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Modulating the processing of complex sounds: How inhibition of nitric oxide alters evoked responses in the bullfrog torus semicircularis

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Modulating the processing of complex sounds: How inhibition of nitric oxide alters evoked responses in the bullfrog torus semicircularis

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DEDICATION

To my wife and daughter, Jessica and Emmeline Stafford, for all of their support through my years as a graduate student and for years to come.

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Thank you to my wife Jessica for her tireless devotion throughout my entire graduate school tenure. To my daughter Emmeline for helping me keep perspective on what's important and understanding when I had to work. To my PI Dr. Jim Hall for the countless hours of mentoring and patience editing not only this dissertation but every poster and presentation I've made while in grad school. To my peers Dr. Joanna, Dr. Jonathan and Dr. Yuki, who were always there with advice and encouragement. Finally, to my friend Kate, without your friendship I don't know if I could have done this. I'm glad we went through this together.

ABSTRACT

Nitric Oxide (NO) is a gaseous molecule that functions as a retrograde messenger in several regions of the brain. Activation of glutamate N-methyl-D-aspartate (NMDA) receptors stimulates NO production via the activity of nitric oxide synthase (NOS). NO is released and subsequently enhances the presynaptic release of glutamate. Staining for β nicotinamide adenine dinucleotide phosphate diaphorase, an indicator of NO production, as well as immunohistochemical studies have revealed the presence of NOS-labeled neurons in a number of vertebrate brain structures including the inferior colliculus (IC), an important auditory processing center. These neurons presumably produce and release NO. **However, the function of nitric oxide in auditory processing at the level of the IC is not known**. Here we address this issue using extracellular single-unit recording combined with microiontophoresis to investigate the role of NO in how different species calls are analyzed by neurons in the IC of the American bullfrog, *Lithobates catesbeiana*. Of particular interest was if NO modulates the responses of IC neurons to conspecific and heterospecific mating calls. *In vivo* iontophoretic application of L-NAME (a NOS inhibitor), and L-Arg (a NOS substrate), was used to evaluate the effect of NO on the sound-evoked responses of neurons (n=35) in the IC. We found that NO modulated neuronal responses in a call dependent manner. Upon application of L-NAME we observed changes in neuronal responses with respect to spike counts, first-spike response latencies, and interspike intervals. Recovery of original response was seen after application of the NOS substrate, L-Arg after cessation of L-NAME application. Our data suggest a role for NO in regulating both gain control and response selectivity in the IC which may influence the output of neural circuits engaged in the analysis of behaviorally relevant acoustic signals, such as speech.

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INTRODUCTION AND GENERAL INFORMATION

1. Analyzing behaviorally relevant sounds

1.1 Spectral and temporal cues in auditory stimuli

As we engage with the world around us our auditory system uses different spectral and temporal cues in the sound stimuli we encounter to distinguish behaviorally relevant sounds such as speech. Within those speech sounds there are cues that allow us to distinguish what is being communicated. There are temporal cues in the form of the differences in the shape of the sound envelope, and spectral cues in the form of frequency content (Figure 1). While cues giving rise to speech perception are well known, the neural mechanisms mediating the recognition of speech sounds are still poorly understood. To fill critical gaps in our knowledge of neural mechanisms underlying speech recognition, we have chosen to explore signal recognition in an anuran (frog) animal model for which vocal communication plays a crucial role in their social behavior in general, and their mating and territorial behavior, in particular.

1.2 Spectral and temporal differences in advertisement calls

Vocal communication plays an important role in the reproductive and territorial behavior of a vast number of species (Gerhardt 2015). For example, during mating season, lakes and ponds are home to a multitude of frog species (anurans), insects and birds all calling to establish territories and find potential mates. Typically, animals have a prominent call that is used by the sender to advertise certain information like location

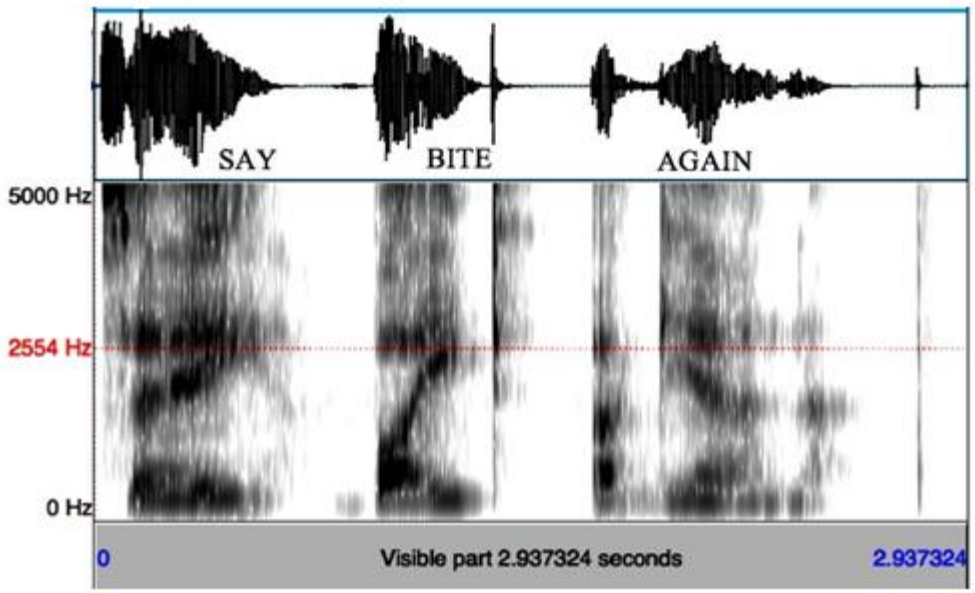

Figure 1. Oscillogram and spectrogram of speech. Top: Oscillogram showing the intensity components of the three words “say”, “bite”, and “again” over time. Bottom: Spectrogram of the same three words showing the frequency components of the same three words over time. Darker regions indicate frequencies with higher energy.

and willingness to mate. These advertisement, or mating, calls are used by females to recognize potential mates, and by males for neighbor/stranger recognition (Bee 2001). Significantly, anuran advertisement calls are relatively fixed and show no evidence of vocal learning (Gringas 2013).

Frogs calling at the same time and in the same area show species-typical differences with respect to the spectral and temporal cues provided by their advertisement calls. Thus, females can readily identify the advertisement call of a conspecific male, reducing the risk of cross-breeding and the production of sterile offspring while males utilize individual differences in the conspecific call for neighbor recognition (Wells 2007). As seen in Figure 2, temporally the bullfrog call is longer in duration (600ms) with longer rise/fall times than the green frog call which has a short rise time, and shorter call duration (250ms). These complex spectral and temporal features are equivalent in complexity to the phonetic components of human speech (Gerhardt 2002). Because their calls play an essential role in mediating social behavior and resemble speech, anurans have been a valuable model system to study how complex sounds, such as speech, are processed in the auditory system. Here, I explore the underlying mechanisms that allow frogs to distinguish between different advertisement calls. Specifically, I examine the role of the neuromodulator, nitric oxide (NO), in the processing of advertisement calls by neurons in the torus semicircularis (TS), an important auditory center of the ascending auditory pathway.

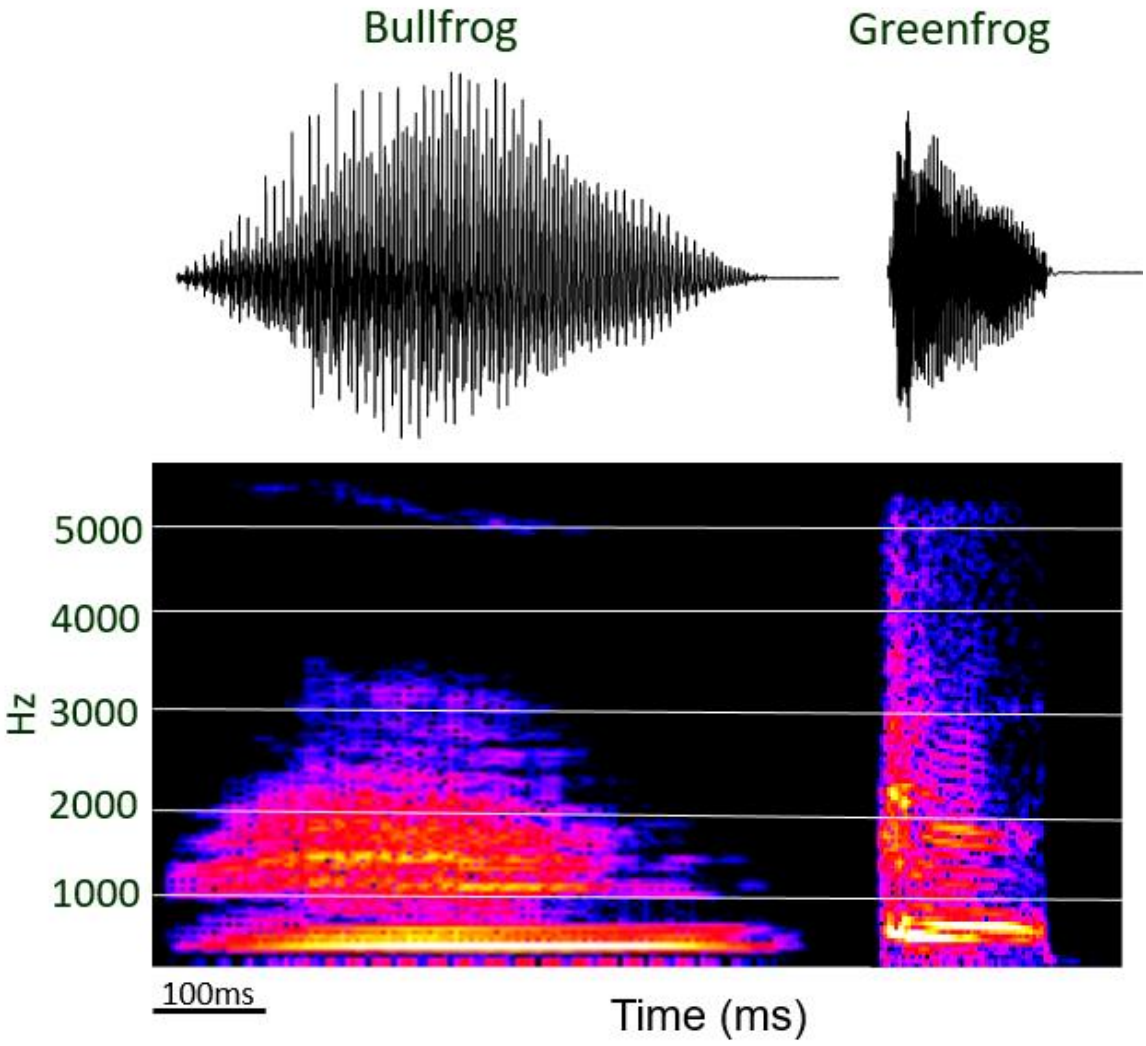

Figure 2. Oscillograms and spectrograms of frog calls. Top: Oscillograms of bullfrog and green frog advertisement calls. The bullfrog call is longer with a symmetrical increase and decrease in sound amplitude. Green frog call is shorter with a fast rise time to peak call intensity, and a gradual fall time. Bottom: Spectrogram of same two calls showing their frequency components over time. The bullfrog call has to primary frequency components around 300Hz and 1300Hz. The green frog call has two primary frequency components around 300Hz and 1700Hz

1.3 Lithobates catesbeiana as a model system

In this study I chose to use the American bullfrog (*L. catesbeiana)* to investigate the role of NO in processing advertisement calls in the torus semicircularis for several reasons:

1) The behavioral significance of the advertisement call is well understood as are the relevant cues for its recognition.

2) There is a great deal of information concerning both the anatomical organization of the auditory system as well as the response properties of its constituent neurons.

3) The presence of nitric oxide and the enzyme responsible for its synthesis (nitric oxide synthase) in the torus semicircularis has been confirmed.

1.4 The bullfrog advertisement call

The most prominent bullfrog vocalization is the advertisement, or mating, call (Capranica 1965, Bee 2007). This call serves to alert females to the presence of a male and communicate information about the caller such as species identity and location (Capranica 1965, Bee 2007). In this context, it is of particular significance as it serves as a premating species isolation mechanism, minimizing interspecific mating and the production of sterile hybrids (Bogert 1960, Capranica 1965, Ralin 1976). The advertisement call is also used for male-male communication, to identify neighbors and maintain territorial distances (Boatright-Horowitz 2000, Bee 2001b).

Spectrally, the bullfrog call consists of two primary frequency bands; a high frequency band centered around 1300Hz and a low frequency band centered around

300Hz (Fig. 2). Behavioral studies utilizing female phonotaxis or the evoked-calling response of male have shown that the simultaneous presence of **both** low and high frequency energy provides the information necessary for call recognition (Capranica 1965, 1966, Rose 2007).

The bullfrog advertisement call also has stereotyped temporal characteristics distinguishing it from those of other species. Temporally, the call is approximately 600ms in duration with symmetrical rise and fall times (Fig. 2). Calls are presented in trains of 6-10 croaks. When dominant frequencies of sympatric species' advertisement calls are similar, temporal differences are sufficient for selecting conspecifics such as pulse repetition rate, pulse number, or envelope rise time (Gerhardt 1988, Arak 1988, Foquette 1975, Narins 1978).

2. The bullfrog auditory system

2.1 Auditory periphery

All vertebrate tetrapods have evolved structures for detecting and processing airborne sound (Wilczynski 1984). Unlike mammals, bullfrogs lack a pinna with the outer ear being represented by an exposed tympanic membrane visible on the side of the head just behind the eyes. A funnel shaped cavity connects the tympanic membrane to the base of the skull where the inner-ear is located (Wilczynski 1984, Mason 2007). Like mammals, the frog tympanic membrane detects variations in air pressure and transmits them to the inner ear, via middle ear structures, where air pressure differences are converted to fluid displacements within two auditory end organs, the amphibian papilla (AP) and basilar papilla (BP). The BP is considered homologous to the cochlea in

mammals while the AP is unique to amphibians (Wilczynksi 1984, Purgue, 2000, Simmons 2007). Hair cells in both papilla are innervated by bipolar cells that, when activated, transmit acoustic information into the auditory system via the 8th nerve (Wilczynski 1984). Tonotopy, the spatial sorting of neurons based on their characteristic frequency (CF), originates in the AP while the BP is broadly tuned and doesn't display tonotopic organization (Lewis 1982, Simmons 2006).

All 8th nerve fibers exhibit spontaneous activity. However, the rate of spontaneous activity is dependent up which auditory end organ they innervate. Those innervating the AP have low, while those innervating the BP have high rates of spontaneous activity.

Fibers from the 8th nerve tuned to low- and mid- frequency sounds (~ 100-1000Hz) innervate hair cells in the AP while fibers tuned to high frequency sounds (~ 1000 – 3000Hz) innervate hair cells in the BP (Feng 1975, Simmons 2007). The frequency tuning of each auditory nerve fiber has a characteristic excitatory V-shaped tuning curve that defines the frequency to which the fiber is most sensitive (the characteristic, or best, frequency). This is determined by the lowest intensity sound at that frequency that elicits a response from the nerve fiber. Additionally, tuning curves also illustrate which frequency/intensity combinations excite the cell. As the intensity of a sound rises the fiber is excited by a larger range of frequencies broadening the tuning curve into its characteristic 'V' shape. Generally, frequency tuning of auditory fibers originating in the AP and BP is directly correlated to animal size and animal sex. Larger frogs have AP and BP fibers tuned to lower frequencies compared to smaller species,

and males generally have fibers tuned to higher frequencies compared to those of females (Lewis 1981, Wilczynski 1992).

The activity of 8th nerve fibers can also be suppressed by the simultaneous presentation of sounds having the appropriate frequency/intensity combinations. This occurs when the response of one fiber to a tone is reduced by another tone of a higher, or lower, frequency (Liff 1970, Rose 1985, Benedix 1994). Single tone suppression is found throughout all fibers of the 8th nerve (Simmons 2007).

In response to temporal stimulus features, auditory nerve fibers typically fire throughout the duration of the stimulus and accurately represent the fluctuations in sound intensity level (amplitude modulation (AM) rate), up to at least 100Hz. Fibers tend to spike at a particular phase of the modulation cycle forming a periodicity code for low frequency amplitude modulation. As the frequencies increase the fibers are no longer able to phase lock and therefore become non-selective for AM coding (Feng 1975, Capranica 1978, Rose and Capranica 1983, Dunia and Narins 1988, Ronken 1993).

2.2 Central auditory system

The central auditory system of the bullfrog follows an evolutionarily conserved organization like the auditory systems of birds and mammals. The following sections are to provide evidence that the results from this study can be extrapolated to pertain to mammals. Also, the source of NO production in the TS is not necessarily limited to the TS because of abundance of afferents coming to the TS from other auditory regions that stain positive for NOS (Boyd 2006).

2.2.1 Dorsal medullary nucleus (DMN)

The central auditory pathway of bullfrogs reflects that of auditory pathways conserved in other tetrapods and starts with the dorsal medullary nucleus (DMN) located in the medulla (Wilczynski 2007**)**. The DMN is the first auditory nucleus, and the only one that receives projections from the 8th nerve (Gregory 1972, Fuzessary 1981) (Figure 3). Incoming 8th nerve fibers distribute throughout the ipsilateral DMN with BP fibers terminating in the dorsomedial region of the nucleus and AP fibers terminating throughout but represented more ventrally thereby forming a ventrolateral-to-dorsomedial tonotopic map (Wilczynski 2007, Fuzessary 1981, Feng 1996).

Cells in the DMN project to the contralateral DMN, bilaterally to the superior olivary nucleus (SON) and to the contralateral torus semicircularis (TS). (Pettigrew 1981, Feng 1986). There are descending reciprocal projections from both the torus and superior olive. Moreover, the DMN receives descending input from the nucleus of the lateral lemniscus (Feng 1986 (Feng 1986, 1991).

The DMN's chemoarchitecture can also be used to differentiate its different regions. Gamma-aminobutyric acid- (GABA-) cells are concentrated in the medial and ventral aspect of the DMN while acetylcholinesterase positive cells are found throughout the DMN **(**Hall & Bunker 1994, Simmons & Chapman 2002**)**. Most relevant to this study is the presence of nicotinamide adenine dinucleotide phosphate diaphorase (NADPHd positive cells), which indicate the presence of cells with nitric oxide synthase (Hope 1991). These NADPHd positive cells are concentrated in the medial portion of the DMN (Munoz 1996).

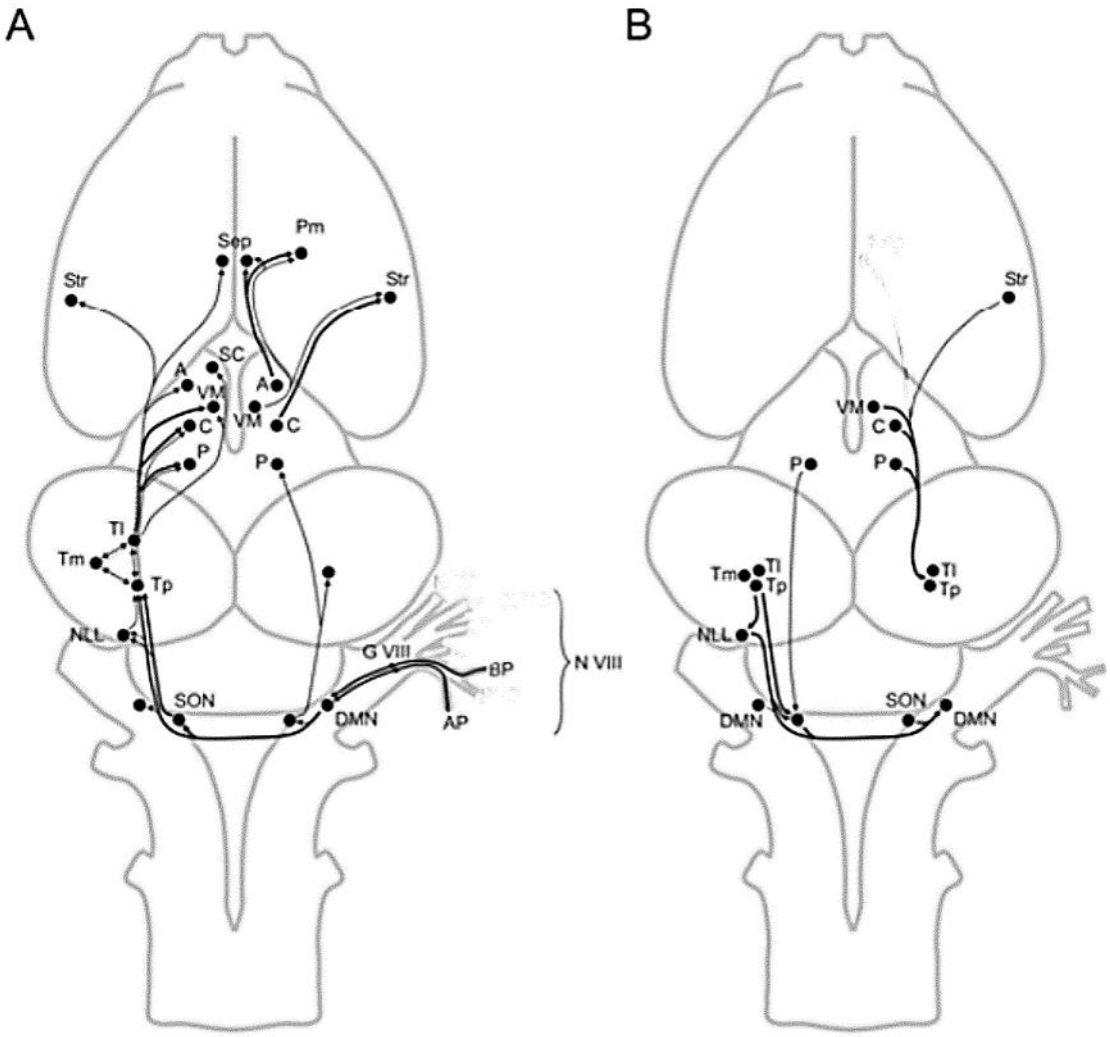

Figure 3. Ascending and descending auditory pathways. A: ascending auditory pathway. B: descending auditory pathway. N VIII 8th nerve, AP amphibian papilla, BP basilar papilla, G VIII vestibulocochlear ganglion, DMN dorsal medullary nucleus, SON superior olivary nucleus, NLL nuclei of lateral lemniscus, T_p principal nucleus of TS, T_m magnocellular nucleus of TS, T_L laminar nucleus of TS, P posterior thalamic nuclei, C central thalamic nuclei¸ VM ventromedial thalamic nucleus, A anterior thalamic nucleus, Str striatum, Sep septal complex, Pm medial pallium (Figure adapted from Wilczyinski 2007)

Neurons in the DMN can be characterized based upon differences in morphology and discharge patterns to tone bursts (Feng 1996). Moreover, they have V-shaped excitatory tuning curves resembling those of the auditory-nerve fibers that innervate them and exhibit two-tone suppression (Feng 1976, Fuzessery 1983).

Differences between the activity of DMN neurons from those of 8th nerve fibers include the following: 1) the majority of DMN neurons show little spontaneous activity, 2) both phasic and phasic-burst response patterns emerge at the level of the DMN as does 3) selectivity for stimulus rise time, duration and amplitude modulation rate. DMN neurons' responses tend to be strongest when amplitude of a stimuli rises very quickly indicating a sensitivity to call rise-time (Hall and Feng, 1991). Thus, the DMN contributes significantly to the first stage at which behaviorally relevant temporal parameters of the advertisement call are processed (Wilczynski 2007, Hall 1988, 1991, 1996).

2.2.2 Superior olivary nucleus (SON)

The SON is positioned medial and ventral to the DMN but extends along the same axis (Wilczynski 2007). It receives direct bilateral input from both the contra- and ipsilateral DMN, with the primary input coming from the contralateral side (Feng 1986b) (Fig.3). The input is ordered tonotopically with higher frequencies represented in the ventral, lower frequencies in the dorsal, aspect of the nucleus.

Unlike the DMN there are no obvious chemoarchitecture subdivisions in the SON with GABA, acetylcholinesterase, and NADPHd labeled neurons spread throughout (Simmons & Chapman 2002, Hall and Bunker 1994, Boyd 2007). One significant

difference in the chemoarchitecture of the SON compared to the DMN is the presence of dopaminergic input (Gonzalez and Smeets 1991, 1993).

The SON sends projections to the ipsilateral nucleus of the lateral lemniscus and the torus, the latter being primarily ipsilateral (Fig. 3). Descending input to the SON is derived from the ipsilateral posterior thalamic nucleus as well as the ipsilateral torus and nucleus of the lateral lemniscus. (Feng 1986b).

Most neurons in the SON have V-shaped frequency tuning curves similar to those in the DMN (Zheng 2000). However, about 20% have complex tuning curves having an asymmetric V-shape, U shape, or W shape, while others are closed. Moreover, all SON neurons contain inhibitory flanking regions above or below their characteristic frequency (Zheng 2000) which stands in contrast to that seen in the DMN where flanking inhibition is seen only at frequencies higher than the cell's CF. Thus, there is a major transformation in the representation of stimulus frequency at the level of the SON.

Neurons in the SON and DMN exhibit similar discharge patterns to tone burst stimuli, however discharge patterns vary between the two nuclei; cells having phasic burst response patterns are more numerous in the SON. (Condon 1991, 1995).

Similar to the DMN, neurons in the SON play an important role in the processing of the temporal features of complex sounds including rise time, duration and amplitude modulation rate. With one important exception, equivalent neuronal response functions to these temporal features are similar in both structures. However, there is a population of neurons in the SON, but not DMN, that shows band-pass response functions to the

rate of amplitude modulation (Condon, 1991). This represents the first appearance of neurons tuned specifically to the rate of amplitude modulation, an important temporal cue for call recognition.

2.2.3 Nucleus of the lateral lemniscus (LL)

Fibers from the DMN and SON gather into the lateral lemniscus (LL) before proceeding to the TS. It receives projections from and connects reciprocally to the DMN and SON. The LL projects to the central part of the torus semicircularis (Fig. 3). It has no obvious subdivisions like the lower brainstem nuclei and contains GABA, acetylcholinesterase and NADPHd positive cells (Simmons and Chapman 2002, Hall and Bunker 1994, Boyd 2007). Single unit recordings from LL neurons reveal that they respond to either tones or amplitude modulated white noise and their thresholds are comparable to those found in the TS. (Rose and Wilczynski 1984).

2.2.4 Torus semicircularis (inferior colliculus)

The TS is the amphibian homologue to the mammalian inferior colliculus (IC). The TS is the only midbrain auditory structure in amphibians and an obligate relay center, receiving inputs from all auditory nuclei below and above it in the auditory pathway (Wilczynski 1981, 2007, Endepols 2001) (Fig. 3). It sends ascending projections to both the posterior and central thalamic nuclei, and descending projections to all lower auditory nuclei (Fig.3). Based upon differences in cytoarchitecture, the TS is divided into different subdivisions: the laminar, magnocellular and principal nuclei (Potter 1965).

Cells in the TS show selectivity for both the temporal and spectral features of the advertisement call. It is the first site along the ascending auditory pathway where neurons serving as putative mating call detectors appear (Fuzessery 1983, Rose 1983, Gooler 1992) making it a logical target for investigating the neural basis of signal recognition.

Principal nucleus

The cells of the principal nucleus (T_p) are organized into clusters (Potter 1965, Wilczynski 2007). It is the primary target of ascending auditory fibers from the contralateral DMN, and ipsilateral SON and LL (Feng 1991, Kulik 1994, Walkowiak 1994. Matesez 1996**)**. The T_p receives ascending afferents from other sensory systems as well: 1) somatosensory input from the contralateral dorsal column nucleus, 2) vestibular input from the contralateral ventral and caudal octaval nuclei, and 3) visual input from layer 7 of the optic tectum **(**Munoz 1994, 1997, Donkelaar 1998, Matesz and Kulik 1996**)**. Thus, in addition to being a major auditory processing center, it is also involved in multisensory integration.

Descending afferents to the T_p originate from different brain regions including the thalamus and suprachiasmatic nucleus (Wilczynski 1981, Feng and Lin 1991). This is important since bullfrog mating behaviors start at dusk, with responses of TS neurons potentially being modulated by inputs from the SCN depending on time of day (Backwell 1988, Gerhardt 2002). While ascending auditory efferents project to the central and posterior thalamic nuclei, descending efferents project to the ipsilateral LL and SON, and bilaterally to the DMN **(**Walkowiak 1998, Feng and Lin 1991**)**.

Somata of T_p neurons are either medium (9x10µm) or small (6x9µm), with small being predominant. Medium-sized cells have spiny dendrites covering large areas of the entire torus while small-sized cells have mostly smooth dendrites distributed locally throughout the principal nucleus. A variety of morphologically distinct cell types have been identified including unipolar, bipolar, multipolar, spherical, ovoidal and triangular (Feng 1983, Luksch 1998).

There are no unique chemoarchitecture differences to distinguish the T_p from the other toral nuclei. T_p neurons are immunoreactive for acetylcholine, aspartate, substance P, and most significantly for this study, NADPHd (Inagaki 1981, Hall and Bunker 1994, Endepols 2000, Bruning and Mayer 1996).

Laminar nucleus

The laminar nucleus (T_L), lying directly beneath the tectal ventricle and above the principal nucleus, is characterized by its alternating layers comprised of somata and fibers (Wilczynski 2007). In contrast to the T_p, the T_L receives weak auditory input, however the dendrites of T_L neurons extend into the T_p most likely receiving some input from lower auditory nuclei (Wilczynski 2007). Like the T_p the T_L receives ascending projections from the vestibular, somatosensory and visual systems.

Efferents from the T_L project bilaterally to the optic tectum and tegmental nuclei, and to diencephalic and telencephalic targets. Descending efferents terminate in the lower auditory regions, as well as motor regions in the medulla (Strake 1994).

As with the T_p, unipolar, bipolar, multipolar, spherical, ovoidal and triangular shaped cells can be identified in the T_L (Feng 1983). In contrast to the T_p, T_L somata are mostly medium sized (8 x 10µm to 9 x15 µm) (Luksch and Walkowiak 1998).

Cells in the T_L are immunoreactive for the neurotransmitters acetylcholine, GABA, and aspartate, as well as staining positive for NADPHd and a host of neuropeptides (Hall and Bunker 1994, Endepols 2000, Merchenthaler 1989, Adli 1999, Boyd 2007). A significant difference between the T_L and other toral nuclei is the presence of estrogen binding proteins and cytosolic progestin receptors, with the former being implicated in female phonotaxis (Roy 1986, Endepols 2000, Chakraborty 2015).

Magnocellular nucleus

The magnocellular nucleus (T_m) is in the caudal part of the TS occupying the space ventral to the T_p (Wilczynski 2007). It receives weak auditory input but receives fibers from the dorsal horn and vestibular system (Wilczynski 2007). Like the other two TS nuclei, the magnocellular nucleus sends projections to all the lower auditory nuclei (Feng 1991).

The T_m consists of neurons with large (14 x 18µm) and very large (16 x 20µm) somata (Luksch 1998, Wilczynski 2007). The large cells are located in the medial portion of the T_m and their spiny dendrites extend into the T_p, while the very large cells are more frequent in the lateral T_m where their dendrites extend into the ventral torus and tegmentum (Wilczynski 2007).

Similar to the other toral nuclei, the T_m receives somatosensory input from the dorsal column nucleus and vestibular inputs from the octaval nuclei (Wilczynski 1981,

Will 1985b). Like the T_L, it is innervated by fibers arising from the contralateral dorsal horn at the level of the spinal cord. Compared to the other toral nuclei, the T_m receives very little descending input (Wilczynski 2007).

Ascending efferents from the T_m project bilaterally to the optic tectum and tegmental nuclei, and to diencephalic and telencephalic targets like the T_L (Neary 1988, Feng and Lin 1991). Descending T_m efferents project to the lower auditory nuclei (Matesz and Kulik 1996, Feng and Lin 1991).

The T_m uses GABA, aspartate and acetylcholine as neurotransmitters like the other toral subdivisions. There is also a significant amount of NADPHd staining indicating the possibility that NO is used as a neuromodulator, altering the release and sensitivity to the various neurotransmitters present in the TS (Endepols 2000, Hall and Bunker 1994, Boyd 2007).

Spectral stimulus processing in the TS

Spectral processing of stimuli is more complex in the TS compared to lower nuclei. A good portion of the neurons (37%) are sensitive to single frequencies and display V-shaped tuning curves like the lower auditory nuclei. TS units with V-shaped tuning functions possess inhibitory flanking regions above or below their excitatory regions, which is mediated by bicuculline (Hall 1999). Approximately 16% of toral units have closed tuning functions. Their excitatory receptive fields are a discrete region in frequency-amplitude space. Some TS units (9%) contain W- shaped tuning curves indicating the merging of information from the basilar and amphibian papilla. While units with closed and W-shaped tuning curves are present in the SON, they are far more

common in the TS. The neurons with W-shaped tuning curves will respond when energy is present in only one of the excitatory regions, but some are AND neurons. These neurons respond most robustly when energy from both of its excitatory receptive fields is present (Rose 2007). This response pattern correlates to the prominent frequencies in the advertisement call and represent the matched filter specialization of the anuran auditory system. A major transformation in spectral coding is that approximately 1/5 of toral neurons show level-tolerant frequency sensitivity. This means the frequency band of excitation remains the same regardless of stimulus intensity.

Temporal stimulus processing in the TS

Temporal stimulus processing is what really sets the TS apart from the brain stem auditory regions. There are some similarities such as the temporal discharge patterns of TS units which fall into three categories, primarylike (67%) which respond throughout the duration of the stimulus, phasic (19%) which respond with a spike or two at the very beginning of the stimulus, and phasic-burst (14%) which respond with a cluster of action potentials at the beginning of the stimulus. These discharge patterns resemble those in the DMN and SON with phasic units responding best to short rise times and being insensitive to stimulus duration. Units with primarylike responses are nonselective for rise-time and reflect the duration of a stimulus in their response (Gooler 1992, Penna 1997, 2001).

However, there are several unique processing tasks carried out by TS neurons. First, some TS neurons (20%) show duration-selective responses. This means stimuli of particular durations elicit the maximal response from these units. Even though stimulus

duration is represented in lower nuclei, this type of stimulus length preference is novel in the TS, representing a major transformation in information processing seen in the midbrain (Narins 1980, Gooler 1992, Penna 1997). TS neurons are also sensitive to stimulus rise time. (Gooler and Feng 1992).

Next, amplitude modulation (AM) representation is more refined in the TS compared to the DMN and SON. (Walkowiak 1980, 1984, Epping 1985, Gooler 1992). Modulation tuning functions (response level vs AM rate) are level tolerant and categorized as low-pass, high-pass, band-suppression, or band-pass. Neurons that are classified as high pass respond to faster AM rates, neurons that are classified as low-pass respond to slower AM rates, band-suppression neurons are inhibited by certain ranges of AM rates, and band-pass neurons are sensitive to certain ranges of AM rate. The distribution of AM tuning functions is species-specific and related to the range of pulse repetition rates observed in the species-specific advertisement calls (Rose and Capranica 1984, Rose 1985). In direct contrast with auditory nerve fibers, there is little periodicity coding of AM rate in the TS. Units that do encode AM rate in time-locked discharges only do so up to 50Hz of AM (Eggermont 1990). This is consistent with what is seen in other vertebrates where AM representation transforms from a periodicity code in the auditory nerve fibers to a temporal filter ensemble in the midbrain (Rose and Capranica 1985, Dunia and Narins 1989, Wilczynski 2007**)**.

There are two mechanisms that are currently proposed to account for AM selectivity: integration and recovery processes. Bandpass and low-pass units that respond to each stimulus pulse at slow AM and pulse repetition rates (PRR) are called

“recovery neurons”. These units respond phasically to each pulse and require a recovery period before another pulse can excite the cell. This process allows a frog to differentiate between intraspecific calls that differ primarily in PRR, but not pulse rise/fall time or duration. Integration processes underlie the other mechanism, which gives rise to AM selectivity and are selective for intermediate or fast PRRs. Neurons of this type only respond after a threshold number of pulses, each separated by cell specific interpulse interval times (Alder 1998, 2000, Edwards 2002). As a whole, AM selectivity in the TS is derived from a combination of integration and recovery processes, along with rise/fall times and duration sensitivity.

2.2.5 Auditory thalamus

The posterior and central thalamic nuclei receive projections from the TS and are sensitive to acoustic stimuli (Fig. 3). However, the anuran thalamus receives afferents of roughly equal strength from auditory, somatosensory, vestibular and visual systems (Hall and Feng 1987, Munoz 1994, 1995; Roth 2003, Westhoff 2004). Because of its multisensory inputs none of the thalamic nuclei are specific to auditory processing. The central thalamic nucleus plays a role in the transmission of multimodal and limbic information rather than segregated auditory processing (Endepols 2003).

The posterior thalamic nucleus is innervated from the T_L and projects to the striatum, but mostly has descending efferents that terminate in several areas including the TS. Like the T_L, the poster thalamic nucleus contains sex steroids and express progestrin receptors. Females have higher estrogen receptor expression than males, while estradiol has been shown to modulate phonotaxis in females (Morell 1975, Kelley

1981, Roy 1986, Chakraborty 2009, 2010). The central thalamic nucleus is also innervated from the T_p and T_m (Hall 1987).

Descending projections from the thalamus arise from both the central and posterior nucleus. The most prominent projections come from the posterior thalamus and terminate in the T_L and T_p (Wilczynski 1981) (Fig. 3). Also, from the posterior thalamus is a weak auditory projection to the ipsilateral SON, and a projection to motor areas in the medulla **(**Matesz and Kulik 1996, Dicke 1998**)**.

Spectral processing in the thalamus

Spectral processing occurs primarily in the posterior thalamic nucleus. To date there is no evidence that toral inputs are topographically organized to form a tonotopic map. A large portion of thalamic units are spectrally tuned to both dominant frequency ranges in the advertisement call. Approximately 33% of the neurons respond only when low- and high-frequency energy from the advertisement call is presented simultaneously (Fuzessary 1983). These represent “AND” neurons and is a significant increase from the approximately 9% of toral units that respond in similar fashion.

Temporal processing in the thalamus

Temporal processing happens in the central thalamic nucleus and selectivity for pulse duration and AM becomes more prominent (Fuzessary 1983, Hall 1986, 1987). Approximately 60% of units respond to tone bursts only if they are a short duration compared to 9% in the torus. Likewise, 20% of thalamic units are sensitive to particular tone burst duration, while only 12% of toral units are (Hall and Feng 1986).

The transformation of AM coding for a periodicity code to a rate-based code is particularly evident in the thalamus. There is little evidence of thalamic neurons coding AM rate in the temporal discharge pattern of their action potential response. This means that like the torus, thalamic units are either low-pass (22%), high-pass (26%), bandpass (45%), or band-suppression (7%) (Feng and Hall 1986, Feng 1990**)**. This is indicative of a more complex form of signal processing than is seen in the lower auditory nuclei, because neurons are now tuned to specific parameters of acoustic stimuli. This also shows that the highest level of auditory processing that occurs in the frog's brain initially happens in the TS.

2.3 Summary

Acoustic communication plays an important role in the social behavior of anurans. Anuran vocalizations are highly species-specific, and their auditory system is tuned to specialize in extracting biologically relevant information from a noisy acoustic background. Tuning properties of neurons become increasingly complex as information ascends through the auditory system. This is evidenced by simple V-shaped tuning curves at the auditory periphery to highly selective AND neurons or counting neurons in the inferior colliculus. Likewise, the transformation of AM processing starts as a periodicity code at the 8th nerve level and transforms into a rate code by the level of the thalamus.

While there has been much work done on evoked calling and phonotaxis to hetero- and conspecific calling, how those distinctions are coded in the auditory system is poorly understood. Moreover, virtually no work has been done on if nitric oxide

modulates those responses to species calls in the auditory system even though NO is present throughout. The TS is an obligate relay in the central auditory system, sending and receiving projections from all other auditory nuclei with nitrergic neurons present in its nuclei. This makes it an ideal location to study the effects of NO on the coding of species-specific calls and the discrimination of those calls in the auditory system.

3. Nitrergic modulation of sensory processing

3.1 Nitric Oxide

Nitric Oxide (NO) is a pleiotropic, membrane permeable, neuronal messenger that carries out diverse signaling tasks in the central nervous system (CNS) such as learning and memory formation, feeding, sleeping, reproductive behaviors, aggression, sensory, and motor function (Schuman 1994, Garthwaite 2008, Stevenson 2015, Jacklet 2004, Kemenes 2002). These roles for NO have been evolutionary conserved dating back to animals with some of the most primitive nervous systems (Moroz 2004, Garthwaite 2008). The distribution of NOS is present throughout multiple phyla providing evidence for an ancient role as a neuronal messenger and a great deal of conserved evolutionary function across species. Significantly, NOS has been confirmed to be present in the central nervous systems of humans, rats, cats, mice, hamsters, leeches, crickets, salamanders, turtles, and frogs (Hinova-Pilova 2017, Druga 1993, Paloff 1998, Leake 1996, Eldred 2005, Huynh 2006, Reuss 2000, Stevenson 2015). This provides confidence that the findings we make in the bullfrog auditory system regarding NO’s role in stimulus processing, can be applied to other species.

In the brain, NO is produced by neuronal nitric oxide synthase (nNOS) and can freely diffuse from its production sites without the aid of specialized cellular machinery (Schuman 1994, Liu 1996, Cudeiro 1999, Alderton 2001, Denningher 1999). Unlike other neurotransmitters, NO is not stored in vesicles prior to release, but is newly synthesized in cells as cytosolic calcium concentrations increase. This independence from cellular docking and release machinery allows it to readily function as a postsynaptic and presynaptic volume messenger (Cudeiro 1994).

3.2 Nitric oxide synthase

There are three different isoforms of neuronal nitric oxide synthase (nNOS) present in the nervous system: nNOSα, nNOSβ and nNOSγ. The nNOSα isoform accounts for 95% of expressed nNOS activity in mouse brain (Huang 1993). The other two nNOS isoforms, nNOSβ and nNOSγ lack the PDZ domain and the γ variant lacks enzymatic activity. It is also noteworthy that the β variant is upregulated in mice lacking the NOSα isoform, especially in the striatum and the cerebral cortex (Langnaese 2006). This could suggest some function under pathological or cerebral injury conditions, however the nNOSα isoform variant is the predominantly active nNOS isoform present in the central nervous system (Langnaese 2006, Hunag, 1993). Before producing NO, nNOS is activated by the calcium-calmodulin complex. This process is started through the interaction of NOS with *N*-methyle-D-aspartate receptor (NMDA) receptors via postsynaptic density protein-95 (PSD-95) through specialized PDZ domains (Brenman 1996, Garthwaite, 1995, 1988). Glutamate binds to the NDMA receptor inducing calcium influx which activates the calcium calmodulin complex. The complex binds and activates

NOS in a reversible calcium dependent manner, which converts L-arginine to citrulline and NO, with NADP acting as an electron donor (Figure 4) (Bredt 1990, Liu 1996, Lima 2014). NO is then free to act on many intra- and extracellular targets (Garthwaite 2008, Steinart 2010, Schuman 1994, Bredt 1991).

There is evidence supporting a close proximity between nNOS and NMDARs. First, nNOS contains several putative sites for phosphorylation allowing for post-translational modifications, and NMDAR-dependent phosphorylation of NOS S1412 by protein kinase B is required for NOS activation (Komeima 2000, Rameau 2007). Second, NMDAR1 subunit expression is higher in nNOS positive neurons compared to non-positive neurons (Price 1993). However, there are positive nNOS cells that do not express NMDAR1 receptors, but instead NMDAR2D receptors are expressed (Dunah 1996). Finally, Burette et al. demonstrated through triple immunostaining that NR2A/B-containing NMDARs, nNOS, and PSD-p5 all co-localize with each other in the hippocampus dendritic spines (Burette 2002).

3.3 Diffusion of nitric oxide

Unlike other neurotransmitters NO is a volume transmitter; i.e., it diffuses from its point of production and can migrate to affect the activity of any combination of presynaptic, postsynaptic, and neighboring neurons (Steinart 2008, Agnati 2010) (Figure 5). Its low molecular weight (30g mol^{-1}), hydrophobic nature, and high diffusion constant (3.3µm/s^2) make it well suited to pass seamlessly and quickly through membranes in an estimated 3 nanoseconds (Lancaster 1994, 1996, Chachlaki 2017).

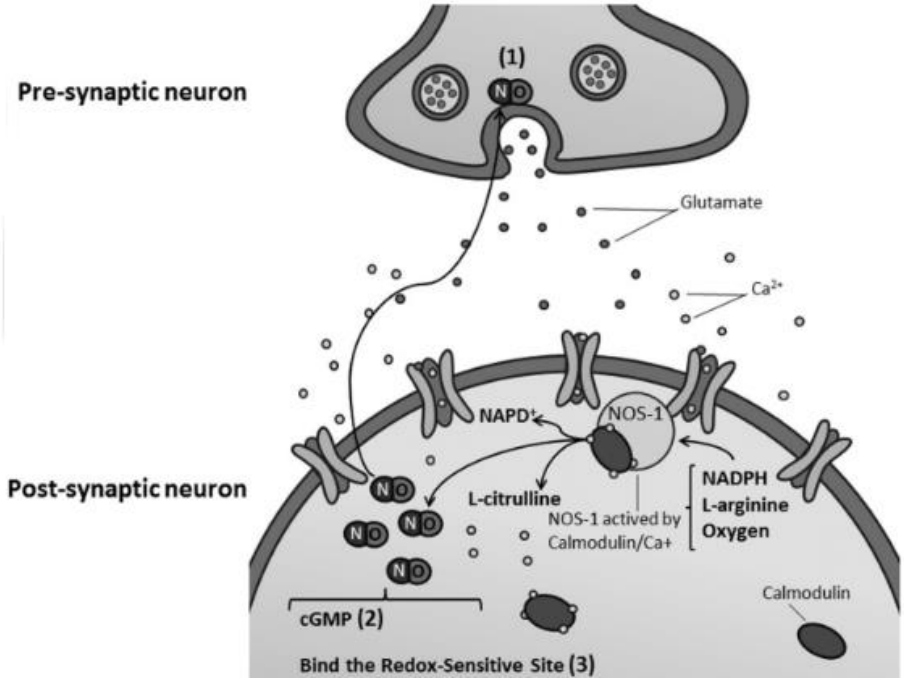

Figure 4. NO synthesis. Glutamate binds to NDMA receptor consisting of NR1 and NR2B subunits. Ca^{2+} flows into the cell forming a complex with calmodulin (CaM). CaM binds neuron nitric oxide synthase (nNOS) converting L-Arginine (L-Arg) to nitric oxide (NO) and L-citrulline. (Lima 2014)

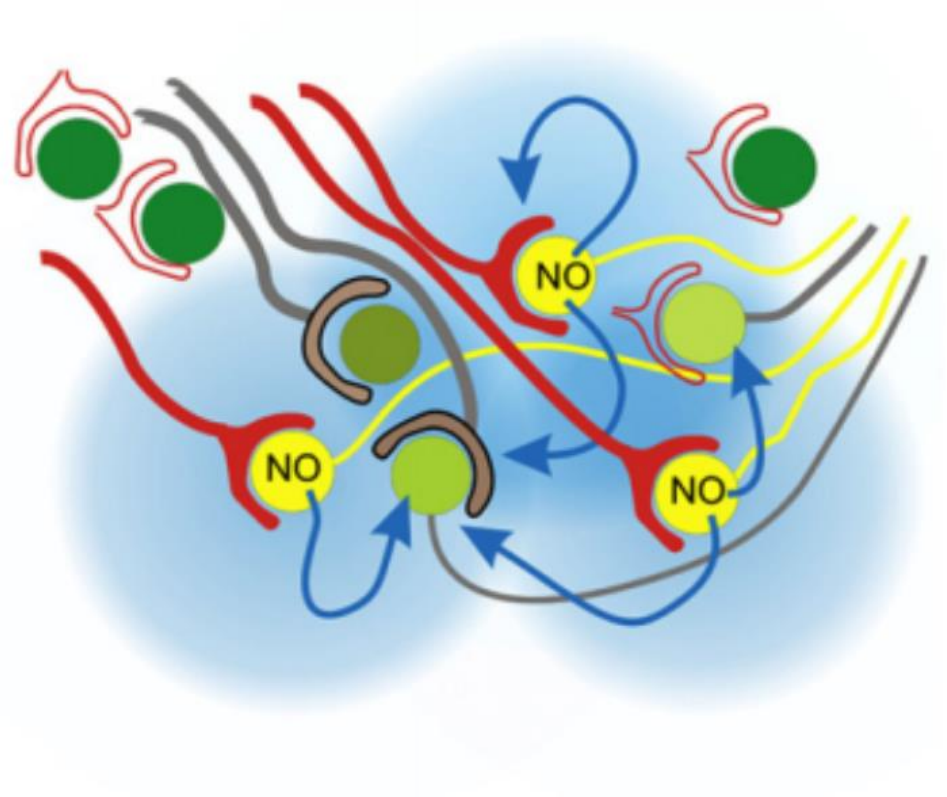

Figure 5. Volume transmission of NO. Depiction of NO diffusion from cells of synthesis. Yellow colored cells represent NO producing neurons. The blue surrounding those cells represents NO's spread. Arrows indicate which cells can be influenced by NO. NO's sphere of influence can modulate firing of cells that aren't directly connected to NO producing cell (Adapted from Steinart 2008).

These characteristics also give NO the ability to spread in three dimensions to neighboring cells since NO spreads equally in all directions from its point of production. Voltammetry experiments and mathematical diffusion models have determined that NO's spread has a maximal radius of 500µm, from a single source **(**Wood 1994, Meulemans 1994). However, because of its short half-life (~5 secs), and the fact that it can be scavenged by molecules such as hemoglobin, NO's sphere of influence doesn't typically reach over 100 - 200µm (Hill 2010, Lancaster 1996, Malinski 1993, Santos 2011, Schuman 1994). Other than hemoglobin, metalloproteins such as soluble guanylyl cyclase and cytochrome *c* oxidase and free radicals such as O_2^- and lipid peroxyl radicals are also targets for NO (Hill 2010). NO's sphere of influence can encompass the area of a synapse ($20nm^3$) or even entire somata ($10\mu m^3$). However, if its source is within a neuropil, NO could spread over a volume that contains roughly 2 million synapses (Wood 1994). *In vivo* experiments in rat hippocampal cells using a NO sensor showed a maximum range of 400µm from its source, corroborating the model's conclusions (Ledo 2005). Recently, using ultrasensitive detector cells, there is now evidence that NO produced at a given synapse may only affect neuronal function within submicrometer distances due to the fact that very low concentrations are produced physiologically (Wood, 2011). Combined these studies seem to show that NO's modulatory capabilities are defined by the environment in which it is released.

Overall, all the research agrees that the influence of NO is not limited to the cell in which it is produced. It is important to note that NO's spread is not a linear trajectory since the spread of the molecules is a random process, meaning the molecule is just as

likely to diffuse in one direction as another, so the maximal distance a NO molecule travels is not necessarily the distance it would travel unhindered in the brain. Ultimately, the diffusion of NO depends on the amount of NO produced, the number of targets in the area, and the number of scavenging molecules, such as hemoglobin, present. Currently, no studies have been performed measuring the diffusion of NO in the torus semicircularis, or any auditory region, to this author's knowledge.

3.4 Mechanisms of NO modulation

NO stimulates soluble guanylate cyclase (sGC) to produce cyclic guanosine3'-5'-monophosphate (cGMP) is the most well defined NO signal transduction pathway within the nervous system **(**Billar 1992, Bryan 2009). Newly synthesized NO diffuses rapidly and binds the heme site of sGC, which then catalyzes the conversion of cGTP to cGMP (Denninger 1999). As a second messenger, cGMP goes on to activate a variety of effectors like cyclic nucleotide gated ion channels, cGMP-dependent kinases, and cGMP regulated phosphodiesterases (Bryan 2009, Francis 2010). This pathway allows small amounts of generated NO to be amplified substantially since cGMP will be produced until NO is released from sGC (Francis 2010). Subunits of soluble guanylyl cyclase, the enzyme that produces cGMP, have been identified in the rat inferior colliculus providing evidence that it would be a target in the mammalian auditory system for NO (Gibb 2001). While no experiments confirming the presence of soluble guanylyl cyclase in the TS have been performed, the presence of NO is a good indication that it is there since that is the primary target of NO.

3.4.1 Depolarization of resting membrane potential

One common way NO modulates neuronal activity is through depolarization of the cells' resting membrane potential. In the rat CNS, NO increases neuronal excitability by elevating intracellular cGMP, which increases membrane conductance, and depolarizes resting membrane potentials (Yang 1999, Yassin 2014, Tozer 2012). In the paraolivary nucleus, NO depolarizes resting membrane potential by cGMP-dependent suppression of the Cl^- channel KCC2. When regulated by cGMP, the internal Cl^- concentration inside the cell decreases, which then depolarizes the resting membrane potential, making the neuron more likely to fire (Yassin 2014). In the rat medial nucleus of the trapezoid body (MNTB) neuronal excitability is increased via cGMP potentiation of voltage gated calcium channels (Tozer 2012). The activity of the feeding circuit in the mollusk *Aplysia* is modulated by the NO-cGMP pathway (Jacket 2004). When SNAP (a NO donor) is applied to the cell, depolarization of the resting membrane potential occurs, and applying a cGMP analog mimics the effect elicited by applying the NO donor.

3.4.2 Facilitation of neurotransmitter release

In the process of long-term potentiation (LTP) in the hippocampus, NO works as a retrograde signaling molecule to enhance presynaptic neurotransmitter release (Arancio 1996). In the olfactory system postsynaptic NO release enhances presynaptic release of glutamate and GABA (Kendrick 1997) (Figure 6). Experiments in the guinea pig cerebral cortex also show that NMDA receptor-mediated release of glutamate and norepinephrine is blocked when production of NO is inhibited, or NO scavengers are

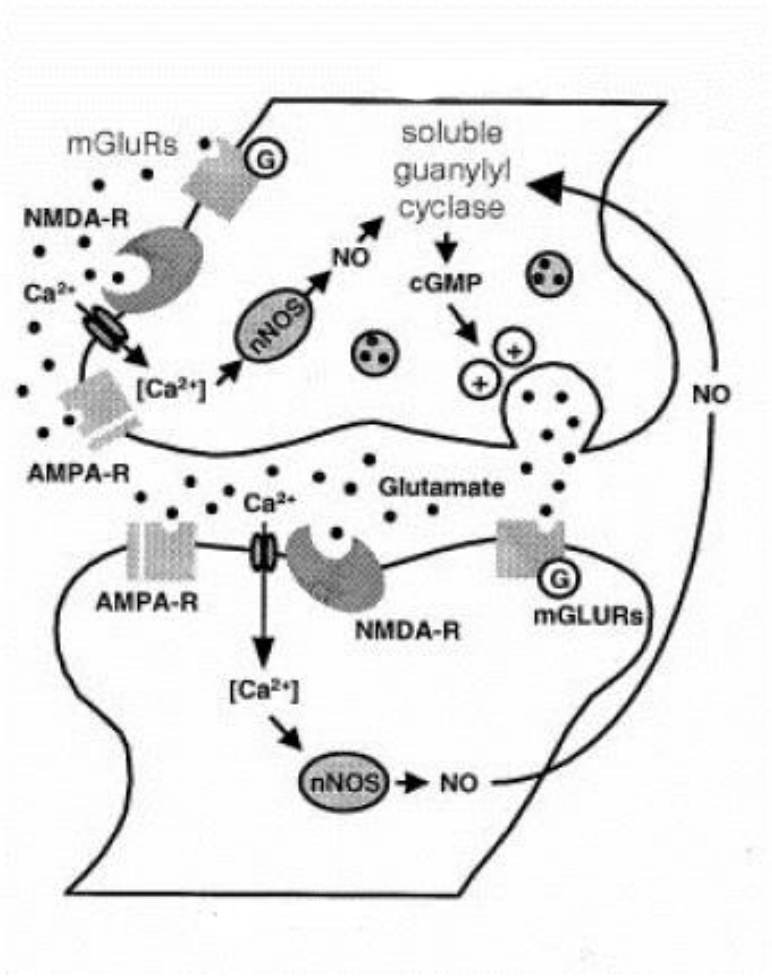

Figure 6. Presynaptic modulation of neurotransmitter release. Nitric oxide synthesized in post-synaptic cell diffuses back to the pre-synaptic cell activating soluble guanylyl cyclase enhancing the release of neurotransmitter (Adapted from Kendrick 1997).

introduced to the system indicating NO's function in presynaptic modulation of neurotransmitter release (Montague 1994). Increased levels of cGMP also affect transport of monoamine neurotransmitters in or out of neurons via cGMP dependent kinases. Sodium nitroprusside (SNP), a NO donor, increases the release of dopamine (DA) in a dose dependent manner via phosphorylation of DA transporters in the cell membrane (Zhu 1992). When this transport is affected, the amount of DA present in the extracellular space increases which then goes on to affect the firing dynamics of neighboring neurons. This is one way which NO modulates the firing of neurons through extra-synaptic interactions.

3.4.3 Other mechanisms of nitrergic modulation

S-nitrosylation mechanism

In higher concentrations (nano- to micromolar) NO also acts on thiol groups of membrane receptors modulating their function. This form of posttranslational modification is called s-nitrosylation, involving the covalent and reversible attachment of a NO molecule to a thiol group (-SH) on cysteine residues with the general structure R-S-N=O (Foster 2003, Bryan 2009). The likelihood of S-nitrosylation increases as the hydrophobicity of the environment increases because lipids partition NO and O_2 molecules raising the concentrations of NO high enough for s-nitrosylation to occur (Liu 1998). Membrane receptors and ion channels are ideal candidates for S-nitrosylation with NO either potentiating or suppressing the activity of K^+, Na^+, Ca^{2+}, NMDA, and cyclic-nucleotide-gated ion channels (Nakamura 2008, Ahern 2002). The most prominent postsynaptic receptor to undergo S-nitrosylation is the NMDAR (Bradley

2016). This is due to a negative feedback pathway which helps protect the cell from excitotoxicity. As calcium continues to enter the cell and activates NOS, intracellular NO levels increase. NO levels eventually get high enough in the cell that the NDMAR gets s-nitrosylated, dampening NMDAR-mediated responses (Ahern 2002).

3.5 Nitric oxide donors and inhibitors

Being a gaseous molecule, NO cannot be applied directly, onto neurons using traditional techniques like iontophoresis to study its effects. To overcome this shortcoming, different donor, substrate, or inhibitory molecules are introduced to the system. NO donors and substrates are compounds that release NO upon degradation or promote its synthesis by providing components of the synthesis pathway respectively. NO donors, which are applied pharmacologically, include compounds such as sodium nitroprusside (SNP), S-nitroso-N-acetylpenicillamine (SNAP), and 2-(N,N-diethylamino)-diazenolate-2-oxide (DEA/NO). In this way, exogenous NO is supposed to mimic the signaling effects of endogenous NO, in the absence of stimuli initiating the synthesis of the latter. One must be careful using donors since they can raise NO levels far above physiological levels. Alternatively, L-Arginine, the substrate for NOS can be applied. This method more accurately reflects the amount of NO that will be produced by cells since its production is limited by the amount of NOS in the area.

Another way of observing the effects of NO in a system, is to remove NO from the system, or block its receptors, and record the results. This can be accomplished by either introducing compounds that will scavenge NO out of the system, such as hemoglobin, or by preventing the production of NO in the first place. This can be

accomplished by introducing compounds that inhibit NOS, preventing the release of endogenous NO at a target source. The most commonly used inhibitors of NOS are L-N^G monomethyl arginine (L-NMMA), N^G-nitro-L-arginine (L-NOArg), and L-N^G nitro arginine methyl ester (L-NAME). These compounds serve as competitive inhibitors to L-Arginine binding of NOS and thus prevent the synthesis of NO. Due to NO's short half-life (a few seconds) NO is cleared from the system shortly after NOS inhibition.

3.6 Location of nitric oxide producing neurons in the torus semicircularis

Before studying the effects of NO in any part of the CNS it is reasonable to ensure NO is present in the first place. Since NO is a gaseous molecule with a very short half-life, identifying NO-expressing neurons must be done involve indirect methods. The most common techniques used to identify neurons for the presence of NO is to label cells for the presence of NOS. NOS immunocytochemistry (IHC) and NADPH diaphorase (NADPHd) histochemistry are the two most common techniques used to identify neurons that contain NOS. For IHC staining the primary antibody was raised in rabbit against an amino acid sequence in the neuronal isoform of rat NOS (Bredt 1991). The evolutionarily conserved form of neuronal nitrergic signaling is evidenced by the fact there is only one amino acid difference between the amino acid target sequence in rat and frog neuronal NOS (Huynh 2006).

NADPHd histochemistry works because there is a codistribution of NOS and its cofactor, NADPH. The technique does not actually stain for NADPH but for the electron receptor nitroblue tetrazoliom (NBT), which gets reduced to NBT formazan (i.e., NOS-positive cells) (Spessert 1994, Scherer-Singler 1983). NBT formazan filled cells can

then be visualized under a light microscope. In the bullfrog brain both NOS ICC and NADPHd staining provide faithful representations of NOS distribution (Huynh 2006). All toral nuclei have NOS/NADPH labeled neurons. Most of the cells are small and periform shaped, while some are medium sized and multipolar. The T_m and T_L stain more heavily for cell bodies, while the T_p has some cell bodies stained but many more fibers (Huynh 2006).

3.7 Roles of nitric oxide in sensory signal processing

There is not a lot of literature available describing the role nitric oxide plays in sensory processing. The most abundant source of information comes from the visual system. For instance, in the dorsal lateral geniculate nucleus of the thalamus in the cat, inhibition of NO suppresses all visual responses (Cudeiro 1994a, b). NO produces this effect by being synthesized in the presynaptic neuron and reducing the transmission of visual information by the gating of NMDA mediated excitation. In rod and cone cells of the retina, NO exhibits differential effects depending on the photoreceptor. In rod cells, increased levels on NO decreases evoked responses, while inhibition of NO with L-NAME increases evoked responses. In cone cells, increased level of NO increases evoked responses while inhibition of NO has no effect on evoked responses (Sato 2010). Discharge patterns of ON and OFF retinal ganglion cells are differentially modulated by NO in response to flash stimuli in ferret retinas. ON cell discharge rate is reduced by 40% while OFF cells' discharge rates are completely blocked (Wang 2003). Similarly, in light adapted retinal ganglion cells, NO is required for maintenance of sensitivity to light stimulation. Inhibiting NO production, with L-NAME, reduces peak

firing rate of ON and OFF retinal ganglion cells by reducing postsynaptic excitatory currents (Nemargut 2009).

In the olfactory system, NO can activate cyclic nucleotide-gated ion channels via cGMP dependent and independent mechanisms, allowing olfactory sensory transduction in periphery olfactory neurons (Breer 1993 Broillet 1996). The activation of these channels initiates the response cascade induced by odorants binding to chemoreceptors on olfactory cilia.

3.8 Summary

Nitric oxide is a ubiquitous volume transmitter found throughout the animal kingdom. It acts primarily through cGMP dependent mechanisms, either through presynaptic potentiation of neurotransmitter release or through postsynaptic modulation of ion channel function. Histo- and cytochemical staining show that it is present throughout the nervous system especially in sensory systems where its role has been demonstrated in stimulus processing.

In the rat, NO plays a role in the auditory gating of repetitive sounds in the hippocampus. Inhibition of NO prevents the inhibition of hippocampal cells to repetitive auditory stimuli (Adams 2000). In the rat auditory cortex there is an increase of NO as the rat ages while there is a decrease in the inferior colliculus (Sanchez-Zuriaga 2007, Lee 2008). In the auditory cortex NO suppresses activation of K^+ currents, which suppresses neuronal excitability hindering auditory sensitivity (Lee 2008). There is also evidence for an increase of nitric oxide production with age in the auditory brainstem (Reuss 2000). While these studies provide evidence that NO plays a role in auditory

processing, the actual exploration of its role in signal processing has not been assessed. This is especially relevant due to age-related changes in NO production and the decrease in auditory sensitivity that occur as organisms age.

3.9 Central Hypothesis

The aim of this study was to investigate the role nitric oxide plays in the processing of complex sounds resembling speech, in the torus semicircularis of the American bullfrog. This was accomplished by recording the evoked responses of neurons to bullfrog and green frog advertisement calls before, and after, inhibition of nitric oxide synthase using iontophoresis.

CHAPTER 2
EXPERIMENTAL METHODS AND DESIGN

1. General methods

Animal care and all surgical procedures were approved by the University of Tennessee IACUC.

1.1 Animal care

Adult male American bullfrogs (10 – 15 cm snout-vent length), *Lithobates catesbeiana* were used in this study. Animals were group housed in two plexiglass tanks (8”H x 18”W x 24”L), maintained on a 16hrL:8hrD light cycle (mimics time of day during breeding season), supplied with fresh water and antibiotics daily and fed crickets once a week.

1.2 Surgical procedures

Animals were placed in a small water tight container and anesthetized by immersion in 200mL of 0.1% ethyl 3-aminobenzoate methanesulfonate (MS-222) buffered with 3.5g of sodium bicarbonate for 40 minutes. The frog was then removed from the MS222 and wrapped in moist gauze for surgery. An intramuscular injection of xylazine (10mg/kg body weight) was administered, serving as an analgesic. Betadine was applied to the skin at the surgical area as a disinfectant and lidocaine was applied as a local anesthetic. A dorsal surgical approach was used to expose the optic tectum. Briefly, a small flap of skin was removed directly between the animal’s tympanic membranes. The muscles were reflected away from the skull and a small hole drilled through the bone. The exposed dura was cut away and the arachnoid and pial

membranes removed using a sharpened tungsten needle formed into a hook. Frogs were allowed to recover from anesthesia, then immobilized with an intramuscular injection of d-tubocurarine chloride (10µg/g body weight), or succinyl choline chloride (22µg/mg body weight), wrapped in fresh moist gauze, then placed on a vibration isolation table in a sound attenuating chamber. Curare was administered as needed via intramuscular injections, but succinyl choline chloride proved to be a more effective paralytic and only had to be administered once immediately following surgery. Awake animals were used because anesthetics have been shown to alter functional properties of central auditory neurons including spontaneous activity, frequency tuning and threshold (Epping 1985). All surgical procedures and experiments were conducted at 22-24^{o}C. Recordings were started at the beginning of the dark period of the frog's light:dark cycle.

1.3 Acoustic stimulus calibration and presentation

Acoustic stimuli were presented free field using a speaker (RCA 5 inch) placed 30cm directly in front of the animal. Before performing any experiments, the speaker was calibrated and sound pressure level (SPL) was measured at the location of the animal's ears. The output of the speaker was flat (+/- 3dB SPL) over a frequency range of 100-2500Hz. Background was generally around 20dB SPL.

Tones and white noise were generated by custom software and Tucker-Davis Technologies System II hardware and could be presented singly or in combination. Vocalizations were presented using Windows Media Player on a Windows 10 operating

system. Frog vocalizations were field recordings of calling males provided by the Megela-Simmons lab.

1.4 Electrodes for recording and iontophoresis

Six-barrel “piggy-back” electrodes were constructed as described by Havey and Caspary (1980) and used to record sound evoked activity from single neurons and for iontophoretic application of pharmacological agents. Briefly, electrodes were made by using a Sutter Instrument Company (model P-87) pipette puller to pull a single barrel micropipette (1.2mm barrel diameter) to a tip diameter of approximately 1µm. A five barrel micropipette (1.2mm barrel diameter) in an H-configuration was pulled using a Kation Scientific Gravipull-3 pipette puller. The tip of the multibarrel pipette was broken back to a diameter of approximately 10µm. The single barrel micropipette was then mounted onto the back of the multibarrel micropipette with mounting clay, then secured with a small plastic insert super glued at a 20^{o} angle with its tip protruding 10-20µm beyond the tips of the five-barrel micropipette. The single barrel was filled with 3M NaCl and served as the recording barrel. Individual barrels of the multibarrel micropipette were filled with L-N^{G} nitro arginine methyl ester (L-NAME, in DI water, 10mM, pH 4.0), or L-Arginine (L-Arg, in DI water, 10mM, pH 10.0). For all experiments recording micropipettes had a resistance between 5-15MOhms; higher resistance pipettes were discarded. Drug barrels had a resistance of 5-30MOhms. Resistances were recorded before ejections of drugs and monitored during the ejection procedure.

The recording barrel was connected to the probe stage of an extracellular preamplifier (Dagan 2400B) via a silver-silver chloride wire. Amplified spikes were

bandpass filtered (30-3000Hz), monitored audio visually (Grass AM8 audio monitor and LabChart 8), and stored on a lab PC hard drive for analysis. Each of the iontophoretic barrels was connected to one channel of a 6-channel micro-iontophoresis current generator (Dagan 6400) that was used to induce and monitor iontophoretic currents. A retention current (5nA, negative polarity, DC for L-NAME, 5nA positive current for L-Arg) was applied to each drug barrel to prevent leakage of drugs out of the barrels. A balancing current was automatically passed through the middle barrel of the multibarrel pipette, containing 3M NaCl, to avoid electrical stimulation of neurons.

1.5 Extracellular recordings of single neuron activity and iontophoresis

A multibarrel pipette was positioned above the optic tectum with the aid of a binocular stereoscope (Olympus SZ30). The micropipette was lowered using a micropositioner (WPI HS6) and a hydraulic microdrive (KOPF 650). The micropipette was driven through the optic tectum and the tectal ventricle into the IC approximately 700μm below the surface of the brain. Distinct “pops” could be heard and seen when the electrode entered and exited the ventricle. The depth from which individual cells were recorded was obtained from the digital readout of the hydraulic microdrive.

White noise (100-4000Hz) or tone combinations (300 Hz and 1400 Hz), 500ms duration and 20ms rise/fall times, were generated and used as search stimuli. Recordings of the bullfrog advertisement call were also used as search stimuli. All search stimuli, both natural and artificial were presented at 65dB peak SPL and at 2 sec intervals. Single units were selected for recording and subsequent analysis when extracellular action potentials were clearly identifiable above background.

When a single cell was isolated its characteristic frequency (CF), threshold (the frequency at which the lowest intensity tone would produce at least one spike/stimulus) and depth were recorded. American bullfrog and green frog (*Lithobates clamitans*) advertisement calls were then presented (10 - 25 presentations each at 2 sec intervals, 60dB peak SPL) and the cell's action potential response recorded. Pharmacological agents were then applied using successive iontophoretic currents of 20nA, 40nA and 60nA having the appropriate polarity, and sound-evoked responses recorded. At each level of drug current, both advertisement calls were presented to the animal (10-25 presentations at 2 sec intervals). For each experiment, the bullfrog call was presented first followed by the green frog call. After the final current injection, the cell was allowed to recover for 30 minutes and a recovery recording taken for both advertisement calls. This was to ensure that NOS inhibition had ceased and therefore would not to interfere with other subsequent recordings from the same animal. At the conclusion of the experiment animals were euthanized by MS-222 bath.

1.6 Iontophoretic controls

To determine if the pH of iontophoretic solutions was influencing neuronal firing, vehicle alone was applied iontophoretically. After control recordings were taken, responses to both calls were recorded just with vehicle being applied. This procedure was performed on three animals. No current or pH effects on neuronal responses were detected. Since the experimental setup was the same for each animal these controls were discontinued once it was determined there were no vehicle or pH effects.

1.7 Statistical Analysis

Quantitative assessment of the effect of call type and drug treatment on the number of spikes elicited in the evoked response was conducted using Labchart 8 software and Graphpad Prism software. Unless otherwise specified, mean spike counts per stimulus presentation were calculated and 2-way ANOVAs were used to determine if differences between the means of control and treatment groups were significant. A $p < 0.05$ was considered significant. Standard error of the mean is indicated in the text by ±, and in the graph with error bars. Qualitative assessment of discharge patterns was made using raster plots and post stimulus time histograms (PSTH) with 5ms bin widths.

CHAPTER THREE
RESULTS

1. General response properties of TS neurons

1.1 Spectral characteristics

Results were compiled from 35 different cells sampled from 21 different frogs. Characteristic frequencies (CFs) ranged from 200-1400 Hz, being distributed across three different frequency ranges encompassing low (60-510 Hz, n=20), mid (511-900 Hz, n=5), and high (1100-1900 Hz, n=10) frequencies (Figure 7). As stated earlier, the bullfrog advertisement call consists of two spectral peaks at approximately 300 and 1300 Hz (Capranica 1965). The bulk of recorded CFs were centered around those two frequency bands. The majority of units responded to single tones (34/35), with only one unit requiring both spectral peaks of the advertisement to be present to evoke response (representing an AND neuron), which is consistent with previously reported response characteristics of TS cells (Fuzessery and Feng 1982).

1.2 Discharge Patterns of TS neurons

Temporal discharge patterns were characterized according to the shape of PSTHs. Discharge patterns for the bullfrog advertisement call responses included primarylike (65%), phasic (28%), or phasic-burst (3%). In response to the green frog advertisement call discharge patterns were primarylike (59%), phasic (15%), and phasic burst (26%) (Figure 8). Primarylike units fired throughout the duration of the stimulus, phasic units responded with 1-2 spikes typically at the onset of the stimulus, phasic burst units responded with a brief burst of spikes at stimulus onset. The frequency

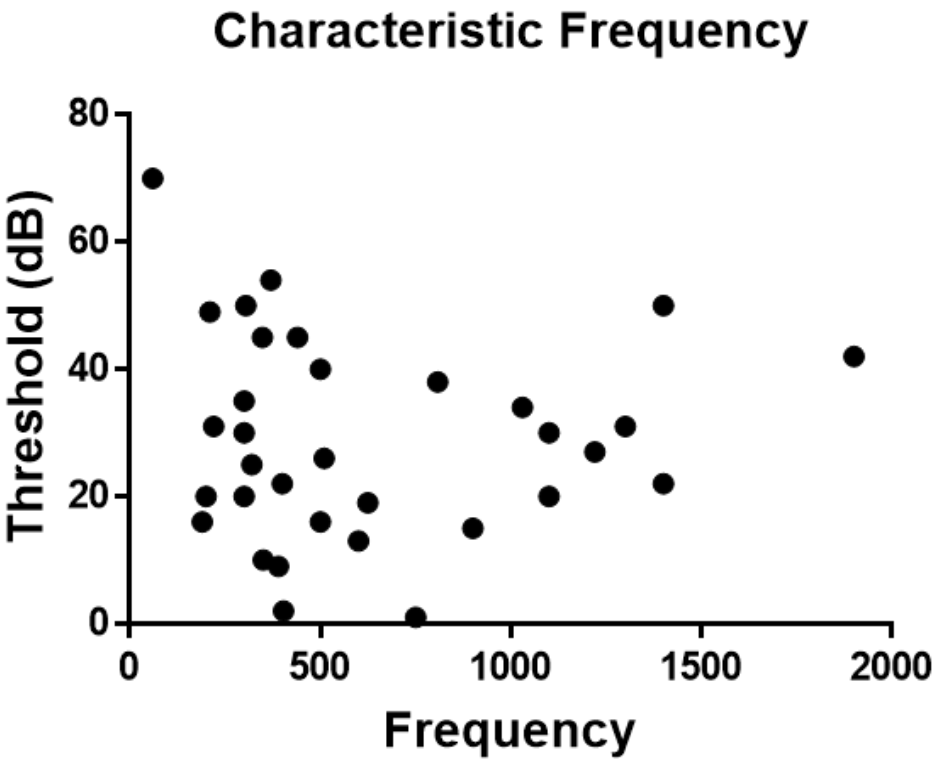

Figure 7. Characteristic frequencies and thresholds. CFs and thresholds for 35 units in the TS. The frequency at which the lowest intensity tone produced a response was considered as unit's CF

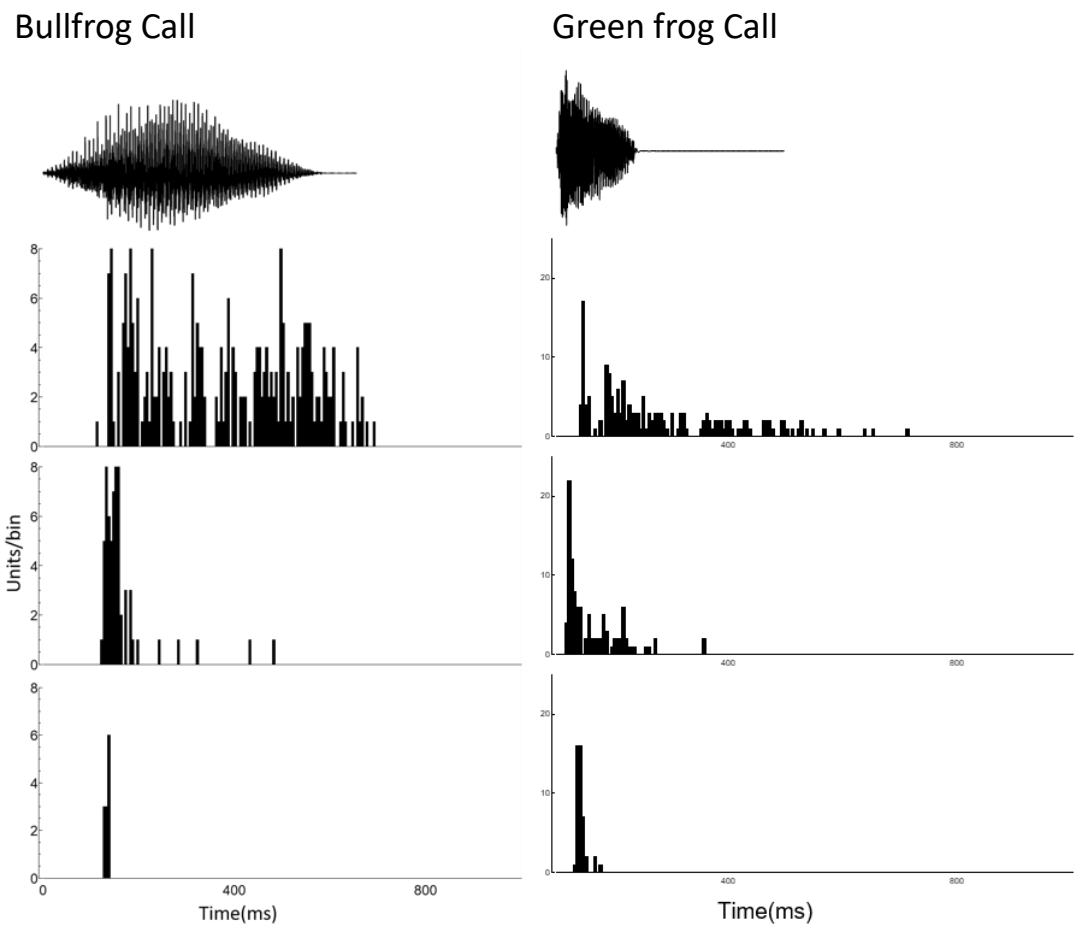

Figure 8. Discharge patterns of TS cells. Top: Oscillograms for bullfrog and green frog advertisement calls. PSTHs showing different discharge patterns evoked by TS cells. Top PSTH: primarylike discharge pattern, Middle PSTH: phasic burst discharge pattern, Bottom PSTH: phasic discharge pattern

discharge patterns observed are consistent with previously described work (Rose 2007, Gooler 1992).

1.3 Call independent effects of L-NAME on evoked response

Of the 35 neurons examined, L-NAME application had no effect on the response of 6 neurons to either the bullfrog or green frog call. Of the remaining 29 cells, L-NAME application either increased and/or decreased response magnitude to one or both call types.

For 9 neurons, L-NAME application resulted in an increase (n=3) or decrease (n=6) in firing rate independent of the call presented. An example of a cell showing a call-independent increase is shown in Figure 9. A two-way ANOVA revealed the influence of L-NAME on the evoked response to both calls was significantly different ($F_{1,23}$=48.09, p<0.0001). While the influence of call type was also significant ($F_{1,23}$=0.0491 p=0.0491), a post-hoc Sidak's multiple comparisons test revealed no significant differences between the two call types before (t_{23}=2.842, p=0.0542) and after (t_{23}=1.536, p=0.5903) drug treatment. There was no significant interaction effect between L-NAME and the advertisement call type ($F_{1,23}$=0.8523, p=0.3655).

L-NAME application caused the response to the bullfrog call to increase 30%, from 6±0.3 to 9±0.3 spikes/stimulus (T_{23}=5.606, p<0.0001); while that to the green frog call increased 20%, from 7±0.8 to 10±0.1 spikes/stimulus (T_{23}=4.301, p<0.01).

An example of a cell showing a call-independent decrease in response is shown in Figure 10. A two-way ANOVA revealed the influence of L-NAME on the evoked response to both calls was significantly different ($F_{2,48}$=239.6, p<0.0001). While the

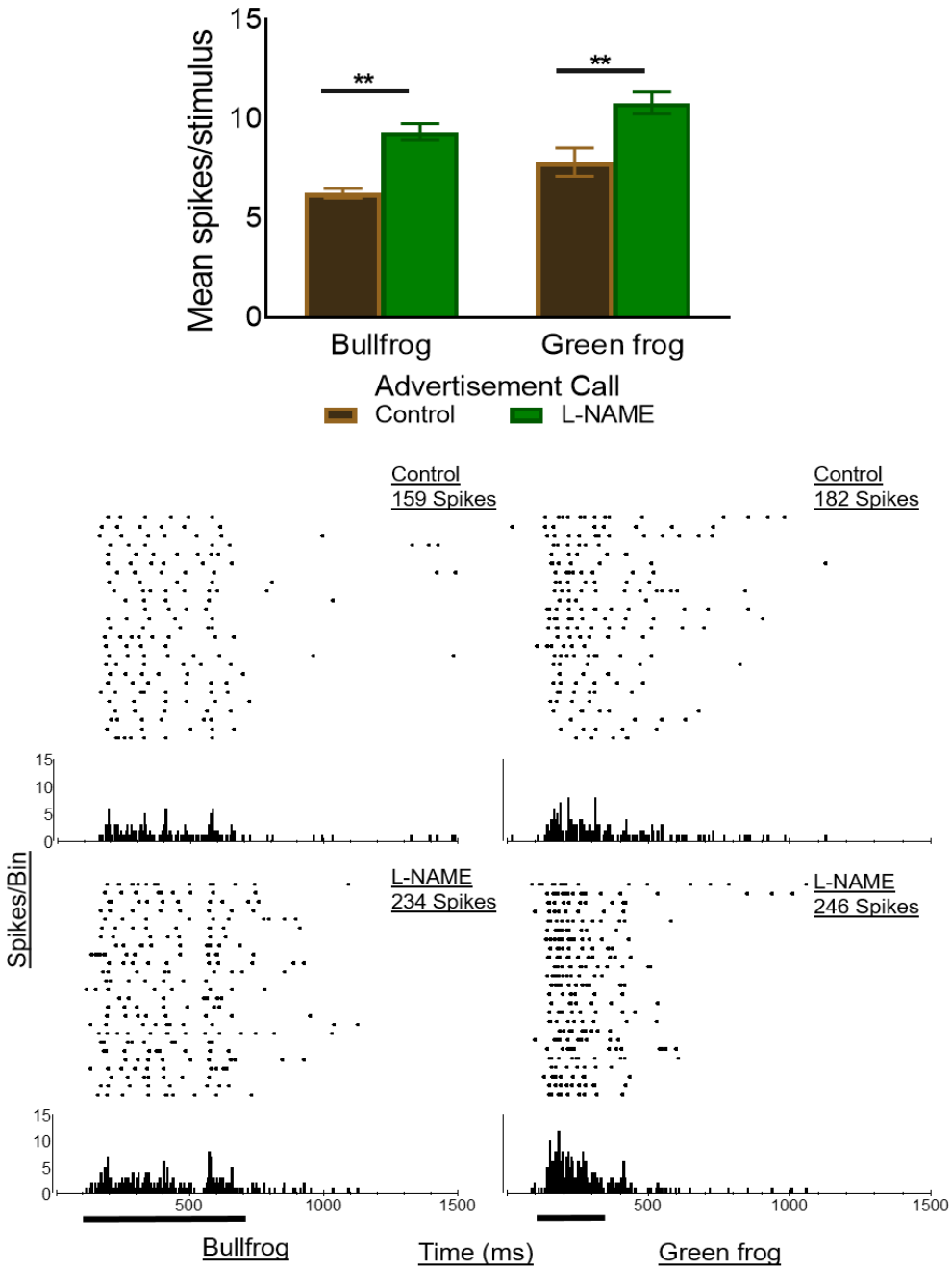

Figure 9. Call independent increase in evoked response. L-NAME application caused a significant increase in evoked response to both advertisement calls. Black bars indicate stimulus lengths. ** $p<0.01$

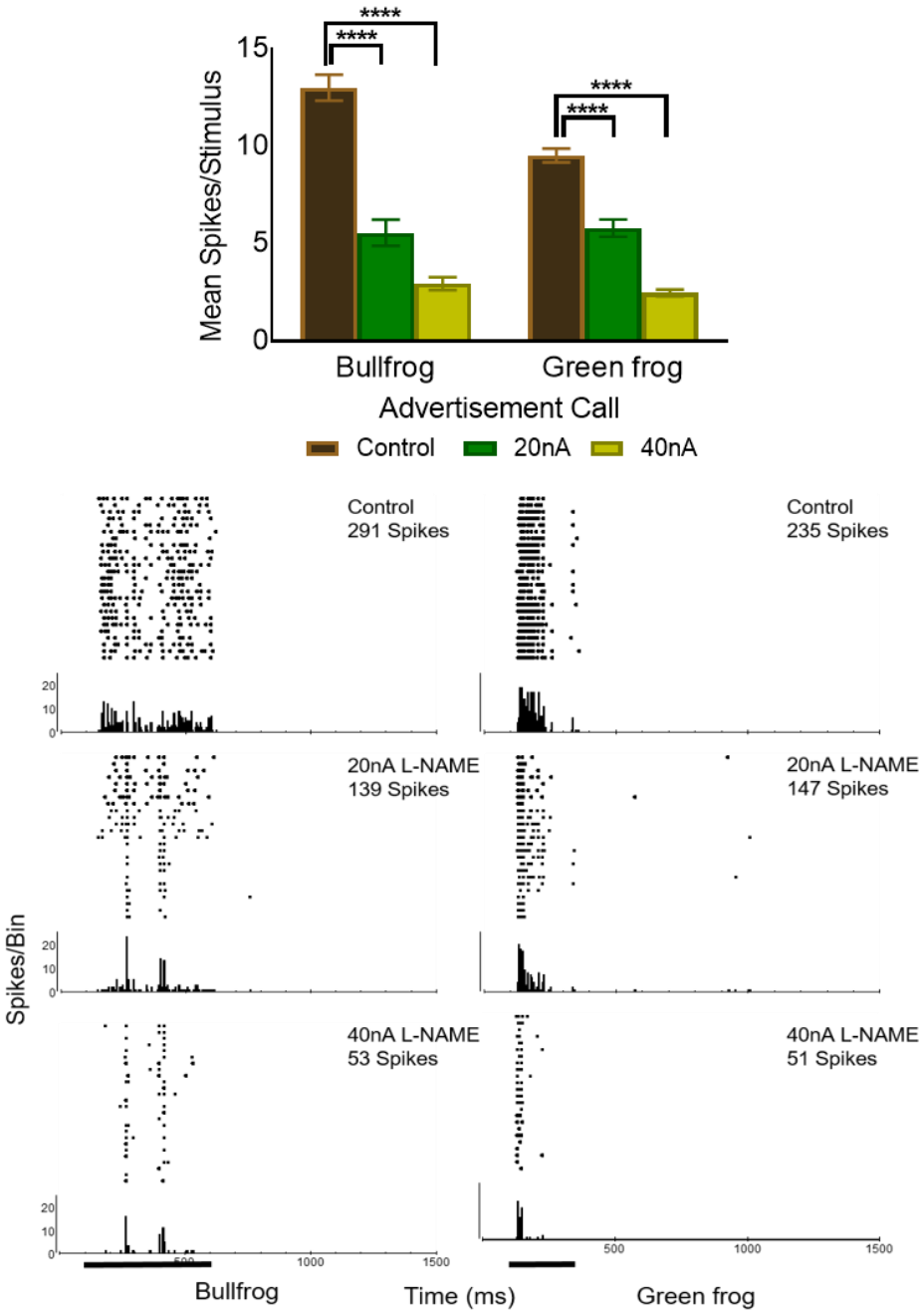

Figure 10. Call independent decrease in evoked response. L-NAME application caused a significant decrease in evoked response to both advertisement calls. Black bars indicate stimulus lengths. **** $p<0.0001$

influence of call type was also significant ($F_{1,24}$=6.678 p<0.05). There was a significant interaction effect between L-NAME and the advertisement call type ($F_{2,48}$=10.04, p<0.001).

A Tukey's post-hoc test revealed that application of L-NAME using a 20nA injection current decreased the response to the bullfrog call by 50%, from 13±0 to 5±0.6 spikes/stimulus (Q_{48}=15.77, p<0.0001); while that to the green frog call decreased by 40% from 10±0.4 to 6±0.4 spikes/stimulus (Q_{48}=7.887, p<0.0001). Doubling the injection current to 40nA further reduced the evoked response compared to control by 75%, from 13±0 to 2±0.3 spikes/stimulus (Q_{48}=22.56, p<0.0001); while that to the green frog was decreased by 60% compared to control 10±0.4 to 4±0.2 spikes/stimulus (Q_{48}=14.93, p<0.0001).

1.4 Call dependent effects of L-NAME on evoked responses

For 20 neurons, L-NAME application had call dependent effects on the evoked responses of TS neurons. Some neurons had a change in response to only the bullfrog call (n=12), others (n=5) only the green frog call.

1.4.1 L-NAME modulates responses to bullfrog call

For neurons in this first group L-NAME increased (N=5) or decreased (N=7) the evoked responses to bullfrog advertisement calls only. An example of a cell showing a call dependent increase in response to just the bullfrog call is shown in Figure 11. A two-way ANOVA revealed the influence of L-NAME on the evoked response was significantly different ($F_{2,18}$=14.15, p<0.001). While the influence of call type was also significant ($F_{1,9}$=36.6 p<0.001). A Tukey's post hoc test revealed no significant

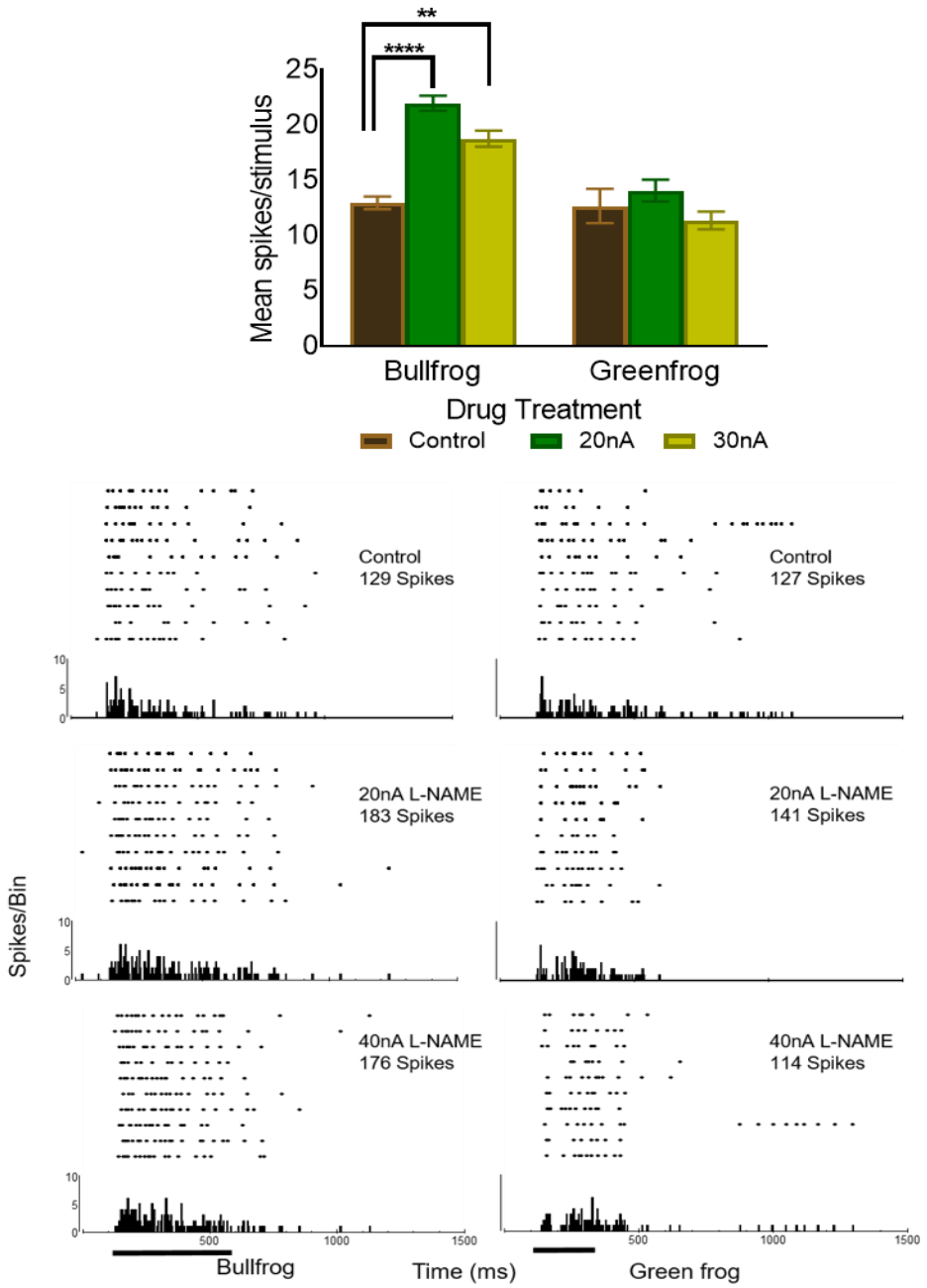

Figure 11. Call dependent increase in evoked response to bullfrog call. L-NAME application caused a significant increase in evoked responses to just the bullfrog call. Black bars indicate stimulus lengths. ** $p<0.01$ **** $p<0.0001$

differences between the two call types before drug application (Q_{18}=0.339, p=0.9999). There was a significant difference between evoked response to the two calls after the 20nA (Q_{18}=8.927, p<0.0001), and 30nA (Q_{18}=8.362, p<0.001) drug treatments. There was a significant interaction effect between L-NAME and the advertisement call type ($F_{2,18}$=11.54, p<0.001).

Application of L-NAME using a 20nA injection current increased the response to the bullfrog call by 70%, from 13±0.6 to 22±0.7 spikes/stimulus (Q_{18}=10.17, p<0.0001); while that to the green frog call increased a slight yet non-significant 8%, from 13±1.6 to 14±1.0 spikes/stimulus (Q_{18}=1.582, p=0.8674). Increasing application current to 30nA increased the response to the bullfrog call 46%, from 13±0.6 to 19±0.7 spikes/stimulus (Q_{18}=6.554, p<0.01); while that to the green frog decreased a non-significant 15%, from 13±1.6 to 11±0.8 (Q_{18}=1.469, p=0.3037).

Figure 12 is an example of a cell showing a call dependent decrease in evoked response to just the bullfrog advertisement call. A two-way ANOVA revealed the influence of L-NAME on the evoked response was significant ($F_{2,48}$=3.534, p=0.037). While the influence of call type was also significant ($F_{1,24}$=254.3 p<0.0001). A Tukey's post hoc test revealed a significant difference between the two call types before drug application (Q_{18}=5.221, p<0.01). There was a significant difference between evoked response to the two calls after the 10nA (Q_{48}=14.36, p<0.0001), and 20nA (Q_{48}=14.57, p<0.001) drug treatments. There was a significant interaction effect between L-NAME and the advertisement call type ($F_{2,48}$=14.25, p<0.0001).

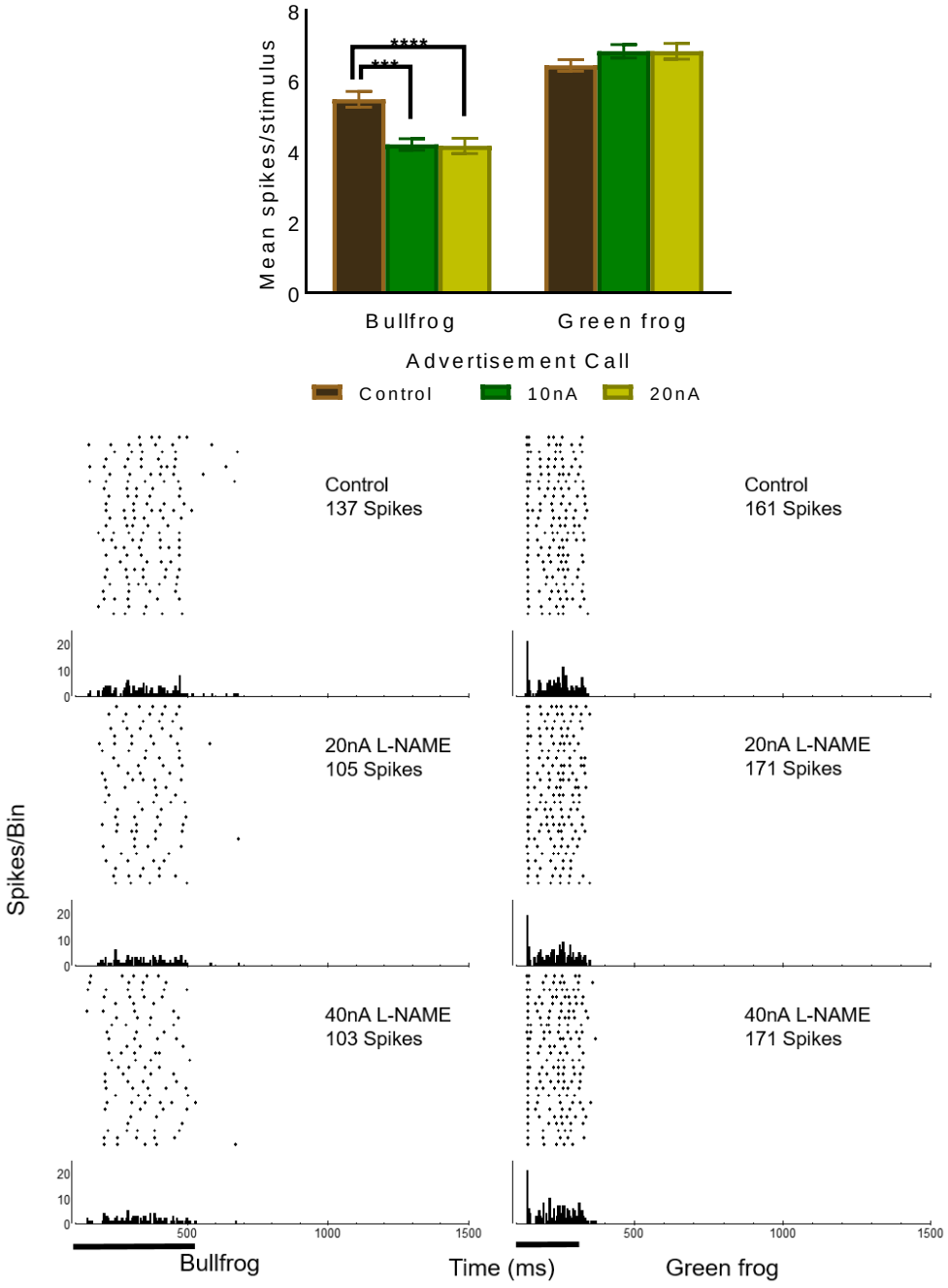

Figure 12 Call dependent decrease in evoked response to bullfrog call. L-NAME application caused a significant decrease in evoked responses to just the bullfrog call. Black bars indicate stimulus lengths. *** $p<0.001$, **** $p<0.0001$

For this cell application of L-NAME using a 10nA injection current decreased the response to the bullfrog call by 20%, from 5.5±0.2 to 4.2±0.2 spikes/stimulus (Q_{48}=6.961, p=0.0001); while that to the green frog call increased a slight yet non-significant 8%, from 6.4±0.2 to 6.8±0.2 spikes/stimulus (Q_{48}=2.175, p=0.6419). Doubling application current to 20nA decreased the response to the bullfrog call 20%, from 5.5±0.2 to 4.2±0.2 spikes/stimulus (Q_{48}=7.179, p<0.0001); while that to the green frog increased a non-significant 8%, from 6.4±0.2 to 6.8±0.2 (Q_{48}=2.175, p=0.6419).

1.4.2 L-NAME modulates responses to green frog advertisement call

In the second group of neurons (n=5) L-NAME application caused the evoked response to the green frog advertisement call to decrease for 4 units compared to control, while the evoked response to the bullfrog call remained unaffected. A single unit (n=1) had the evoked response to the green frog call increase compared to control while the evoked response to the bullfrog call remained unaffected.

An example of a cell showing a call dependent change in response to just the green frog call is shown in Figure 13. A two-way ANOVA revealed the influence of L-NAME on the evoked response was significant ($F_{1,24}$=8.783, p<0.01). While the influence of call type was also significant ($F_{1,24}$=6.665 p<0.05). A Tukey's post hoc test revealed a significant difference between the two call types before drug application (T_{24}=1.003, p<0.0001). After drug application there was no significant difference between evoked response to the two calls (T_{24}=4.812, p=0.9063). There was a

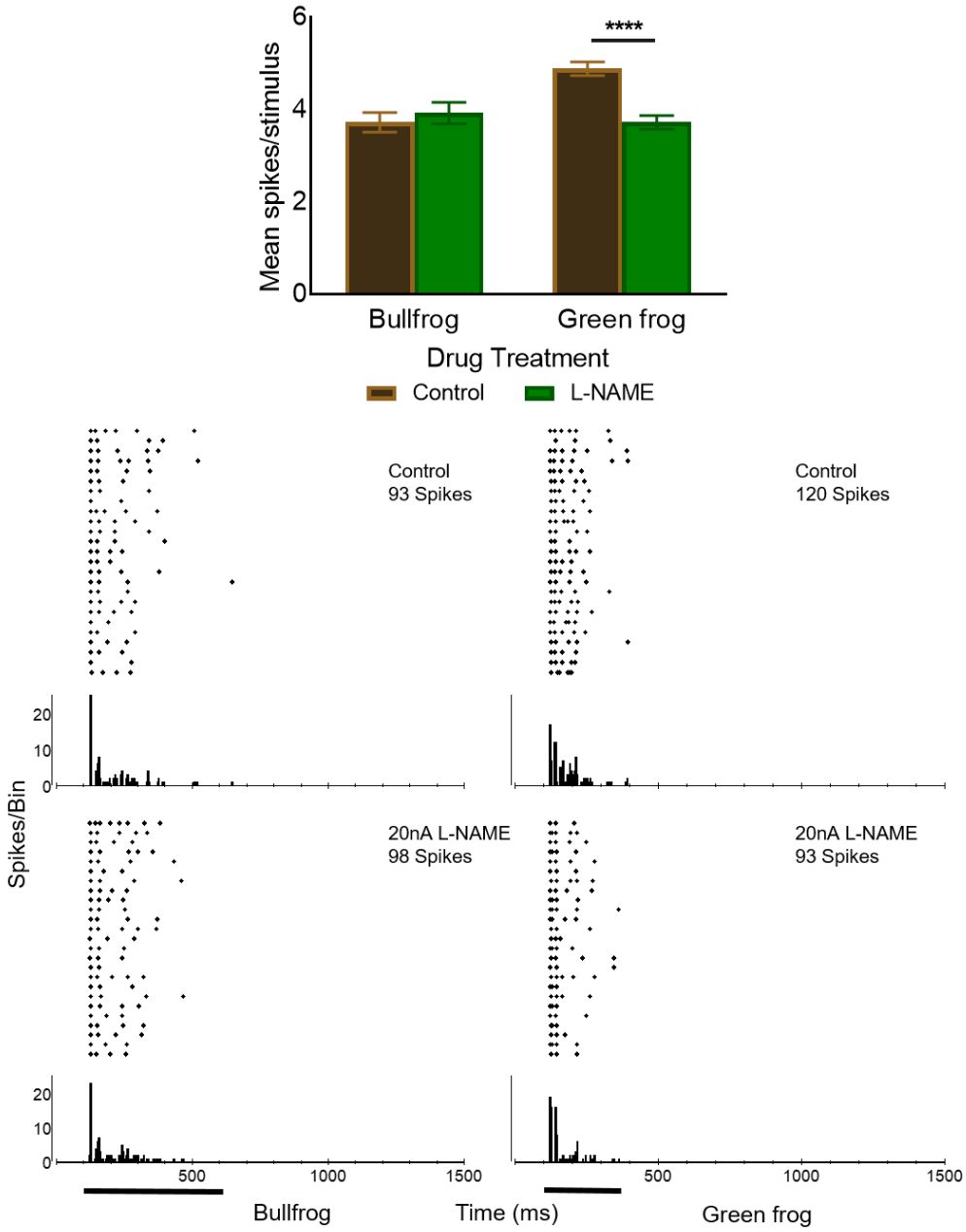

Figure 13. Call dependent decrease in evoked response to green frog call. L-NAME application caused a significant decrease in evoked responses to just the green frog call. Black bars indicate stimulus lengths. **** $p<0.0001$

significant interaction effect between L-NAME and the advertisement call type ($F_{1,24}$=23.24, p<0.0001).

For this cell application of L-NAME using a 20nA injection current increased the response to the bullfrog call by a non-significant amount, from 3.7±0.2 to 3.9±0.2 spikes/stimulus (T_{24}=1.003, p=0.9063); while that to the green frog call decreased 20%, from 4.9±0.1 to 3.7±0.1 spikes/stimulus (T_{48}=5.815, p<0.0001).

The data from a single cell showing a call dependent change in response to just the green frog call where the evoked response increased is shown in Figure 14. A two-way ANOVA revealed the influence of L-NAME on the evoked response was significant ($F_{3,72}$=3.484, p<0.05). While the influence of call type was also significant ($F_{1,24}$=490.9 p<0.0001). A Tukey's post hoc test revealed a significant difference between the two call types before drug application (Q_{72}=1.582, p<0.0001). There was a significant difference between evoked responses to the two calls after the 20nA (Q_{72}=21.62, p<0.0001), 40nA (Q_{72}=23.34, p<0.001) and 60nA (Q_{72}=23.07, p<0.001) drug treatments. There was a significant interaction effect between L-NAME and the advertisement call type ($F_{3,72}$=9.748, p<0.0001).

L-NAME caused the evoked response to the bullfrog call to decrease by 14% compared to control at the 20nA injection current, from 2.8±0.2 to 2.4±0.2 spikes/stimulus (T_{48}=1.713, p=0.0932); while that to the green frog call increased 28%, from 7.1±0.4 to 8.9±0.6 spikes/stimulus (T_{48}=2.604, p=0.0122). Doubling application current to 40nA decreased response to the bullfrog call 16%, from 2.8 ±0.2 to 2.3±0.1 spikes/stimulus (T_{48}=2.224, p=0.0309); while that to the green frog increased 30%,

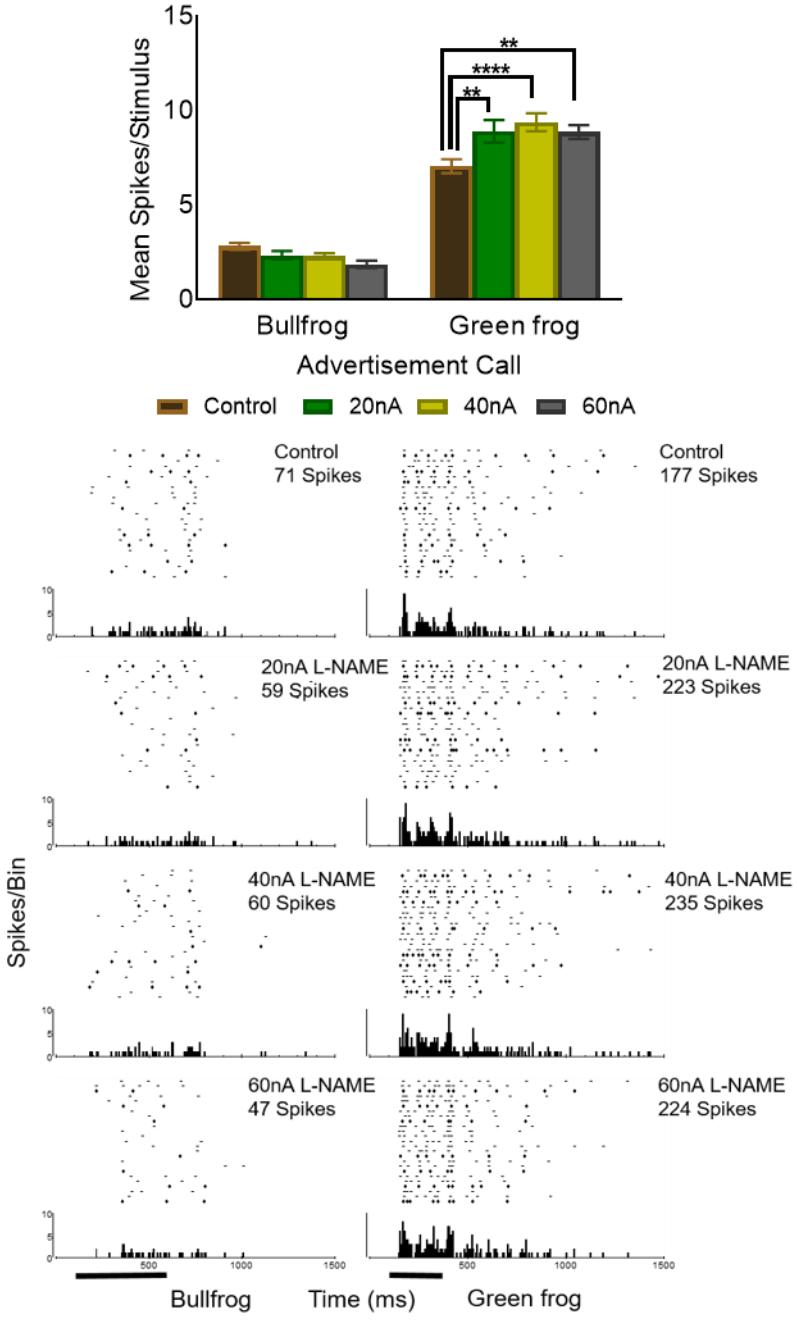

Figure 14. Call dependent increase in evoked response to green frog call. L-NAME application caused a significant decrease in evoked responses to just the green frog call. Black bars indicate stimulus lengths. ** $p<0.01$, **** $p<0.0001$

from7.1±0.4 to 9.4±0.5 (T_{48}=3.833, p=0.0004). Raising the application current to 60nA further decreased the evoked response to the bullfrog call by 32%, from 2.8±0.2 to 1.9±0.2 spikes/stimulus (T_{48}=3.545, p=0.0009); while that to the green frog increased 28%, from 7.1±0.4 to 8.9±0.4 (T_{48}=3.48, p=0.0011).

1.4.3 L-NAME modulates responses to advertisement calls in differential manner

In the final group of neurons (n=4) L-NAME modulated the response to both advertisement calls but in different ways. For 3 units, application of L-NAME decreased the evoked response to the bullfrog call while simultaneously increasing the evoked response to the green frog call (Figure 15). A two-way ANOVA revealed the influence of L-NAME on the evoked response was significant ($F_{1,9}$=6.639, p<0.05). While the influence of call type on evoked response was not significant ($F_{1,9}$=3.093 p=0.1125). A Tukey's post hoc test revealed a significant difference between the two call types before drug application (Q_9=13.27, p<0.0001). A significant difference between evoked responses to the two calls after the 20nA (Q_9=7.003, p<0.01) drug treatment was also found. There was a significant interaction effect between L-NAME and the advertisement call type ($F_{1,9}$=102.7, p<0.0001).

L-NAME caused the evoked response to the bullfrog call to decrease by 33% compared to control at the 20nA injection current, from 10.6±0.3 to 7.1±0.4 spikes/stimulus (Q_9=12.9, p<0.0001); while that to the green frog call increased 22%, from 7.0±0.3 to 9.0±0.3 spikes/stimulus (Q_9=7.372, p=0.01).

For the other unit in this group, L-NAME application caused the evoked response to the bullfrog call to increase, while it caused the evoked response to the green frog

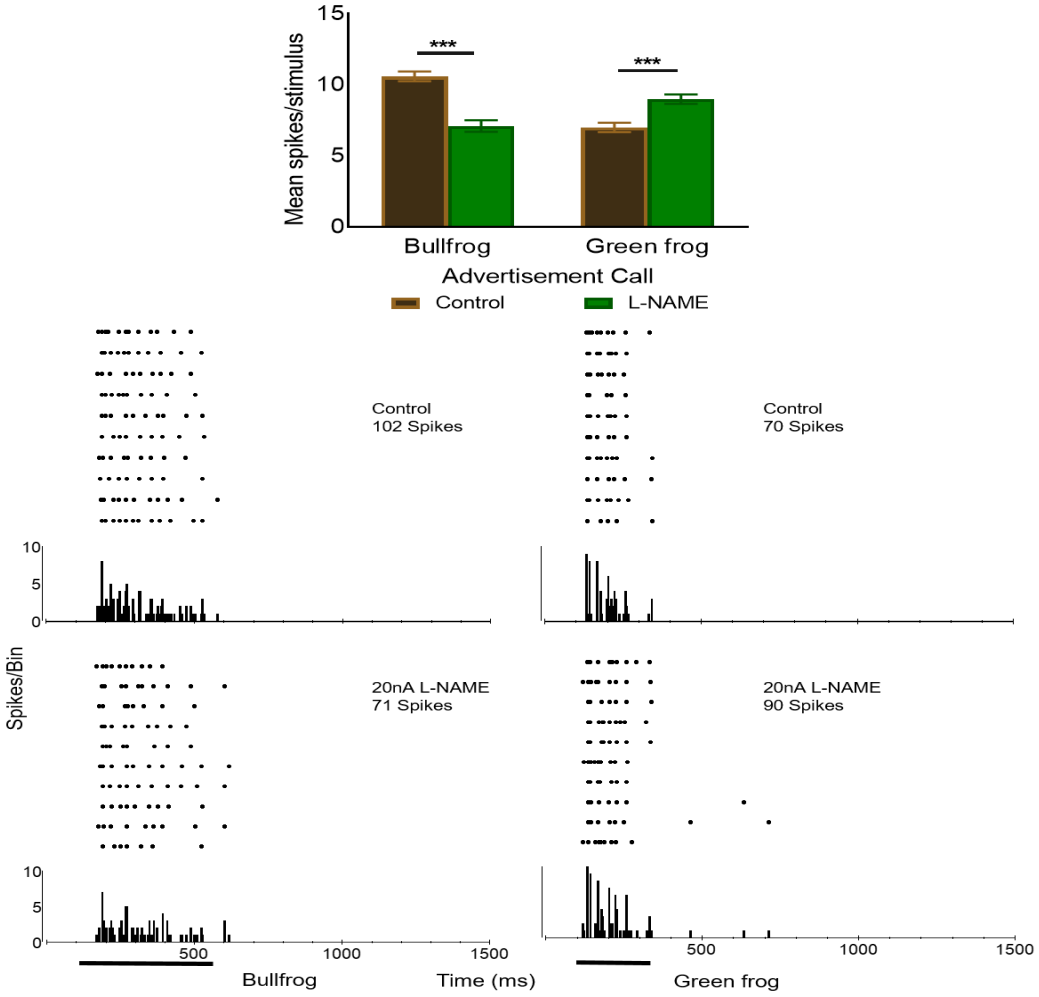

Figure 15. Differential call independent response in evoked response. L-NAME application caused a significant decrease in evoked responses to the bullfrog call while simultaneously increasing the evoked response to the green frog call. Black bars indicate stimulus lengths. *** $p<0.001$

call to decrease as depicted in Figure 16. A two-way ANOVA revealed the influence of L-NAME on the evoked response was not significant ($F_{2,48}$=1.755, p=0.1838) when considering the difference between the two calls' evoked responses. While the influence of call type on evoked response was not significant ($F_{1,24}$=72.56 p<0.0001). A Tukey's post hoc test revealed a significant difference between the two call types before drug application (Q_9=13.29, p<0.0001). A significant difference between evoked responses to the two calls was not found after the 20nA (Q_{48}=1.727, p=0.8243) and 40nA (Q_{48}=1.594, p=0.8675) drug treatments. There was a significant interaction effect between L-NAME and the advertisement call type ($F_{2,48}$=30.51, p<0.0001).

For this unit, application of L-NAME using a 20nA injection current increased the response to the bullfrog call by 125%, from 1.2±0.1 to 2.8±0.3 spikes/stimulus (Q_{48}=5.315, p<0.01); while that to the green frog call decreased 34%, from 5.2±0.5 to 3.4±0.2 spikes/stimulus (Q_{48}=6.245, p<0.001). Doubling the application current to 40nA increased response to the bullfrog call 175%, from 1.2±0.1 to 3.1±0.3 spikes/stimulus (Q_{48}=4.651, p<0.001); while that to the green frog decreased 15%, from 5.2±0.5 to 2.6±0.2 (Q_{48}=8.637, p<0.0001).

2. L-Arg reverses effects of L-NAME

To verify that the effects we were seeing were the result of L-NAME inhibiting NO production, we also iontophoretically applied the nNOS substrate, L-Arg to cells and recorded its effect. If L-NAME was indeed eliciting the above modulations in evoked responses, then L-Arg should negate or reverse those effects. L-Arg was applied to 7

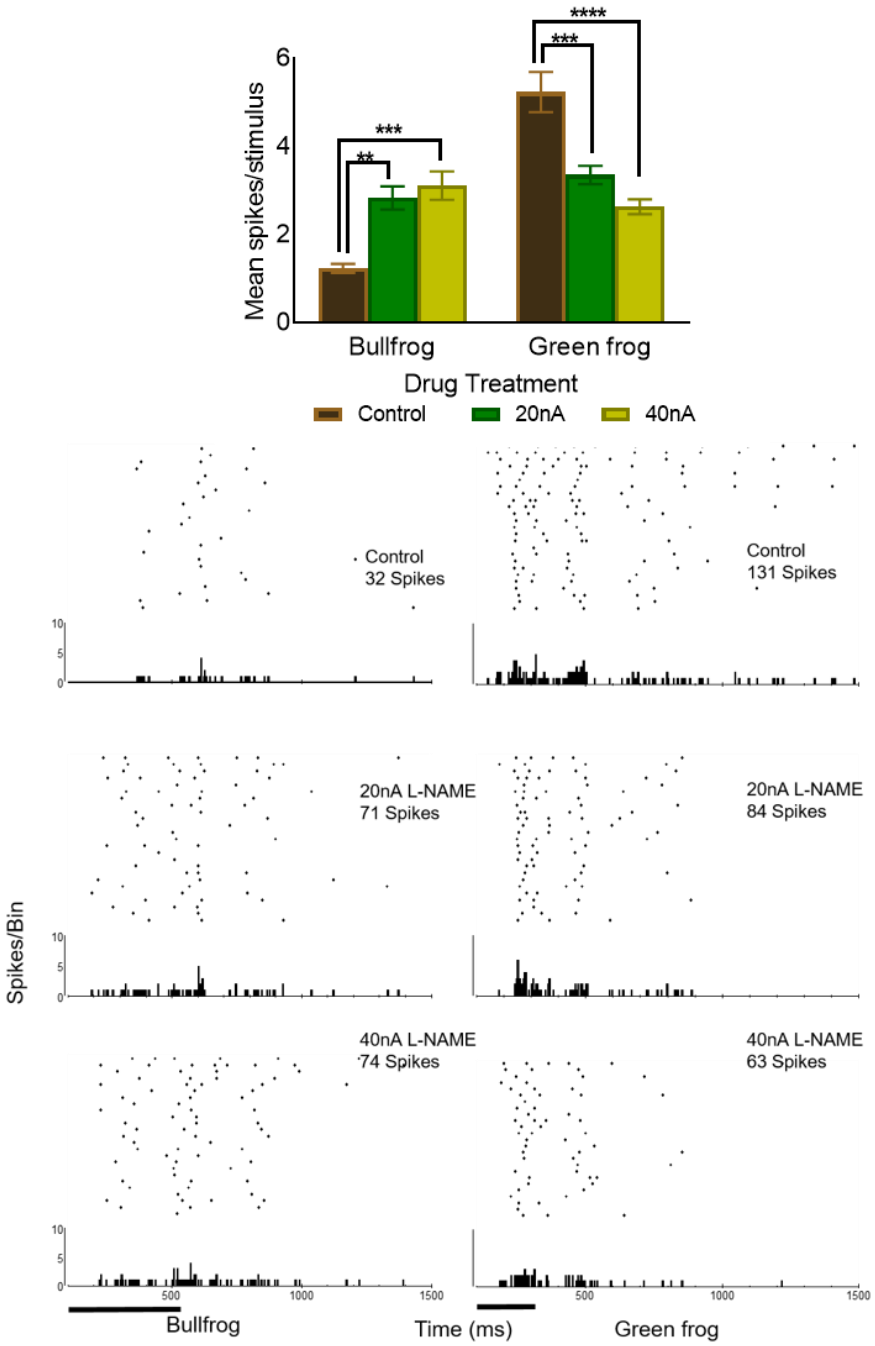

Figure 16. Differential call independent response in evoked response. L-NAME application caused a significant increase in evoked responses to the bullfrog call while simultaneously decreasing the evoked response to the green frog call. Black bars indicate stimulus lengths. ** $p<0.01$, **** $p<0.0001$

units and in each case, we saw either an abolition of L-NAME modulation or an effect that was opposite of L-NAME.

The neuron shown in Figure 17 is an example of a unit where L-NAME application decreased the evoked response to both the bullfrog and the green frog call, but simultaneous application of L-Arg with L-NAME restored control levels of firing. A two-way ANOVA revealed the influence of L-NAME on the evoked response was significant ($F_{3,72}$=3.484, p<0.05). While the influence of call type was also significant ($F_{1,24}$=490.9 p<0.0001). A Tukey's post hoc test revealed a significant difference between the two call types before drug application (Q_{72}=1.582, p<0.0001). There was a significant difference between evoked responses to the two calls after the 20nA (Q_{72}=21.62, p<0.0001), 40nA (Q_{72}=23.34, p<0.001) and 60nA (Q_{72}=23.07, p<0.001) drug treatments. There was a significant interaction effect between L-NAME and the advertisement call type ($F_{3,72}$=9.748, p<0.0001).

Here L-NAME application caused the evoked response to the green frog call to decrease by 55% compared to control at the 20nA injection current, from 6±0.8 to 3±0.5 spikes/stimulus (Q_{20}=3.648, p=0.1486); while that to the bullfrog call decreased 20%, from 10±0.8 to 8±0.7 spikes/stimulus (Q_{20}=2.345, p=0.5725). Simultaneous application of L-NAME with L-Arg returned the evoked response to control levels for the green frog call, from 6±0.7 to 5±0.8 spikes/stimulus (Q_{20}=2.692, p=0.4283); while that to the bullfrog also returned to control levels, from 10±0.8 to 9±1.2 spikes/stimulus (Q_{20}=1.042, p=0.9747).

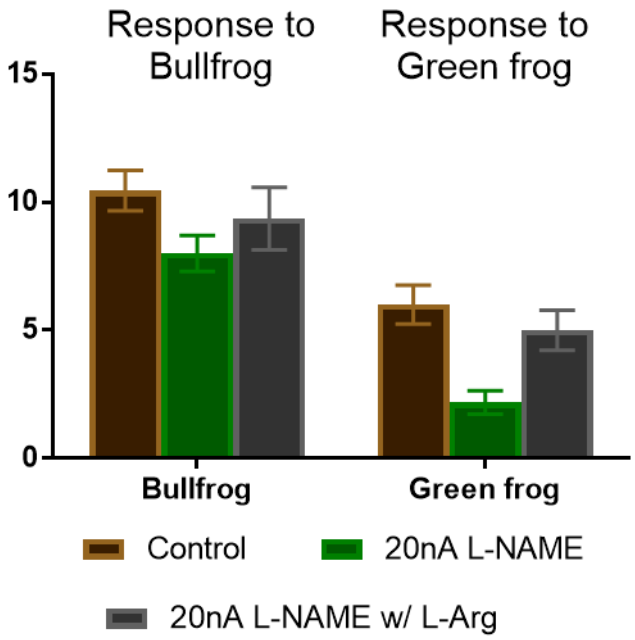

Figure 17. Effects of L-NAME countered by L-Arg Application of L-NAME reduced evoked response to the green frog call. However, L-Arg application in conjunction with L-NAME rescued the response to control levels. This indicated blockade of NO is responsible for the response seen.

CHAPTER FOUR
DISCUSSION AND CONCLUSIONS

1. Role of NO modulation in information processing in the TS

1.1Technical Considerations

Some considerations must be taken when analyzing data obtained by *in-vivo* iontophoresis: 1) exact concentrations of drugs expelled from the iontophoresis barrel cannot be measured but only inferred by the amount of current being applied; and 2) diffusion of drug means it could be affecting other neurons in the vicinity of the neuron being recorded, and therefore, changes in evoked responses aren't necessarily because the drug is modulating the response of the recorded neuron directly.

1.2 Sound discrimination

Nitrergic signaling has been shown to modulate the activity of single neurons and local microcircuits in a number of species including the cat, rat, mouse, and guinea-pig (Wang 2017, Cudeiro 1994, Shaw 1997, Friedlander 1996). The role NO plays in different sensory systems is diverse, but it seems to accentuate the relay of information from peripheral receptors to central processing centers. In frogs, there is a clear progression in the complexity of sound processing as the information ascends through the auditory system. Specifically, at the level of the TS, NO seems to play a role in sound discrimination based on the call-dependent modulation of evoked responses.

Two-way ANOVAs revealed an interaction between call type and L-NAME application for 22/35 units. This means that L-NAME was having a significant effect on response magnitude to one call compared to the other. However, only for 13/35 units did L-NAME application affect response preference for one call over the other compared to control.

Experiments have shown bullfrogs are capable of discriminating between stimuli that are representative of their advertising call versus stimuli that lack appropriate temporal or spectral components. Further complicating the processing task is the fact that vocalizations are frequently presented in a noisy environment masking the advertisement call. In these scenarios, the receiver has to 1) identify behaviorally relevant vocalizations in 2) background noise and 3) respond in a behaviorally appropriate manner. The TS is involved in all three of these processes through auditory signal processing and sensory-motor integration. Immunohistochemistry NADPHd staining indicates NO is produced throughout the TS (Boyd 2006). Therefore, NO could play a major role in the underlying mechanisms of these processes in the anuran auditory midbrain.

The results of this study clearly demonstrate NO plays a role in the processing of information in the TS. This conclusion is based on change in evoked responses to different species' calls after NO was removed from the system via inhibition of its production with L-NAME. In this discussion I will address is 1) the mechanism/s through which NO is acting, 2) which feature of

the advertisement call NO seems to enhance, 3) NO's possible role in sensory-motor integrations, and 4) broader implication for other species beyond anurans.

1.3 Mechanisms underlying generation of NO mediated modulation

NO can modulate neuronal activity through either pre- or post-synaptic mechanisms. Though this study is insufficient to definitively reveal which mechanisms may be employed in the TS, it does provide sufficient evidence to support pursuing such experiments in the future.

Figure 18 shows the various ways NO can modulate neuronal activity. Due to the nature of our experiments and the results we found, some of the modulatory pathways can be eliminated without further experimentation. For instance, since we were inhibiting NO production, the actions of neurotoxic peryoxynitrites ($ONOO^-$) are unlikely candidates for modulated activity since those are produced in environments containing excess NO (Steinart 2010). The same case can be made for S-Nitrosylation, which occurs when NO reaches micromolar concentrations around the cell (Steinart 2010). Again, since we were inhibiting NO production, concentrations of NO would not have been high enough to induce S-Nitrosylation. Finally, due to the time course of our experiments and the rapid nature in which the modulation of activity occurred (within one minute), modulation of gene expression or receptor trafficking to the membrane are also unlikely due to the longer timeframe required for those activities to take place. There are two cGMP dependent mechanisms to consider: pre-synaptic

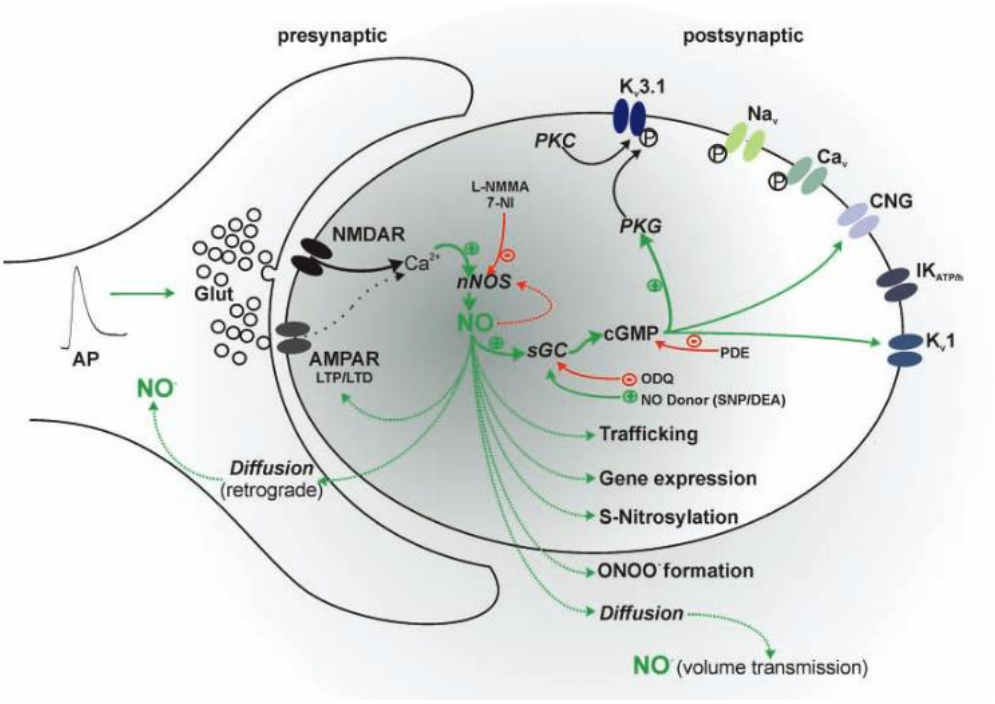

Figure 18. Various pre- and post-synaptic mechanisms of NO modulation. NO binds and activates sGC which produces cGMP. In the post-synaptic cell cGMP can go on to activate protein kinase G (PKG) or bind directly to cyclic nucleotide gated ion channels affecting membrane potential. NO can also have concentration dependent effects on trafficking, gene expression, membrane receptors, and peryoxynitrite formation (Adapted from Steinart 2010).

modulation of neurotransmitter release and post-synaptic modulation of resting membrane potential. Future experiments that can be performed to tease apart the mechanisms through which NO is acting will be explored later in the discussion.

1.4 Mechanisms mediating call-independent effects of NO modulation

1.4.1 Nitrergic modulation of pre-synaptic GABA release

GABA plays a prominent role in shaping the response properties of TS neurons to spectral and temporal sound characteristics. For example, experiments blocking GABAergic signaling reduce or abolish inhibitory flanking regions such that frequency-intensity combinations that are inhibitory under control conditions are excitatory under experimental conditions. Significantly, these effects were observed for AND neurons which are considered putative mating call detectors because they require frequency in both the high and low ranges found in the mating call to respond (Hall 1999). Neural responses to temporal call characteristics, like call duration, are also controlled by GABA dependent mechanisms. In the brown bat, duration tuning is greatly reduced or abolished by application of the GABA antagonist bicuculline (Casseday 1994, 2000). Significantly, there are neurons in the anuran TS that fire preferentially to particular stimulus durations, and a common mechanism establishing duration tuning is likely (Narins and Capranica 1980, Gooler and Feng 1992).

GABA has also been shown to affect temporal response properties such as direction-dependent frequency tuning, AM tuning, and gain control

mechanisms that enhance mating call detection over background noise (Hall 1994 1999, Zhang and Feng 1999). Taken together, GABA-mediated processes have been shown to contribute significantly to the processing and identification of advertisement calls. Of the different NO modulatory mechanisms, one candidate to explain the results seen in our experiments is the pre-synaptic modulation of GABA release.

An observed increase in response magnitude during L-NAME application is compatible with the hypothesis that NO modulates GABA release. In cases where the responses to both calls is increased, such as the cell depicted in figure 9, decreasing pre-synaptic GABA release should result in an increase in excitability. Whether pre-synaptic GABA release can be totally blocked by L-NAME application is not known at present.

Pre-synaptic modulation of glycine and acetylcholine release could also mediate call-independent effects of L-NAME modulation via inhibitory mechanisms. Both nicotinic and muscarinic acetylcholine agonists have been shown to suppress single unit activity, and cells stain positive for acetylcholinesterase throughout the TS (Farley, 1983, Habbicht 1996, Hall & Bunker 1994, Simmons & Chapman 2002).

1.4.2 Nitrergic modulation of pre-synaptic excitatory neurotransmitter release

NO has been shown to increase pre-synaptic glutamate release through a cGMP dependent interaction via hyperpolarization-activated cyclic nucleotide gated ion channels (Guevara-Guzman 1994, Neitz 2011, Wang 2017).

Furthermore, NO has been implicated in modulating the pre-synaptic release of acetylcholine, which can mediate excitation through nicotinic and muscarinic acetylcholine receptors (Guevare-Guzman 1994, Trabace 2000 Prast 2001, Habbicht 1996, Farley 1983). Significantly, both glutamatergic and cholinergic neurons have been identified in the TS (Hall personal communication, Hall and Bunker 1994, Endepols 2000**)**. Thus, it is hypothesized that L-NAME application reduces the pre-synaptic release of glutamate and/or ACh release, thereby decreasing the magnitude of sound-evoked responses of post-synaptic neurons. Our findings are consistent with this hypothesis as illustrated by results presented in figure 10.

1.4.3 Modulation of postsynaptic membrane potentials

NO has been implicated in the depolarization of resting membrane potentials as described in the introduction. If NO depolarizes the resting membrane potential of a cell under control conditions, then inhibiting NO production should cause a call-independent decrease in response magnitude as illustrated in figure 10. This mechanism of modulation would mimic a decrease in excitatory neurotransmitter release or increase in inhibitory neurotransmitter relase, so further experiments would be required to determine which mechanism is occurring.

1.5 Mechanisms mediating call-dependent effects of L-NAME

A possible explanation for the results seen when the effects of L-NAME are call-independent is that it affects modulation of neurotransmitter release or

resting membrane potential. Call-dependent effects were also seen in this study and they require further explanation. Modulation of sound duration sensitivity could explain some of the call-dependent results. Sound duration is a biologically important component found in animal communication sounds and speech and there are neurons in the TS that are tuned to it. These cells' tuning is correlated to conspecific vocalization pulse length (Potter 1965, Narins and Capranica 1980, Feng 1990, Hall and Feng 1991, Condon 1991, Gooler and Feng 1992). Similar types of neurons are found in the bat auditory midbrain and their tuning sensitivity is mediated by GABA (Pinheiro 1991, Casseday 1994, Fuzessery 1994, Ehrlich 1997, Casseday 2000). As stated previously, NO plays a direct role in mediating pre-synaptic GABA release and could play a direct role in duration tuning of TS neurons.

Bullfrog and green frog advertisement calls are spectrally similar, but the bullfrog call has an approximately 400ms longer duration (Fig. 2). It can be hypothesized that differences in a unit's duration response tuning could account for the modulation of evoked responses seen in call-dependent units after L-NAME application. L-NAME could modulate neurotransmitter release shaping duration preference, and duration tuning could be lost or gained. For instance, the cell depicted in Fig. 13 could initially be tuned to stimuli with shorter durations and the response specificity is mediated by GABA. After L-NAME is applied, GABA release is reduced and the tuning preference to the green frog call is lost

because the cell no longer prefers the shorter call over the longer. The same could be applied to the cell depicted in figure 15.

1.6 NO as a volume transmitter

Explanations for some of the results reported here cannot be explained by alterations of neurotransmitter release at a single synapse. A major issue in understanding central auditory processing is how multiple synaptic inputs converge and interact to shape the response properties of TS neurons. In some cases, the evoked responses to both calls are affected in completely opposite ways after NO production is blocked via L-NAME (Figure 15, 16). While it has already been postulated that loss of call duration sensitivity may account for these effects, another possibility is that NO volume transmission modulated multiple inputs to the neuron from which the recording was made.

TS cells are highly differentiated regarding their soma morphology, dendritic arborization and axonal projections (Luksch 1998). While some cells are smaller, many have axons and dendrites that extend hundreds of microns into different toral subdivisions or out of the torus altogether (Feng 1993, Luksch 1998) (Figure 19). Being a volume transmitter, NO can modulate the activity of entire microcircuits and not just individual synapses. However, the extent of NO's sphere of influence is typically no more than a couple hundred microns (Hill 2010, Lancaster 1996). That distance is not enough to cover the entire range of inputs converging on a cell. Therefore, the spatial arrangement of inputs on the

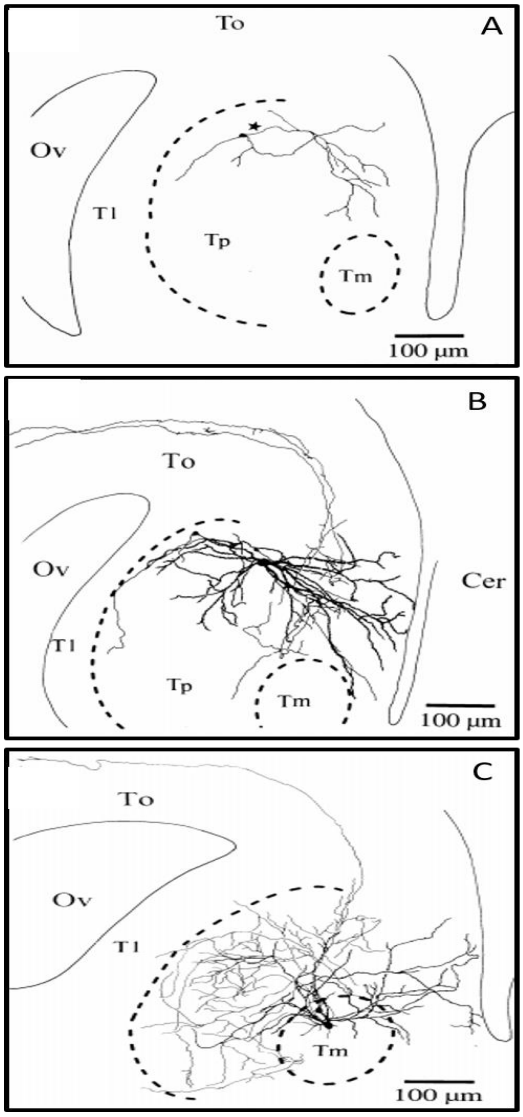

Figure 19. Intracellularly stained TS neurons. A: principal nucleus neuron. Star indicates origin of axon. B: principal nucleus neuronal ensemble. C: magnocellular nucleus neuronal ensemble. Ov: optic ventricle, To: optic tectum, Tl: laminar nucleus, Tp: principal nucleus. Tm: magnocellular nucleus. Cer: cerebellum (Figure adapted from Luksch 1998).

cell for each call could vary in such a way that L-NAME application disproportionately affects the evoked response to one call. This arrangement could explain the call-dependent modulations in evoked responses to one call as seen in figures 11 and 12, or the differential responses seen in figures 15 and 16. For the cells represented in both figures 11, and 12, only the evoked response to the bullfrog call was affected by L-NAME. This could indicate inputs to the cell which create its response to the bullfrog call are arranged more distally to the cell body, since these responses are being modulated while the evoked responses to the green frog are not. Likewise, for the cell represented in figure 16, the increasing application currents have increasing effects on evoked responses. This could be because as more drug is applied it spreads further, affecting more proximal inputs to the cell we recorded from.

1.7 Contributing to existing knowledge/Implications for other species

This study builds on the pre-existing knowledge we have of nitric oxide in the auditory system. Histo- and cytochemical staining has revealed large quantities of NOS in the TS, and voltammetry experiments demonstrate that NO is indeed produced. The findings of this study start to give us some idea of what role it could be playing in auditory processing.

There is an abundance of information regarding where nNOS is located in the brains from different animal taxa, and the way it modulates neuronal responses shown using *in vitro* experiments and data. However, there is a paucity of information regarding how NO effects neuronal processing *in vivo*

using biologically relevant stimuli (Hinova-Palova 2017). In accordance with data from the visual, olfactory and somatosensory systems of other species, NO directly influences signal processing. While the role it plays is not yet entirely evident, NO does seem to influence call discrimination between conspecific and heterospecific advertisement calls.

Results from this study could have broader reaching implications due to the ubiquity of nNOS in the auditory system. NOS staining techniques have revealed NO to be present in the TS of other anuran and bufo species, as well as the inferior colliculi of mammals, including humans (Mizukawa 1989, Vincent and Kimura 1992, Moreno-Lopez 1998, Sazon and Beitz 2000, Dzhambazova 2008, Hinova-Palova 2017). Like the anuran TS, the human IC serves as a neural hub integrating spectral, and temporal features of sound and NADPHd positive cells vary according to cell morphology (Hinova-Palova 2017). However, one should be careful with comparisons due to the anatomical differences between the two brain regions and the different distributions of NADPHd positive neurons within the subdivisions of frogs represents more closely the NADPHd staining distribution in the rat compared to humans (Wu 2008, Boyd 2006, Hinova-Pilova 2017).

1.8 Future directions and experiments

While this study sheds some light on the role NO is playing in signal processing in the anuran IC there are still several questions than need to be explored. First, through what mechanism is NO producing the results seen in this

study? As stated above NO works through both pre- and postsynaptic pathways to influence a neuron's excitability.

Determining if NO acts pre-synaptically modulates neuronal activity requires the removal of extracellular NO from the system. This can be accomplished through application of a NO scavenger like hemoglobin (Guevara-Guzman 1994). If evoked responses change after application of hemoglobin, it can be inferred that NO is acting through pre-synaptic alterations of neurotransmitter release on the target cell. Moreover, depending on whether postsynaptic activity increases or decreases, the type of neurotransmitter being released can be further narrowed to being either excitatory or inhibitory. However, if no change in activity is recorded after hemoglobin application, one can infer post-synaptic NO mechanisms are influencing the evoked responses of a cell.

A cGMP-dependent post-synaptic mechanisms of action can be investigated by the application of the membrane permeable cGMP analogue, 8-bromo-cyclic GMP, or the cGMP antagonist, 1H- [1,2,4] oxadiazolo [4.3-a] quinoxalin-1-one (ODQ) (Guevera-Guzman 1994, Wang 2017). If NO is acting through postsynaptic cGMP mechanisms, applying the analog should have the same effect as applying a NO donor, and applying the antagonist should mimic the effect of an NOS antagonist such as L-NAME. Further experiments antagonizing the activity of different cGMP targets e.g. cyclic nucleotide gated ion

channels or protein kinases, would further reveal the route NO is taking to mediate its effect on neuronal activity.

Second, an assumption made in this study is that the difference in call duration between the bullfrog and green frog advertisement calls is the critical characteristic driving the differential effects of NO on evoked responses to the calls. Experiments using tones of different durations could be used to confirm this is correct. Using tones would eliminate any spectral differences between stimuli and temporal differences such as rise/fall time and AM rate could also be controlled for.

Finally, anurans typically chorus near bodies of water where masking of conspecific calls by other species can be problematic. While species have evolved to produce calls that are spectrally and temporally different this doesn't completely mitigate the overlap of advertisement calling. NO could play a role in enhancing conspecific calls in noisy environments. To answer this question, recordings of TS neurons' responses to conspecific advertisement calls presented alone, and with background noise could be made before and after L-NAME application. Differences in responses could be measured and further conclusions about NO's role in auditory processing could be elucidated.

LIST OF REFERENCES

Adams, C., Stevens, K., Kem, W., Freedman, R. (2000) Inhibitino of nitric oxide synthase prevents alpha 7 nicotinic receptor-mediated restoration of inhibitory auditory gating in rat hippocampus. *Brain Research*. **877,** 235-244

Ahern, G., Klyachko, V., Jackson, M. (2002) cGMP and S-nitrosylation: two routes for modulation of neuronal excitability by NO. *TRENDS in Neurosciences.* **25**. 510-517

Alder, T., Rose, G. (1998) Long-term temporal integration in the anuran auditory system. *Nature Neuroscience*. **1**. 519-522

Alder, T., Rose, G. (2000) Integration and recovery processes contribute to the temporal selectivity of neurons in the midbrain of the northern leopard frog, *Rana pipiens*. *Journal of Comparative Physiology A*. **186**. 923-937

Alderton, W., Cooper, C., Knowles, G. (2001) Nitric oxide synthases: structure, function and inhibition. *Biochemical Journal*. **357**, 593-615.

Arak, A (1988) Female mate selection in the natterjack toad: active choice or passive attraction? *Behavio. Ecol. Sociobiol.* **22**. 317-327

Arancio, O., Kiebler, M., Lee, C., Lev-Ram, V., Tsien, R., Kandel, E., Hawkins, R. (1996) Nitric oxide acts directly in the presynaptic neuron to produce long-term potentiation in cultured hippocampal neurons. *Cell*. **87**. 1025-1035

Backwell, P. (1988) Functional partitioning in the two-part call of the leaf-folding frog, *Afrixalus delicates*. *Herpetologica*. **47**:226-229

Beckman, J., Koppenol, W. (1996) Nitric Oxide, superoxide, and peryoxynitrite: the good, the bad, and the ugly. *American Physiological Society*. **271**. C1424-C1437

Bee, M. Perrill, S., Owen, P. (1999) Size assessment in simulated territorial encounters between male green frogs (*Rana clamitans*). *Behavioral Ecology Sociobiology*. **45**. 177-184

Bee, M., Gerhardt, C. (2001b) Neighbor-stranger discrimination by territorial male bullfrogs (*Rana catesbeiana*): I. Acoustic basis. *Animal Behaviour*. **62**. 1129-1140

Benedix, J., Pedemonte, M., Velltie, R., Narins, P. (1994) Temperature dependence of two-tone rate suppression in the northern leopard frog, *Rana pipiens pipiens*. *Journal Acoustical Society America*. **96**. 2738-2745

Billar, T., Curran, R., Harbrecht, B., Stadler, J., Williams, D., Ochoa, J., Di Silvio, M., Simmons, R., Murray, S. (1992) Association between synthesis and release of cGMP and nitric oxide biosynthesis by hepatocytes. *American Journal of Physiology*. **262**. 1077-1082

Boatright-Horowitz, S., Horowitz, S., Simmons, A. (2000) Pattern of vocal interactions in a Bullfrog (*Rana catesbeiana)* chorus: preferential responding to far neighbors. *Ethology*. **106**. 701-712

Bogert, C. (1960) The influence of sound on the behavior of amphibians and reptiles. *Animal sounds and communication.* pp 137-320

Bradley, S., Steinert, J. (2016) Nitric Oxide-mediated posttranslational modifications: Impacts at the synapse. *Oxidative medicine and cellular longevity*. **2016**. 1-9.

Bredt, D., Glatt, C., Hwang, P., Fotuhi, M., Dawson, T., Snyder, S. (1991) Nitric oxide synthase protein and mRNA are discretely localized in neuronal populations of the mammalian CNS together with NADPH diaphorase. *Neuron*. **7**. 615-624

Bredt, D., Snyder, S. (1990) Isolation of nitric oxide synthase, a calmodulin-requiring enzyme. *Proc. Natl. Acad. Sci. USA.* **87**. 682-685

Breer, H., Shepherd, G. (1993) Implications of the NO/cGMP system for olfaction. *TINS*. **16**. 5-9.

Brenman, J., Chao, D., Gee, S., MsGee, A., Craven, S., Santillano, D., Wu, Z., Huang, F., Xia, H., Peters, M., Froehner, S., Bredt, D. (1996) Interaction of nitric oxide synthase with the postsynaptic density protein PDS-95 and α1-syntrophin mediated by PDZ domains. *Cell*, **84**, 757-767.

Briollet, M., Firestein, S. (1996). Direct activation of olfactory cyclic nucleotide-gated channel through modification of sulfhydryl groups by NO compounds. *Neuron*. **16**. 377-385.

Bryan, N., Bian, K., Murad, F. (2009) Discovery of nitric oxide signaling pathway and targets for drug development. *Frontiers in Bioscience*. **14**, 1-18

Burette, A., Zabel, U., Weinberg, R., Schmidt, H., Valtschanoff, J. (2002) Synaptic localization of nitric oxide synthase and soluble guanylyl cyclase in the hippocampus. *Journal of Neuroscience*. **22**. 8961-8970

Capranica, R. (1965) The evoked vocal responses of the bullfrog. Cambridge, Massachusetts: MIT Press

Capranica, R. (1966) Vocal response of the bullfrog to natural and synthetic mating calls. *Journal of the Acoustical Society of America*. **40**. 1131-1139

Capranica, R. (1978) Auditory Processing in Anurans. *Fed Proc* **37**. 2324-2328

Capranica, R., Moffat, A. (1983) Neurobehavioral correlates of sound communication in anurans. *Advances in Vertebrate Neuroethology*. pp. 701-730

Casseday, J., Covey E. (1995) Mechanisms for analysis of auditory temporal patterns in the brainstem of echolocating bats. In: *Neural representations of temporal patterns*. pp. 25-52

Casseday, J., Ehrlich, D., Covey, E. (2000) Neural measurements of sound duration: control by excitatory-inhibitory interaction in the inferior colliculus. *The American Physiological Society*. 1475-1487

Chakraborty, M., Burmeister, S. (2009) Estradiol induces sexual behavior in female tungara frogs. *Hormones and Behavior*. **55**. 106-112

Chakraborty, M., Burmeister, S. (2010) Sexually dimorphic androgen and estrogen receptor mRNA expression in the brain of tungara frog. *Hormones and Behavior*. **58**. 619-627

Chakraborty, M., Burmeister, S. (2015) Effects of estradiol on neural responses to social signals in female tungara frogs. *The Company of Biologists*. **218**. 3671-3677

Condon, C., Chang. S., Feng, A. (1991) Processing of behaviorally relevant temporal parameters of acoustic stimuli by single neurons in the superior olivary nucleus of the leopard frog. Journal *of Comparative Physiology*. **168**. 709-725

Condon, C., Chang, S., Feng, A. (1995) Classification of the temporal discharge patterns of single auditory neurons in the frog superior olivary nucleus. *Hearing Research*. **83**. 190-202

Covey, E. (2000) Neural population coding and auditory temporal pattern analysis. *Physiology and behavior*. **69**. 211-220

Cudeiro, J., Grieve, K., Rivadulla, C., Rodriguez, R., Martinez-conde, S., Acuna, C. (1994) The role of nitric oxide in the transformation of visual information within the dorsal lateral geniculate nucleus of the cat. *Neuropharmacology*. **33**. 1413-1418

Cudeiro, J., Grieve, K., Rivadulla, C., Rodriguez, R., Martinez-conde, S., Acuna, C., Alonso, J. (1994) Modulatory influence of putative inhibitors of nitric oxide synthesis on visual processing in the cat lateral geniculate nucleus. *Journal of Neuroscience*. **71**. 146-149.

Cudeiro, J., Rivadulla, C. (1999) Sight and insight – on the physiological role of nitric oxide in the visual system. *Trends in Neurosciences*. **22**, 109-116

Denninger, J., Marletta, M. (1999) Gunaylate cyclase and the NO/cGMP signaling pathway. *Biochimica et Biophysica Acta 1411*. 334-350

Do, K., Binns, K., Salt, T. (1994) Release fo the nitric oxide precursor arginine from the thalamus upon sensory afferent stimulation and its effect on thalamic neurons *in vivo*. *Neuroscience.***60**. 581-586.

Druga, R., Syka, J. (1993) NADPH-diaphorase activity in the central auditory structures of the rat. *NeuroReport*. **4**. 999-1002.

Dunah, A., Yasuda, R., Wang, Y., Luo, J., Davila-Garcia, M., Gbadegesin, M., Vicini, S., Wolfe, B. (1998) Regional and ontogenetic expression of NMDA receptor subunit NR2D protein in rat brain using subunit-specific antibody. *Journal of Neurochemistry*. **71**. 1926-1934

Dunia, R., Narins, P. (1988) Temporal resolution in the frog auditory nerve fibers. *Journal of the Acoustical Society of America*. **85**. 1630-1638

Edwards, C., Alder, T., Rose, G. (2002) Auditory midbrain neurons that count. *Nature Neuroscience*. **5**. 934-936

Eggermont, J. (1989) Coding of free field intensity in the auditory midbrain of the leopard frog, I. Results for tonal stimuli. *Hearing Research*. **40**. 147-166

Ehrlich, D., Casseday, J., Covery, E. (1997) Neural tuning to sound duration in the inferior colliculus of the big brown bat, *Eptesicus fuscus*. *Journal of Neurophysiology*. **77**. 2360-2372

Eldred, W., Blute, T. (2005) Imaging of nitric oxide in the retina. *Vision Research*. **45**. 3469-3486

Endepols H, Walkowiak, W. (2001) Integration of ascending and descending inputs in the auditory midbrain of anurans. *Journal of Comparative Physiology*. **186**, 1119-1133

Epping, W., Eggermont, J. (1985) Relation of binaural interaction and spectro-temporal characteristics in the auditory midbrain of the grassfrog. *Hearing Research*. **19**. 15-28

Farley, G., Morley, B., Javel, E., Gorga, M. (1983) Single-unit responses to cholinergic agents in the rat inferior colliculus. *Hearing Research*. **11**. 73-91

Feng, A. (1983) Morphology of neurons in the torus semicircularis of the northern leopard frog *Rana pipiens pipiens*. *Journal of Morphology*. **175**. 253-269

Feng, A. (1986) Afferent and efferent innervation patterns of the superior olivary nucleus of the leopard frog. *Brain Research*. **364**. 167-171

Feng, A. (1986a) Afferent and efferent innervation patterns of three divisions of the cochlear (dorsal medullary nucleus) of the leopard frog. *Brain Research*. **364**. 183-191

Feng, A., Capranica, R. (1976) Sound localization in anurans. I. Evidence of binaural interaction in dorsal medullary nucleus of bullfrog (*Rana catesbeiana*). *Journal Neurophysiology*. **41**. 43-54

Feng, A., Hall, J., Gooler, D. (1990) Neural basis for sound pattern recognition in anurans. *Prog. Neurobiol*. **34**. 313-329

Feng, A., Lin, W. (1991) Differential innervation patterns of three divisions of the frog auditory midbrain (torus semicircularis). *Journal of Comparative Neurology*. **306**. 613-603

Feng, A., Lin, W. (1996) Neuronal architecture of the dorsal nucleus (cochlear nucleus) of the frog, *Rana pipiens*. *Journal Comparative Neuroscience*. **366**. 320-334

Foquette, M. (1975) Speciation in chorus frogs. I. Reproductive character displacement in the *psuedacris nigrita* complex. *Syst Zool*. **24**. 16-23

Foster, M., McMahon, T., Stamler, J. (2003) S-nitrosylation in health and disease. *TRENDS in Molecular Medicine*. **9**. 160-168

Francis, S., Busch, J., Corbin, J. (2010) cGMP-dependent kinases and cGMP phosphodiesterases in nitric oxide and cGMP action. *Pharmacological Reviews*. **65**, 525-563

Fuzessery, Z., Feng, A. (1981) Frequency representation in the dorsal medullary nucleus of the leopard frog, *Rana p. pipiens*. *Journal Comparative Physiology*. **143**. 339-349

Fuzessery, Z., Feng, A. (1983) Mating call selectivity in the thalamus and mid brain of the leopard frogs (*Rana p pipiens*): single and multiple analyses. *J Comp Physiol*. **150**. 333-344

Fuzessery, Z. (1994) Response selectivity for multiple dimensions of frequency sweeps in the pallid bat inferior colliculus. *Journal Neurophysiology*. **72**. 1061-1079

Fuzessery, Z., Feng, A. (1983) Frequency selectivity in the anuran medulla: Excitatory and inhibitory tuning projections of single neurons in the dorsal medullary and superior olivary nuclei. *Journal Comparative Physiology*. **150**. 107-119

Garthwaite, J. (2008) Concepts of neural nitric oxide-mediated transmission. *European Journal of Neuroscience*. **27**, 2783-2802

Garthwaite, J., Boulton, J. (1995) Nitric oxide signaling in the central nervous system. *Annual Review Physiology*. **57**. 683-706

Garthwaite, J., Charles, S., Chess-Williams, R. (1988) Endothelium-derived relaxing factor release on activation of NMDA receptors suggests role as intercellular messenger in the brain. *Nature*. **336**, 385-388

Gerhadrt, H. (1988) Acoustic properties used in call recognition by frogs and toads. In. *The evolution of the amphibian auditory system*. pp. 455-483

Gerhardt, C., Bee, M. (2007) Recognition and Localization of Acoustic Signals. *Hearing and Sound Communication in Amphibians* pp. 113-147

Gerhardt, H. (2015) Auditory selectivity for the acoustic properties of conspecific mate-attracting signals in lower vertebrates and songbirds. *Open Access Animal Physiology*. **7**. 73-85

Gerhardt, H., Diekemp, B., Ptacek, M. (1989) Inter-male spacing in choruses of the spring peeper, *Pseudacris (Hyla) crucifer. Animal Behviour.* **38**. 1012-1024

Gerhardt, H., Schwartz, J. (2001) Auditory and frequency preferences in anurans. In: Ryan, M. (ed) Anuran Communication. Washington DC. *Smithsonian Instiution Press.* 73-85

Gibb, B., Garthwaite, J. (2001) Subunits of the nitric oxide receptor, soluble guanylyl cyclase, expressed in the rat brain. *European journal of Neuroscience*. **13**. 539-544

Gonzalez, A., Smeets, W. (1991) Comparative analysis of dompamine and tyrosine hydroxylase immunoreactivities in the brain of two amphibians, the anuran *Rana ribibunda* and the urodele *Pleurodeles waltlii*. *Journal Comparative Neurology*. **303**. 457-477

Gonzalez, A., Smeets, W. (1993) Noradrenaline in the brain of the South African clawed frog *Xenopus laevis*: A study with antibodies against noradrenaline and dopamine-β-hydroxylase. *Journal Comparative Neurology*. **331**. 363-374.

Gooler, D., Feng, A. (1992) Temporal coding in the frog midbrain: The influence of duration and rise-fall time on the processing of complex amplitude-modulated stimuli. *Journal of Neurophysiology*. **67**. 1-22

Gregory, K. (1972) Central projections of the eight nerve in frogs. *Brain Behaviour and Evolution*. **5**. 70-88

Gringas, B., Mohandesan, E., Boko, D., Fitch, W. (2013) Phylogenetic signal in the acoustic parameters of the advertisement calls of four clades of anurans. *Evolutionary Biology*. **13**. 134

Guevera-Guzman, R., Emson, P., Kendrick, K. (1994) Modulation of *in vivo* striatal transmitter relase by nitric oxide and cyclic GMP. *Journal of Neurochemistry*. **62**. 807-810

Habbicht, H., Vater, M. (1996) A microiontophoretic study of acetylcholine effects in the inferior colliculus of horseshoe bats: implications for a modulatory role. *Brain Research*. **724**. (169-179)

Hall, J. Feng, A. (1988) Influence of envelope rise time on neural responses in the auditory system of anurans. *Hearing Research*. **36**. 261-276

Hall, J., Bunker, M. (1994) Acetycholinerase staining in the brainstem nuclei of the leopard frog, *Rana pipiens*. *Neuroscience Letters*. **182**. 222-226

Hall, J., Feng, A. (1986) Neural analysis of temporally patterned sounds in the frog's thalamus: processing of pulse duration and pulse repetition rate. *Neuroscience Letters*. **63**. 215-220

Hall, J., Feng, A. (1987) Evidence for parallel processing in the frog's auditory thalamus. *Journal of Comparative Neurology*. **258**. 407-419

Hall, J., Feng, A. (1991) Temporal processing in the dorsal medullary nucleus of the northern leopard frog (*Rana pipiens pipiens*) *Journal of Neurophysiology*. **66**. 955-973

Hall, J., Feng, A. (1996) Classification of the temporal discharge patterns of single auditory neurons in the dorsal medullary nucleus of the northern leopard frog. *Journal Neurophysiology*. **64**. 1460-1473

Hill, B., Dranka, B., Bailey, S., Lancaster, J., Darley-Usmar, V. (2010) What part of NO don't you understand? Some answers to cardinal questions in nitric oxide biology. *Journal of Biological Chemistry*. **26**. 19699-19704

Hinova-Palova, D., Landzhov, B., Dzhambazova, E., Edelstein, L., Minkov, M., Fakih, K., Minkov, R., Paloff, A., Ovstsharoff, W. (2017) NADPH-diaphorase-positive neurons in the human inferior colliculus: morphology, distribution and clinical implications. *Brain Structure Function*. **222**. 1829-1846

Hope, B., Michael, G., Knigge, K., Vincent, S. (1991) Neuronal NADPH diaphorase is a nitric oxide synthase. *PNAS*. **88**. 2811-2814

Huang, P., Dawson, T. Bredt, D., Snyder, S., Fishman, M. (1993) Targeted disruption of the neuronal nitric oxide synthase gene. *Cell*. **74**. 1273-1286

Jacklet, J., Tieman, D. (2004) Nitric oxide and histamine induce neuronal excitability by blocking background currents in neuron MCC of *Aplysia*. *Journal of Neurophysiology.* **91**. 656-665.

Kemenes, I., Kemenes, G., Andrew, R., Benjamin, P., O'Shea, M. (2002) Critical time-window for NO-cGMP-dependent long-term memory formation after one-trial appetitive conditioning. *Journal of Neuroscience*. **22**. 1414-1425.

Kendrick, K., Guevra-Guzman, R., Zorrilla, J., Hinton, M., Broad, K., Mimmack, M., Ohkura, S. (1997) Formation of olfactory memories mediated by nitric oxide. *Nature*. **388**. 670-674

Kulik, A., Matesez, K., Szekely, G. (1994) Mesencephalic projections of the cochlear nucleus in the frog, *Rana esculenta*. *Acta Biologica Hungarica*. **45.** 323-335

Lancaster, J. (1994) Simulation of the diffusion and reaction of endogenously produced nitric oxide. *Proc. Natl. Acad. Sci.* **91**. 8137-8141.

Lancaster, J. (1996) A Tutorial on the diffusibility and reactivity of free nitric oxide. *Nitric Oxide: Biology and Chemistry.* **1**. 18-30

Langnaese, K., Richter, K., Smalla, K., Krauss, M., Thomas, U., Wolf, G., Laube, G. (2006) Splice-isoform specific immunolocalization of neuronal nitric oxide synthase in mouse and rat brain reveals that the PDZ-complex-building nNOSα β-finger is largely exposed to antibodies. *Developmental Neurobiology*. **67**. No. 4. 422-437

Leake, L., Moroz, L. (1996) Putative nitric oxide synthase (NOS)-containing cells in the central nervous system of the leech, *Hirudo medicinalis*: NADPH-diaphorase histochemistry. *Brain Research*. **723**. 115-124

Lee, J., Cho, Y., Huh, Y., Cha, C., Yeo S. (2008) Effect of nitric oxide on auditory cortical neurons of aged rats. *Neuroscience Letters*. **447**. 37-41

Ledo, A., Barbosa, R., Gerhardt, G., Cadenas, E., Laranjinha, J. (2005) Concentration dynamics of nitric oxide in rat hippocampal subregions evoked by stimulation of NMDA glutamate receptor. *PNAS*. **102**. 17483-17488

Lewis, E. (1981) Suggested evolution of tonotopic organization in the frog amphibian papillae. *Scan Electron Microscope*. **11** .633-642

Lewis, E., Leverenz, E., Koyama, H. (1982) The tonotopic organization of the bullfrog amphibian papilla, an auditory organ lacking a basilar membrane. *Journal Comparative Physiology*. **145**. 437-445

Liff, H., Goldstein, M. (1970) Peripheral inhibition in the auditory fibers in the frog. *J Acoustic Society America.* **47**. 1538-1547

Lima, M.,Maximo, C., Matos, K., Brasil, A., Crespo-Lopez, M., Batista, E., Rocha, F., Picano-Diniz, D., Herculano, A. (2014) Nitric oxide as a regulatory molecule in the processing of the visual stimulus. *Nitric Oxide*. **36**. 44-50

Liu, Q. Gross, S. (1996) Binding sites of nitric oxide synthases. *Methods in enymology*. **268**, 311-324

Liu, X., Miller, M., Joshi, M., Thomas, D., Lancaster, J. (1998) Accelerated reaction of nitric oxide with O2 within the hydrophobic interior of biological membranes. *PNAS*. **95**. 2175-2179

Luksch, H., Walkowiak, W. (1998) Morphology and axonal projection patterns of auditory neurons in the midbrain of the painted leopard frog, *Discolossus pictus*. *Hearing Research*. **122**. 1-17

Komeima, K., Hayashi, Y., Naito, Y., Watanabe, Y. (2000) Inhibition of nNOS by calcium/calmodulin-dependent protein kinase II alpha through Ser 847 phosphorylation in NG108-15 neuronal cells. *Journal of Biological Chemistry*. **275**. 28139-28143

Malinski, T., Taha, Z., Grunfield, S., Patton, S., Kapturczak, M., Tomboulian, P. (1993) Diffusion of nitric oxide in the aorta wall monitored *in situ* by porpyrinic microsensors. *Biochem. Biophys. Res. Commun.***193** 1076-1082

Martin, W. (1972) Evolution of vocalizations in the genus *Bufo*. *Evolution of the genus Bufo.* 279-309

Mason, M. (2007) Pathways for sound transmission to the inner ear in amphibians. *Hearing and Sound Communication in Amphibians* pp. 147-183

Matesez, C., Kulik, A. (1996) Connections of the torus semicircularis and oliva superior in the frog, *Rana esculenta*. *Acta biologica Hungarica*. **47**. 287-301

Megela-Simmons, A., Moss, C., Kimberly, D. (1985) Behavioral audiograms of the bullfrog (*Rana catesbeiana*) and the green tree frog (*Hyla cinerea*). *Journal of the Acoustical Society of America*. **78**. 1236-1244

Meulemans, A. (1994) Diffusion coeffecients and half-lives of nitric oxide and N-nitroso-L-arginine in rat cortex. *Neuroscience Letters*. **171**. 89-93

Montague, P., Gancyo, C., Winn, M., Marchase, R., Friedlander, M. (1994) Role of NO production in NMDA-receptor mediated neurotransmitter release in cerebral cortex. *Science*. **263**. 973-977

Munoz, A., Munoz, M., Marin, O., Alonso, J., Porteros, A., Gonzalez, A. (1996) Topographical distribution of NADPH-diaphorase activity in the central nervous system of the frog *Rana perezi*. *Journal of Comparative Neurology*. **367**. 54-69

Nakamura, T., Lipton, S. (2008) Protein S-nitrosylation: purview and parameters. *Nature Reviews Cell Biology*. **10**. 87-101.

Narins, P., Capranica, R. (1980) Neural adaptations for processing the two-note call of the Puerto Rican treefrog, *Elutherodactylas coqui*. *Brain and Behavior Evolution*. **17**. 48-66

Narins, P., Capranica, R. neural adaptations for processing the two-note call of the treefrog *Eleutherodactylus coqui*. *Journal of Comparative Physiology*. **127**. 1-9

Nemargut, J., Wang, G. (2009) Inhibition of nitric oxide synthase desensitizes retinal ganglion cells to light by diminishing their excitatory synaptic currents under light adaptation. *Vision Research*. **49**. 2936-2947

Paloff, A., Hinova-Palova, D. (1998) Topographical distribution of NADPH-diaphorase-positive neurons in the cat's inferior colliculus. *Journal Brain Research*. **39**. 231-243

Penna, M. (1997) Selectivity of evoked vocal responses in the time domain by frogs of the genus Batrachlya. *Journal Herpatology*. **31**. 202-217

Penna, M., Lin, W., Feng, A. (2001) Temporal selectivity by single neurons in the torus semicircularis of Batrachyla anatartandica (Amphibia: Leptodactylidae). *Journal Comparative Phsyiology A.* **187**. 901-912

Pettigrew, A. (1981) Brainstem afferents to the torus semicircularis of the Queensland toad (*Bufo marinus*). *Journal Comparative Neurology*. **202**. 59-68

Pinheiro, A., Wu, M., Jen, P. (1991) Encoding repetition rate and duration in the inferior colliculus of the big brown bat, *Eptesicus fuscus*. *Journal of Comparative Physiology A*. **169**. 69-85

Potter, H. (1965) Mesencephalic auditory regions of the bullfrog. *Journal Neurophysiology*. **28**. 1132-1154

Price, R., Mayer, B., Beitz, A. (1993) Nitric Oxide synthase neurons in rat brain express more NMDA receptor mRNA that non-NOS neurons. *NeuroReport*. **4**. 807-810

Purgue, A., Narins, P. (2000) Mechanics of the inner ear of the bullfrog (*Rana, catesbeiana*): the contact membranes and the periotic canal. *Journal Comparative Physiology*. **186**. 481-488

Ralin, D. (1976) Comparative hybridization of diploid-tetraploid cryptic species pair of treefrogs, *Copeia*. 191-196

Rameau, G., Chiu, L., Ziff, E. (2007) Bidirectional regulation of neuronal nitric-oxide synthase phosphorylation at serine 847 by the *N*-methyl-D-aspartate receptor. *Journal of Biological Chemistry*. **279**. 14307-14314

Reuss, S., Reimann, R. (2000) Distributino and projections of nitric oxide synthase neurons in the rodent superior olivary complex. *Microscopy research and technique*. **51**. 318-329

Reuss, S., Schaeffer, D., Laages, M., Riemann, R. (2000) Evidence for increased nitric oxide production in the auditory brain stem of the aged dwarf hamster (Phodopus sunforus): an NADPH-diaphorase histochemical study. *Mechanisms of Ageing and Development*. **112**. 125-134

Ronken, D., Bosch, W., Molnar, C. (1993) Effects of spike discharge history on discharge probability and latency in frog basilar papilla units. *Hearing Research*. **69**. 55-75

Rose, G. Capranica, R. (1985) Sensitivity to amplitude modulated sounds in the anuran auditory nervous system. *Journal Neurophysiology*. **53**. 446-465

Rose, G., Gooler, D. (2007) Function of the amphibian central auditory system. *Hearing and sound communication in amphibians* pp. 250-290

Sanchez-Zuriaga, D., Marti-Gutierrez, N., De la Cruz, M., Peris-Sanchis, M. (2007) Age-related changes of NADPH-diaphorase-positive neurons in the rat inferior colliculus and auditory cortex. *Microscopy Research and technique.* **70**. 1051-1059

Santos, R., Lourenco, C., Gerhardt, G., Cadenas, E., Laranjinha, J., Barbosa, R. (2011) Evidence for a pathway that facilitates nitric oxide diffusion in the brain. *Neurochemistry International*. **59**. 90-96

Sato, M., Ohtsuka, T. (2010) Opposite effects of nitric oxide on rod and cone receptors of rat retina *in situ*. *Neuroscience Letters*. **473**. 62-66.

Scherer-Singler, U., Vincent, S., Kimura, H., McGeer, E. (1983) Demonstration of unique population of neurons with NADPH-diaphorase histochemistry. *Journal of Neuroscience Methods*. **9**. 229-234

Schuman, E., Madison, D. (1994) Nitric oxide and synaptic function. *Annual Review Neuroscience*. **17**, 153-183

Shaw, P., Salt, T. (1997) Modulation of sensory and excitatory amino acid responses by nitric oxide donors and glutathione in the ventrobasal thalamus of the rat. *European Journal of Neuroscience*. **9**. 1507 – 1513

Simmons, A., Chapman, J. (2002) metamorphic changes in GABA immunoreactivity in the brainstem of the bullfrog, *Rana catesneiana*. *Brain behave Evol*. **60**. 189-206

Simmons, D., Meenderink, S., Vassilakis, P. (2007) Anatomy, physiology, and function of auditory end-organs in the frog inner ear. *Hearing and Sound Communication in Amphibians* pp. 184-220

Spessert, R., Wohlgemuth, C., Reuss, S., Layes, E. (1994) NADPH-diaphorase activity of nitric oxide synthase in the olfactory bulb: co-factor specificity and characterization regarding the interrelation to NO formation. *Journal of Histochemistry and cytochemistry*. **42**. No. 5. pp 569-575

Steinart, J., Chernova, T., Forsythe, D. (2010) Nitric oxide signaling in the brain function, dysfunction, and dementia. *The Neuroscientist*, **16**, 435-452.

Steinart, J., Kopp-scheinpflug, C., Baker, C. Chaliss, R., Mistry, R., Haustein, M., Griffin, S., Tong, H., Graham, B., Forsythe, D. (2008) Nitric oxide is a volume transmitter regulating postsynaptic excitability at a glutamatergic synapse. *Neuron*. **60**, 642-656.

Stevenson, P., Rillich, J. (2015) Adding up the odds – Nitric oxide signaling underlies the decision to flee and post-conflict depression of aggression. *Science Advances*, **1**.

Stevenson, P., Rillich, J. (2015) Adding up the odds – Nitric oxide signaling underlies the decision to flee and post-conflict depression of aggression. *Science Advances*. **1**. 1-7

Walkowiak, W. (1980) The coding of auditory signals in the torus semicircularis of the fire-bellied toad and grass frog: Responses to simple stimuli and conspecific calls. *Journal of Comparative Physiology*. **138**. 131-148

Walkowiak, W. (1984) Neuronal correlates of the recognition of pulsed sound signals in the grass frog. *Journal of Comparative Physiology A*. **155**. 57-66

Walkowiak, W., Luksch, H. (1994) Sensory motor interfacing in acoustic behavior of anurans. *American Zoologist*. **34**. 685-695

Wang, G., Liets, L., Chalupa, L. (2003) Nitric oxide differentially modulates ON and OFF responses of retinal ganglion cells. *Journal of Neurophysiology*. **90**. 1304-1313

Wells, K., Schwartz, J. (2007) The behavioral ecology of anuran communications. In: Nairns, P., Feng, A., Fay, R., Popper, A. Hearing and sound communication in amphibians. Springer-Verlag Ney York. 44-86

Wilczynski, W., Capranica, R. (1984) The auditory system of anuran amphibians. *Progress in Neurobiology*. **22**. 1-38

Wilczynski, W., Keddy-Hector, A., Ryan, M. (1992) Call patterns and basilar papilla tuning in cricket frogs. I. Differences among populations and between sexes. *Brain Behaviour and Evolution*. **39**. 229-337

Wood, J., Garthwaite, J. (1994) Models of the diffusional spread of nitric oxide: implications for neural nitric oxide signaling and its pharmacological properties. *Neuropharmacology*. **33**. 1235-1244.

Wood, K., Batchelor, A., Bartus, K., Harris, K., Garthwaite, G., Vernon, J., Garthwaite, J. (2011) Picomolar nitric oxide signals from central neurons recorded using ultrasensitive detector cells. *Journal of Biological Chemistry*. **50**. 43172-43181

Woolley, S., Gill, P., Theunissen, F. (2006) Stimulus-dependent auditory tuning results in synchronous population coding of vocalizations in the songbird midbrain. *Journal of Neuroscience*. **26**. 2499-2512

Yang, Q., Hatton, G. (1999) Nitric oxide via cGMP-dependent mechanisms incrases dye coupling and excitability of rat supraoptic nucleus neurons. *Neuroscience*. **19**. 4270-4279

Zheng, W., Hall, J. (2000) GABAergic inhibition shapes frequency tuning and modifies response properties in the superior olivary nucleus of the leopard frog. *Journal of Comparative Physiology*. **186**. 661-671

Zhu, X., Luo, L. (1992) Effect of nitroprusside (nitric oxide) on endogenous dopamine release from rat striatal slices. *Journal Neurochemistry*. **59**. 932-935

Zoltan, M., Fuzessery, M., Feng, A. (1982) Frequency selectivity in the anuran auditory midbrain: single unit responses to single and multiple tone stimulation. *Journal of Comparative Physiology*. **146**. 471-484

VITA

Andrew Stafford was born in Howell, MI in 1986. In 2005 he graduated from Columbia Central High School in Columbia, TN. He went to college at the University of Tennessee at Knoxville where he majored in microbiology. Here Andrew met his lovely wife, Jessica. Andrew received his BS in biology with a concentration in microbiology in August 2010. In the fall of 2012, Andrew began graduate school, remaining at the University of Tennessee at Knoxville. Andrew rotated in the Burch-Smith, Bembenek, and Hall labs and ultimately chose Dr. Hall's lab due to his interest in neuroscience. Andrew continued his rotation project on the neural mechanisms behind vibration source localization in the fiddler crab. In October 2014 Andrew and Jessica were blessed with a daughter, who they named Emmeline. In 2015 Andrew started a project exploring the effect of NO on stimulus processing in the auditory midbrain. Andrew finally finished his project 6 years after beginning graduate school.