted

area of the caudate nucleus. In the serotonin system, serotonin is produced and released by the raphe nuclei, and is then released all over the brain.

These two systems have been implicated previously in vocal control systems. Monoamine oxidase (MAO) inhibitors specific for the breakdown of serotonin and dopamine decreased the frequency of squirrel monkey isolation calls, but not other behaviors associated with isolation, such as vigilance checking or locomotor behavior (Newman et al., 1991). Also, the MAO inhibitor specific for the breakdown of dopamine but not serotonin increased the duration and decreased the peak frequency of the isolation calls. Another study in rats showed that dopamine depletion decreased the bandwidth of ultrasonic vocalizations, while all other behaviors normally associated with these mating/courtship vocalizations were left intact (Ciucci et al., 2007). Also, occurrence of rat pup isolation calls were significantly decreased following serotonergic lesions, but the animals developed and behaved normally otherwise (Winslow and Insel, 1990). In songbirds, dopamine plays a role in the anterior forebrain pathway of the song system, important for song development in juveniles and vocal plasticity in adults, while also mediating song differences due to social context (Sasaki et al., 2006). In humans, a clinical report indicated that two patients became afflicted with dysphonia, a general term for disorders of the voice, including but not limited to breathiness or hoarseness, due to intake of selective serotonin reuptake inhibitors (Petitpain et al., 2005). The majority of patients with Parkinson's disease exhibit dysarthria, a speech disorder affecting articulation, with common symptoms including nasality, monotone, slurring, and others. Treatment with Levodopa (L-DOPA), the precursor to dopamine, improves

these patients' speech (Sanabria et al., 2001). These results all imply that serotonin and dopamine play a role in the modulation of calls.

Lateral habenula circuit: Possible vocal loop modulator

The lateral habenula is not a known vocal region, but it may be involved in vocal behavior indirectly. It receives input from the striatum and the rest of the basal ganglia by way of the globus pallidus internal segment (Hikosaka et al., 2008). It also receives input from the lateral hypothalamus. It then projects to the substantia nigra pars compacta and ventral tegmental area (SNc/VTA) and also to the dorsal and medial raphe nuclei. The lateral habenula inhibits dopamine neurons of the SNc/VTA and also modulates serotonin release in the raphe nuclei (Hikosaka et al., 2008, Yang et al., 2008). While it is unclear what role this structure plays in vocalization, it was previously reported to be vocalization-induced in tamarins, along with the raphe nuclei (Jürgens et al., 1996). It is possible that the lateral habenula has a modulating role in vocalization by affecting the dopamine system, the serotonin system, or both. In my study, echolocation-induced c-fos activity was also found in the SNc, dorsolateral caudate nucleus, lateral hypothalamus, and raphe nuclei, all regions on the giving inputs to or receiving inputs from the lateral habenula.

Relationship between moving and calling

Our data indicates that there is a weak correlation between moving and echolocating. The fact that these are loosely linked makes sense because echolocation is used for navigation and spatial awareness. A bat that is moving, particularly in the dark, is going to echolocate in order to "see" its surroundings. I cannot say conclusively that all of the c-fos immunoreactivity seen is strictly due to vocalizing, because I cannot completely separate calling from moving in echolocation. However, I also cannot say that all of the c-fos immunoreactivity is strictly due to moving. While I can only make observations about regions involved in echolocation, moving and calling collectively, instead of just vocalizing, I now have several specific target areas with which I can continue to parse together a more concrete mammalian vocal motor network.

Auditory regions and evidence for audio-vocal integration

Audio-vocal integration is important for complex vocal behavior like the ability to modulate calls quickly due to changes in environment, which I see in the free-tailed bat (Tressler and Smotherman, 2009, Jarvis et al., 2010). Additionally, this integration would be important for vocal learning, where a subject modifies vocalizations as a result of experience with vocalizations from other individuals (Janik and Slater, 1997). Vocal learning has been shown previously in bat species (Boughman, 1998, Knörnschild et al., 2006, Knornschild et al., 2010). Additionally, several species exhibit courtship songs similar to those seen in vocal learning birds (Behr and von Helversen, 2004, Bohn et al., 2008, Bohn et al., 2009), but song development in juvenile bats has not yet been studied.

While it is unclear if *Tadarida brasiliensis* exhibits vocal learning in the development of the courtship song, it is a distinct and likely possibility. Therefore, audio-vocal integration is particularly important in this species. This experiment provided no real concrete evidence for audio-vocal integration, but it does loosely support a few of the running theories, in that I found FLI in the proposed cortico-striatal-thalamic loop and also in the ACg.

Pharmacological activation of echolocation-activated regions of interest

To further characterize a few of the ROIs activated by echolocation and to determine whether these regions were vocalization-induced rather than movement-induced, I pharmacologically activated them with two different glutamate agonists. I injected the PAG, ACg, and dlCdN and vocalizations were elicited from all three, strongly supporting a role for these three regions in the non-human mammalian vocal motor pathway.

The vocalizations recorded after PAG stimulation were short duration downward FM sweeps that looked similar to echolocation calls (Figure 29A, B). These calls were similar to reported call types recorded from PAG stimulation in other bats (Suga et al., 1973, Fenzl and Schuller, 2002). Single syllable vocalizations have also been stimulated from this region in monkeys, mice, and other mammals (Jürgens, 2002). The PAG is an established member of the visceromotor vocal motor pathway and these results replicate what has been found in other mammalian species.

Similarly, after injections in the ACg, I recorded natural-looking echolocation calls, similar to results reported previously in another bat species (Figure 29C) (Gooler and O'Neill, 1987). However, the more exciting and unexpected result was that additionally in this region, glutamate agonist injection produced song-like sequences (Figure 29D-F). Several sequences had all of the courtship/territorial song components (Figure 15) that even occurred in the correct order (for a representative sequence, see Figure 29F). My results are particularly interesting because previous reports have indicated that the ACg has a more subdued role in the vocal motor pathway acting only as a vocal initiator through the PAG. Nothing even resembling the complex vocalizations seen in these experiments has been reported in another mammal after stimulation of the ACg. Stimulation of the ACg in the monkey and bat resulted in single syllable vocalizations similar to those elicited after PAG stimulation, supporting the idea that the ACg acts as a vocal initiator through the PAG (Robinson, 1967, Jürgens and Ploog, 1970, Gooler and O'Neill, 1987). Lesions to this region in monkeys reduce spontaneous vocalizations and the ability to master a vocal operant conditioning task (Sutton et al., 1974, MacLean and Newman, 1988). The resulting songs elicited from the free-tailed bat suggest that the ACg has a more significant role in sequence formation and complex vocalizations, along with a role in echolocation. It is possible that the ACg actually contributes to the neocortical pathway along with playing a role in the visceromotor pathway. It is unclear what caused the different call types elicited from this area, but it is possible that dose might play a role. The pneumatic delivery of the drug makes it difficult to control the amount of drug injected, so it is difficult to

confidently report the dosage. Future work with cannula injections will allow me to better control for total amount of drug injected and will allow me to determine any dose effect.

Finally, injections into the dorsolateral caudate nucleus also resulted in song-like sequences. Stimulating calls from this area of the striatum is extremely important because it supports the previous c-fos findings that this region is involved in the vocal motor pathway, particularly since this area has only been hypothesized to be part of the vocal motor pathway in mammals. The sequences produced from excitation of this region consisted of several different syllables, some very similar to the syllables found in courtship/territorial songs (Figure 29G-I). Preliminary observation seemed to indicate that these sequences were more variable that those recorded after ACg stimulation, both in syllable type and syllable order. There are no reports in the literature about vocalizations elicited by stimulation of any region of the striatum in mammals, and Robinson reported no results from stimulations of the caudate nucleus and putamen in the rhesus monkey (Robinson, 1967). However, the avian homologue of the striatum is part of the vocal system in songbirds (Jarvis et al., 2005) and is active during human speech (Schulz et al., 2005). Additionally, the striatum in general is thought to play a role in several human speech disorders, including stuttering and Tourette's syndrome, along with other disorders with speech symptoms, including Parkinson's disease and autism (Graybiel, 2000, Alm, 2004, Langen et al., 2007). These results support a role for the dlCdN in the vocal motor pathway in non-human mammals as well.

Conclusions

C-fos immunohistochemistry revealed echolocation-induced activation of regions in the known and hypothesized vocal-motor pathways, providing support for the neocortical motor loop hypothesis. This work effectively answered both of my questions, vocal regions found in microstimulation studies were activated in naturally vocalizing bats and there were additional areas activated in naturally vocalizing bats that were not previously known that could account for some of the more complex vocal behaviors seen in the free-tailed bat. Additionally, pharmacological excitation of three of these regions produced vocalization, further supporting their involvement in the non-human mammalian vocal motor pathway.

CHAPTER VI

CONCLUSIONS

One of the most compelling questions in science is how and why humans evolved their capacity for speech. This question has been difficult to address experimentally because of the very uniqueness of speech and language. Primates do not possess anything even remotely resembling a "proto-speech" or "sub-speech". Instead, primate vocal communication is largely identical to most other mammals, no more similar to human speech than a cat's meow or a dog's bark. However many mammals exhibit discreet evidence of vocal complexity that when fully understood may, in combination with data from other animals, begin to provide partial answers to the origins and neural basis of speech. Instead of hunting for an evolutionary progression in vocal complexity, investigators must instead explore specific features of vocal control in many different animals and test hypotheses about how and why specific aspects of vocal complexity. Once the behaviors are characterized I can begin to identify the underlying brain regions and specialized neural circuits that may collectively provide an accounting of complex vocal behaviors such as speech. In support of this larger goal, the focus of this dissertation was to identify the neuroanatomical basis of vocal control in a mammal that exhibits more complex and plastic vocal behaviors than any primate other than humans. In this dissertation I have shown that the free-tailed bat, *Tadarida brasiliensis,* has a vast vocal repertoire, including many different call types, the capacity for context-dependent vocal plasticity, and the formation of a complex, stereotyped vocal sequence

(i.e. songs). I have shown that certain aspects of their vocal behavior and syllable usage was seasonal, and since melatonin is known to regulate seasonal behaviors in other animals, I mapped the distribution of melatonin binding sites in the brain. When I found high densities of melatonin receptors present in the striatum (in addition to other more common areas found in other mammals), I examined the distribution of D1- and D2-type dopamine receptors, which showed a strong correlation between the distribution of the melatonin and dopamine receptors. This indicates that melatonin's interactions with dopaminergic pathways may play a role in the seasonal regulation of vocalizing, and thereby indirectly implicates the basal ganglia in vocal control.

The basal ganglia are not included in current models of mammalian vocal control, but there are several additional reasons to suspect their involvement in free-tailed bat vocal behavior. Firstly, the bat songs I described were more similar to birdsongs than to any known mammalian vocalization, and it is well known that birdsong production depends upon basal ganglia pathways for both song learning and production. Secondly, in both songbirds and humans the basal ganglia have been assigned a special role in vocal plasticity. The free-tailed bat's echolocation behavior has become a model of mammalian vocal plasticity, and notably current models of vocal production cannot account for this plasticity. Thus, major aspects of the free-tailed bat's vocal behavior are strongly suggestive of basal ganglia function. My evidence that melatonin-dopamine interactions in the basal ganglia may also contribute to vocal control is supportive of this conclusion. It was based on this evidence that I sought to identify the best available neuroanatomical technique to test the hypothesis that there

should be neuronal activity in the basal ganglia during vocalizing, namely immunohistochemical localization of immediate early gene expression following prolonged bouts of vocalizing.

I studied immediate early gene expression in echolocating, listening, and silent bats to find ROIs activated by the act of vocalizing but not by hearing oneself. C-fos immunohistochemistry showed that a small region of the caudate nucleus was activated during echolocation behavior. Additionally, several other regions were also active, including all the previously identified major vocal centers, such as the ACg, PAG and PB. These results indicate that there were areas of the brain involved in vocalization that had not been identified in previous vocalization research. The reason for this likely has to do with the fact that the primary techniques used in all previous studies, namely electrical stimulation and extracellular recordings in restrained animals, would have been poorly suited to the modulatory nature of the basal ganglia's function in vocal control. In other words, if the primary function of the basal ganglia in vocal control is to mediate the effects of social context and sensory-feedback on vocal plasticity, then these circuits would not necessarily have been activated by electrical stimulations of the cortex or midbrain vocal control centers. Instead, by using c-fos immunohistochemistry I was able to identify novel brain regions that were activated during normal vocal behaviors in freely moving animals.

This work provides evidence that in addition to the well known visceromotor vocal pathway, an extrapyramidal pathway may be involved in more complex mammalian vocal behaviors such as those exhibited by free-tailed bats. *Tadarida*

brasiliensis is capable of more than the simple single syllable vocalizations that have dominated vocal motor pathway research. Using naturally vocalizing bats allowed me to avoid the constraints of other model systems, and while I sacrificed some experimental control with this tactic, I found several brain regions to explore further, such as key regions of the basal ganglia known to be involved in vocal plasticity in other animals. To further characterize the role of several ROIs in vocal behavior, I did some preliminary pharmacology experiments, which have shown that injecting the excitatory drugs such as the glutamate receptor agonist kainic acid and the $GABA_A$ receptor antagonist bicuculline methiodide into either the dorsolateral caudate nucleus or the anterior cingulate cortex resulted in prolific activation of the vocal motor pathway. The vocalizations recorded from these experiments ranged from rapidly repeated single syllables to stereotyped song-like sequences, similar to the courtship/territorial songs recorded in the field and in the lab. Indeed, the diversity of complexity of evoked vocalizations achieved by injecting excitatory drugs into these two brain areas in the free-tailed bat is well beyond anything reported in other mammals or even songbirds. Some of the sequences elicited from ACg stimulation looked remarkably similar to naturally recorded songs (Figures 15, 29). Preliminary observations indicated that the sequences elicited from the CdN were more variable in syllable type and order.

This dissertation provides support for an extrapyramidal motor loop as part of the main vocal motor pathway (Figure 22), and it also poses some new questions. First, what additional role is the ACg playing in the vocal motor pathway? The current model of vocalization has this region acting as a vocal initiator, dependent on the PAG and

reticular formation for vocal production. However, the pharmacology results indicate that this region is involved in more complex vocal behaviors such as the execution of stereotyped sequences. Second, what role do the limbic areas play in vocal behavior? This work has revealed some additional brain regions potentially involved, including the lateral hypothalamus, lateral habenula, substantia nigra pars compacta, and raphe nuclei. These areas have not previously been included in the vocal motor pathway, but the results from the c-fos immunohistochemistry experiment indicate that they are active during vocalization. And finally, third, how does this work relate to what is known about human speech production? These experiments produced evidence for the extrapyramidal pathway in mammals and also revealed some additional areas potentially involved in vocalization. Below I provide answers to these questions, and discuss how these interpretations can provide a roadmap for a better understanding of the evolution and neural basis of human speech.

To address the first question, it is obvious from my results that the anterior cingulate cortex must play a substantial role in the vocal motor pathway. Chemical stimulation of this area produced complex sequences in the free-tailed bat, but previous studies using electrical and pharmacological microstimulations of this region in monkeys and bats reported only simple single syllable calls (Robinson, 1967, Jürgens and Ploog, 1970, Gooler and O'Neill, 1987). It is possible that the ACg is involved in complex vocal behavior in some species but not others, but since several of the previous species studied only use simple single syllable vocalizations, stimulation of this area would probably only result in that type of vocalization. However, stimulations in this region in

the mustached bat, known to exhibit syntax in some of its vocal combinations, produced single syllable vocalizations as well (Kanwal et al., 1994). Still, the simple fact that I were able to stimulate complex vocal sequences from the ACg indicates that this region deserves a second look.

Other evidence also supports the ACg's importance in the vocal motor pathway. This cortical area receives projections from the larynx area of the motor cortex (Jürgens, 1976). Stimulation of the laryngeal motor cortex moves the vocal folds of the larynx, but does not elicit vocalization in the squirrel monkey (Jürgens, 1974). The laryngeal motor cortex projects to the reticular formation, bypassing the PAG (Jürgens and Zwirner, 1996). This is the basis for the hypothesized neocortical vocal motor pathway which is separate from the visceromotor pathway containing the ACg and PAG (Jürgens, 2009). I do not know whether stimulation in the laryngeal area of the motor cortex in the bat would elicit vocalization, but since I was able to trigger complex vocal behaviors from the ACg, it seems possible that this region is also part of a motor loop that coordinates vocal complexity and that this area potentially interacts with the laryngeal motor cortex and associated motor loop. The vocal pathway including the ACg is classically thought to run through the PAG because chemical lesions in the PAG suppressed ACg-induced vocalization (Jürgens and Lu, 1993). However, ACg also projects to the reticular formation, presumably bypassing the PAG similar to the laryngeal motor cortex area, and to areas of motor cortex (Pandya et al., 1981, Wyss and Sripanidkulchai, 1984). The fact that effectively paralyzing the PAG blocked ACg-induced vocalization might also be accounted for by the PAG's role in respiration

(Subramanian et al., 2008). It is unclear whether the chemically-lesioned animals could vocalize at all, because no other vocalization-inducible area was tested.

The ACg interacts with more than just motor cortex. It also projects to the striatum and receives more thalamic afferents than any other cortical area, supporting the idea that it is part of a motor loop (Devinsky et al., 1995). This also suggests that the ACg has an integrative role, since the thalamus is a major relay center of cortical and subcortical regions. In addition to thalamic input, the ACg also receives projections from many cortical areas, including auditory cortex (Paus, 2001). There is some evidence that the ACg is important for audio-vocal integration. In experienced human singers, the ACg is active when making pitch corrections, suggesting that this region is important for vocal modulation, correcting the vocal output based on the correct template (Zarate and Zatorre, 2005, 2008). Both from my experimental results and the evidence present in the literature, it seems likely that the ACg plays a larger role in the vocal motor pathway than just vocal initiation.

To address the second question, the role of limbic areas in vocal behavior, the literature indicates that these areas are all interconnected. The lateral habenula, substantia nigra, lateral hypothalamus, and raphe nuclei are not part of the classic vocal motor pathway models. All of the limbic regions activated during echolocation in my experiments are associated with each other through the lateral habenula, in a circuit thought to incorporate limbic-based emotion and motivation into motor action selection (Hikosaka et al., 2008). The lateral habenula receives input from the lateral hypothalamus and caudate putamen (through globus pallidus). Lateral habenula then

projects to the substantia nigra and raphe nuclei, modulating the release of dopamine and serotonin respectively. The dopaminergic and serotonergic systems interact within the basal ganglia in complex way, making it difficult to dissect specific functions of these systems in the vocal motor pathway. Dopamine has known roles in learning, motivation, and voluntary movement, all of which are important for vocalization. Serotonin is involved in many aspects of cognition and attention, which might be particularly important for seasonal reproductive behaviors such as the bat song. Additionally, several reports in the literature indicate that these neurotransmitters interact with each other in the striatum (Sasaki-Adams and Kelley, 2001, Esposito et al., 2008). It seems likely that these limbic areas are involved in motivation for vocalization, providing input to the vocal motor pathway pertaining to the emotional state of the animal and predicted reward value of the behavior. However, it is unclear whether these areas are involved in vocal behavior at all, although all of these regions except the substantia nigra were also activated by electrical stimulation-induced vocalization (Jürgens et al., 1996). Considering the potentially central role of the habenula for integrating the many diverse inputs regulating vocalizing, more work is certainly called for to determine the role of the lateral habenula circuit in vocalization.

In addressing the third question, it is important to review what is currently known about human speech. Humans possess a direct connection from the motor cortex to the laryngeal motor neurons in the nucleus ambiguus which is supposedly absent in mammals, although few studies have actually been carried out and other mammals do have direct connections from the cortex to the respiratory circuitry and oral articulators,

including the motoneurons controlling the lips, tongue, and jaw (Schulz et al., 2005). Regions of the prefrontal cortex, supplementary motor areas, and anterior cingulate cortex are all activated during human speech, along with the PAG and areas of the basal ganglia and thalamus which form motor loops with the cortex. It seems that in the human vocal pathway, both the ancient visceromotor pathway and an updated and improved version of the neocortical pathway function in tandem to coordinate the diverse suite of motoneurons necessary to produce the complex sounds underlying speech. The fact that there is a direct connection between the motor cortex and the motoneuronal pools coordinating vocalization and respiration affords more fine-tuned vocal control than that seen in non-human mammals. It is unclear whether non-primates have cortical brain regions performing functions analogous to prefrontal cortex in the human vocal control pathway. Preuss (1995) proposes that what was considered to be dorsolateral prefrontal cortex in the rat based on mediodorsal thalamic nuclei and dopaminergic innervations is actually premotor cortex and anterior cingulate cortex, and that these non-primate mammals do not have true prefrontal cortex regions. This is important in my study of the mammalian vocal motor pathway because prefrontal cortex is an important structure for human speech production (Schulz et al., 2005). If bats and other non-primates lack a prefrontal cortex, then the anterior cingulate cortex could form the evolutionary basis for prefrontal vocal centers that evolved in humans. Thinking about the ACg as functionally analogous to a "pre-prefrontal cortex" supports the idea that this area has a more significant role in the vocal motor pathway of non-human mammals.

This work answered some questions about the non-human mammalian vocal motor pathway and created a basic foundation for relating mammalian vocal control to similar circuits in humans and songbirds, but it also created many more questions to be answered. My hypothesized vocal motor pathway is provided in Figure 30, including the limbic vocal areas activated in vocalization and also a more important role for the ACg. This hypothesis sets the stage for future work and hopefully a greater understanding of mammalian vocalization.

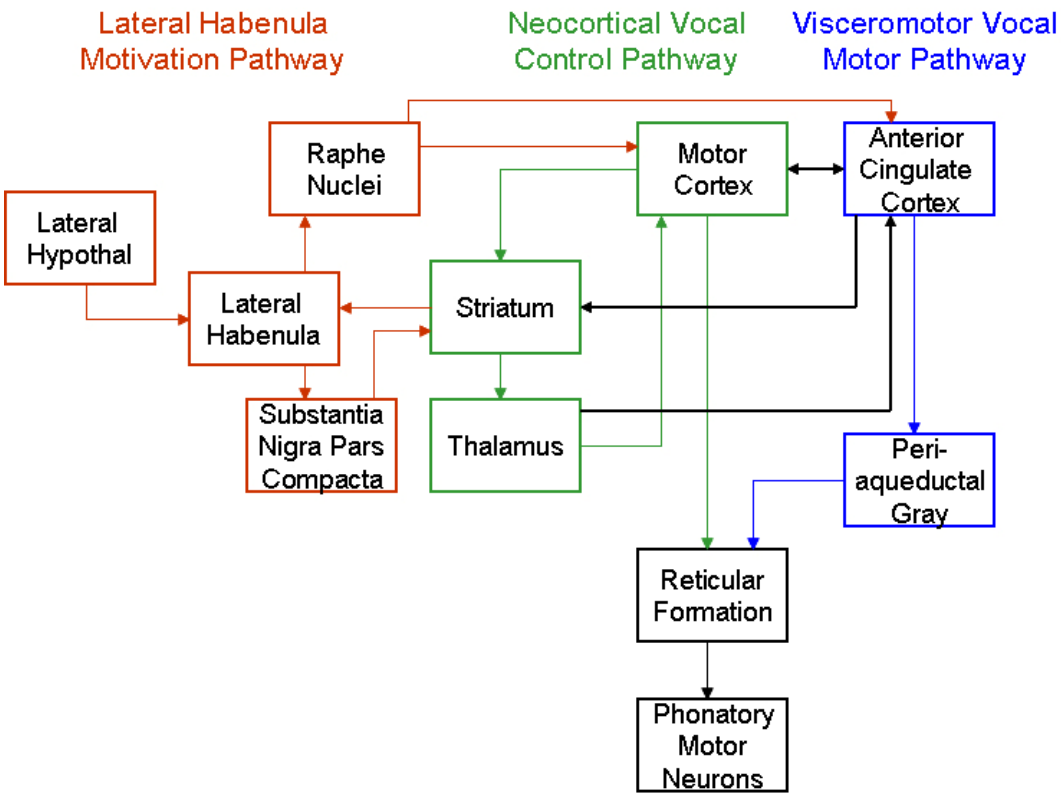

Figure 30. Revised Non-human Mammalian Vocal Motor Pathway.

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