Norepinephrine and Vocal Communication in the Rat

by

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A dissertation submitted in partial fulfillment of the requirements for the degree of

Doctor of Philosophy

(Communication Sciences and Disorders)

at the

UNIVERSITY OF WISCONSIN-MADISON

2015

Date of final oral examination: 9/15/2015

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Abstract

Voice and communication are significantly impaired in Parkinson disease (PD),[1-7] frequently manifesting early in the disease process,[8-11] and negatively impacting social interactions and quality of life.[1,6,12] Voice deficits are refractory to pharmacological and surgical therapies aimed at treating the primary disease pathology of nigrostriatal dopamine depletion.[13-17] This suggests that alternative, non-dopaminergic, mechanisms contribute to these early-onset vocal deficits. Germane to this issue, loss of norepinephrine (NE) in brainstem regions critical to neuromodulation, sensorimotor, and cognitive functions also occurs early in the disease progression in humans with PD[18-20] and has been implicated in the manifestation and treatment of other refractory deficits.[20-23] Consistent with what is observed in humans, vocal communication and NE are compromised in transgenic mouse and genetic rat models of PD,[24,25] indicating that NE loss may contribute to the manifestation of vocal communication deficits in these models. However, in each of these models, there were other pathologies that could be contributing to these vocal deficits. Importantly, the contribution of NE in normal vocal control and in PD related voice deficits has not been well established. The present work employed a rat model to address these gaps in knowledge. In Chapter 2 we establish that NE depletion following the administration of the neurotoxin DSP-4 is sufficient to disrupt pertinent vocal communication parameters. In Chapter 3 we used NE adrenoceptor agonists and antagonists to quantify the contribution of specific NE receptors to pertinent vocalization parameters. This work is significant because results confirm the involvement of NE in vocal control and point to potential pharmacological targets for treating voice deficits in PD.

Acknowledgements

"*Isn't it funny how day by day nothing changes, but when you look back, everything is different …*" C.S. Lewis

This dissertation represents the culmination of 6 years of hard work, late nights and countless hours of studying that hardly seem like anything in and of themselves, but somehow add up to one cohesive accomplishment. An accomplishment that would not have been possible without the unwavering support of some very important people, who, day by day, have helped me shape a goal and a few ideas into something.

Michelle, my mentor in every sense of the word. There really is no way to articulate how grateful I am for all that you have taught me; in the classroom, in the lab and in my life. Your unrelenting positive attitude has shown me what a powerful force one can be if we keep our heads up, look forward and smile. You have instilled in me a confidence that extends beyond academic and scientific knowledge, and provides me with the skills necessary to move on and make a career for myself. Thank you.

I would also like to acknowledge and thank my committee members, Drs. Lyn Turkstra, Sheila Fleming, Nathan Welham, Vaishali Bakshi and Nadine Connor. Thank you for sharing your time, expertise and encouragement. In particular, I want to thank Vaishali for meeting with me and helping me design a pharmacological study, patiently walking me through the details. And to Nadine, who took me under her wings and treated me as one of her own, both formally as my T32 mentor, and informally as she effortlessly modeled what it takes to navigate academia.

The university laboratory is an ever revolving machine, with projects and faces constantly changing, year in and year out. In the past 6 years, I have had the privilege of working alongside some truly remarkable individuals in the Ciucci and Connor Labs (and beyond). I am humbled and honored to count myself as one of “them.” Specifically, I want to thank Jaime Moore, Aaron Johnson, Allison Schaser, John Russell, Heidi Kletzien, Corinne Jones and Kelsey Barth for being outstanding scientists and role models, always ready to talk “science” or help me troubleshoot a problem.

The friendships I have made during this time have had a tremendous impact on who I have become as a scientist and person. A few people deserve an honorable mention and thanks. To Heidi who has been my confidant and sounding board for all things scientific and personal. To Jaime, who has shared this journey with me from the beginning, in so many ways. And to Kelsey, who is meticulous and dedicated and wise beyond her years. You are true friends and colleagues, and I thank you.

A final acknowledgement goes to my husband and biggest supporter, Jason, who arguably has just as much invested in this dissertation as I do. You pushed me to go back to school, you held my hand when I needed encouragement and gave me space when I needed to work. There really are no words to describe how much I appreciate the support and encouragement you provide for me every day. Not least of which includes picking up the slack when I am gone or when I am home, but not always present. I love you and I thank you.

This work was supported by the National Institute on Deafness and other Communication Disorders T32 DC009401 and the Department of Surgery-Division of Otolaryngology.

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Chapter 1: General Introduction and Background

Overview

As many as 90% of individuals with Parkinson disease (PD) experience communication deficits that are devastating to quality of life.[1,2] Unfortunately, these communication deficits have proven difficult to treat because they are not amenable to levodopa or deep brain stimulation.[3-7] These standard treatments for PD are directed at nigrostriatal dopamine depletion, long considered to be the primary pathology of the disease. The relative ineffectiveness of standard treatments suggests that communication deficits in PD may be driven by non-dopaminergic mechanisms. This notion is reinforced by evidence that voice deficits, in particular, are thought to emerge early in the disease process, prior to the onset of significant nigrostriatal dopamine depletion.[8-11] Currently, the only potentially effective treatment that is used for voice and communication deficits in patients with PD is behavioral voice therapy, such as the Lee Silverman Voice Treatment (LSVT).[12-19] Interestingly, while the mechanisms underlying improvement with voice therapy are not known, they do not appear to be dopaminergic.[20]

The pathology in PD is widespread, affecting multiple brainstem and cortical regions and neurotransmitter systems, in addition to depletion of nigrostriatal dopamine.[21-26] Germane to this issue, there is increasing evidence that extra-striatal mechanisms such as broad alpha-synuclein (aSyn) aggregation in the peripheral and central nervous system [27-29] and compromise of other neurotransmitter systems, such as norepinephrine (NE) in the locus coeruleus (LC),[21,22,26,30] may be critical to understanding PD-related deficits that are not amenable to standard, dopaminergic

treatments. However, the contribution of extra-striatal mechanisms to vocal function and management of voice disorders in PD has not been defined. One obstacle to addressing these questions is a lack of a robust PD model to investigate early, non-dopaminergic mechanisms in PD. The recent development of genetic and transgenic PD models in rodents offer a unique opportunity to evaluate the relationship between underlying neural mechanisms associated with phenotypic behaviors such as vocal deficits.[31-37]

The central hypothesis underlying this work is that early norepinephrine loss is related to the manifestation of voice and communication deficits in PD. However, in order to address this hypothesis two important steps must be taken. First, an appropriate model of PD to study early vocal deficits as they relate to extra-striatal pathologies, including NE loss, must be developed and characterized. Towards this end, two important studies from our lab are highlighted and detailed below. Briefly, we quantified ultrasonic vocalizations (USV) in a transgenic mouse model of PD, demonstrating that vocal deficits are present in a pre-manifest, parkinsonian mouse model that includes compromise of noradrenergic cell bodies in the LC.[31] In addition, we also found early and progressive vocalization and oromotor deficits in a genetic rat model of PD, which includes compromise of noradrenergic (NE) neurons in the LC. This foundational knowledge is crucial for understanding the underlying mechanisms of voice disorders in patients with PD and for developing and refining effective treatment interventions.

The second crucial step towards addressing the hypothesis that NE loss is related to voice and communication deficits is to quantify the contribution of NE to vocal

communication. Specifically, we need to address how specific noradrenergic mechanisms relate to pertinent aspects of vocal behavior. The following chapters describe two studies aimed at establishing (Chapter 2) and quantifying (Chapter 3) the contribution of NE in vocal function. Figure 1.1 details an overview on what is known regarding vocal communication deficits in PD, treatment, and noradrenergic mechanisms as they relate to the questions addressed in this dissertation. This work complements recent evidence in rodent models of PD implicating NE loss in the manifestation of vocal deficits [31,36] and points to potential therapeutic targets for attenuating specific vocal communication deficits via noradrenergic mechanisms.

Pathophysiology of Parkinson Disease

Post-mortem studies from humans have demonstrated that the pathology related to PD causes degeneration of neurons that follows a predictable path through the brain.[21,22,28] Long unmyelinated and poorly myelinated projection neurons are most vulnerable to the pathology of PD. Specifically, aggregations called Lewy body inclusions and neurites form throughout the central and peripheral nervous system and are thought to disrupt neuronal function of this demographic of neuron.[22] Lewy bodies and neurites are composed largely of the mis-folded presynaptic membrane binding protein alpha-synuclein (aSyn), which is widely dispersed throughout neurons, though not universally.[38,39] As such, aSyn has been implicated in familial and sporadic forms of PD.[40-42]

In the central nervous system, the neurodegenerative process of PD is thought to advance caudo-rostrally, beginning in the dorsal motor nucleus of the vagus in the

medulla, and extending rostrally through the pons (compromising the LC and raphe nucleus) to the midbrain, affecting the substantia nigra dopaminergic neurons (primary disease pathology) and eventually affecting association and primary cortical areas[22] By the time an individual is symptomatic and a diagnosis is made, the disease has progressed beyond the substantia nigra in the midbrain to the mesocortex and thalamus.[21] Given that the onset of the hallmark clinical symptoms (that lead to a diagnosis) corresponds to this later stage of the disease, much of the research and attention directed at determining the etiology and most appropriate interventions for PD has been devoted to the nigrostriatal pathways of the basal ganglia and its primary neurotransmitter, dopamine. However, not all PD symptoms are amenable to treatments aimed at restoring dopamine or modulating the basal ganglia, including voice and swallowing deficits,[3-6,43-47] underscoring the importance of characterizing alternative neuropathological mechanisms contributing to these deficits.

Recent evidence suggests that aSyn aggregation is not limited to the central nervous system, and is present in both the peripheral and enteric nervous systems as well.[23,25,28,48-51] Relevant to cranial sensorimotor systems, there is evidence of aSyn aggregates in the vagal and pharyngeal branches of Cranial Nerve X as well as at the neuromuscular junctions associated with these nerves [25,27,29] suggesting that peripheral aSyn aggregation may contribute to the manifestation of early voice and swallowing deficits associated with dysfunction of these nerves and muscles, although the time course for this has not been determined. This is also consistent with evidence that both voice and swallow dysfunction in PD are refractory to standard pharmacological and surgical interventions.[3-6,43-47,52]

Signs and Symptoms: Sensorimotor Deficits in Parkinson Disease

The cardinal signs leading to a clinical diagnosis typically include resting tremor, bradykinesia, muscle rigidity, and postural instability,[53] and have long been associated with degeneration of the nigrostriatal, dopaminergic pathway.[54-56] However, deficits in PD are not limited to sensorimotor deficits in the trunk and limbs and may also include compromised olfaction,[24,57] gastrointestinal dysfunction,[57,58] autonomic dysregulation,[59-62] cognitive impairment,[63-68] depression,[24,62,64,69-74] sleep disturbances,[24,57,73,75] dysarthria,[1,47,76-99] and dysphagia.[82,100-113] Specifically, cranial sensorimotor deficits that affect voice,[2,84,91,95,99,114,115] speech,[1,76,78-80,84,85,88,89,95,97,116-118] and swallowing[82,100-113] are frequently present in PD, even in the early stages of the disease.[9,10,47,85,90,102,107] (Discussed more in following section.)

Given that it is known the pathophysiology in PD begins prior to nigrostriatal dopamine loss, it is not surprising that deficits extend beyond those associated with this primary disease pathology. Specifically, many of the deficits in PD that appear to be related to extra-striatal mechanisms frequently appear early in the disease progression and are increasingly being recognized as prodromal deficits (i.e. hyposmia, REM behavioral disorder).[75,119-122] However, given that a diagnosis is typically made following the onset of gross motor deficits in the limb (bradykinesia, tremor, etc.), determining the exact onset and progression of prodromal deficits is inherently difficult as patients are not typically being monitored or followed during this period. Developing animal models of PD that recapitulate the full spectrum behavioral deficits and underlying neuropathology (see discussion below) will be critical to expanding the discussion beyond dopamine depletion and the cardinal motor signs to develop novel

biomarkers to aid in earlier diagnosis. As a testament to the power of prodromal signs in early diagnosis, there is evidence that hyposmia appears very early in the progression of PD and may in fact be a reliable biomarker for early diagnosis.[120-122] This work found that olfaction tests alone were highly predictive of a future diagnosis of PD in a population of individuals already being followed for neurological complaints with undetermined etiology and in combination with other neurological testing, olfactory deficits were a stronger predictive prodromal sign.[120-122]

Broadly speaking, sensorimotor innervation for 'voluntary' movement under direct cortical control can be broken down into the corticospinal and corticobulbar tracts. These tracts generally follow the skeletal system and can be functionally divided into axial (proximal sensorimotor control of the head and neck) and appendicular (distal sensorimotor control of the limbs) systems. There are also other tracts that innervate the head and spine that control axial movements such as posture and head movement, such as the rubrospinal, vestibulospinal, and tectospinal tracts. Not surprisingly, PD affects targets in all of these pathways. Appendicular deficits in the limbs include tremor, muscle rigidity and bradykinesia, and have historically been considered the hallmark, cardinal signs of PD. Notable axial deficits include freezing of gait, postural instability, falling, and cranial sensorimotor dysfunction such as dysphagia (disordered swallowing) and dysphonia (disordered voice). Perhaps not surprisingly, axial deficits frequently dissociate from appendicular deficits in onset,[10,11,36,47,90,98,107] trajectory,[43,123-128] and responsivity to PD treatments such as levodopa and deep brain stimulation.[43,129-133] This includes dysphonia, which will be the focus of this dissertation.

Voice and Communication Deficits in Parkinson Disease

Despite the devastating effects of cranial sensorimotor deficits on communication and quality of life,[1,134] voice and speech are often neglected with respect to rehabilitative services (speech therapy is less common than physical or occupational therapy).[95] Dysarthria in PD is characterized by imprecise articulation,[78,86,91] reduced intelligibility,[76,88] vocal tremor, breathy vocal quality, as well as reductions in vocal loudness, pitch variability, and phonation duration.[1,80,91,115,116] Temporal characteristics of speech rate in individuals with PD indicate a disturbance within the articulatory movements themselves, versus overall timing changes within an utterance, such as increased inter-word pauses.[135] Studies have demonstrated that the amplitude (displacement) and velocity (speed) of lip[136] and jaw [89,118] movements, in particular, are reduced in individuals with PD relative to geriatric controls. Additionally, voice onset time is increased while the duration of vocalic segments, formant transitions,[118] and vowel space [76] are reduced. Importantly, there is evidence to suggest that laryngeal deficits, such as changes in voice, may be present in the early stages of PD [9-11,47,90] along with other subtle motor disturbances that appear before the cardinal signs of PD are present and a diagnosis is made.[24,28,58,75,119,137] These findings support our hypothesis that cranial sensorimotor dysfunction, such as voice deficits, are likely mediated by early, non-dopaminergic mechanisms. As voice and communication deficits frequently result in social withdrawal and isolation for individuals with PD, [1,116] inadequate management can be devastating to quality of life. This is particularly important in light of evidence that factors contributing to quality of life and resilience have profound, positive carryover effects on overall health prognosis.[138-140]

Treatment in Parkinson Disease

The primary disease pathology in PD has long been recognized as loss of nigrostriatal dopamine, and consistent with this, treatments aimed at restoring dopamine (levodopa) or modulating the basal ganglia circuits (deep brain stimulation) have been effective in attenuating the debilitating sensorimotor deficits that characterize PD. However, it is clear from clinical research that deficits do not universally or consistently respond to these treatments, especially with regard to vocal production.[45,47,141-146] Similarly, animal studies indicate that the cranial function (vs. limb) may not be mediated to the same degree by dopamine or the basal ganglia circuits,[127,147-149] indicating that other neural mechanisms may be responsible in addition to sensorimotor control by the basal ganglia. This is not surprising given that the neural and muscle architecture important for voice (and swallowing) is so tightly coupled with the respiratory tract - and vital bodily functions - and likely mediated by redundancies in control that protect them from neurochemical manipulations of higher level neurotransmitters. Complexities and redundancies aside, voice deficits in PD remain largely undertreated and will likely continue to be undertreated until more is known about the underlying neural mechanisms and targeted therapies are developed and implemented.

Ultrasonic Vocalizations in Rodents

Rodent models are ubiquitous in foundational research studies aimed at characterizing complex neurodegenerative conditions that would otherwise be impossible to study in humans due to ethical and experimental control concerns.

Pertinent to this dissertation, rat models have served as an important tool for investigating the mechanisms underlying sensorimotor deficits in PD, including vocal dysfunction.[20,125,126,150-153] Rats produce USV that are qualitatively homologous to voice and speech in humans. Specifically, rat USV are produced by egressive airflow through the larynx,[154,155] are semiotic in nature, elicit responses from the recipient, and can indicate affective state.[156-159] Although rat USV are not the same as human voice and communication, the analogous nature of certain behavioral and anatomical substrates of rat USV[160] make them an appropriate species for addressing the proposed research questions. Evidence indicates that the neural pathways underlying vocal control in rats are complex and include "top-down" control from higher order cortical and forebrain regions that are analogous to regions of vocal control in humans.[160-162] Although neural control is not identical, the neural pathways underlying vocal control in rats parallel human vocal control, and evidence from studies using rat USV are relevant when interpreted based on a knowledge of the similarities and differences. Importantly, USV are vulnerable to both dopamine[149-151,163,164] and NE depletion[165] (see also, Chapter 2) as well as genetic/transgenic manipulations.[31,125]

Modeling Parkinson Disease in Rodents

Characterizing the underlying neural mechanisms related to voice and communication deficits in PD using an animal model has been a challenge. This is largely due to use of PD models that merely recapitulate the primary disease pathology of nigrostriatal dopamine depletion.[166-168] Voice deficits emerge early in the disease process [8-10,85] and are refractory to treatments aimed at restoring dopamine.[2,47,84,146,169]

Thus, there is an obvious limitation to what can be gleaned from models of PD that only model dopamine depletion, which by their very nature, cannot mimic early, extra-striatal mechanisms in PD. However, several models (detailed in the following paragraphs) have been useful in studying vocal dysfunction in PD and, germane to the present work, provide justification for studying the role of NE in the development of early voice deficits.

The neurotoxin 6-hydroxydopamine (6-OHDA) has been a widely used and useful means to model PD. Structurally similar to both dopamine and norepinephrine, 6-OHDA is taken up by the dopamine transporter where it readily oxidizes within the cell to cause oxidative stress and eventually death of the neuron.[166,167] As a neurotoxin that is highly selective for dopamine cells, 6-OHDA's main strength is in its ability to replicate the primary disease pathology of PD; nigrostriatal dopamine depletion. Another strength of the 6-OHDA model is that many measureable sensorimotor deficits (rotation, head bias, disengage task, etc) correlate well with the amount of nigrostriatal dopamine depletion,[148,170,171] making it a reliable and robust model for assessing the relationship between sensorimotor deficits and dopamine loss.

One notable exception to this is that while oromotor [127,128] and vocalizations[150,151] are vulnerable to nigrostriatal dopamine depletion, these deficits do not correlate well with striatal dopamine loss or cortical motor maps.[125,128,147] This is consistent with what is observed clinically with humans, as voice and swallowing deficits emerge prior to severe dopamine loss and are not amenable to dopaminergic drug treatments.[2,9,10,45,47,85,98,146,172,173] Importantly, this work suggests that dopamine alone does not mediate vocal deficits, though it does demonstrate that dopamine loss is sufficient to disrupt some aspects of vocalizations and establishes 6-OHDA as an

appropriate model for evaluating vocalization deficits in PD. It is worth noting that 6-OHDA is toxic to NE as well, and given that, many researchers protect NE neurons using desipramine[174] when using 6-OHDA in order to induce a purely dopaminergic model. However NE was not protected in the 6-OHDA work described above, and it is possible that the vocalization deficits observed were due in part to a loss of NE in addition to dopamine.

Genetic and transgenic models based on known genetic causes of PD have been instrumental in advancing the research aimed at relating underlying neural mechanisms to refractory voice and communication deficits. For example, abnormal aSyn pathology represents another significant neurodegenerative process in PD.[21,22] Mice overexpressing human wild-type aSyn under the Thy1-promoter (Thy1-aSyn) as a model of pre-manifest PD have broad aSyn aggregation throughout brainstem and cortical regions and demonstrate early and progressive sensorimotor deficits beginning at 2 months of age and progressing out to 8 months of age.[175] These deficits appear to be independent of nigrostriatal dopamine depletion, which does not occur until 14 months.[176] To determine if vocal communication is impacted in this model, we evaluated USV in Thy1-aSYn from 2 to 9 months of age.[31] The full manuscript can be found at http://www.ncbi.nlm.nih.gov/pmc/articles/PMC4079049/. This work was the first to demonstrate vocalization deficits (reduced duration and altered call profile) in a transgenic, pre-manifest parkinsonian model, paralleling early vocal deficits in the human condition. Further, vocalization deficits were not associated with nigrostriatal dopamine depletion,[176] but were associated with increased aSyn aggregates in the periaqueductal gray,[31] a region important for vocal control.[160-162,177,178] Interestingly, and

pertinent to this dissertation, Thy1-aSyn mice also have aSyn aggregates in the LC.[179] However the exact contribution of aSyn aggregates in the LC and periaqueductal gray to specific aspects of USV were not evaluated in either of these studies.

Unilateral infusions of 6-OHDA into the medial forebrain bundle in rats (modeling primary disease pathology of dopamine depletion) result in decreased intensity, bandwidth and call complexity of USV.[150,151] Taken together, data from these two models suggest that while some aspects of vocal production appear to be dopamine dependent (bandwidth and intensity) others appear to be vulnerable to aSyn pathology in regions such as the LC and/or periaqueductal gray (duration), underscoring the usefulness of each of the models in approaching cranial sensorimotor deficits in PD. It would be interesting to evaluate vocalizations in the Thy1-aSyn model at a later time-point, past the onset of dopamine loss. This would better recapitulate the human condition, as loss of dopamine is the inevitable hallmark of PD, and would afford the opportunity to assess the combined impact of aSyn pathology in the periaqueductal gray and LC and loss of DA on vocal control. However, a drawback to this model is that all mice, wild-type and Thy1-aSyn, call less as they age, and even at 9 months have drastically reduced call rates.[31] Thus, assessing and characterizing the full impact of PD-related pathologies is limited.

Genetic animal models of PD developed through the manipulation of genes known to cause PD in humans offer a high degree of construct validity. Mutations in the *PINK1* gene are associated with both familial (second most common cause of autosomal recessive PD) and sporadic forms of PD[180] resulting in progressive sensorimotor deficits and significant nigrostriatal dopamine depletion.[181,182] As in

humans, *PINK1* KO rats demonstrate progressive motor deficits. Rats show reduced movement in the open field, reduced forelimb and hindlimb movement, and increased number of steps on the challenging beam beginning at 4 months of age.[34] Additionally, homozygous *PINK1* KO rats do not exhibit reduced dopamine levels until at least 8 months of age, [34] indicating that they would be a good model in which to assess early behavioral deficits associated with non-dopaminergic pathologies. However, cranial sensorimotor function had not been evaluated previously. To determine the onset and progression of cranial sensorimotor deficits in this model, we evaluated vocalization and oromotor function in homozygous and heterozygous *PINK1* KO rats across time and related behavioral change to underlying neural mechanisms.[36] The full manuscript can be found at http://www.ncbi.nlm.nih.gov/pubmed/26234713. We found that homozygous *PINK1* KO rats demonstrate early and progressive vocalization and oromotor deficits. Specifically, homozygous *PINK1* KO rats had reduced intensity beginning at 2 months, and reduced bandwidth and peak frequency beginning at 4 months. Interestingly, while reduced loudness (intensity) appeared early, intensity deficits were stable between 2-8 months, while reduced bandwidth and peak frequency emerged slightly later, but continued to decline over time. Given that a previous study reported a reduction in dopamine in the substantia nigra in the *PINK1* knock-out model, we were surprised that nigrostriatal dopamine was not significantly reduced at 8 months.[34] One possible reason for this discrepancy is that rats in the current study were frequently handled, socialized with females and otherwise provided with what might be considered an enriched environment. There is evidence in neurotoxin models of PD (6-OHDA and 1-methly-4-pheynl-1,2,3,6-tetrahydopyridine - MPTP) that exercise

and environmental enrichment may be neuroprotective and may spare dopaminergic cell loss.[183,184] Whether that is the case in our study is not known.

While dopamine was not significantly compromised at 8 months, homozygous *PINK1* knock-out had aSyn aggregates in the periaqueductal gray, a region important for vocal control,[160-162,177,178] as well as a reduced number of tyrosine hydroxylase-immunoreactive positive cells in the LC, indicating a compromise of NE. Interestingly, this loss was correlated with intensity of frequency modulated calls. Vocal intensity was compromised early (2 months), and evaluating noradrenergic cell loss at 2 months may point to a possible mechanism for early intensity deficits in the *PINK1* knock-out model.

The development of genetic and transgenic models of PD has provided an opportunity for studying refractory voice deficits in the context of early, non-dopaminergic pathologies, particularly regarding compromise of the LC and NE depletion. As noradrenergic mechanisms have been implicated in PD-related deficits [26,30,185-188] and have been effective at treating other refractory deficits,[72,189-191] exploring the contribution of NE in PD-related voice deficits is prudent. As a first step towards understanding how compromise of NE in PD impacts vocal communication, Chapters 2 and 3 are focused on establishing and characterizing the contribution of NE in acoustic vocalization parameters pertinent to voice deficits in PD.

Norepinephrine and Parkinson Disease

The hallmark disease pathology is loss of dopamine, and there is a wealth of PD-related research that has focused on dopaminergic pathways, but cellular loss extends beyond the nigrostriatal dopamine neurons in PD and compromises other

neurotransmitter systems such as acetylcholine, serotonin, and NE.[30,186,188,192] NE, in particular, has been implicated in the sensorimotor and cognitive deficits associated with PD [26,187,193,194] and the LC, a noradrenergic rich area of the pons in the brainstem, has been implicated in the pathology of PD.[26,30,186] In humans, degeneration of the LC may precede that of the basal ganglia and is vulnerable to the aSyn dense inclusion bodies that characterize PD.[21,23,28,195]

Importantly, modulation of noradrenergic (NE) mechanisms is effective for treating other refractory deficits in PD, such as depression,[189,196] cognitive dysfunction[72] and dyskinesias associated with chronic dopamine replacement therapy.[197] Evidence suggests that widespread and diffuse noradrenergic projections are neuromodulatory in nature,[30] making degradation of NE early in the disease process[21] (prior to dopamine loss) an important and viable neural substrate to consider with respect to early, pharmacologically refractory voice deficits. In addition, noradrenergic mechanisms contribute to directing attentional resources, adjusting the signal-to-noise ratio, and mediating goal-directed behavior,[198,199] all of which are important cognitive faculties for effective behavioral interventions, such as voice therapy. However, the contribution of specific noradrenergic receptor subtypes to vocal communication parameters pertinent to PD-related voice deficits is unknown.

Although the roles that NE and the LC play in the disease pathology of PD are not fully understood, one potential mechanism is neuromodulation. Modulation of striatal dopamine and subsequent motor/behavioral deficits via LC - mediated noradrenergic pathways has been established in several animal models of PD. In a mouse MPTP model, lesions to the LC caused enhanced striatal dopamine loss.[200]

Similar lesions to the LC in monkeys, prior to treatment with MPTP, resulted in persistent motor deficits that did not improve over time as compared with sham controls (MPTP, no lesion), which did improve 6-9 weeks post treatment.[201] In rats, NE depletion using the selective noradrenergic neurotoxin N-ethyl-2-bromobenzylamine (DSP-4) prior to bilateral 6-OHDA lesions of the medial forebrain bundle resulted in an exacerbation of motor deficits and potentiation of dopamine loss versus 6-OHDA lesions alone.[193,202] Furthermore, this dual depletion model produced differential responses to the dopamine agents levodopa (precursor to dopamine) and D-Amphetamine (stimulates presynaptic release of dopamine) versus 6-OHDA lesions alone. In rats with a single dopaminergic lesion, levodopa and D-Amphetamine reversed catalepsy and hypoactivity in the open field. In rats with a dual dopaminergic-noradrenergic lesion, levodopa reversed catalepsy (as it did in the dopamine only lesion group) and resulted in hyperactivity in the open field (vs. simply attenuating hypoactivity as in the dopamine group). However, D-Amphetamine only partially reversed catalepsy and hypoactivity in the dual lesion group. This may be because the damage imparted on dopamine neurons following a noradrenergic and dopaminergic lesion renders them less responsive to D-Amphetamine. Alternatively, because levodopa is also a precursor to norepinephrine, increasing the levels of both neurotransmitters in concert (with the administration of levodopa) may facilitate a reversal of catalepsy and hypoactivity.[202] These results suggest that depleting NE along with dopamine may result in more severe neurodegeneration of dopamine neurons.

In addition to behavioral evidence that NE may modulate dopamine-mediated behaviors, there is electrophysiological and neuropharmacological evidence that NE

interacts with dopamine and the nigrostriatal pathway. In studies with rats, the activity of dopamine neurons has been shown to be manipulated by NE α_1-AR antagonists and with lesions to the LC.[203] Dopaminergic lesions with 6-OHDA to the nigrostriatal pathway[204] and ventral tegmental area [203] resulted in increased firing in the LC, a pattern of firing that indicates that noradrenergic influence on dopaminergic brain regions may be inhibitory in nature. Further, α_2 -AR antagonists reversed levodopa-induced dyskinesias in mice,[205] monkeys,[191] and humans.[197] Clearly, noradrenergic mechanisms have a role in the sensorimotor deficits in animal models of PD, and given this, may also be involved in the deficits seen in humans, particularly those that do not respond to traditional treatments rooted in the nigrostriatal/dopamine deficiency framework.

The contribution of NE to vocal control and the manifestation of voice deficits in PD is not known. There is evidence that modulation of noradrenergic mechanisms via α_1-, α_2-, and β- AR agonists and antagonists have differential effects on general, qualitative aspects of USV, such as call profile and rate.[165] Chapter 2 in this document summarizes a preliminary study demonstrating that NE depletion in rats with the selective neurotoxin DSP-4 results in acute decreases in bandwidth (highest frequency - lowest frequency) peak frequency, and intensity. Given that vocal dysfunction in PD typically involves alterations in acoustic properties, such as reductions in loudness (intensity), pitch variability, and harmonics-to-noise ratio,[1,2,10,79,116] determining how receptor subtypes contribute to these pertinent acoustic parameters is essential to developing effective targeted pharmacological treatments. Chapter 3 in this document is devoted to evaluating the contribution of noradrenergic receptor subtypes in

modulating pertinent acoustic vocalization parameters. The central hypothesis of this work is that NE contributes to aspects of vocal control that are pertinent to voice deficits in PD, which are devastating to quality of life and currently undertreated.

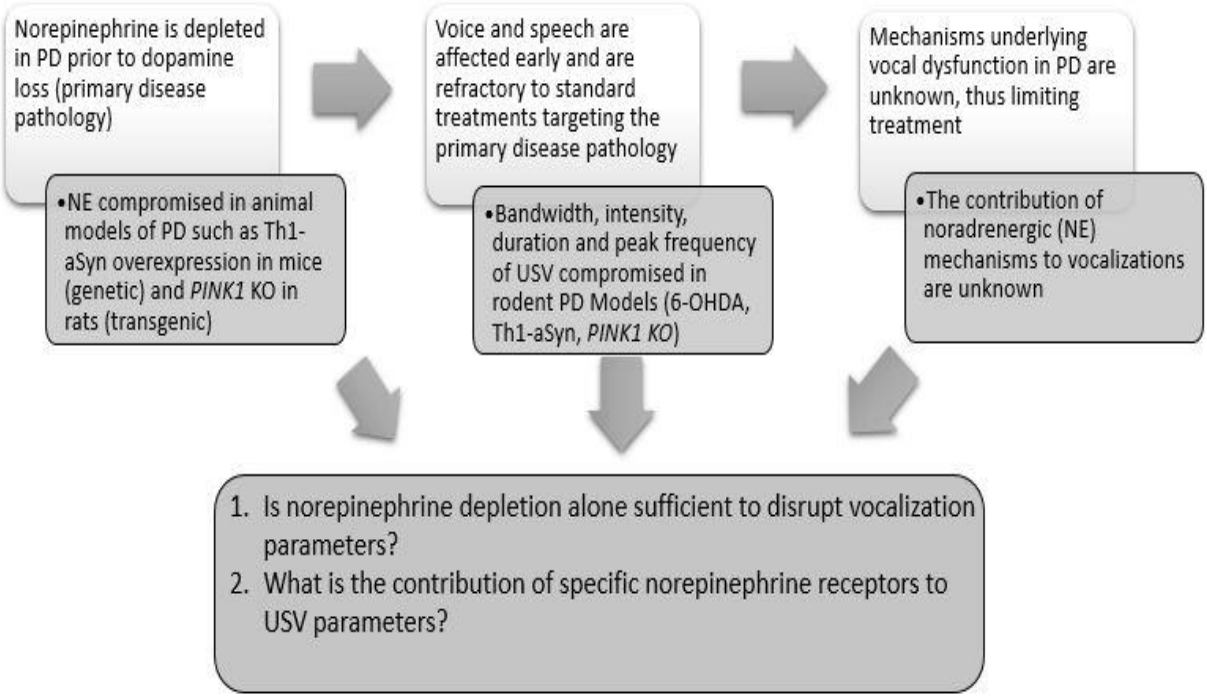

Figure 1.1. Overview of Voice deficits in Parkinson disease (PD) and the Contribution of Norepinephrine (NE) to Vocal Function.

Chapter 2: Norepinephrine - Establishing Sufficiency

Introduction

The hallmark signs of Parkinson disease (PD) (tremor, bradykinesia, and muscle rigidity) result in severe sensorimotor impairments that greatly impact quality of life.[56,206,207] In addition, as many as 90 % of individuals with PD also experience cranial sensorimotor deficits such as difficulty swallowing and communicating, [47,2,116,208,209] which can be equally devastating to social interactions, ability to work, and health. Aspiration pneumonia is the leading cause of death in PD.[106,111,134,210,211] Communication deficits may include disordered speech (dysarthria) and/or disordered voice (dysphonia).[1,2,86,94,116,208] In contrast to motor deficits in the extremities, which have a clear relationship with dopaminergic loss in PD (namely, respond very well to levodopa), voice deficits in PD emerge early and while dopamine depletion is sufficient to disrupt vocalizations,[149-151,153] dopamine replacement does not attenuate voice deficits,[2,173] indicating that other mechanisms are responsible.

Germane to this issue, the pathology in PD includes widespread neurodegeneration affecting multiple brainstem nuclei, cortical regions and neurotransmitters.[21,22,212] The locus coeruleus (LC) and its primary neurotransmitter, norepinephrine (NE), are compromised early in PD, and have been implicated in PD deficits[26,30,185-188] and treatment.[72,189-191] The contribution of NE to refractory voice deficits in PD, however, has not been established. In a rat model of PD, infusions of the neurotoxin 6-hydroxydopamine (6-OHDA) into the substantia nigra, striatum or medial forebrain bundle results in a reduction in dopamine as well as concomitant motor deficits reminiscent of those observed in humans with PD (tremor, hunched posture,

rigidity).[213] This includes vocalization deficits reminiscent of those in PD, such as reduced bandwidth and frequency range.[150,214] However, this model is limited as it does not recapitulate other aspects of the pathophysiology of PD, such as severe compromise of NE in the LC, early in the disease process. Given that NE has been implicated in the cognitive and motor deficits associated with PD,[30,186] several groups have attempted to create a dual NE + dopamine loss rat model of PD by administering the selective noradrenergic neurotoxin N-ethyl-2-bromobenzylamine (DSP-4) prior to bilateral 6-OHDA administration. The result is an exacerbation of motor deficits and potentiation of dopamine loss versus 6-OHDA lesions (dopamine) alone.[193,202,204] While still limited as a model of PD, in that it does not fully encompass all of the pathological processes that impact cognition and sensorimotor function, it does establish a link between NE loss and motor impairments, allowing us to appreciate how the combined loss of two crucial catecholaminergic neurotransmitters contribute to PD-related sensorimotor deficits. While it has been found that differentially modulating specific noradrenergic receptors results in alterations to call profile and rate,[165] the impact of NE depletion in LC on pertinent vocalization properties like intensity, bandwidth, peak frequency and duration has not been investigated. Given that NE loss begins prior to dopamine loss in PD,[21,22,26] and voice deficits do not respond to dopaminergic replacement therapies (distinguishing them from gross motor deficits in the extremities), determining the impact of NE loss on vocalizations, independent of dopamine loss, is a necessary first step towards understanding if and how compromise of NE in PD contributes to refractory voice deficits.

Thus the purpose of this study was to determine if depletion of NE is sufficient to disrupt vocalizations, independent of dopamine loss in a rat model. We evaluated ultrasonic vocalizations (USV), gross motor function (spontaneous activity and catalepsy), and motivation (latency to mount) following an injection of DSP-4 or saline. We hypothesized that call rate, profile, duration, bandwidth, intensity and peak frequency would be reduced in DSP-4 treated rats compared to saline controls at day 1 and day 6 post-injection. We also hypothesized that there would not be any differences between DSP-4 and saline treated rats for spontaneous activity measures, catalepsy or latency to mount.

Methods

Forty-four male Long-Evans rats (Charles River) between the ages of 8-12 weeks at the time of testing were used. A portion of this cohort went on to receive 6-OHDA infusions for a subsequent study (N=32). Eighteen females between the ages of 2 and 7 months were used for sexual experiencing and to elicit USV. Prior to all testing, all rats were handled, acclimated to the vocalization set-up and sexually experienced for 5 days a week for 2 weeks. All rats were housed in pairs in standard polycarbonate cages, on a reverse 12:12 hour light cycle. Food and water were available *ad libidum*. Acclimation and testing took place under partial red-light illumination during the dark period. All procedures were approved by the University of Wisconsin School of Medicine and Public Health Animal Care and Use Committee and were conducted in accordance with the United States Public Health Service Guide for the Care and Use of Laboratory Animals (National Research Council).[215]

Overview of testing

Rats were randomly assigned to receive an intraperitoneal (IP) injection of DSP-4 (n=24) or saline (n=20). USV, cataleptic descent, latency to mount, and spontaneous activity were recorded at baseline and at day 1 and day 6 post DSP-4 injection. Following day 6 testing, a sub-set of 12 rats (8 DSP-4 treated, 4 saline treated) were euthanized for immunohistochemical analysis to evaluate markers of NE in the LC. The remaining 32 rats (16 DSP-4, 16 saline) were used for another study.

Ultrasonic Vocalizations Recording & Acoustic Analysis: Vocalizations were recorded with an ultrasonic microphone with a flat frequency response up to 150 kiloHertz (kHz), a working frequency response range of 10-180 kHz (CM16, Avisoft, Germany), 16-bit resolution and sampled at 250 kHz. The microphone was mounted 15 cm above a standard polycarbonate rat cage. Each male was isolated from his cage-mate and an estrous female placed in his home-cage. After the male demonstrated interest in the female (sniffing, mounting, chasing), the female was removed and vocalizations were recorded for 60 seconds (sec). Offline acoustic analysis was done by a rater masked to condition with a customized automated program using SASLab Pro (Avisoft, Germany). Spectrograms were built from each waveform with the frequency resolution set to an FFT of 512 points, a frame size of 100% and flat top window and the temporal resolution set to display 75 % overlap. Extraneous low frequency noise was removed from the spectrogram using a high pass filter set to exclude sounds below 25-kHz.[20] Individual calls were isolated, classified based on complexity (frequency modulated, harmonic, or simple – see Figure 2.1)[20,151,216,217] and the following measures were

analyzed for each call: maximum and average duration (start time minus end time) in seconds, maximum and average intensity in decibels (dB), maximum and average bandwidth (highest frequency minus lowest frequency, Hz), and maximum and average peak frequency (peak frequency at the loudest part of the call, kHz)**.**

Cataleptic Descent: The cataleptic descent assay measures gross motor function and general limb motor impairment following pharmacological manipulations.[127,218] Catalepsy was assessed prior to USV testing on each testing day. To assess catalepsy, each male rat was isolated from its cage mate and placed in a standard polycarbonate cage with a stable bar (1 cm) affixed 8 cm above and running parallel to the floor of the cage. The rats' forelimbs were placed on the bar and cataleptic descent time was defined as the latency between initial forelimb contact with the bar and contact with both forelimbs to the cage floor. Catalepsy was assessed for 3 trials, and the average and fastest descent times (seconds) were calculated and analyzed.

Latency to Mount: Latency to mount is a standard measure of sexual interest and is sensitive to impairments in motivation that may affect the mating-vocalization paradigm. Latency to first mount with the female (seconds) was recorded during USV testing to quantify the males' interest in the female. In our experience, well acclimated and experienced males will typically mount within 1-2 minutes. However, if on testing days, the male had not mounted within 5 minutes, the female was removed and recording commenced.

Spontaneous activity: Spontaneous activity was measured in a transparent cylinder (20 x 30 cm), adapted from previous studies.[175,219] The cylinder was placed on a piece of glass and a camera (Sony HDR-CX210, New York, NY) was positioned below to allow a clear view of movements along all surfaces of the cylinder. Video recordings were analyzed offline and rated in slow motion by a rater masked condition. The number of rears, forelimb steps while up (rearing), forelimb steps while down, and hindlimb steps over a one-minute period was measured for each rat. If a full 60 seconds of spontaneous activity was not obtained for an animal, that recording was not used for this analysis.

DSP-4 injection: DSP-4 was obtained from Sigma Aldrich (St. Louis, MO) and dissolved in sterile saline (0.9% NaCl) to reach a concentration of 50 mg/ml. One day following baseline behavioral testing, rats received an IP injection of DSP-4 (50 mg/kg) or saline (1 ml/kg).

Immunohistochemical analysis: At the completion of the study a subset of rats were euthanized to confirm loss of NE in the LC (N=12 rats). Rats were deeply anesthetized with 5% isoflurane, transcardially perfused with 200 ml of cold saline followed by 500 ml of cold 4% paraformaldehyde. Brains were removed, post-fixed for 1-4 hr in 4% paraformaldehyde, cryoprotected in 0.02% sodium azide in 0.1M PBS solution and sliced at 60 microns on a freezing microtome. Free-floating sections were stained for tyrosine hydroxylase-immunoreactivity (TH-IR) over every 5th (1/5) section. Sections containing LC were blocked in 20% normal goat serum solution, incubated overnight in

primary solution: polyclonal rabbit anti-TH at 1:2000 (AB152, Millipore, Billerica, MA) as described previously.[220] Subsequently, samples were incubated in conjugated biotinylated secondary solution at 1:500 (Millipore, Billerica, MA) for 3 hr, incubated in an avidin biotin solution (Vector Laboratories, Burlingame, CA) for 1 hr, and the complex was visualized using filtered 3, 3-diaminobenzidine (Sigma Aldrich, St. Louis, MO) with 0.02% hydrogen peroxide for 8 minutes (min). All sections were float mounted onto gel-coated slides, dehydrated in a series of alcohols and Histo-Clear™ (National Diagnostics®, Atlanta, Georgia) and coverslipped using Eukitt mounting medium (Electron Microscopy Sciences, Hatfield, PA).

Images were acquired for tyrosine-hydroxylase immunoreactive labeling in the LC. Sections containing the LC were visualized using a Spot camera (Diagnostic Instruments, Inc) connecting a microscope (Olympus BX60, Center Valley, PA, USA) to a computer. The number of tyrosine-hydroxylase immunoreactive labeled cells within the LC was qualified by two raters masked to condition. The total number of tyrosine-hydroxylase immunoreactive cells was determined by summing the number of tyrosine-hydroxylase immunoreactive cells on the left and right LC. This was done on 3 sections for each animal. A mean was determined by summing the totals from 3 sections and dividing by 3, so that an average number of tyrosine-hydroxylase immunoreactive cells for each animal was determined. If 3 sections were not available, that sample was not included in this analysis.

Statistical Analysis: Data were tested to ensure they met the assumptions for ANOVA, and if they were not, those data were transformed (rank or squared). A repeated measures ANOVA was used to test for differences between the DSP-4 and saline treated groups (treatment) at baseline, day 1 post injection and day 6 post injection (testing day) for the following dependent variables: average and maximum duration, average and maximum intensity, average and maximum bandwidth and average and maximum peak frequency of USV, average and fastest latences to descend for cataleptic descent, latency to mount, and rears, forelimb steps while up and forelimb steps while down and hindlimb steps for spontaneous activity. Post-hoc comparisons were made with Fisher's LSD. To test for differences in tyrosine-hydroxylase immunoreactive labeling in the LC, an unpaired t-test was used to compare the saline and DSP-4 treated groups. For all comparisons, an *a priori*, critical alpha level of 0.05 was used.

Effect Sizes: To relate the relative magnitude of the effects of DSP-4 versus other USV altering drugs, effect sizes were calculated using the means and standard deviation of bandwidth and intensity at day 1 and compared to effect sizes following the dopamine antagonists SCH-23390 (D1 and D5 receptor antagonist) and eticlopride (D2 and D3 receptor antagonist),[149] and 6-OHDA.[150]

Results

Ultrasonic Vocalizations

Representative calls from DSP-4 and saline treated rats at day 1 are in Figure 2.2.

Complexity

There was not an interaction between treatment and testing day [$F(2,77)=1.035$, $p=0.36$]. There was a main effect of treatment [$F(1,77)=4.64$, $p=0.037$]. The percent of complex calls was significantly reduced in the DSP-4 treated group compared to saline ($p=0.038$), independent of testing day. There was also a main effect of testing day [$F(2,77)=14.13$, $p<0.001$]. Regardless of treatment, rats produced fewer complex calls at day 1 compared to baseline ($p<0.001$) and day 6 $p<0.001$).

Call Rate

There was an interaction between treatment and testing day [$F(2,77)=4.67$, $p=0.012$]. On day 1, call rate was significantly reduced in the DSP-4 treated group compared to saline ($p=0.019$). Call rate was significantly reduced in the DSP-4 treated group at day 1 compared to baseline ($p<0.001$) and day 6 ($p=0.005$). (Figure 2.3a)

Duration

Average: There was an interaction between treatment and testing day [$F(2,77)=3.56$, $p=0.033$]. Average duration was significantly reduced in the saline-treated group on day 1 ($p=0.025$) and day 6 ($p=0.042$) compared to baseline (Figure 2.3b). There were no significant differences between the saline and DSP-4 treated groups on any of the testing days, nor was the DSP-4 treated group different across the testing days ($p>0.05$ for all comparisons; Table 2.1).

Maximum: There was not an interaction between treatment and testing day for maximum duration [$F(2,77)=1.22$, $p=0.302$]. There were also no main effects of treatment [$F(1,77)=1.54$, $p=0.222$] or testing day [$F(2,77)=3.001$, $p=0.056$] (Table 2.1).

Bandwidth

Average: There was an interaction between treatment and testing day for average bandwidth [F(2,77)=8.57, *p<0.001*]. Average bandwidth was significantly reduced in the DSP-4 treated group at day 1 compared to baseline ($p<0.001$) and day 6 ($p<0.001$). Average bandwidth was significant reduced in the DSP-4 treated group compared to saline at day 1 ($p<0.001$) and day 6 ($p=0.048$). (Figure 2.4a).

Maximum: There was an interaction between treatment and testing day for maximum bandwidth [F(2,77)=8.55, *p<0.001*]. Maximum bandwidth was significantly reduced in the DSP-4 treated group at day 1 compared to baseline ($p<0.001$) and day 6 ($p<0.001$) and compared to the saline treated group at day 1 ($p<0.001$). (Figure 2.4b).

Intensity

*Average: T*here was an interaction between treatment and testing day for average intensity [F(2,77)=10.67, *p=0.026*]. Average intensity was reduced in the DSP-4 treated group at day 1 ($p<0.001$) and day 6 ($p=0.049$) compared to baseline (Figure 2.5a).

Maximum: There was an interaction between treatment and testing day for maximum intensity [F(2,77)=3.82, *p<0.001*]. Maximum intensity was significantly reduced in the saline treated group on day 6 compared to baseline ($p=0.023$) and in the DSP-4 treated group at day 1 compared to baseline ($p<0.001$) and day 6 ($p=0.003$). Maximum intensity was significant reduced in the DSP-4 treated group compared to saline at day 1 ($p<0.001$) and day 6 ($p=0.036$). (Figure 2.5b).

Peak Frequency

*Average: T*here was an interaction between treatment and testing day for average peak frequency [F(2,77)=25.19, *p<0.001*]. Average peak frequency was reduced in the DSP-

4 treated group at day 1 (p<0.001) and day 6 (p<0.001) compared to baseline and to the saline treated group at day 1 (p<0.001). (Figure 2.6a).

*Maximum: T*here was an interaction between treatment and testing day for maximum peak frequency [F(2,77)=23.64, *p<0.001*]. Maximum peak frequency was reduced in the DSP-4 treated group at day 1 compared baseline (p<0.001) and day 6 (p<0.001) and to the saline treated group at day 1 (p<0.001). (Figure 2.6b).

Gross Motor and Motivation

Latency to Mount

There was not an interaction between treatment and testing day for latency to mount [F(2,51)=0.0202, p=0.98]. There was not a main effect for treatment [F(1,51)=0.31, p=0.58] or testing day [F(1,51)=0.62, p=0.54]. (Data not shown).

Catalepsy

Average decent time: There was not an interaction between treatment and testing day for the average time to descend for catalepsy [F(2,20)=0.056, p=0.95]. There was a main effect of testing day on the average time to descend [F(2,20)=3.88, p=0.038]. All rats were significantly faster at days 1 (p=0.019) and 6 (p=0.03) compared to baseline. There was not a main effect of treatment [F(1,20)=0.86, p=0.36]. (Data not shown).

Fastest descent time: There was not an interaction between treatment and testing day for the average time to descend for catalepsy [F(2,20)=0.014, p=0.99]. There was a main effect of testing day on the average time to descend [F(2,20)=4.63, *p=0.022*]. All rats were significantly faster at day 6 (p=0.007) compared to baseline. There was not a main effect of treatment [F(1,20)=0.86, p=0.36]. (Data not shown).

Spontaneous Activity

Rears: There was not an interaction between treatment and testing day for the number of rears [F(2,43)=0.87, *p*=0.43]. There was a main effect of testing day on the number of rears [F(2,43)=9.38, *p*<0.001]. Regardless of treatment condition, rats made significantly fewer rears on day 1 (*p*<0.001) and day 6 (*p*=0.003) compared to baseline. There was not a main effect of treatment [F(1,43)=0.66, *p*=0.42]. (Figure 2.7a).

Forelimb Steps While Up: There was not an interaction between treatment and testing day for the number of forelimb steps while up [F(2,43)=0.58, *p*=0.56]. There was a main effect of testing day [F(2,43)=14.48, *p*<0.001]. Rats made significantly fewer steps on day 1 (*p*<0.001) and day 6 (*p*<0.001) compared to baseline. There was not a main effect of treatment [F(1,43)=0.104, *p*=0.75]. (Figure 2.7b).

Forelimb Steps While Down: There was not an interaction between treatment and testing day for the number of forelimb steps while down [F(2,43)=0.15, *p*=0.86]. There was a main effect of testing day [F(2,43)=4.67, *p*=0.015]. Rats made significantly fewer steps on day 6 compared to baseline (*p*=0.005). There was not a main effect of treatment [F(1,43)=0.41, *p*=0.52]. (Figure2.7d).

Hindlimb Steps: There w*as* not an interaction between treatment and testing day for the number of hindlimb steps [F(2,43)=1.18, *p*=0.32]. There was a main effect of testing day [F(2,43)=4.12, *p*=0.023]. Independent of treatment, rats made significantly fewer steps on day 6 compared to baseline (*p*=0.012). There was also a main effect of treatment [F(1,43)=5.37, *p*=0.024]. Regardless of testing day, DSP-4 treated rats made significantly fewer hindlimb steps (*p*=0.035). (Figure 2.7c).

Tyrosine hydroxylase - Immunoreactivity in the LC

There was no difference between saline and DSP-4 treated rats for the number of tyrosine-hydroxylase immunoreactive cells in the LC ($t(7)=0.40$, $p=0.70$) (Figure 2.8).

Effect Sizes:

The effect size for the magnitude of change following DSP-4 was 0.48 for intensity and 1.87 for bandwidth. Table 2.2 shows these effect sizes relative to 3 other USV-altering compounds. The effect size intensity following DSP-4 was 0.48 and considered small-moderate (according to Cohen [221]), and was not as large as the effect size for 6-OHDA (0.1.4) or eticlopride (1.5) both of which are considered strong. The effect size for bandwidth following DSP-4 was 1.87, which is considered a strong effect, and was larger than effect sizes for 6-OHDA (1.13), SCH-22390 (1.46), and eticlopride (1.16).[149,217]

Discussion

Compromise of NE following the neurotoxin DSP-4 resulted in acute, transient changes in acoustic properties of USV. These findings are significant because they establish that NE depletion alone is sufficient to disrupt USV parameters such as duration, intensity, peak frequency, and bandwidth. This work provides the preliminary evidence necessary to warrant further investigation into the contribution of noradrenergic mechanisms in vocal control and PD-induced voice deficits. As the mechanisms underlying voice and communication deficits in PD are currently not well understood, this is an important first step towards understanding how compromise of NE in the neurodegenerative processes associated with PD may contribute to voice and communication deficits.

DSP-4 resulted in significant reductions in multiple USV parameters at the day 1 time point. Specifically, average and maximum intensity, bandwidth, and peak frequency were all reduced in the DSP-4 treated group at day 1. In contrast, duration did not change for DSP-4 treated rats across time, but it did for saline treated rats, indicating that a reduction in duration across time reflects normal function. Overall, these results indicate that noradrenergic mechanisms contribute to not only call rate and profile [165], but also USV features that are pertinent to parkinsonian models of vocal deficits [31,125,149-153] and dysphonia in humans with PD, such as reduced loudness and frequency range.[1,2,84,94,116] At the day 6 time point, nearly all of these features had returned to baseline (the exception being maximum intensity, which was still significantly reduced at day 6) indicating the effects of DSP-4 on vocalization were acute and transient.

In contrast to dopaminergic neurotoxins and antagonists [31,149-151], there was not a significant interaction between treatment and time point for call complexity. Although DSP-4 treated rats produced fewer complex calls overall, it was independent of testing day, indicating that they two groups were not equal for this parameters at the start. Moreover, call complexity was reduced at day 1 compared to baseline and day 6, regardless of treatment condition. Given the proximity of baseline and day 1 testing time points (only a few days apart), it is possible that the observed effects are due in part to some degree of habituation with the USV assay, which manifests as a decreased amount of complex calls at that time point. As this effect occurred independent of treatment condition, it seems more likely that it is due to temporal effects related to testing day, rather than drug treatment.

In general, maximum and average values for parameters were affected equally. The two exceptions for this were duration and intensity. Average duration of frequency modulated calls was only reduced for saline treated rats, a trend that suggests normal changes in that parameter as a function of repeated testing would be shorter calls. We did not observe reductions in duration in the DSP-4 treated rats, nor was maximum duration affected at all. While both average and maximum intensity of frequency modulated calls were both reduced at day 1 in DSP-4 treated rats, average intensity essentially returned to baseline at day 6, while maximum values were still significantly different than baseline and from saline treated rats at day 6. While the mechanisms underlying these differential effects on maximum and average values for acoustic parameters are not clear, these findings underscore the importance evaluating both average and maximum values when trying to capture the full spectrum of USV deficits.

Consistent with behavioral findings, there were no lasting changes in the amount of tyrosine hydroxylase-immunoreactivity in the LC for animals treated with DSP-4. Given this, we cannot rule out the possibility that the observed behavioral changes were not the result of NE loss. However we do not believe this is the case. DSP-4 is widely used [202,222-230] and has been well established as a neurotoxin selective for noradrenergic neurons originating in the LC.[227,231-237] More likely, we may be observing a temporal effect as there is evidence that lasting depletions of NE in the LC may require repeated DSP-4 injections.[238] Current theories suggest that DSP-4 is taken up by a reuptake mechanism at the axon terminal, thus reducing NE levels and compromising the axon before retrograde actions on the soma.[237] Thus, while single injections result in transient reductions in NE levels,[225,239] cell bodies are not affected

unless repeated doses of DSP-4 are administered.[238] Given that even single doses of DSP-4 temporarily reduces NE levels [225] and that the purpose of this study was to establish whether NE depletion alone was sufficient to disrupt pertinent USV parameters, only one injection was used. The findings in this experiment indicate that NE depletion is sufficient to disrupt USV parameters, and the immunohistochemical findings are consistent with behavioral findings. However, to truly establish causal link between a reduction in NE in the LC and deficits in USV parameters, a more comprehensive experiment with repeated injections (to induce a lasting NE depletion in the LC) would be necessary. In addition, measuring cortical NE levels using more sensitive assays (such as high pressure liquid chromatography) would allow for correlations between the amount of NE loss and the degree of change in specific parameters.

Based on these results it appears that NE depletion is sufficient to disrupt USV parameters, and a more prudent course of action might be to characterize the contribution of specific noradrenergic mechanisms by systematically examining the effect of distinct noradrenergic receptors on USV. This would provide information regarding which receptors influence specific USV attributes (eg, intensity, peak frequency, bandwidth). Determining the contribution of specific receptors on pertinent vocalization parameters could point to viable pharmacological targets for treating refractory voice deficits in PD.

Consistent with our hypotheses, measures of gross locomotor function and motivation were not differentially altered following DSP-4 administration, however they did change across time. In general, spontaneous movement variables (number of

rears, forelimb, and hindlimb steps) decreased across testing days, independent of treatment condition. This assay capitalizes on the fact that rats naturally rear and explore novel environments[240] and these results suggest that rats became habituated to the task and were not as motivated to explore their environment. There was a difference between DSP-4 and saline treated rats for the number of hindlimb steps, but this was independent of testing day suggesting that the two groups were not equal for this parameter. Interestingly, while most groups have not reported alterations in overall motor function following DSP-4 alone, one recent report showed reductions in spontaneous activity 1 week following DSP-4 administration.[241] While the reason for this discrepancy is unclear, it is possible, given the short time course of this study that more profound locomotor deficits would manifest after the day 6 time point. Additionally, it could be that our locomotor assay was not sensitive enough to detect differences between the DSP-4 and saline treated animals, as doses were equivalent across the two studies.

In general, however, we found that all rats generally habituated, or improved across testing days, and given the short time course of this experiment (1 week), this is not surprising. Similarly, cataleptic descent times increased, indicating an improved performance as time went on, which again could be due to the short time course of the experiment. Latency to mount was also not different between DSP-4 and saline treated rats suggesting that sexual motivation for the mating paradigm used to elicit USV was not affected by the reduction in NE. Importantly, these measures of gross motor function along with latency to mount did not vary as function of treatment, indicating that

the impact of NE on USVs in this study were not impacted by extraneous factors such as overall reduced locomotor activity or motivation.

It has been demonstrated that vocalizations in rats are vulnerable to a number of pharmacological manipulations and disease and aging models.[36,125,149-152,164,165,216,242-249] To appreciate the relative magnitude of the effects of DSP-4, and concomitant reduction of NE, we compared effect sizes for the change in bandwidth and intensity across several compounds (Table 2.2). We found that the magnitude of change for intensity was small to moderate, relative to 6-OHDA (catecholaminergic neurotoxin) and eticlopride (D2 and D3 receptor antagonist) induced changes. Following DSP-4, the magnitude of change for bandwidth was greater than those for SCH-23390 (D1 and D5 receptor antagonist), eticlopride,[149] and 6-OHDA.[217] While it is well established that dopamine depletion[125,150,151] and antagonism[149,151] result in alterations to the intensity and bandwidth of USV, this is the first study to show that NE depletion results in similar effects. Although the effects for intensity were not as strong, they were only just shy of the cut off for moderate effects [221], and the effects on bandwidth following DSP-4 were stronger than any of the other models examined. These results suggest that NE loss is on par with dopamine with respect to USV-altering effects.

Voice and communication deficits in PD are currently undertreated, in large part because they do not respond to pharmacological and surgical interventions designed to treat dopamine depletion.[5,47] Along with evidence that voice deficits present prior to the onset of cardinal motor signs in PD that have long been associated with the primary disease pathology (tremor, rigidity, bradykinesia), the refractory nature of voice deficits suggests that non-dopaminergic mechanisms may be responsible. Several rodent

models of PD have found vocalization deficits reminiscent of those in PD (reduced loudness and frequency range) in the absence of dopamine loss,[31,36] further validating theories that voice deficits, along with other axial deficits in PD, are the result of extrastriatal pathologies in PD. This includes aSyn aggregations in areas of the brainstem important for vocal control like the periaqueductal gray and pathological findings in the LC in Thy1-aSyn model of pre-manifest PD[31,250] and *PINK1* knock-out model of PD.[36] In light of this, the present findings are significant as they point to a specific neurochemical mechanism (NE loss) that might partially account for PD related vocal deficits.

Conclusion

NE depletion following DSP-4 administration resulted in significant reductions in the intensity, bandwidth and peak frequency of USV. These changes occurred independent of gross motor and sexual motivation impairments. Effect sizes indicated that the magnitude of the effects of NE reduction on intensity and bandwidth are comparable to those following dopamine depletion or antagonism. Given evidence that extrastriatal, non-dopaminergic pathologies likely contribute to voice deficits in PD, these findings indicate that one of those contributing mechanisms might be NE loss. Fully characterizing the contribution of noradrenergic mechanisms to vocal control will be necessary to determine if treatments aimed at restoring or modulating NE would be beneficial for treating voice deficits in PD, which are currently refractory to standard treatments.

		Call Rate	*Percent Complex*	*Bandwidth (Hertz)*	*Peak Frequency (Hertz)*	*Intensity (decibels)*	*Duration (seconds)*
Saline	*Baseline*	1.85 (0.16)	71.39 (2.56)	27877 (1001) 59445 (1996)	57169 (598) 71450 (1108)	-44.29 (0.59) -28.29 (0.79)	0.062 (0.004) 0.21 (0.21)
	Day 1	1.88 (0.16)	64.65 (7.12)	28433 (989) 55922 (2277)	56394 (964) 72056 (1495)	-44.039 (0.72) -27.35 (1.11)	0.057 (0.004) 0.17 (0.021)
	Day 6	1.9 (0.17)	71.89 (3.33)	26814 (1236) 59095 (2688)	56932 (968) 71970 (1173)	-43.73 (0.77) -26.82 (1.1)	0.061 (0.006) 0.21 (0.031)
DSP- 4	*Baseline*	1.95 (1.32)	67.98 (3.13)	25508 (806) 54857 (2045)	56363 (842) 70252 (1210)	-43.83 (0.51) -27.55 (0.64)	0.059 (0.0056) 0.17 (0.023)
	Day 1	1.23 (0.17)	51.67 (4.14)	19888 (1029) 35083 (2858)	48070 (1333) 57870 (1423)	-45.66 (0.77) -33.68 (1.42)	0.057 (0.003) 0.15 (0.017)
	Day 6	1.70 (0.14)	66.05 (2.77)	24621 (996) 55605 (2554)	55503 (911) 69118 (1024)	-44.74 (0.49) -28.7 (0.63)	0.057 (0.005) 0.19 (0.024)

Table 2.1. **Summary of Ultrasonic Vocalization Data**. Mean and SEM for the call rate and percent complex and mean and SEM for average (top) and maximum (bottom) bandwidth, peak frequency, intensity, and duration for DSP-4 and saline treated rats at baseline, day 1 and day 6.

		Cohen's d	
Compound	Main Effect	Intensity USV	Bandwidth USV
DSP-4	NE Neurotoxin	0.48	1.87
6-OHDA[150]	Catecholamine neurotoxin	1.4	1.13
SCH-23390[149]	Dopamine receptor antagonist (selective for D1 and D5)	-0.27	1.46
Eticlopride[149]	Dopamine receptor antagonist (selective for D2 and D3)	1.50	1.16

Table 2.2. Comparison of Effect Sizes across Studies. Effect sizes of various ultrasonic vocalization (USV)-altering compounds. According to Cohen,[221] effect sizes of greater than 0.5 can be considered moderate and greater than 0.8 are considered strong.

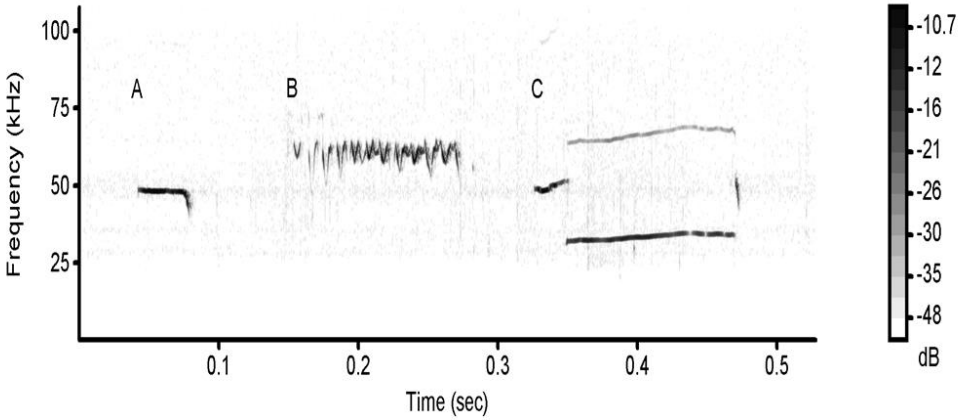

Figure 2.1. Representative call types: simple (A), frequency modulated (B) and harmonic (C). To determine overall call complexity, frequency modulated and harmonic calls are considered complex. Reprinted from Johnson et al, 2011 (permission pending).

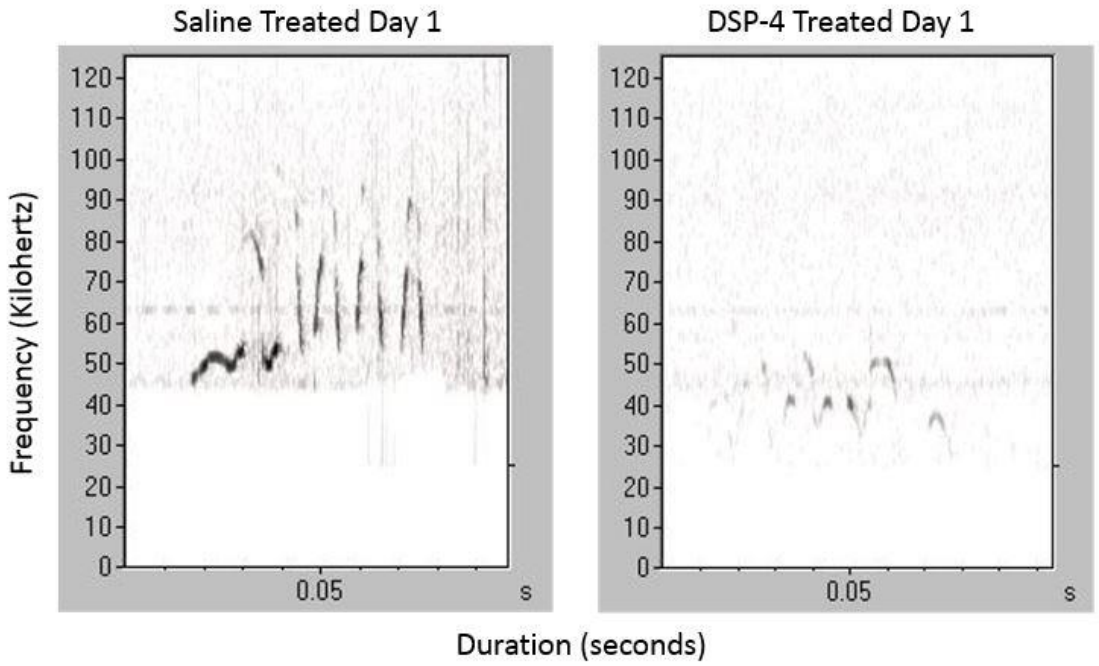

Figure 2.2. Representative Frequency Modulated Calls at Day 1. a) Call rate was significantly reduced in DSP-4 treated rats at day 1 compared to baseline, day 6 and saline treated rats at day 1. b) Duration was significantly reduced for the saline treated rats at day 1 and day 6 compared to baseline. Solid line = $p<0.05$; dashed line = $p<0.01$.

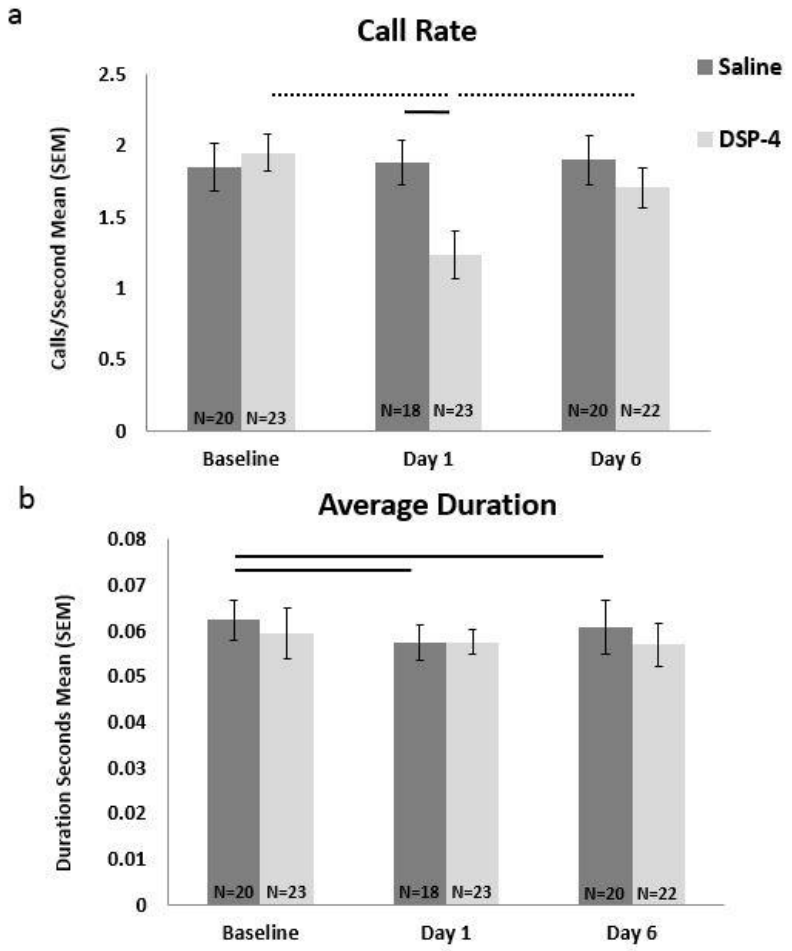

Figure 2.3. The Effects of DSP-4 on Call Rate and Duration. a) Call rate was significantly reduced in DSP-4 treated rats at day 1 compared to baseline, day 6 and saline treated rats at day 1. b) Duration was significantly reduced for the saline treated rats at day 1 and day 6 compared to baseline. Solid line = $p<0.05$; dashed line = $p<0.01$.

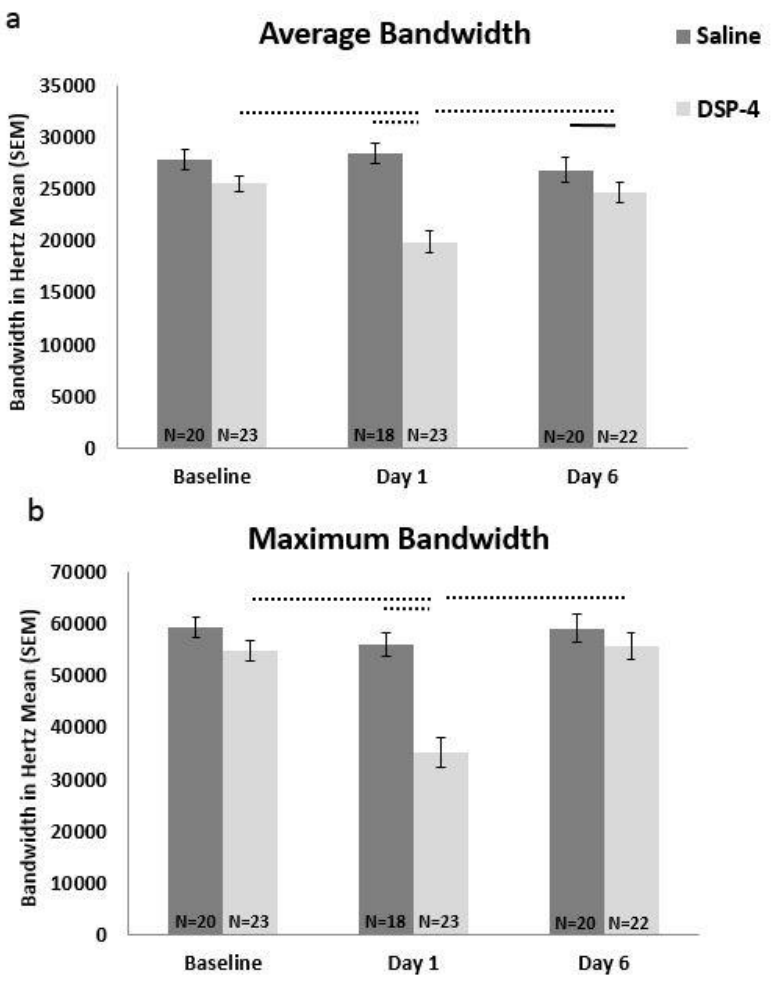

Figure 2.4. Effects of DSP-4 on Bandwidth of Frequency Modulated Calls. a) Average bandwidth of frequency modulated calls was significantly reduced in DSP-4 treated rats at day 1 compared to baseline and day 6. Bandwidth was reduced in DSP-4 treated rats at day 1 and day 6 compared to saline treated rats at the same time points. b) Maximum bandwidth of frequency modulated calls was significantly reduced in DSP-4 treated rats at day 1 compared to baseline and day 6 and saline treated rats at day 1. Solid line = $p<0.05$; dashed line = $p<0.01$.

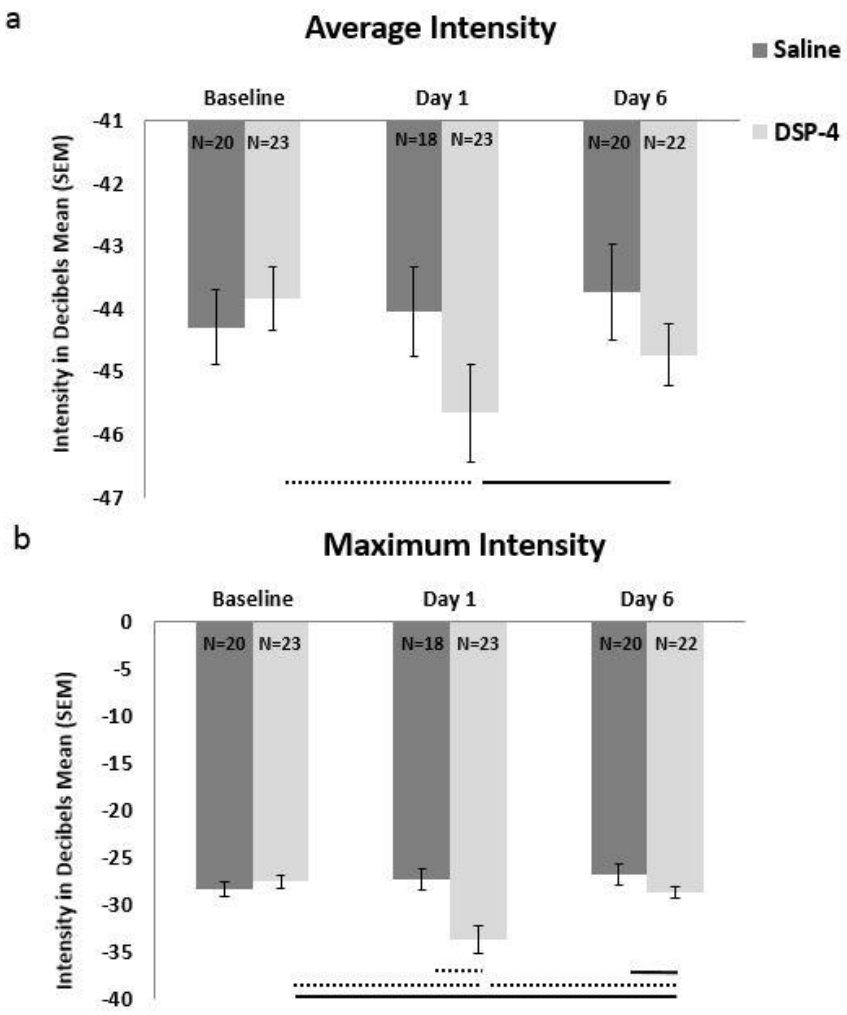

Figure 2.5. Effects of DSP-4 on Intensity of Frequency Modulated Calls. a) Average intensity was significantly reduced in DSP-4 treated rats at day 1 compared to baseline and day 6. b) Maximum intensity of frequency modulated calls was significantly reduced in DSP-4 treated rats at day 1 compared to saline and compared to baseline and day 6. Additionally, maximum intensity was also reduced for DSP-4 treated rats at day 6 compared to baseline and to saline at day 6. Solid line = $p<0.05$; dashed line = $p<0.01$.

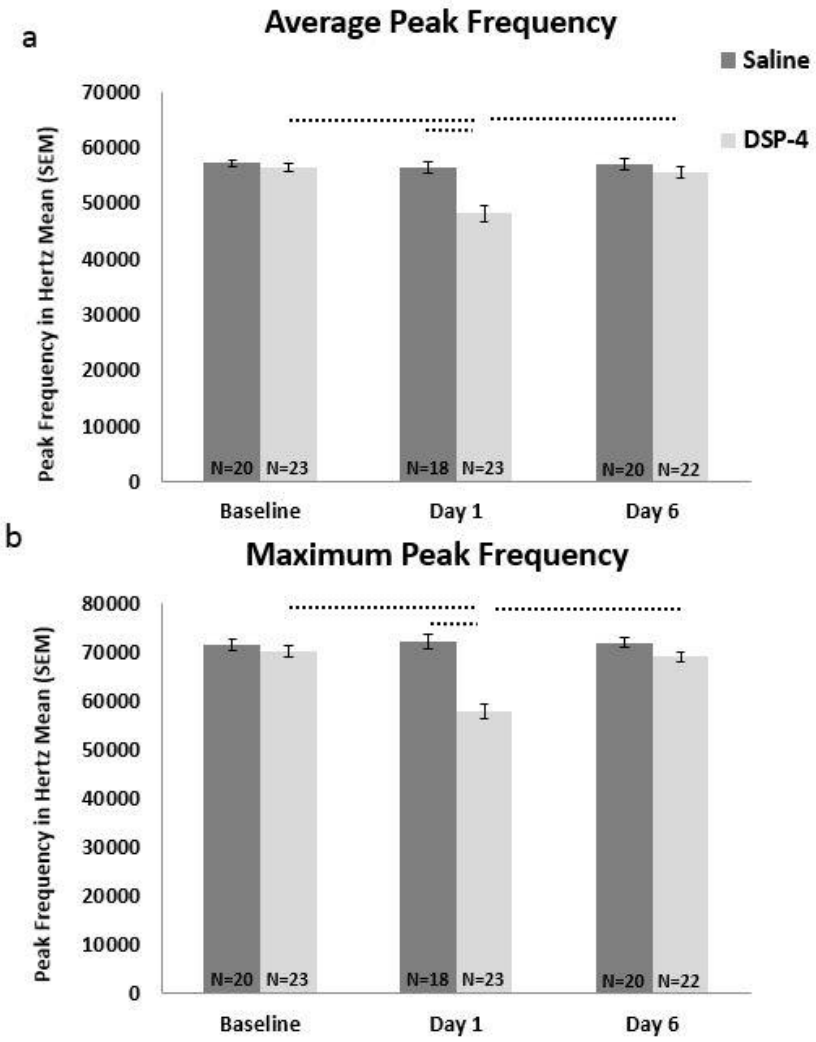

Figure 2.6. Effects of DSP-4 on Peak Frequency of Frequency Modulated Calls. Average (a) and maximum (b) peak frequency was significantly reduced in DSP-4 treated rats at day 1 compared to saline treated rats, and compared to baseline and day 1. Solid line = $p<0.05$; dashed line = $p<0.01$.

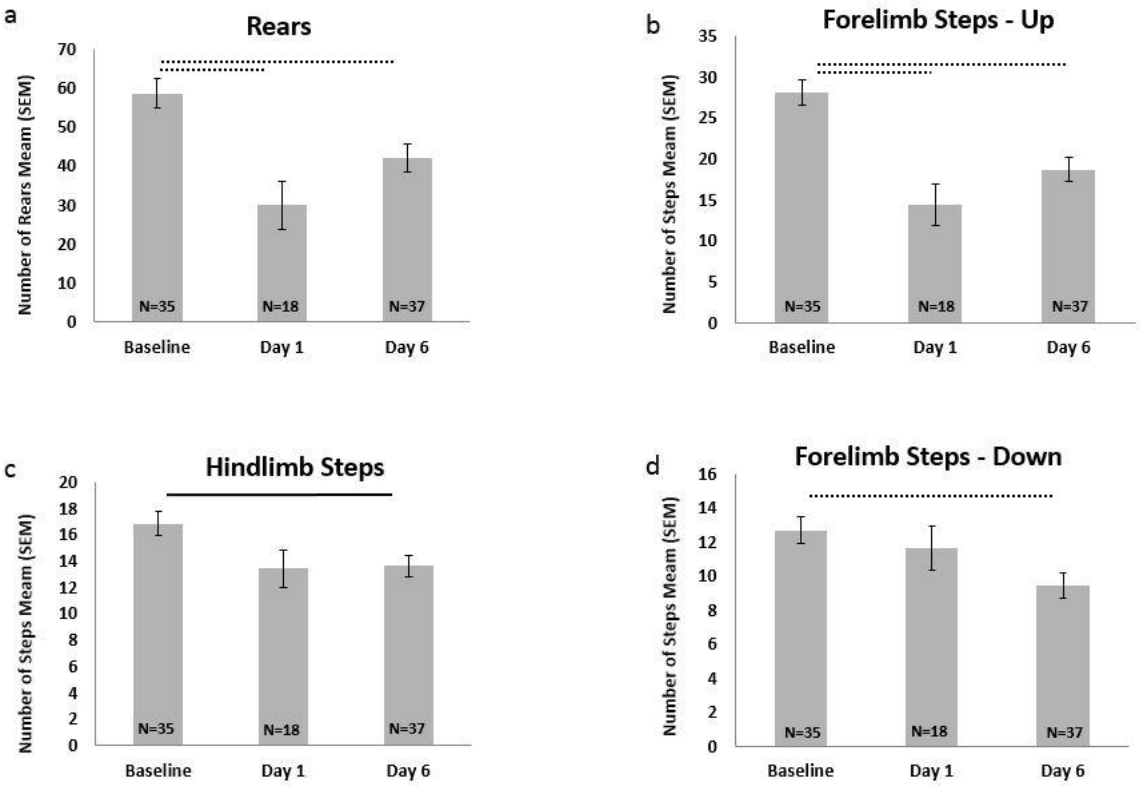

Figure 2.7. Effects of DSP-4 on Spontaneous Activity. The number of rears (a) and forelimb steps while up (rearing) were significantly reduced at day 1 and day 6 compared to baseline, regardless of treatment condition. The number of hindlimb steps (c) and forelimb steps while down (not rearing) (d) were significantly reduced at day 6 compared to baseline. Solid line = $p<0.05$; dashed line = $p<0.01$.

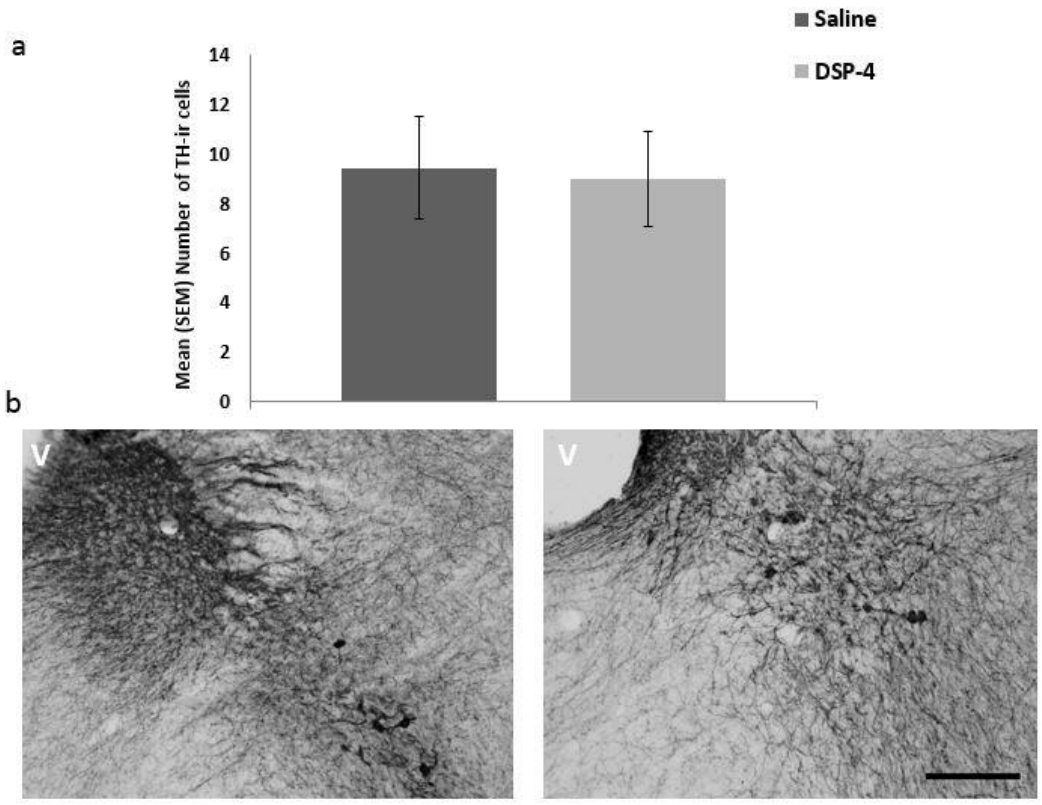

Figure 2.8: Tyrosine hydroxylase-immunoreactivty in the Locus Coeruleus. a) Mean (SEM) number of TH-immunoreactive (TH-ir) in the locus coeruleus. b) Photomicrographs of TH-immunoreactivty in the locus coeruleus in saline (a) and DSP-4 (b) treated rats. Magnification 10x. Scale bar = 500um, V= 4th ventricle

Chapter 3: Contribution of Norepinephrine to Vocal Control

Introduction

Worldwide, PD affects approximately 1% of the population over 65 and 10% of the population over 80 years of age, and is devastating to sensorimotor function.[53,207,251] In addition to the hallmark signs in PD of tremor, rigidity and bradykinesia, as many as 90% of individuals experience voice and communication deficits that severely impact social interactions and quality of life.[1,2] PD-related voice deficits, or dysphonia, may include a harsh, breathy vocal quality, vocal tremor, and reduced pitch range and loudness.[1,2,79,115,116,208] Despite the negative impact dysphonia has on quality of life for individuals with PD, voice and communication deficits remain grossly undertreated. This is because standard pharmacological and surgical interventions aimed at treating the primary disease pathology of nigrostriatal dopamine depletion, provide little or no benefit for voice and communication deficits.[3-7] The refractory nature of dysphonia along with evidence that voice deficits may emerge early in the progression of PD,[9-11,90,98,114] prior to the onset of cardinal motor signs typically associated with nigrostriatal dopamine loss, suggest that the neuropathology underlying dysphonia may be distinct from the primary disease pathology. However, the exact neurodegenerative mechanisms underlying voice and communication deficits in PD are not known.

There is increasing evidence that extrastriatal mechanisms may contribute to the complex nature of autonomic, cognitive, and motor deficits in PD. Norepinephrine (NE), in particular, has been implicated in cognitive and motor dysfunction in PD.[30,186] Modulation of noradrenergic (NE) mechanisms is effective for treating other refractory deficits in PD, such as depression,[189,196] cognitive dysfunction,[72] orthostatic

hypertension,[252] and dyskinesias associated with chronic dopamine replacement therapy.[197] The locus coeruleus (LC), a noradrenergic brainstem nucleus, is compromised prior to the substantia nigra, making degradation of NE early in the disease process[21] an important and viable neural substrate to consider with respect to early, pharmacologically refractory deficits such as vocal communication.

Consistent with what is observed in humans, several rodent models of PD demonstrate vocal communication deficits (including reduced loudness, reduced pitch range).[31,36,125,150-152] Unilateral infusions of the neurotoxin 6-hydroxydopamine (6-OHDA) into the medial forebrain in the rat result in reduced intensity, bandwidth (pitch range), and complexity of ultrasonic vocalizations (USV).[125,150,151] Given that 6-OHDA is toxic to both dopamine and NE, many researchers protect noradrenergic cells during the administration of 6-OHDA with the selective norepinephrine reuptake inhibitor desipramine in order to study the effects of dopamine depletion alone.[174] However, desipramine was not used in those 6-OHDA USV studies,[125,150,151] making it possible that deficits observed were partially due to compromise of norepinephrine in addition to dopamine. Interestingly, in rats, unilateral injections of rAAV2/5-aSyn directly into the substantia nigra (resulting in dopamine depletion alone) results in reduced intensity and call rate, but not bandwidth, peak frequency, or duration.[152] This suggests that compromise of specific acoustic parameters may be site and neurotransmitter specific. Vocal communication deficits have also been observed in transgenic and genetic models of PD.[31,36] Mice overexpressing human wild-type alpha-synuclein (aSyn; Thy1-aSyn) show alterations in call profile, intensity and duration of USV,[31] while *PINK1* knock-out rats with a genetic form of PD demonstrate reduced intensity, bandwidth, and

peak frequency of USV.[36] In both the transgenic mouse and genetic rat model of PD, vocalization deficits emerge early, independent of nigrostriatal dopamine loss, and each have neuropathological findings in the LC; aSyn aggregates in the Thy1-aSyn mouse model[250] and aSyn aggregates and reduced tyrosine hydroxylase-immunoreactivity in *PINK1* knock-out rat model.[36] Interestingly, reductions in tyrosine hydroxylase-immunoreactivity in the LC of *PINK1* knock-out rats is significantly correlated with reductions in intensity, indicating that reductions in norepinephrine may contribute to decreased loudness in this model.[36] However, these studies merely indicate a relationship between norepinephrine and vocal control and the exact nature of how noradrenergic mechanisms contribute to the manifestation of voice deficits in PD remains to be determined.

A first step towards understanding how NE loss impacts voice deficits in PD is to characterize the role of norepinephrine in normal vocal control. One study found that modulation of specific noradrenergic receptor subtypes (α_1-, α_2- and β- adrenoceptors (AR) with agonists and antagonists results in differential effects on call rate and call profile, or the types of calls produced.[165] Specifically, this study found that drugs targeting receptors that suppress noradrenergic signaling (clonidine and prazosin) resulted in decreased call rate or alterations in call profile (propranolol), while drugs that increase NE transmission (cirazoline and atipamezole) had no effect on rate. However, while this work establishes that modulation of AR results in changes in general USV features such as call rate and profile,[165] it is not known if or how specific receptor subtypes contribute to pertinent aspects of USV such as intensity, bandwidth, duration or peak frequency. As these pertinent features are compromised in rodent models of

PD and analogous to features of voice deficits experienced by humans with PD, determining the nature of noradrenergic mechanisms in vocal control may point to novel pharmacological targets for treating refractory voice deficits in PD. Preliminary work in our lab has shown that following the administration of the NE neurotoxin N-(2-chloroethyl)-N-ethyl-2-bromobenzylamine (DSP-4), rat USV demonstrate acute decreases in call rate, bandwidth, intensity, and peak frequency (See Chapter 2).

Thus, while it is clear that loss of NE is sufficient to disrupt pertinent aspects of USV, it is not known how specific receptor subtypes modulate features of USV such as intensity, peak frequency, duration and bandwidth. Vocal dysfunction in individuals with PD typically involves alterations in acoustic properties, such as reductions in loudness (intensity), pitch variability, and harmonics-to-noise ratio.[1,2,10,79,116] Determining how receptor subtypes contribute to specific acoustic parameters is essential to developing effective targeted pharmacological treatments.

There are 3 main receptors for NE: α_1-AR, α_2-AR, and β-AR.[253] An overview of these receptors, their location, and the net effect of agonists and antagonists is in Table 3.1. While all of these receptors can be found post-synaptically throughout the central and peripheral nervous system, the α_2-AR is primarily located pre-synaptically as an autoreceptor, regulating the release of NE.[30,254] Agonists for post-synaptic ARs mimic the endogenous ligand, NE when they bind (α_1-AR and β-AR). In contrast, antagonists for post-synaptic ARs block the effects of the endogenous ligand. The opposite is true for the pre-synaptic autoreceptor (α_2-AR), which when agonized with a drug, halts the release of NE into the synapse, while the antagonist blocks this effect, allowing the continued release of norepinephrine. [255,256] NE ARs can more generally be thought of in

terms of their net effects of increasing NE signaling (α_1-AR and β-AR agonists and α_2-AR antagonists) or decreasing NE signaling (α_1-AR and β-AR antagonists and α_2-AR agonists). Noradrenergic ARs are widely distributed throughout the central nervous system, where they are thought to be neuromodulatory, regulating attention, vigilance, signal-noise-ratio and broadly influencing a wide range of behavioral functions.[186,198,199,257] It has been found that both α_2-AR antagonists (promote NE release) and α_1-AR and β-AR antagonists (block NE transmission) are effective at reducing freezing of gait [258-260] and levodopa induced dyskinesias [190,191,197,261,262] implicating noradrenergic ARs in the manifestation and treatment of PD related motor deficits.

The purpose of this study was to determine the manner in which distinct noradrenergic ARs mediate vocal control. We hypothesized that selectively targeting noradrenergic receptors would result in the differential modulation of pertinent acoustic properties of USV - intensity, bandwidth, peak frequency, duration in addition to general USV parameters such as call profile and call rate. Previous USV work has shown that drugs that reduce NE signaling reduce call rate (α_1-AR antagonist and α_2-AR agonist) and alter call profile (β-AR antagonist).[165] However, drugs that *block* NE signaling and drugs that *promote* NE release both reduce sensorimotor deficits such as freezing of gait and levodopa induced dyskinesias.[190,191,197,258-260,262] Thus, while we expected to see a differential effect of norepinephrine AR agonists and antagonists on specific USV parameters, we did not specify a direction of change in our hypotheses. Establishing how NE mediates vocal function is crucial to the development and application of novel and specific pharmacological interventions for voice deficits. This is an essential first

step to bridging the current gap in treatment efficacy which leaves communication deficits in PD undertreated.

Methods

Sixty male Long-Evans (Charles River) rats, aged 4-5 months, were used. An additional 8 females, aged 2-12 months were used to elicit USV. Prior to all testing, male rats were handled and acclimated to the USV recording set-up, 5 days a week for 2 weeks. USV acclimation involved familiarizing rats to the testing room and behavioral assay (as described below), but not recording. All rats were on a reverse 12:12 hour light cycle, with recording occurring under partial red-light illumination during the dark cycle. Food and water were available *ad libidum*. All procedures were approved by the University of Wisconsin School of Medicine and Public Health Animal Care and Use Committee and were conducted in accordance with the United States Public Health Service Guide for the Care and Use of Laboratory Animals (National Research Council)[215] as well as the Use of Animals and Humans in Research published by the Society for Neuroscience.

Overview of experimental timeline and testing

USV were recorded from rats following the administration of α_1-, α_2- and β- AR agonists, antagonists, and vehicle control (Table 3.2). Separate rats were used for testing each drug condition (n=10/drug for a total of n=60). The sample size for each condition was based on power calculations for the difference in bandwidth following administration of DSP-4 (depletes NE; Chapter 2) or saline. Based on the difference in means, in order to reach a level of at least .84 power, with an alpha of <0.05, the

sample size was 8 for each sub-study. However, due to attrition (non-vocalizers), an additional 2 rats were tested per sub-study, for a total of 10 rats per drug condition (60 total). To determine dose response effects, each rat received 3 different doses of a given drug (Table 3.2) and 1 injection of vehicle control for a total of 4 injections across 2 weeks. A counterbalanced within subjects design was used and each rat received only 1 injection (dose) on a given day with a 3-day washout period between each drug dose. Testing order/day was determined using a Latin Square design. On each testing day, rats were placed individually in the test cage (homecage with bedding) for 30 min to acclimate to the testing room. Following this period of acclimation, each rat received an injection, was placed back in their home cage 20-30 minutes (Table 3.2). Locomotor activity was measured (described below) followed immediately by USV testing (also described below).

Drug Treatments

Doses, receptor targets, and timings for drug administration are shown in Table 3.2. Drug sources and preparation are described below. Propranolol (Sigma Aldrich), cirazoline (Sigma Aldrich), clonidine (Sigma Aldrich), isoproterenol (Sigma Aldrich) and atipamezole (Sigma Aldrich) were all dissolved in 0.9% sterile isotonic saline and injected i.p. at a volume of 1 ml/kg. Prazosin (MP Biomedicals, LLC) was prepared in distilled water with 5 % DMSO (Sigma Aldrich), sonicated until dissolved, and injected intraperitoneally (i.p.) at a volume of 1 ml/kg. Drug doses and pharmacological windows were based on published work.[165,263-267]

Ultrasonic Vocalization Recording & Acoustic Analysis

USV were recorded with an ultrasonic microphone with a flat frequency response up to 150 kHz, a working frequency response range of 10-180 kHz (CM16, Avisoft, Germany), 16-bit resolution and sampled at 250 kHz. The microphone was mounted 15 cm above a standard polycarbonate rat cage. Each male rat was isolated from his cage-mate and an estrous female placed in his home-cage. After the male demonstrated interest in the female (sniffing, mounting, chasing), the female was removed and vocalizations were recorded for 90 sec. Offline acoustic analysis was done by two raters masked to experimental condition with a customized automated program using SASLab Pro (Avisoft, Germany). Spectrograms were built from each waveform with the frequency resolution set to an FFT of 512 points, a frame size of 100% and flat top window and the temporal resolution set to display 75 % overlap. Extraneous low frequency noise was removed from the spectrogram using a high pass filter set to exclude noise below 25 kHz.[268] General vocalization properties examined included: latency to the first call (seconds), call rate (calls per second; calculated as the total number of calls for the first 60 seconds after calling commenced divided by 60), overall call profile (% of individual call type; described below), and standard deviation of intensity (described below). Individual calls were isolated, visually and acoustically inspected, and a call profile was determined by classifying each call as none of eight call types described in Table 3.3 so that a call profile could be determined. Calls were classified using a modified version of the call profile classification scheme developed and used previously.[165,216] However, after call profile was determined, calls were grouped together into three broad categories for detailed acoustic analysis on similar calls- simple, complex and compound calls (Table 3.3) and the following acoustic

variables were analyzed for each: average and maximum bandwidth in Hz, average and maximum peak frequency in kHz, average and maximum duration in seconds (sec), and average and maximum intensity (loudness) in decibels (dB).[269-272] These broad categories for detailed acoustic analysis were based on previous work demonstrating differential effects on simple versus complex calls.[125,149-151] During recording, rats moved freely around the cage, resulting in variable mouth-to-mic distance which may affect intensity (loudness) measures. In our experience, the impact of this movement and inherent variability in mouth-to-mic distance is negligible as all rats are tested in the exact same manner and produce calls in all parts of the cage, resulting in equally heterogeneous samples of call intensity across animals. However, to account for this quantitatively, we calculated the standard deviation of intensity (loudness) during each recording session as a gross indication of the range of intensities produced by each rat under each drug condition.

Locomotor Activity and Latency to Mount

To measure alterations in gross motor function resulting from the drugs, we assayed locomotor activity. Following the administration of each drug (20-30 minutes, see Table 3.2), each rat was recorded in their homecage using a digital camera (Sony HDR-CX210, New York, NY) for exactly 20 minutes. Videos were analyzed offline by 3 raters masked to condition for the following variables: total number of cage crossings, total number of rears, time spent grooming (seconds), and time spent eating (seconds).

Latency to first mount (sec) with the female was recorded during USV testing to quantify the males' interest in the female. Typically, well acclimated rats will

demonstrate interest and attempt to mount within 1-2 minutes of exposure to a female. However, if a rat did not mount within 5 minutes, the female was removed and vocalizations were recorded.

Statistical analysis

All variables under each drug were examined separately using a within subjects 1 factor (drug dose) repeated measures analysis of variance (rmANOVA) to determine dose-response effects. All data were tested to ensure that the assumptions of ANOVA were met. If the data failed equal variance or normality tests, rank or log transformations were used. Post-hoc comparisons were made using Fisher's LSD. The critical level of significance was determined *a priori* and set at $p < 0.05$. Given the number of comparisons, we acknowledge the risk of Type 1 error. However, after weighing the risks and benefits, we decided *a priori* not to correct for multiple comparisons due to the exploratory nature of this work and the risk of beta error. We have provided the data to be transparent in our analyses and presentation.

To determine the degree to which USV and locomotor data were reliably analyzed, an intraclass correlation coefficient (ICC) was calculated on 5 % of all of the files to determine the inter- and intra-rater reliability. A two-way ANOVA was run on each data set to determine mean squares and degrees of freedom, from which the ICC was computed.

Results

An overview of the effects of each drug on USV parameters is displayed in Table 3.4. Additionally, means and standard error of the mean for call rate, latency to call, latency to mount and standard deviation of intensity for all of the drugs are displayed in Table 3.5. The results of the ANOVA for all of the USV and locomotor variables are detailed below for each experiment.

Experiment 1: Cirazoline (post-synaptic α_1- AR agonist, increases NE transmission)

General Vocalization Properties

There was no effect of cirazoline on call rate, latency to call, or standard deviation of intensity ($p>0.05$; Table 3.5). There was a main effect of drug treatment on the percent of flat calls [$F(3,27)=5.78$, $p=0.003$]. Compared to vehicle, the percent of flat calls was reduced with 0.125 mg/kg ($p=0.014$), 0.25 mg/kg ($p<0.001$), and 0.50 mg/kg ($p=0.024$) (Figure 3.1a). There were no other significant effects on call profile ($p>0.05$; data not shown).

Duration

There was a main effect of drug treatment on average duration of compound calls [$F(3,27)=4.24$, $p=0.014$]. Average duration of compound calls was increased with the 0.50 mg/kg dose of cirazoline compared to vehicle ($p=0.011$) and 0.125 mg/kg ($p=0.003$) (Figure 3.1b). There were no main effects of cirazoline on maximum duration of compound calls or average or maximum duration of simple or complex calls ($p>0.05$; Table 3.6).

Bandwidth

There was a main effect of drug treatment on maximum bandwidth of complex calls [F(3,27)=3.63, *p*=0.026]. Maximum bandwidth of complex calls was reduced in the 0.125 mg/kg (*p*=0.007) and 0.50 mg/kg (*p*=0.01) doses compared to vehicle (Figure 3.1c). There were no main effects of cirazoline on average bandwidth of complex calls or average or maximum bandwidth of simple or compound calls (*p*>0.05; Table 3.6).

Intensity

There were no main effects of cirazoline on average or maximum intensity for simple, complex or compound calls (*p*>0.05; Table 3.6).

Peak Frequency

There was a main effect of drug treatment on maximum peak frequency of compound calls [F(3,27)=3.49, *p*=0.029]. Maximum peak frequency was reduced with the 0.25 mg/kg dose compared to vehicle (*p*=0.24), 0.125 mg/kg (*p*=0.027), and 0.50 mg/kg (*p*=0.006) (Figure 3.1d). There were no main effects of drug treatment on average peak frequency of compound calls or average or maximum simple or complex calls (*p*<0.05; Table 3.6).

Locomotor and Latency to mount

There was a main effect of cirazoline on the number of rears [F(3,27)=4.29, *p*=0.013]. The total number of rears was significantly decreased following 0.125 mg/kg (*p*=0.019), 0.25 mg/kg (*p*=0.02), and 0.50 mg/kg (*p*=0.002) compared to vehicle (Figure 3.2). There were no effects on number of cage crossings, time spent eating, or time spent grooming (*p*<0.05; Table 3.11). There was not a main effect of cirazoline on latency to mount (*p*>0.05; Table 3.5).

Experiment 2: Prazosin (post-synaptic α_1- AR antagonist, decreases NE transmission)

General Vocalization Properties

There was a main effect of drug treatment on call rate [F(3,27)=15.26, p<0.001]. Call rate was significantly decreased with 0.1 mg/kg (p=0.048), 0.3 mg/kg (p<0.001), and 1.0 mg/kg (p<0.001) of prazosin compared to vehicle, and 0.3 mg/kg and 1.0 mg/kg were also significantly decreased compared to 0.1 mg/kg (p=0.006 and p<0.001, respectively) (Figure 3.3a). There was also a main effect of drug treatment on the standard deviation of intensity [F(3,26)=5.6, p=0.004]. Standard deviation of intensity was significantly reduced with 1.0 mg/kg of prazosin compared to vehicle (p=<0.001) and 0.3 mg/kg (p=0.035) and with 0.1 mg/kg compared to vehicle (p=0.034) (Figure 3.3b).

There was a main effect of drug treatment on the percent of compound [F(3,27)=3.79, p<0.022] and frequency modulated [F(3,27)=4.86, p=0.008] calls. The percent of compound calls was significantly reduced with the 0.3 mg/kg (p=0.005) and 1.0 mg/kg (p=0.022) doses compared to vehicle treatment (Figure 3.3c). The percent of frequency modulated calls was significantly reduced with the 0.1 mg/kg (p=0.013), 0.3 mg/kg (p<0.001), and 1.0 mg/kg (p=0.049) doses compared to vehicle treatment (Figure 3.3d). There was not a main effect of drug treatment on latency to call (Table 3.5) or any other call types (p>0.05; data not shown).

Duration

There were no main effects of drug treatment on average or maximum duration for simple, complex or compound calls (p>0.05; Table 3.7).

Bandwidth

There was a main effect of drug treatment on average bandwidth of simple calls [$F(3,26)=3.76$, $p=0.023$]. Average bandwidth of simple calls was reduced with 1.0 mg/kg of prazosin compared to vehicle ($p=0.003$) and 0.3 mg/kg ($p=0.046$) (Figure 3.4a). There was no effect of prazosin on maximum bandwidth of simple calls or average or maximum bandwidth of complex or compound calls ($p>0.05$; Table 3.7).

Intensity

There was a main effect of drug treatment on average [$F(3,26)=6.37$, $p=0.002$] and maximum [$F(3,26)=10.60$, $p<0.001$] intensity of simple calls. Average intensity of simple calls was reduced with the 0.1 mg/kg ($p=0.027$), 0.3 mg/kg ($p=0.005$), and 1.0 mg/kg ($p<0.001$) doses of prazosin compared to vehicle (Figure 3.5b). Maximum intensity of simple calls was also reduced with the 0.1 mg/kg ($p=0.001$), 0.3 mg/kg ($p<0.001$), and 1.0 mg/kg ($p<0.001$) doses of prazosin compared to vehicle (Figure 3.5a).

There was a main effect of drug treatment on average [$F(3,27)=12.14$, $p<0.001$] and maximum [$F(3,27)=10.13$, $p<0.001$] intensity of complex calls. Average intensity of complex calls was reduced with the 0.1 mg/kg ($p=0.004$), 0.3 mg/kg ($p=0.005$), and 1.0 mg/kg ($p<0.001$) doses of prazosin compared to vehicle and the 1.0 mg/kg dose was also significantly reduced compared to the 0.1 mg/kg ($p=0.007$) and 0.03 mg/kg ($p=0.006$) doses (Figure 3.5c). Maximum intensity of complex calls was also reduced with the 0.1 mg/kg ($p=0.022$), 0.3 mg/kg ($p<0.005$), and 1.0 mg/kg ($p<0.001$) doses of prazosin compared to vehicle and the 1.0 mg/kg dose was also significantly reduced compared to the 0.1 mg/kg ($p=0.005$) and 0.03 mg/kg ($p=0.023$) doses (Figure 3.5d).

There was a main effect of drug treatment on maximum intensity of compound calls [F(3,24)=7.10, *p*=0.001]. Maximum intensity of compound calls was reduced with the 1.0 mg/kg dose of prazosin compared to vehicle (*p*<0.001) and 0.1 mg/kg (*p*=0.009) and the 0.3mg/kg dose was significantly reduced compared to vehicle (*p*=0.015) (Figure 3.5e). There was not an effect of prazosin on average intensity of compound calls (*p*>0.05; Table 3.7).

Peak Frequency

There was a main effect of drug treatment on maximum peak frequency of compound calls [F(3,24)=3.03, *p*=0.049]. Maximum peak frequency of compound calls was reduced with 0.3 mg/kg of prazosin compared to vehicle (*p*=0.007) (Figure LL). There was no effect of prazosin on average peak frequency of compound calls or average or maximum bandwidth of complex or simple calls (*p*>0.05; Table 3.7).

Locomotor and Latency to Mount

There was not a main effect of prazosin on latency to mount (*p*>0.05; Table 3.5). There were also no main effects of drug treatment on the number of cage crossings, number of rears, time spent eating or time spent grooming or any of the locomotor variables (*p*>0.05; Table 3.11).

Experiment 3: Clonidine (primarily pre-synaptic α_2- AR agonist, decreases NE transmission)

General Vocalization Properties

There was a main effect of drug treatment on latency to call [F(3,26)=5.02, *p*=0.007]. Rats commenced calling significantly later following the 0.1 mg/kg (*p*<0.001) and 0.02

mg/kg (p=0.023) doses of clonidine compared to vehicle. Latency to call was also significantly increased with the 0.1 mg/kg dose compared to the 0.01 mg/kg dose (p=0.035). (Figure 3.6a). There was a main effect of drug treatment on call rate [$F(3,25)=48.221$, $p<0.001$]. Clonidine dose dependently decreased call rate ($p<0.001$ for all comparisons except 0.01 mg/kg compared to 0.02 mg/kg was p=0.004; Figure 3.6b).

There was a main effect of drug treatment on the percent of frequency modulated [$F(3,25)=9.572$, $p<0.001$] and ramp calls [$F(3,25)=5.97$, p=0.003]. Rats produced fewer frequency modulated and ramp calls at the highest dose (0.1 mg/kg) compared to vehicle ($p<0.001$; p=0.002, respectively), 0.01 mg/kg (p=0.001; p=0.023, respectively), and 0.02 mg/kg (p=0.002; $p<0.001$, respectively) (Figure 3.7a, c). In addition, there was main effect of drug treatment on the percent of harmonic [$F(3,25)=4.0$, p=0.019] and short calls [$F(3,25)=3.07$, p=0.046]. Harmonic and short calls were abolished at the highest dose, which was significantly different than vehicle (p=0.008) and 0.01 mg/kg (p=0.011) for harmonic calls and significantly different than vehicle (p= 0.011) and 0.02 mg/kg (p= 0.022) for short calls (Figure 3.7b, d). There were no other main effects of drug treatment on call type ($p>0.05$; data not shown).

Duration

There was a main effect of drug treatment on average duration of compound calls [$F(3,19)=3.18$, p=0.048]. Average duration was increased with the highest dose of clonidine (0.1 mg/kg) compared to vehicle (p=0.01), 0.01 mg/kg (p=0.011), and 0.02 mg/kg (p=0.033) (Figure 3.6c). There were no main effects of drug treatment on

maximum duration of compound calls or average or maximum duration for simple or complex (p>0.05; Table 3.8).

Bandwidth

There was a main effect of drug treatment on average [F(3,19)=7.37, p=0.002] and maximum [F(3,19)=9.73, p<0.001] bandwidth of compound calls. Average bandwidth of compound calls was reduced in the 0.01 mg/kg (p=0.018), 0.02 mg/kg (p=0.001), and 0.1 mg/kg (p<0.001) doses of clonidine compared to vehicle (Figure 3.8c). Maximum bandwidth of compound calls was reduced in the 0.01 mg/kg (p=0.006), 0.02 mg/kg (p=0.007), and 0.1 mg/kg (p<0.001) doses of clonidine compared to vehicle. Maximum bandwidth was also reduced at the highest dose, 0.1 mg/kg, compared to the 0.01 mg/kg (p=0.011) and 0.02 mg/kg (p=0.032). (Figure 3.8a).

There was a main effect of drug treatment on average [F(3,21)=12.13, p<0.001] and maximum [F(3, 21)=15.51, p<0.001] bandwidth of complex calls. Average bandwidth of complex calls was reduced in the 0.02 mg/kg and 0.1 mg/kg doses of clonidine compared to vehicle (p=0.001 and p<0.001, respectively) and 0.01 mg/kg (p<0.001 and p=0.003, respectively) (Figure 3.8d). Maximum bandwidth of complex calls was significantly reduced in the 0.01 mg/kg (p=0.031), 0.02 mg/kg (p<0.001) and 0.1 mg/kg (p<0.001) doses of clonidine compared to vehicle. The 0.1 mg/kg dose of clonidine was also significantly reduced compared to 0.01 mg/kg (p<0.001) and the 0.02 mg/kg dose was significantly reduced compared to the 0.01 mg/kg dose (p=0.004) (Figure 3.8b).

There was no effect of clonidine on average or maximum bandwidth of simple calls (p>0.05; Table 3.8).

Intensity

There was a main effect of drug treatment on average [$F(3,23)=12.19$, $p<0.001$] and maximum [$F(3, 23)=8.94$, $p<0.001$] intensity of simple calls. Average intensity of simple calls was significantly reduced with the 0.1 mg/kg dose compared to vehicle ($p<0.001$) 0.01 mg/kg ($p<0.001$), and 0.02 mg/kg ($p=0.014$) while the 0.02 mg/kg dose was significantly reduced compared to vehicle ($p=0.004$) and the 0.01 mg/kg dose ($p=0.046$) (Figure 3.9b). Maximum intensity of simple calls was also reduced with the highest dose (0.1 mg/kg) of clonidine compared to vehicle ($p<0.001$), 0.01 mg/kg ($p<0.001$), and 0.02 mg/kg ($p=0.01$) and the 0.02 mg/kg dose was significantly reduced compared to the 0.01 mg/kg dose ($p=0.042$) (Figure 3.9a).

There was a main effect of drug treatment on average [$F(3,21)=4.34$, $p=0.016$] and maximum [$F(3, 21)=7.78$, $p=0.001$] intensity of complex calls. Average intensity of complex calls was significantly reduced with the 0.02 mg/kg dose compared to vehicle ($p=0.002$) and 0.01 mg/kg ($p=0.029$) (Figure 3.9d). Maximum intensity of complex calls was significantly reduced with the 0.1 mg/kg dose compared to vehicle ($p<0.001$), 0.01 mg/kg ($p=0.006$), and 0.02 mg/kg ($p=0.034$) while the 0.02 mg/kg ($p=0.008$) and 0.01 mg/kg dose ($p=0.047$) doses were significantly reduced compared to vehicle (Figure 3.9e).

There was a main effect of drug treatment on maximum intensity of compound calls [$F(3, 19)=4.15$, $p=0.02$]. Maximum intensity of compound calls was significantly reduced with 0.1 mg/kg of clonidine compared to vehicle ($p=0.003$) and 0.01 mg/kg ($p=0.47$) (Figure 3.9e). There was no effect of clonidine on average intensity of compound calls ($p>0.05$; Table 3.8).

Peak Frequency

There was a main effect of drug treatment on average [F(3,23)=4.6, *p*=0.012] and maximum [F(3, 23)=3.38, *p*=0.036] peak frequency of simple calls. Average peak frequency of simple calls was significantly reduced with the 0.1 mg/kg dose compared to vehicle (*p*=0.002), 0.01 mg/kg (*p*=0.006), and 0.02 mg/kg (*p*=0.021) (Figure 3.10b). Maximum peak frequency of simple calls was reduced with the 0.1 mg/kg dose compared to vehicle (*p*=0.01), 0.01 mg/kg (*p*=0.008), and 0.02 mg/kg (*p*=0.047) (Figure 3.10a).

There was a main effect of drug treatment on average [F(3,21)=4.68, *p*=0.012] and maximum [F(3, 21)=4.5, *p*=0.014] peak frequency of complex calls. Average peak frequency of complex calls was significantly reduced with the 0.1 mg/kg dose compared to vehicle (*p*=0.003), 0.01 mg/kg (*p*=0.008), and 0.02 mg/kg (*p*=0.003) (Figure 3.10d). Maximum peak frequency of complex calls was reduced with the 0.1 mg/kg dose compared to vehicle (*p*=0.002), 0.01 mg/kg (*p*=0.028), and 0.02 mg/kg (*p*=0.008) (Figure 3.10c).

There was a main effect of drug treatment on average [F(3,19)=4.08, *p*=0.021] and maximum [F(3,19)=4.25, *p*=0.019] peak frequency of compound calls. Average peak frequency of compound calls was significantly reduced with the 0.1 mg/kg dose compared to vehicle (*p*=0.004), 0.01 mg/kg (*p*=0.018), and 0.02 mg/kg (*p*=0.008) (Figure 3.10f). Maximum peak frequency of compound calls was reduced with the 0.1 mg/kg dose compared to vehicle (*p*=0.003), 0.01 mg/kg (*p*=0.007), and 0.02 mg/kg (*p*=0.041) (Figure 3.10e).

Locomotor and Latency to Mount

There was no effect of clonidine on latency to mount (p>0.05; Table 3.4). There was a main effect of clonidine on the total number of cage crossings [F(3,26)=9.39, p<0.001] and rears [F(3,26)=16.55, p<0.001]. The number of cage crossings was significantly reduced with 0.1 mg/kg clonidine compared to vehicle (p<0.001), 0.01 mg/kg (p<0.001), and 0.02 mg/kg (p=0.007) mg/kg (Figure 3.11a). The number of rears was significantly reduced with 0.1 mg/kg clonidine compared to vehicle (p<0.001), 0.01 mg/kg (p<0.001), and 0.02 mg/kg (p=0.003) mg/kg and with 0.02 mg/kg compared to vehicle (p=0.003). (Figure 3.11c). There was a main effect of drug treatment on time spent grooming [F(3,26)=13.64, p<0.001] and eating [F(3,26)=6.56, p=0.002]. Time spent grooming was significantly reduced with 0.01 mg/kg (p=0.004), 0.02 mg/kg (p<0.001), and 0.1 mg/kg (p<0.001) compared to vehicle and with 0.1 mg/kg compared to 0.01 mg/kg (Figure 3.11b). Time spent eating was significantly reduced with 0.1 mg/kg of clonidine compared to vehicle (p=0.015), 0.01 mg/kg (p=0.007), and 0.02 mg/kg (p<0.01) (Figure 3.11d).

Experiment 4: Atipamezole (primarily pre-synaptic α_2- AR antagonist, increases NE transmission)

General Vocalization Properties

There was a main effect of drug treatment on the percent of step calls [F(3,26)=4.18, p=0.015]. Rats produced significantly more step calls with 0.3 mg/kg (p=0.028) and 1.0 mg/kg (p=0.003) of atipamezole compared to vehicle and significantly more with 1.0 mg/kg compared to 0.1 mg/kg (p=0.035) (Figure 3.12a). Atipamezole did not have any

other significant effects on call profile (p>0.05). There were no main effects of atipamezole on latency to call, call rate, or standard deviation of intensity (p>0.05; Table 3.5).

Duration

There were no main effects of drug treatment on average or maximum duration for simple, complex or compound calls (p>0.05; Table 3.9).

Bandwidth

There was a main effect of drug treatment on maximum bandwidth of compound calls [$F(3,25)=4.85$, p=0.009]. Bandwidth was reduced with the highest dose of atipamezole, 1.0 mg/kg, compared to the vehicle (p=0.013), 0.1 mg/kg (p=0.013), and 0.3 mg/kg (p=0.002) treatments (Figure 3.12c). There were no effects of drug treatment on average bandwidth of compound calls or average or maximum bandwidth of simple and complex calls (p>0.05; Table 3.9).

Intensity

There was a main effect of drug treatment on average intensity of simple calls [$F(3,26)=3.7$, p=0.024]. Average intensity was increased with the highest dose of atipamezole, 1.0 mg/kg (p=0.008), and middle dose, 0.3 mg/kg (p=0.006, compared to the lowest dose, 0.1 mg/kg (Figure 3.12b).

Peak Frequency

There were no main effects of drug treatment on average or maximum peak frequency for simple, complex or compound calls (p>0.05 for all; Table 3.9).

Locomotor and Latency to Mount

There were no main effects of drug treatment on the number of cage crossings, number of rears, time spent eating or time spent grooming (*p*>0.05 for all; Table 3.11). There was not an effect of atipamezole on latency to mount (p>0.05; Table 3.9).

Experiment 5: Isoproterenol (post-synaptic β-AR agonist, increases NE transmission)

Following locomotor and USV testing on the first day of the experiment, multiple rats that received the lowest and highest doses of isoproterenol went into cardiac arrest. Consequently, this experiment was terminated.

Experiment 6: Propranolol (post-synaptic β- AR antagonist, decreases NE transmission)

General Vocalization Properties

There was a main effect of drug treatment on the call rate [F(3,27)=3.00, *p*=0.048]. Call rate was significantly increased with all the doses compared to vehicle (*p=0.024, p= 0.035, and p*=0.013 for 0.3, 1.0, and 3.0 mg/kg, respectively) (Figure 3.13a). There was no effect of propranolol on latency to call or standard deviation of intensity (*p*>0.05; Table 3.5).

Duration

There was a main effect of drug treatment on the duration of simple calls [f(3,27)=3.52, p=0.028]. Duration was increased with the highest dose, 3.0 mg/kg propranolol compared to baseline (Figure 3.13c). There were no main effects of drug treatment on maximum duration of simple or average or maximum duration of complex or compound calls (*p*>0.05; Table 3.10).

Bandwidth

There were no main effects of drug treatment on average or maximum bandwidth for simple, complex or compound calls (p>0.05; Table 3.10).

Intensity

There was a main effect of propranolol on the average intensity of complex calls [F(3,27)=3.540, p=0.028]. Average intensity of complex calls was significantly increased with the 1.0 mg/kg and 3.0 mg/kg doses of propranolol (p=0.035 and p=0.004, respectively) (Figure 3.14a). While there was no main effects of drug treatment on maximum intensity of complex calls or average or maximum intensity of simple or compound calls (p>0.05; Table 3.10), there was a general trend towards increasing intensity with dose (Figure 3.14b, c).

Peak Frequency

There was a main effect of drug treatment on the average peak frequency of simple [F(3,27)=3.67, p=0.025] and complex [F(3,27)=3.66, p=0.025] calls. There was a significant decrease in average peak frequency of simple calls at 1.0 mg/kg (p=0.036) and 3.0 mg/kg (p=0.003) (Figure 3.13b). Similarly, there was a significant decrease in average peak frequency of complex calls at 1.0 mg/kg (p=0.007) and 3.0 mg/kg (p=0.012) (Figure 3.13d).

Locomotor and Latency to Mount

There was no effect of clonidine on latency to mount (p>0.05; Table 3.4) or the number of cage crossings, number of rears, time spent eating or time spent grooming (p>0.05 for all; Table 3.11).

Discussion

The purpose of this study was to determine the contribution of noradrenergic mechanisms on pertinent vocalization parameters. To this end, we evaluated USV in rats following systemic injections of cirazoline (α_1- AR agonist, increases NE transmission), prazosin (α_1- AR antagonist, decreases NE signaling), atipamezole (α_2- AR antagonist, increases NE transmission), clonidine (α_2- AR agonist, decreases NE transmission), and propranolol (β- AR antagonist, decreases NE signaling). We hypothesized that selectively agonizing or antagonizing noradrenergic receptors would result in differential effects on properties of USV such as intensity, bandwidth, peak frequency, and duration that are compromised in rodent models of PD. Our hypothesis was partially confirmed, as we observed differentially altered acoustic properties of USV with some of these drugs. However, almost paradoxically, we also observed that changes in USV parameters were not strictly dependent on the absolute increase/decrease in NE transmission, as the antagonist and agonist for a given receptor frequently resulted in similar effects of a given USV parameter. Overall, these findings indicate that specific noradrenergic mechanisms do contribute to vocal control, but the relationship is not linear. USV intensity (loudness) was increased following the administration of two distinct drugs (atipamezole, an α_2-AR antagonist that promotes NE release, and propranolol a β-AR antagonist that blocks NE signaling). As reduced loudness is a hallmark feature in both humans with PD[1,2,116,208] and rodent models of PD,[31,36,125,150-152] these could be important pharmacological targets for improving voice deficits in PD, which are currently undertreated.

Noradrenergic AR are widely distributed throughout the central nervous system and may be located pre- or post-synaptically.[253,273] The α_1-AR is located post-synaptically and α_1-AR agonists typically increase NE signaling (cirazoline) or decrease NE signaling (prazosin). We found that cirazoline (α_1- AR agonist, increases NE signaling), resulted in a dose dependent decrease in the number of flat calls and the number of rears, without affecting call rate. In addition, we observed dose dependent effects of cirazoline on maximum bandwidth of complex calls (decreased), maximum peak frequency of compound calls (decreased), and average duration of compound calls (increased).

Prazosin (α_1- AR antagonist, decreases NE signaling), on the other hand, dose dependently decreased call rate, significantly reducing the percent of compound and frequency modulated calls, and decreased average bandwidth and maximum peak frequency of compound calls. Both maximum and average intensity were reduced for simple and complex calls as well as maximum intensity of compound calls following prazosin. These findings indicate that the post-synaptic α_1-AR is important for mediating not only the number of calls produced,[165] but may also contribute to the control of bandwidth, peak frequency, duration, and intensity. Interestingly, both the agonist (cirazoline) and antagonist (prazosin) resulted in similar decreases on bandwidth and peak frequency, indicating that alterations in NE transmission, regardless of the direction (increase or decrease in NE transmission) have similar end results on these acoustic parameters.

Although the α_2-AR may also be located post-synaptically, it is primarily a pre-synaptic auto receptor responsible for regulating the release of NE from the terminal.

Given this, drugs that agonize (clonidine) the α_2-AR result in a reduction of NE release, while antagonists (atipamezole) typically result in an increase in NE release. We found that atipamezole (α_2- AR antagonist) which promotes the release of NE resulted in dose dependent increases in the number of step calls and average intensity of simple calls, but decreased maximum bandwidth of compound calls. In contrast, clonidine, (α_2- AR agonist) which blocks the release of NE, resulted in increased latency to call, dose dependent decreases in call rate, the percent of frequency modulated, ramp, harmonic and short calls. In addition, average duration of compound calls was increased, while maximum and average bandwidth was dose dependently reduced for compound and complex calls, maximum and average intensity were reduced for simple and complex calls, and maximum and average peak frequency was reduced for all calls. All locomotor parameters were dose dependently decreased following clonidine as well. Given that clonidine has been shown to have sedative effects,[274] it is possible that the broad locomotor and USV effects observed here are related to that. However, atipamezole (α_2- AR antagonist) increases NE release, and also had similar effects on bandwidth (decreases) but increased intensity (loudness), which was the opposite effect of clonidine, the α_2- AR agonist. Thus it is likely at the least bandwidth and intensity effects are independent of any sedative effects of clonidine.

The third noradrenergic AR examined was the β receptor. Propranolol (β-AR antagonist), which results in a decrease in NE transmission, was the only drug examined that dose dependently increased call rate as well as average intensity of complex calls (average intensity of simple and compound were also increased, though not significantly). We also saw decreases in average peak frequency of simple and

complex calls, and increased average duration of simple calls. Again, these findings suggest that NE-ARs, specifically the β-AR and α_2- AR, may be important for mediating intensity, or loudness, of USV.

Drugs that agonize or antagonize noradrenergic ARs can be grouped in terms of the net effects on NE signaling with increases in NE transmission following α_1-AR and β-AR agonists and α_2-AR antagonists or decreases in NE transmission following α_1-AR and β-AR antagonists and α_2-AR agonists. Generally speaking, in the current study we found that drugs that antagonize post-synaptic α_1-AR noradrenergic receptors (prazosin) or reduced the amount of norepinephrine available by agonizing the pre-synaptic AR (clonidine), resulted in significant reductions in multiple USV parameters. Clonidine, in particular, resulted in decreased complexity of call profile (frequency modulated, harmonic, ramp, and short calls were all reduced), dose dependently reduced call rate, and reduced intensity, bandwidth, and peak frequency. Similarly, prazosin, which also antagonizes the post-synaptic α_1-AR resulting in a decrease in NE transmission, altered call profile (reduced compound and frequency modulated calls) and reduced call rate, bandwidth, intensity, and peak frequency. This trend was not universal, however, as propranolol (post-synaptic β-AR antagonist, which decreases NE transmission) resulted in increased call rate and intensity (discussed below). Despite this exception, the results of this study indicate that decreasing NE transmission at the α_1-AR (prazosin) and α_2-AR (clonidine) results in quantitatively negative effects on call rate, intensity, peak frequency, and bandwidth.

However, reductions in vocal parameters were not limited to drugs that effectively reduce NE signaling. Atipamezole (α_2-AR antagonist, increases NE release via the

pre-synaptic autoreceptor) and cirazoline (post-synaptic, increase NE transmission via the post-synaptic α_1-AR agonist) both decreased bandwidth (as was the case for each of their respective antagonists) and had either no effect on peak frequency (atipamezole) or decreased (cirazoline) peak frequency. In short, agonizing and antagonizing the α_1-AR resulted in similar effects on bandwidth and peak frequency (reduced in both cases) while agonizing and antagonizing the α_2-AR resulted in similar effects on bandwidth, but not peak frequency or intensity (discussed below). These seemingly conflicting findings suggest that while noradrenergic ARs contribute to specific aspects of vocalizations like bandwidth and peak frequency, the relationship is not linear. Specifically, the results of this study suggest that there may be an optimal set-point/range for noradrenergic signaling within which these behaviors function best such that deviations from this range (in this case via AR agonists/antagonists) compromise the integrity of that particular faculty. This is consistent with the Yerkes-Dodson (inverted-U) principle that for a given behavior/function, there exists an optimal level of performance, and that shifts from this (via endogenous or exogenous mechanisms) result in impairments.[257]

The results of this study generally support previous work looking at amphetamine-induced rat USV and add to what is known about the contribution of noradrenergic mechanisms to vocal control. As was the case in Wright et al (2012)[165] we found reductions in call rate with drugs that effectively "antagonize" norepinephrine ARs (clonidine and prazosin) and no changes in call rate with drugs that promote norepinephrine transmission (atipamezole and cirazoline). In addition, we also observed alterations in USV intensity, bandwidth and peak frequency with all of these

drugs. Wright et al (2012) saw no changes in call rate with atipamezole (α_2- AR antagonist, promotes NE activity) without amphetamine and few changes in call profile (increase in step and short calls) with amphetamine. Our results are partially consistent with this, as we did not observe changes in call rate and the only change in call profile was a decrease in step calls. Taken together, these results indicate that the α_2-AR may contribute to the types of calls produced, but that contribution may be dependent on context. Specifically, call profile changes observed when amphetamine is used to induce USV[165] differ from those observed when a social, mating paradigm is used (current study). Given that amphetamine alone can induce changes in USV production, [164,165,216,243,275,276] it is possible that the combined effects of atipamezole and amphetamine account for these differences. An alternative, but not mutually exclusive explanation, is that context matters. Given the role of norepinephrine in goal directed behaviors,[198,199,257] it would not be surprising if alterations to USV following AR modifying drugs enhanced/diminished vocal behaviors as a function of context.

Interestingly, we found that both atipamezole (α_2-AR antagonist at pre-synaptic autoreceptor, promotes NE release) and propranolol (β-AR antagonist, reduces NE activity at the post-synaptic receptor) increased intensity, or loudness, of USV. These findings are exciting because reduced loudness is a hallmark feature of dysphonia in humans with PD[1,2,116,208] and likewise, is almost universally affected in rodent models of PD.[31,36,125,150-152] While the mechanisms underlying this increase are not clear, some potential explanations include increased arousal and/or attention [199,277] and increased sexual motivation. [278,279] However, while atipamezole (α_2- AR antagonist, promotes NE activity) has been shown to increase sexual activity and NE activity in regions important

for mediating sexual behavior,[278,279] we did not observe differences in latency to mount for any of the drugs used in this study. Propranolol (β-AR antagonist, reduces NE activity at the post-synaptic receptor) also increased call rate, which was not the case in previous work.[165] This discrepancy is likely due to methodological differences in how calls are elicited (discussed above), as the dose range used in Wright et al (2012)[165] included all the doses used in the current study.

The findings in the present study indicate that noradrenergic receptors (specifically the presynaptic α_2- AR and post-synaptic β-AR) may be viable targets for attenuating voice deficits in PD, particularly vocal intensity. Future studies in rodent models of PD to test whether these drugs improve vocal intensity are warranted. Specifically, *PINK1* knock-out rats experience early deficits in vocal intensity that correlate with the amount of tyrosine hydroxylase-immunoreactivity in the LC, indicating that noradrenergic mechanisms may contribute to this vocal deficit. Given that both atipamezole (α_2- AR antagonist of presynaptic autoreceptor, promotes NE activity) and propranolol (post-synaptic β-AR antagonist, reduces NE activity) increase vocal intensity in a wild-type, non PD rat, it is possible that they would also increase loudness in this diseased state as well.

In contrast the bandwidth, peak frequency, and intensity, which were frequently reduced following NE-AR manipulation, duration was increased at the highest doses of cirazoline (post-synaptic α_1-AR agonist, promotes NE transmission), clonidine (pre-synaptic α_1-AR agonist, reduces NE transmission), and propranolol (post-synaptic β-AR antagonist, reduces NE activity). Duration is not altered following unilateral 6-OHDA administration,[150,151] though antagonizing specific dopamine receptor subtypes does

result in decreased duration.[149] While DSP-4 (NE neurotoxin) does not decrease duration, control animals treated with saline in the same condition, did show reductions in duration across the study, indicating that NE loss in DSP-4 treated animals differentially prevented this shortening of calls. The results discussed here show that 3 separate drugs each targeting a different NE - AR result in increased duration, and indicate that duration, along with peak frequency, intensity, and bandwidth, is vulnerable to alterations in NE signaling.

In addition to vocalizations, we also evaluated locomotor activity as a measure of gross sensorimotor function. We found that prazosin (α_1- AR antagonist, decreases NE signaling), atipamezole (α_2- AR antagonist, increases NE signaling), and propranolol (β-AR antagonist, decreases NE signaling) did not have any effect on the number of cage crossings, number of rears, times spent eating, or time spent grooming. The number of rears was dose dependently decreased following cirazoline (α_1- AR agonist, increases NE signaling) while clonidine (pre-synaptic α_2- AR agonist which decreases NE release) dose dependently affected all aspects of locomotor activity. In addition, latency to call was only affected with clonidine. Given that rats vocalize when moving around, we cannot rule out the possibility that the effects of clonidine on latency to call and USV parameters were due to general sedation effects of clonidine.[274] However, locomotion alone does not in and of itself result in vocalizations,[280] and while it is possible that gross motor effects on locomotor behavior may affect the acoustic properties of USV, it is equally possible that the mechanisms underlying each behavioral change are not dependent on each other.

In humans with PD, axial and appendicular deficits tend to dissociate with respect to onset, trajectory and response to treatment (with axial deficits tending to be refractory to standard treatments like levodopa and deep brain stimulation). Although we did not evaluate the effects of NE-ARs agonists and antagonists in an animal model of PD, it is worth noting that there tends to be a dissociation between appendicular and axial deficits in these models as well. Specifically, in rats with unilateral 6-OHDA lesions to the medial forebrain bundle or the striatum, cranial sensorimotor deficits do not correlate well with the degree of dopamine loss or gross motor deficits in the limb.[125,148] With the exception of clonidine, which has known sedative effects,[274] we saw only one other alteration in gross motor function; cirazoline (α_1- AR agonist, increases NE signaling) decreased the number of rears. Thus the data here indicate that while alterations in NE signaling (increases and decreases) impact multiple USV parameters, gross locomotor behaviors are left relatively intact, underscoring a possible dissociation of USV (axial deficits) from gross motor function in the limb (appendicular).

The mating paradigm used to elicit calls in the present study is dependent on the males' interest in the female. Thus, in order to assess whether the drugs used impaired sexual motivation, we recorded the latency to first mount on testing day. We found that latency to mount was not significantly affected by any of the drugs or doses examined, indicating that sexual motivation was not impaired in the mating paradigm we use to elicit calls. Typically, well acclimated and experienced males will mount within the first few minutes of exposure to the female. However, if a male had not mounted by 5 minutes, we removed the female and recorded vocalizations as we normally would. Thus for that rat on that day, latency to mount was recorded as 300 seconds (5

minutes). Given that our males had all mounted at some point, and consistently called regardless of whether or not they mounted, we do not believe this affected our USV parameters. However, it is worth keeping in mind. Additionally, it is possible that latency to mount may not be a sensitive enough measure to detect changes. An alternate measure such as the number of intromissions, time spent sniffing/interacting may provide a more sensitive index of sexual interest and motivation.[281] However, given that none of the drugs or doses abolished calling, it seems more likely that motivation to call was not negatively impacted and provides validation for the use a sexual paradigm for eliciting calls.

There are several limitations with the current study and findings. First of all, the effects (or lack of effects) observed are limited to the dose ranges specifically tested in this study. It is likely that higher doses might affect parameters differently, particularly in the case of variables that were not altered following drugs. In addition, one possible explanation for the lack of effects of “agonists” or drugs that mimic the effects of endogenous norepinephrine in the brain is because of a ceiling effect (no room to go up) – for particular parameters (e.g., peak frequency for atipamezole was not increased). However, given that some parameters were increased following drugs (atipamezole and propranolol both increased intensity) it seems unlikely that this is the case for all acoustic variables. Finally, although the variables affected by modulation of noradrenergic ARs were chosen to be relevant to vocal deficits observed in both rodent models of PD and voice deficits in humans with PD (intensity, bandwidth, peak frequency, and duration), the current study used wild-type animals. It is possible that in a diseased state, the effects of these drugs and doses might not be the same due to the

up/down regulation of noradrenergic-ARs as a result of the disease process. However, it is clear based on the results of this study that noradrenergic mechanisms are involved in the modulation of pertinent vocal parameters and in some cases (atipamezole and propranolol) result in desirable effects (increased call rate and intensity).

Conclusion

The results of this study clearly indicate that specific acoustic properties of USV can be modulated by noradrenergic mechanisms. These findings validate and extend previous work demonstrating that general features such as call profile and rate are affected following the administration of NE agonists and antagonists. In addition, modulation at distinct receptor subtypes has the potential to alter USV parameters in similar ways suggesting that there might be an optimal range of noradrenergic signaling in which vocal motor control functions best. Finally, two drugs (atipamezole which acts on the pre-synaptic α_2 AR resulting in an increase in NE transmission and propranolol which acts at the post-synaptic β-AR resulting in a decrease in NE transmission) increased vocal loudness (intensity), indicating that NE-modulating drugs may be effective at attenuating PD-induced voice deficits, which are currently undertreated.

Receptor	***Location***	***Net Effect***	
α_1- adrenoceptor	Post-synaptic	agonist	Promotes NE signaling
		antagonist	Blocks NE signaling
α_2- adrenoceptor	(primarily) Pre-synaptic	agonist	Reduces functional NE release
		antagonist	Promotes functional NE release
β- adrenoceptor	Post-synaptic	agonist	Promotes NE signaling
		antagonist	Blocks NE signaling

Table 3.1: Overview of Receptors. NE = norepinephrine

Experiment	*Drug Name*	*Receptor Target*	*Doses (route)*	*Time Before Testing*
1	Cirazoline	α_1- AR agonist	0, 0.125, 0.25, 0.50 mg/kg (IP)	20 min
2	Prazosin	α_1- AR antagonist	0, 0.1, 0.3, 1.0 mg/kg (IP)	30 min
3	Clonidine	α_2- AR agonist	0, 0.01, 0.02, 0.1 mg/kg (IP)	20 min
4	Atipamezole	α_2- AR antagonist	0, 0.1, 0.3, 1.0 mg/kg (IP)	30 min
5	Isoproterenol	β- AR agonist	0, 1.25, 2.5, 5 mg/kg (IP)	30 min
6	Propranolol	β- AR antagonist	0, 0.3, 1.0, 3.0 mg/kg (IP)	20 min

Table 3.2: Overview of Drug Conditions. AR=adrenoceptor IP=intraperitoneal, min=minutes, mg/kg= milligrams/kilogram.

	Call Type	*Description*
Simple	Flat	Flat, unmodulated with not more than a 0.2 kHz/second rate of frequency change
	Step	Two adjoining frequencies with a jump (or step) between them
	Short	Very brief, unmodulated call
	Ramp	Slow change in frequency of at least 0.2 kHz/second
Frequency Modulated	Trill	Rapid frequency modulations
	Harmonic	Very intense flat call that breaks into the upper and lower harmonic frequencies
	Frequency Modulated	Call consisting of at least 3 frequency changes.
Compound	Compound	Two or more calls combined. (ex, Flat+Trill, Ramp+Trill)

Table 3.3: Call Classification. Descriptions of each of the 9 call types. Broad call categories used for detailed analysis of vocalizations (Simple, Frequency Modulated, and Compound) indicated in the left column.

Drug, target, effect		*Latency to Call*			*Call Rate*			*Duration*			*Intensity*			*Bandwidth*			*Peak Frequency*		
		L	M	H	L	M	H	L	M	H	L	M	H	L	M	H	L	M	H
Atipamezole α_2- AR antagonist	Λ																		
Cirazoline α_1- AR agonist	Λ																		
Clonidine α_2- AR agonist	V																		
Prazosin α_1- AR antagonist	V																		
Propranolol β- AR antagonist	V																		

Table 3.4 Overview of Vocalization Results. Alterations following the administration of each drug as compared to saline. Smallest cells within each column designate low (L), medium (M), high (H) dose for each respective drug. Solid black indicates a decrease at a particular dose, light shading indicates an increase, and blank indicates no change. AR: adrenoceptor, V "Decreases" norepinephrine transmission, Λ "Increases" norepinephrine transmission.

		Call Rate (calls per sec)	*StDev Intensity*	*Latency to Call (sec)*	*Latency to Mount (sec)*
Atipamezole	*Vehicle*	1.87 (0.08)	7.25 (0.29)	1.78 (0.83)	160.1 (46.8)
	0.1 mg/kg	2.29 (0.19)	7.31 (0.27)	1.19 (0.37)	129.6 (41.55)
	0.3 mg/kg	2.03 (0.21)	7.41 (0.31)	0.57 (0.18)	73.8 (37.6)
	1.0 mg/kg	2.18 (0.25)	7.01 (0.26)	0.97 (0.28)	119.1 (39.39)
Clonidine	*Vehicle*	1.77 (0.17)	6.24 (0.34)	0.70 (0.24)	260.6 (27.07)
	0.01 mg/kg	1.17 (0.17)	6.23 (0.26)	3.30 (1.45)	275.7 (24.3)
	0.02 mg/kg	0.73 (0.13)	5.89 (0.56)	5.48 (1.74)	282.2 (17.8)
	0.1 mg/kg	0.17 (0.06)	6.34 (0.7)	24.85 (11.24)	300 (0)
Prazosin	*Vehicle*	1.54 (0.1)	6.59 (0.16)	2.16 (1.02)	31.4 (16)
	0.1 mg/kg	1.2 (0.12)	5.8 (0.23)	2.68 (0.76)	34.5 (24.6)
	0.3 mg/kg	0.8 (0.13)	5.91 (0.32)	5.21 (1.21)	97.4 (41.5)
	1.0 mg/kg	0.6 (0.17)	5.03 (0.44)	2.94 (1.2)	44 (28.52)
Cirazoline	*Vehicle*	2.03 (0.17)	7 (0.15)	1.23 (0.51)	36.3 (29.33)
	0.125 mg/kg	2.033 (0.22)	6.97 (0.22)	2.26 (0.94)	39 (29.23)
	0.25 mg/kg	2.11 (0.15)	6.84 (0.12)	0.7 (0.35)	42.1 (28.98)
	0.5 mg/kg	1.91 (0.22)	7 (0.26)	1.73 (0.8)	43.4 (29.03)
Propranolol	*Vehicle*	1.8 (0.19)	6.8 (0.26)	1.78 (1.1)	43.7 (28.73)
	0.3 mg/kg	2.2 (0.24)	7.3 (0.42)	1.61 (0.6)	70 (38.38)
	1.0 mg/kg	2.2 (0.16)	7.2 (0.18)	1.35 (0.8)	88.5 (39.62)
	3.0 mg/kg	2.3 (0.13)	7.5 (0.23)	0.45 (0.2)	83.5 (36.94)

Table 3.5 General Ultrasonic Vocalization Parameters. Mean (SEM) for call rate, StDev Intensity, Latency to Call and Latency to Mount for each drug and dose. StDev= Standard Deviation, Sec=Seconds.

Cirazoline		*Bandwidth (Hertz)*	*Peak Frequency (Hertz)*	*Intensity (decibels)*	*Duration (seconds)*
Simple Calls	*Vehicle*	9476 (926) 26920 (3828)	52814 (1215) 64160 (1907)	-47.62 (0.98) -32.8 (1.86)	0.028 (0.0017) 0.063 (0.0092)
	0.125 mg/kg	8053 (556) 22980 (1863)	51822 (1157) 63570 (1596)	-48.97 (0.85) -32.8 (1.86)	0.028 (0.0028) 0.062 (0.0084)
	0.25 mg/kg	8786 (608) 33740 (4220)	51259 (1186) 61780 (1231)	-49.27 (0.75) -34.5 (2.29)	0.028 (0.0015) 0.075 (0.012)
	0.5 mg/kg	8868 (416) 25660 (1583)	51838 (1517) 65180 (2366)	-49.34 (0.76) -34.45 (1.65)	0.029 (0.0024) 0.072 (0.011)
Complex Calls	*Vehicle*	23123 (1693) 53090 (2811)	57393 (1580) 69380 (1881)	-46.34 (0.88) -30.71 (1.36)	0.046 (0.004) 0.13 (0.23)
	0.125 mg/kg	20031 (1408) 43700 (2954)	56746 (1614) 67860 (2238)	-46.96 (0.66) -30.2 (1.29)	0.042 (0.0033) 0.11 (0.022)
	0.25 mg/kg	20525 (1548) 47270 (4531)	56475 (1147) 70120 (2481)	-47.079 (0.59) -31.42 (0.087)	0.045 (0.0024) 0.16 (0.023)
	0.5 mg/kg	20326 (1905) 44200 (3759)	57217 (1654) 70030 (3004)	-48.16 (0.53) -34.4 (1.18)	0.045 (0.0029) 0.11 (0.016)
Compound Calls	*Vehicle*	30759 (1795) 53420 (3323)	54067 (1779) 65340 (2602)	-42.67 (1.3) -30.6 (1.48)	0.064 (0.0035) 0.15 (0.02)
	0.125 mg/kg	29459 (1440) 51020 (4314)	54328 (1631) 65240 (2441)	-43.97 (1.17) -31.82 (1.83)	0.062 (0.006) 0.132 (0.023)
	0.25 mg/kg	29053 (2174) 45020 (5824)	52744 (1303) 61230 (2428)	-42.42 (0.88) -33.4 (1.42)	0.072 (0.007) 0.18 (0.032)
	0.5 mg/kg	29636 (2083) 51160 (5928)	53648 (1754) 66360 (2806)	-42.83 (1.67) -32.15 (2.62)	0.079 (0.0064) 0.017 (0.021)

Table 3.6 Summary of Data for Cirazoline. Data are expressed as a mean (SEM) for the average (top) and maximum (bottom) values for each parameter.

Prazosin		*Bandwidth (Hertz)*	*Peak Frequency (Hertz)*	*Intensity (decibels)*	*Duration (seconds)*
Simple Calls	*Vehicle*	9157 (666) 25680 (2632)	52171 (1303) 63570 (2003)	-48.69 (0.67) -34.44 (0.82)	0.027 (0.003) 0.052 (0.005)
	0.1 mg/kg	7845 (729) 24750 (3368)	51963 (1888) 59480 (2357)	-50.51 (0.74) -40.28 (1.3)	0.027 (0.002) 0.055 (0.009)
	0.3 mg/kg	9277 (1676) 22130 (2679)	54386 (1927) 66130 (2911)	-51.087 (0.46) -41.046 (1.51)	0.029 (0.003) 0.072 (0.014)
	1.0 mg/kg	6409 (413) 17378 (1972)	53175 (1797) 61089 (3659)	-52.25 (0.92) -44.047 (1.88)	0.027 (0.004) 0.067 (0.017)
Complex Calls	*Vehicle*	20041 (1547) 44220 (3802)	56515 (1653) 67590 (2678)	-46.84 (0.84) -32.99 (1.02)	0.04 (0.002) 0.1 (0.011)
	0.1 mg/kg	20578 (999) 44490 (2373)	55567 (2152) 68740 (3042)	-49.72 (0.83) -37.76 (1.59)	0.042 (0.003) 0.095 (0.016)
	0.3 mg/kg	20331 (1702) 40040 (2886)	56230 (1168) 66640 (2547)	-49.66 (0.75) -39.015 (2.082)	0.048 (0.006) 0.092 (0.014)
	1.0 mg/kg	19353 (1567) 37650 (2641)	57176 (2053) 67080 (2851)	-52.45 (0.83) -43.76 (2.15)	0.042 (0.003) 0.079 (0.012)
Compound Calls	*Vehicle*	28892 (1775) 44980 (2446)	53329 (1395) 63920 (2543)	-44.86 (1.11) -34.69 (1.35)	0.061 (0.002) 0.11 (0.006)
	0.1 mg/kg	29878 (1699) 41070 (3077)	51960 (2079) 61430 (3819)	-46 (1.13) -38.9 (0.72)	0.064 (0.007) 0.1 (0.02)
	0.3 mg/kg	28833 (3410) 38067 (5585)	53357 (974) 56478 (1641)	-46.9 (1.53) -41.1 (2.19)	0.071 (0.013) 0.13 (0.053)
	1.0 mg/kg	27772 (1788) 35450 (2680)	54938 (2737) 59838 (1917)	-50.3 (1.99) -46.2 (2.96)	0.072 (0.01) 0.096 (0.015)

Table 3.7 Summary of Data for Prazosin. Data are expressed as a mean (SEM) for the average (top) and maximum (bottom) values for each parameter.

Clonidine		*Bandwidth (Hertz)*	*Peak Frequency (Hertz)*	*Intensity (decibels)*	*Duration (seconds)*
Simple Calls	*Vehicle*	13166 (1875) 27990 (294)	55762 (1381) 64050 (2625)	-48.24 (0.9) -38.40 (2.8)	0.04 (0.0044) 0.099 (0.025)
	0.01 mg/kg	12673 (2259) 30516 (4684)	55173 (1382) 64420 (1746)	-49.19 (0.8) -36.8 (1.7)	0.045 (0.0058) 0.12 (0.019)
	0.02 mg/kg	14113 (2166) 28022 (4692)	54620 (1165) 63256 (2022)	-50.81 (0.7) -41.18 (2.6)	0.043 (0.0053) 0.093 (0.015)
	0.1 mg/kg	10802 (2686) 18043 (2990)	50939 (2542) 55414 (1975)	-52.53 (0.9) -48.62 (3.0)	0.054 (0.0084) 0.094 (0.023)
Complex Calls	*Vehicle*	16375 (1748) 40980 (2736)	55943 (1350) 67060 (2255)	-48.59 (0.76) -32.88 (2.04)	0.042 (0.0042) 0.12 (0.015)
	0.01 mg/kg	17333 (2838) 34020 (2802)	53412 (2915) 61920 (3988)	-49.71 (0.74) -38.21 (1.68)	0.055 (0.011) 0.12 (0.015)
	0.02 mg/kg	11709 (1781) 23233 (2340)	55360 (1626) 64456 (1883)	-52.25 (0.98) -41.07 (3.2)	0.046 (0.0071) 0.084 (0.015)
	0.1 mg/kg	12375 (2189) 19540 (2765)	50165 (2565) 53160 (2721)	-50.85 (2.07) -47.24 (3.1)	0.051 (0.0091) 0.073 (0.016)
Compound Calls	*Vehicle*	30954 (1479) 50910 (2265)	55478 (1875) 64220 (1029)	-45.4 (0.92) -34.9 (1.53)	0.079 (0.006) 0.19 (0.03)
	0.01 mg/kg	26547 (1266) 39840 (3367)	54517 (847) 63030 (2342)	-46.73 (0.83) -38.13 (1.39)	0.079 (0.0076) 0.17 (0.035)
	0.02 mg/kg	23997 (1462) 40586 (3969)	55234 (863) 62114 (1427)	-48.25 (1.22) -38.05 (2.05)	0.087 (0.013) 0.18 (0.042)
	0.1 mg/kg	22942 (1600) 31040 (3648)	50596 (2073) 54340 (2873)	-48.87 (0.94) -43.21 (1.2)	0.11 (0.024) 0.21 (0.072)

Table 3.8 Summary of Data for Clonidine. Data are expressed as a mean (SEM) for the average (top) and maximum (bottom) values for each parameter.

Atipamezole		*Bandwidth (Hertz)*	*Peak Frequency (Hertz)*	*Intensity (decibels)*	*Duration (seconds)*
Simple Calls	*Vehicle*	9455 (883) 26560 (2576)	54022 (974) 36870 (1154)	-47.15 (0.68) -32.85 (1.48)	0.039 (0.006) 0.11 (0.026)
	0.1 mg/kg	8822 (730) 26089 (2982)	52089 (1154) 63578 (1135)	-48.24 (0.77) -33.69 (1.57)	0.034 (0.003) 0.094 (0.015)
	0.3 mg/kg	9360 (575) 26850 (3265)	54106 (1129) 65070 (1443)	-46.52 (0.69) -31.20 (1.01)	0.032 (0.004) 0.13 (0.042)
	1.0 mg/kg	9359 (765) 28990 (2630)	53693 (1201) 64840 (1297)	-46.58 (1.11) -30.29 (1.88)	0.031 (0.004) 0.087 (0.015)
Complex Calls	*Vehicle*	20445 (412) 45120 (3523)	57455 (1371) 68510 (1775)	-47.95 (0.93) -33.31 (1.9)	0.047 (0.002) 0.13 (0.011)
	0.1 mg/kg	21285 (896) 49878 (2743)	55805 (1128) 70044 (2811)	-47.39 (0.68) -31.58 (1.38)	0.047 (0.004) 0.14 (0.031)
	0.3 mg/kg	19686 (1162) 48150 (4455)	57169 (1430) 70550 (1767)	-47.36 (1.09) -30.45 (1.58)	0.04 (0.002) 0.11 (0.013)
	1.0 mg/kg	21857 (1022) 46000 (2561)	57271 (1684) 71610 (2781)	-46.79 (1.1) -32.03 (1.68)	0.045 (0.004) 0.15 (0.048)
Compound Calls	*Vehicle*	30980 (1528) 54950 (2026)	54502 (1138) 66110 (1789	-42.06 (0.91) -29.13 (1.57)	0.089 (0.011) 0.22 (0.035)
	0.1 mg/kg	30138 (1280) 55767 (3321)	52483 (968) 63433 (1558)	-41.33 (0.87) -28.09 (1.47)	0.081 (0.01) 0.26 (0.063)
	0.3 mg/kg	31820 (1511) 59589 (2573)	53614 (1485) 64767 (2249)	-40.99 (1.05) -27.85 (0.72)	0.076 (0.005) 0.19 (0.028)
	1.0 mg/kg	29208 (1542) 45140 (3407)	54196 (1000) 65820 (1991)	-41.77 (1.04) -30.75 (1.57)	0.074 (0.006) 0.18 (0.042)

Table 3.9 Summary of Data for Atipamezole. Data are expressed as a mean (SEM) for the average (top) and maximum (bottom) values for each parameter.

Propranolol		*Bandwidth (Hertz)*	*Peak Frequency (Hertz)*	*Intensity (decibels)*	*Duration (seconds)*
Simple Calls	*Vehicle*	8928 (483) 23880 (1657)	54527 (677) 64800 (1990)	-48.58 (1.15) -37.03 (1.89	0.022 (0.0015) 0.049 (0.005)
	0.3 mg/kg	9043 (495) 27150 (1212)	52966 (1243) 62450 (1156)	-47.16 (1.60) -321.38 (2.31)	0.026 (0.0028) 0.062 (0.0098)
	1.0 mg/kg	8370 (409) 24180 (1829)	52271 (846) 64840 (1239)	-47.14 (0.49) -33.14 (1.28)	0.027 (0.0036) 0.085 (0.022)
	3.0 mg/kg	9899 (619) 29260 (3496)	51229 (1157) 61790 (1819)	-46.33 (0.83) -31.26 (1.52)	0.026 (0.0021) 0.069 (0.014)
Complex Calls	*Vehicle*	20909 (9980) 48080 (3226)	58130 (401) 69170 (1370)	-47.67 (0.58) -31.49 (1.34)	0.041 (0.002) 0.11 (0.02)
	0.3 mg/kg	21299 (1410) 47500 (2878)	56640 (1183) 70510 (2822)	-45.94 (1.1) -29.056 (1.43)	0.043 (0.0029) 0.11 (0.011)
	1.0 mg/kg	22220 (1078) 56250 (2838)	54889 (1127) 69970 (1961)	-45.58 (0.66) -29.91 (1.28)	0.044 (0.0028) 0.11 (0.01)
	3.0 mg/kg	21957 (945) 49820 (2600)	55108 (979) 68160 (2423)	-44.68 (0.61) -27.63 (0.89)	0.047 (0.0029) 0.15 (0.021)
Compound Calls	*Vehicle*	31581 (1755) 50600 (5301)	54602 (761) 64000 (1947)	-43.19 (0.85) -31.75 (2.04)	0.071 (0.0065) 0.16 (0.029)
	0.3 mg/kg	32741 (1987) 49760 (4748)	54408 (1585) 65180 (1032)	-41 (1.46) -29.7 (2.68)	0.07 (0.01) 0.17 (0.042)
	1.0 mg/kg	33498 (1496) 54850 (3137)	52039 (1414) 59870 (1827)	-41.99 (0.7) -28.29 (1.82)	0.07 (0.0055) 0.17 (0.03)
	3.0 mg/kg	31818 (1821) 52740 (2586)	52383 (1210) 63660 (1325)	-41.48 (0.9) -29.73 (1.61)	0.071 (0.0045) 0.19 (0.037)

Table 3.10 Summary of Data for Propranolol. Data are expressed as a mean (SEM) for the average (top) and maximum (bottom) values for each parameter.

		Number of Cage Crosses	*Number of Rears*	*Time Spent Grooming (sec)*	*Time Spent Eating (sec)*
Atipamezole	*Vehicle*	25.88 (4.92)	55.5 (9.61)	84.88 (22.67)	48.38 (20.07)
	0.1 mg/kg	36.5 (5.97)	60.5 (6.88)	85.83 (14.22)	45.67 (13.68)
	0.3 mg/kg	44.4 (4.01)	80.4 (4.55)	104.2 (40.66)	98 (47.48)
	1.0 mg/kg	32.5 (5.32)	67.25 (16.98)	102.75 (32.74)	27.75 (12.07)
Clonidine	*Vehicle*	28.2 (2.25)	61.5 (4.91)	120.3 (21.82)	50.6 (15.58)
	0.01 mg/kg	22.9 (5.39)	48.3 (7.03)	59.4 (14.24)	91.7 (36.47)
	0.02 mg/kg	19.22 (2.71)	37.11 (6.23)	29.11 (6.07)	134.2 (38.37)
	0.1 mg/kg	7.6 (1.15)	15.8 (2.53)	3.2 (2.16)	5.7 (3.73)
Prazosin	*Vehicle*	31.11 (5.95)	53.56 (5.68)	107.78 (33.68)	33.44 (18.45)
	0.1 mg/kg	28.6 (5.48)	46.9 (7.78)	94.7 (13.29)	78 (26.77)
	0.3 mg/kg	23.9 (5.73)	40.2 (5.97)	89.4 (29.42)	99.2 (28.36)
	1.0 mg/kg	19.33 (2.92)	43 (5.18)	55.89 (10.09)	157 (37.37)
Cirazoline	*Vehicle*	28.2 (4.48)	59.5 (5.81)	106.7 (16.85)	57.2 (17.65)
	0.125 mg/kg	24 (2.45)	47.7 (6.65)	141.6 (22.26)	36.6 (21.91)
	0.25 mg/kg	25.6 (2.73)	47.8 (3.63)	143 (26.15)	24.6 (14.4)
	0.5 mg/kg	23.8 (2.24)	43.3 (3.98)	113.1 (35.68)	28.9 (19.76)
Propranolol	*Vehicle*	31.3 (5.12)	58.9 (5.51)	93.3 (21.99)	67 (27.31)
	0.3 mg/kg	36.2 (4.2)	65.9 (5.99)	108.4 (27.67)	89.5 (33.53)
	1.0 mg/kg	30.8 (2.73)	62.3 (6.83)	109.5 (33.09)	80.1 (24.55)
	3.0 mg/kg	32 (5.48)	56.5 (4.87)	92.6 (17.57)	83.7 (27.4)

Table 3.11 Overview of Locomotor Effects. Mean (SEM) for the number of cage crosses, number of rears, time spent grooming, and time spent eating. Sec=Seconds.

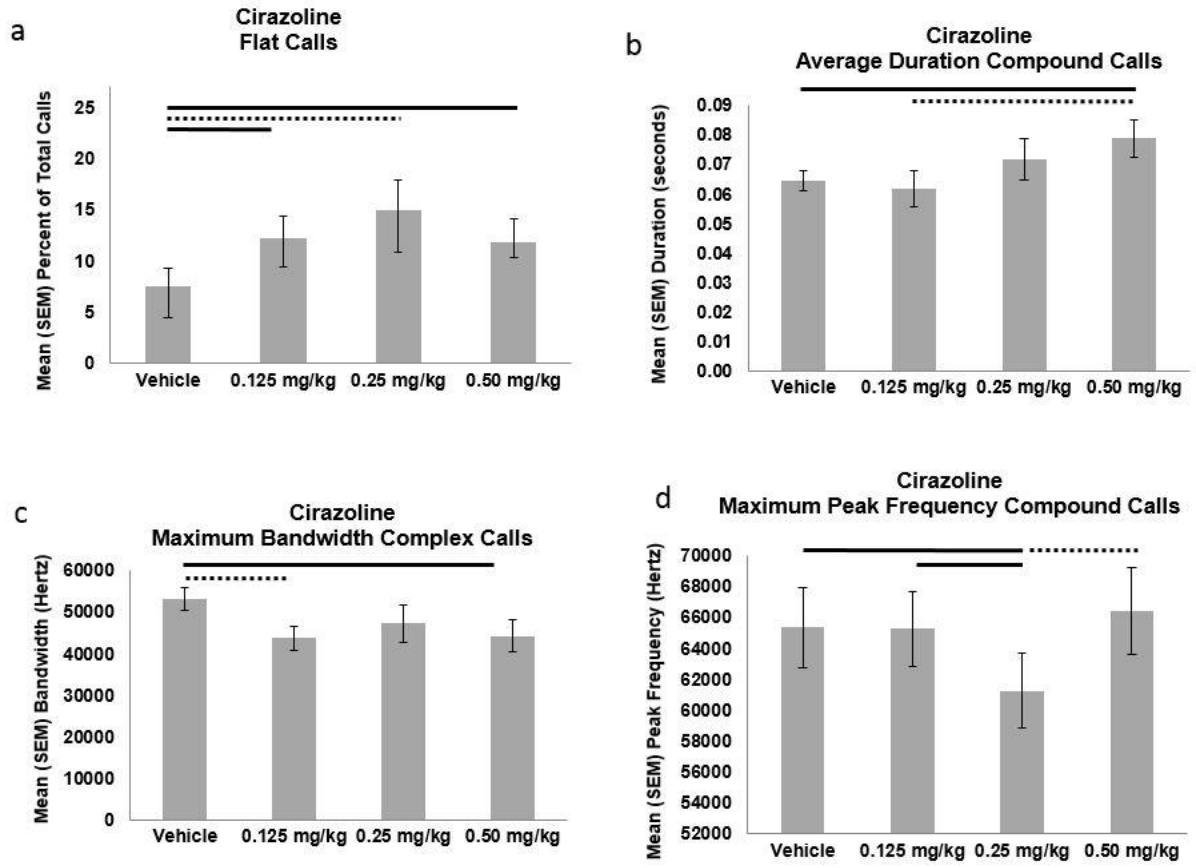

Figure 3.1: Effects of Cirazoline on Ultrasonic Vocalizations. a) The percent of flat calls was significantly increased following each dose of cirazoline compared to vehicle. b) Average duration of compound calls was increased with the 0.5 mg/kg dose of cirazoline compared to vehicle and 0.125 mg/kg. c) Maximum bandwidth of complex calls was significantly reduced with 0.125 mg/kg and 0.50 mg/kg of cirazoline compared to vehicle. d) Maximum peak frequency was reduced with 0.25 mg/kg of cirazoline compared to all other drug treatments. Solid line: $p<0.05$, dashed line: $p<0.01$.

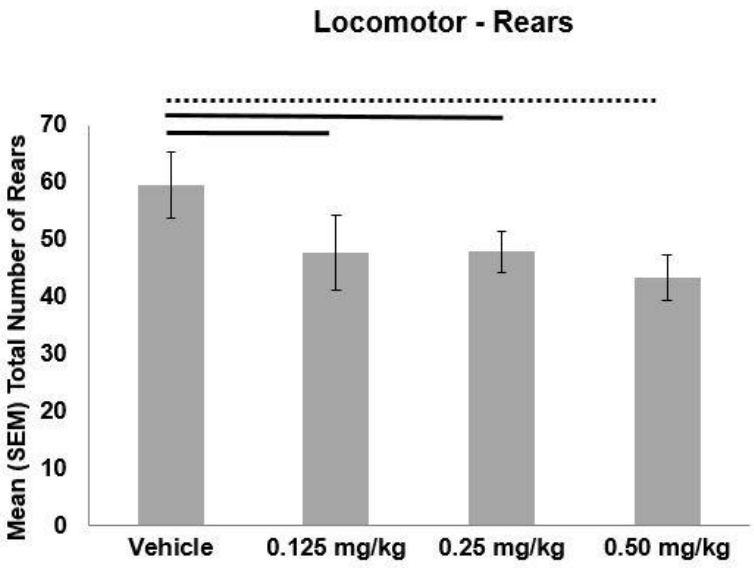

Figure 3.2. Effects of Cirazoline on Locomotor Behavior. The total number of rears was significantly decreased with each of the doses compared to vehicle. Solid line: $p<0.05$, dashed line: $p<0.01$.

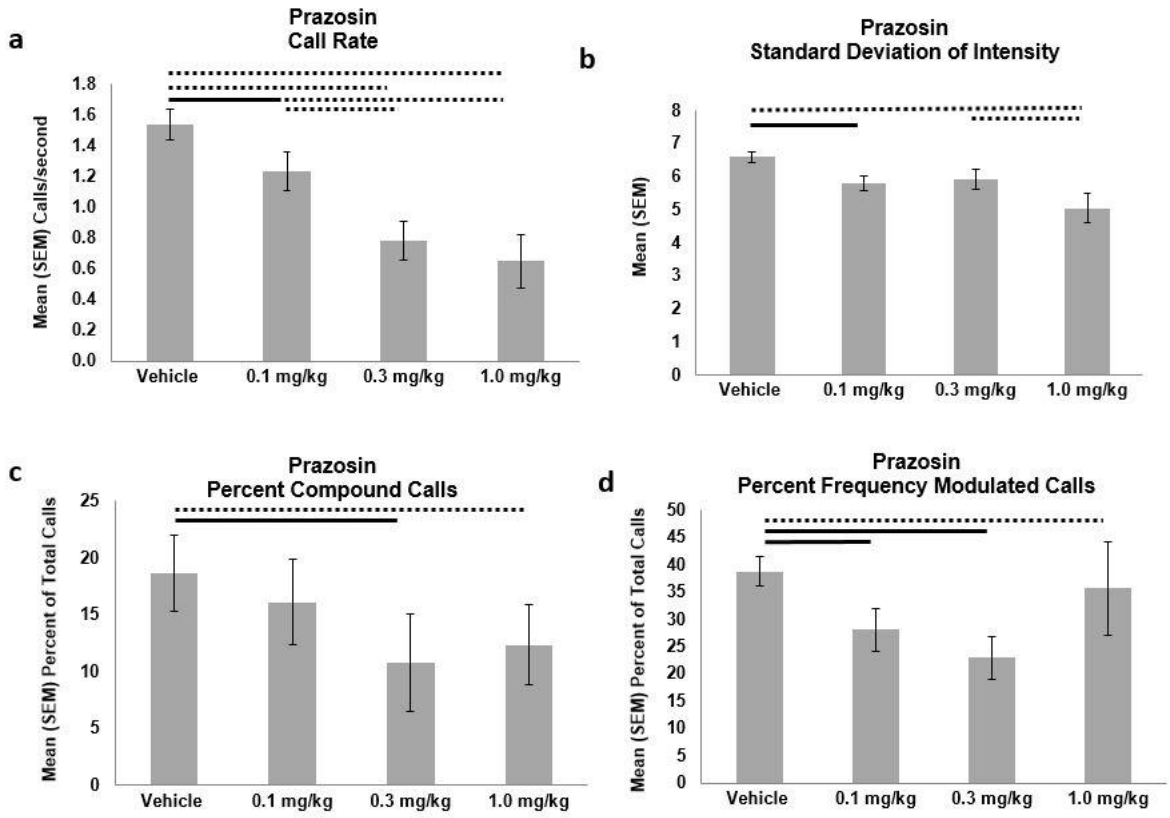

Figure 3.3: Effects of Prazosin on General Vocalization Parameters. a) Call rate was significantly decreased following each dose of prazosin. b) The standard deviation of intensity was significant reduced with 0.1 mg/kg and 1.0 mg/kg of prazosin compared to vehicle. c) The percent of compound calls was significantly decreased following the two highest doses of prazosin compared to vehicle. d) The percent of frequency modulated calls was significantly reduced with all doses of prazosin compared to vehicle. Solid line: $p<0.05$, dashed line: $p<0.01$.

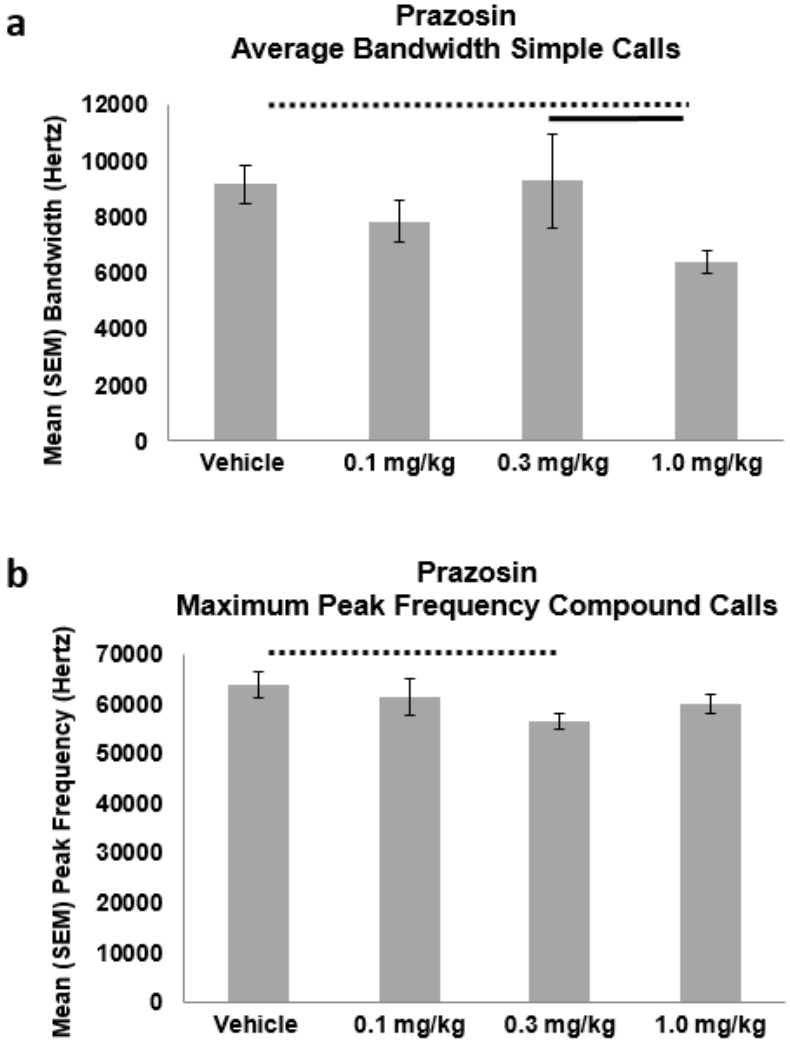

Figure 3.4: Effects of Prazosin on Bandwidth and Peak Frequency of Ultrasonic Vocalizations. a) Average bandwidth of simple calls was significantly reduced with 1.0 mg/kg prazosin. b) Maximum peak frequency was reduced with 0.3 mg/kg prazosin compared to vehicle. Solid line: $p<0.05$, dashed line: $p<0.01$.

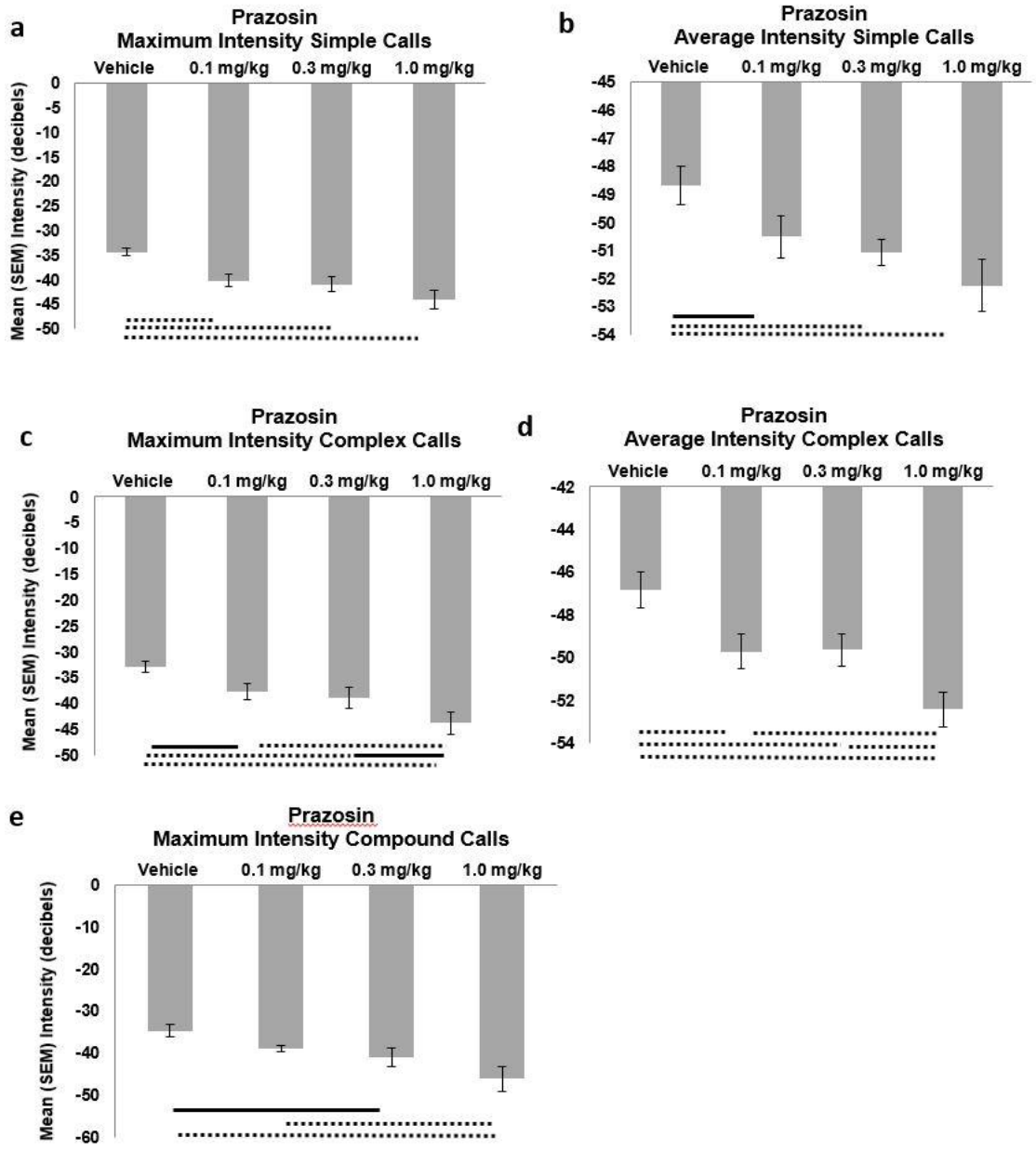

Figure 3.5: Effects of Prazosin on Intensity of Ultrasonic Vocalizations. Maximum (a) and average (b) intensity of simple calls were significantly reduced with all doses of prazosin. Maximum (c) and average (d) intensity of complex calls were significantly reduced with all doses of prazosin and maximum intensity of compound calls was also reduced at the two highest doses (e). Solid line: $p<0.05$, dashed line: $p<0.01$.

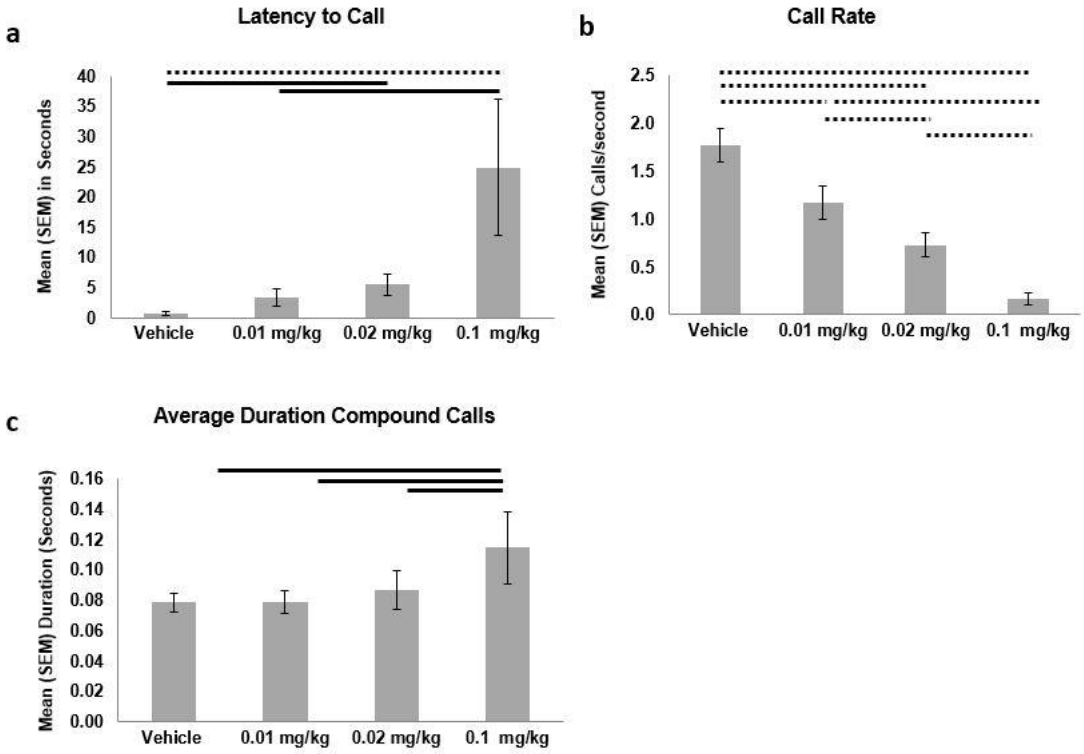

Figure 3.6: Effects of Clonidine on Ultrasonic Vocalizations. a) Latency to call was significantly increased with the 0.02 mg/kg and 0.1 mg/kg doses of clonidine. Call rate was significantly reduced with all doses of clonidine. c) Average duration of compound calls was significantly increased with 0.1 mg/kg clonidine. Solid line: $p<0.05$, dashed line: $p<0.01$.

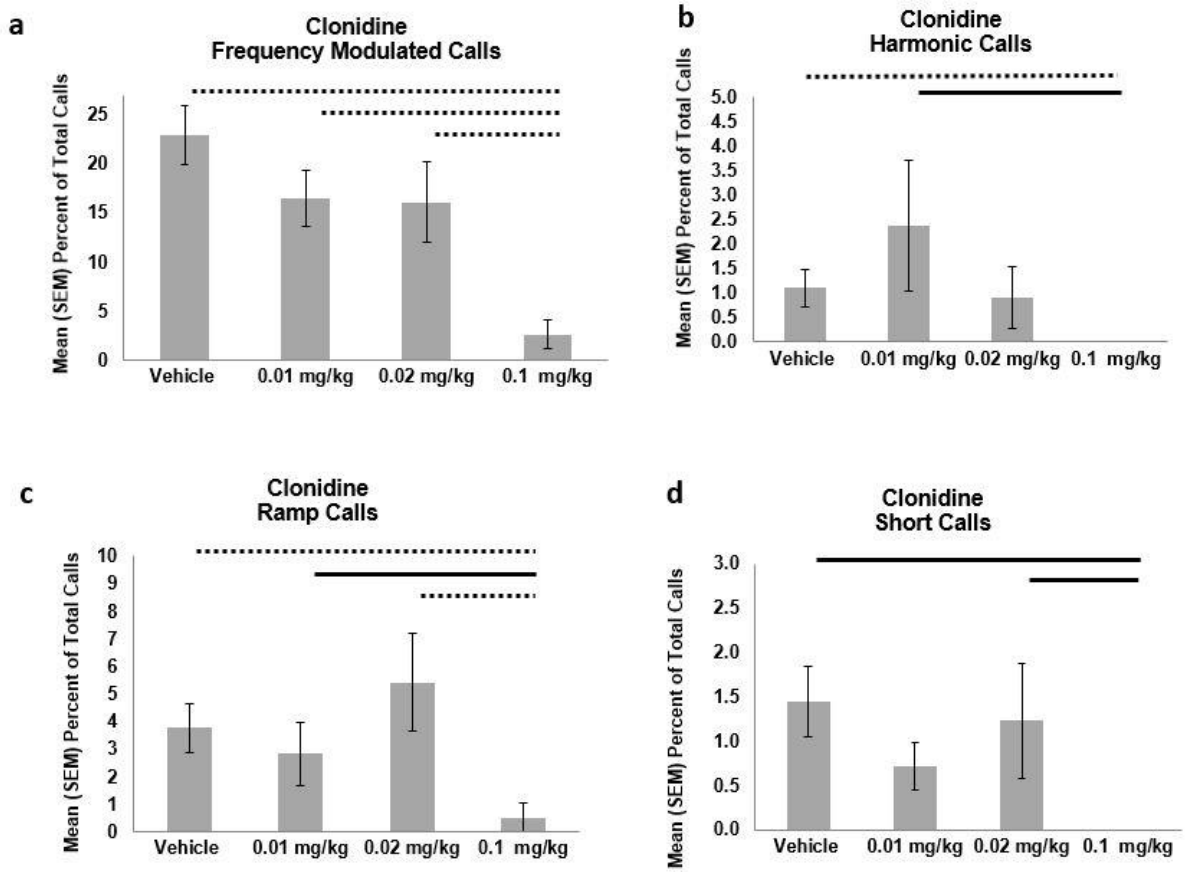

Figure 3.7: Effects of Clonidine on Call Profile. The percent of frequency modulated (a), harmonic (b), ramp (c), and short calls was reduced at the highest dose of clonidine, 0.1 mg/kg. Solid line: $p<0.05$, dashed line: $p<0.01$.

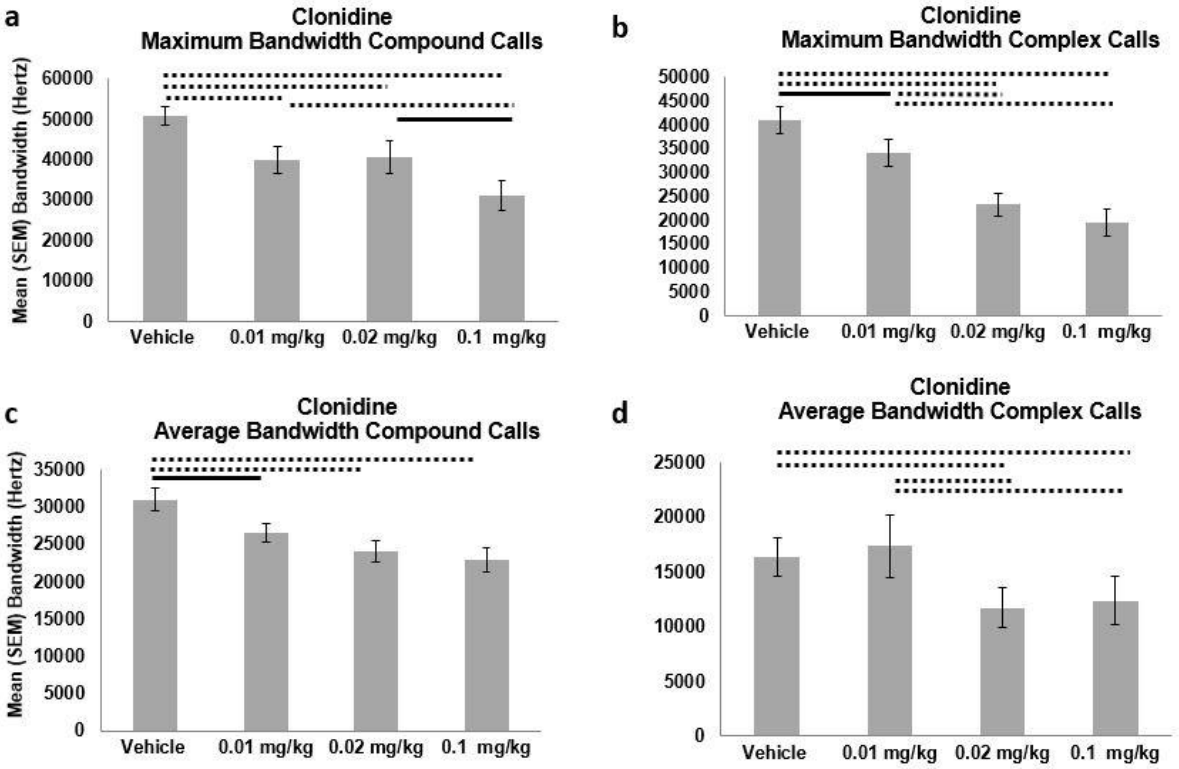

Figure 3.8: Effects of Clonidine on Bandwidth of Ultrasonic Vocalizations.

Maximum (a) and average (c) bandwidth of compound calls was significantly reduced at all doses of clonidine. Maximum (c) bandwidth of complex calls was reduced at all doses of clonidine while average bandwidth was reduced at the two highest doses (d). Solid line: $p<0.05$, dashed line: $p<0.01$.

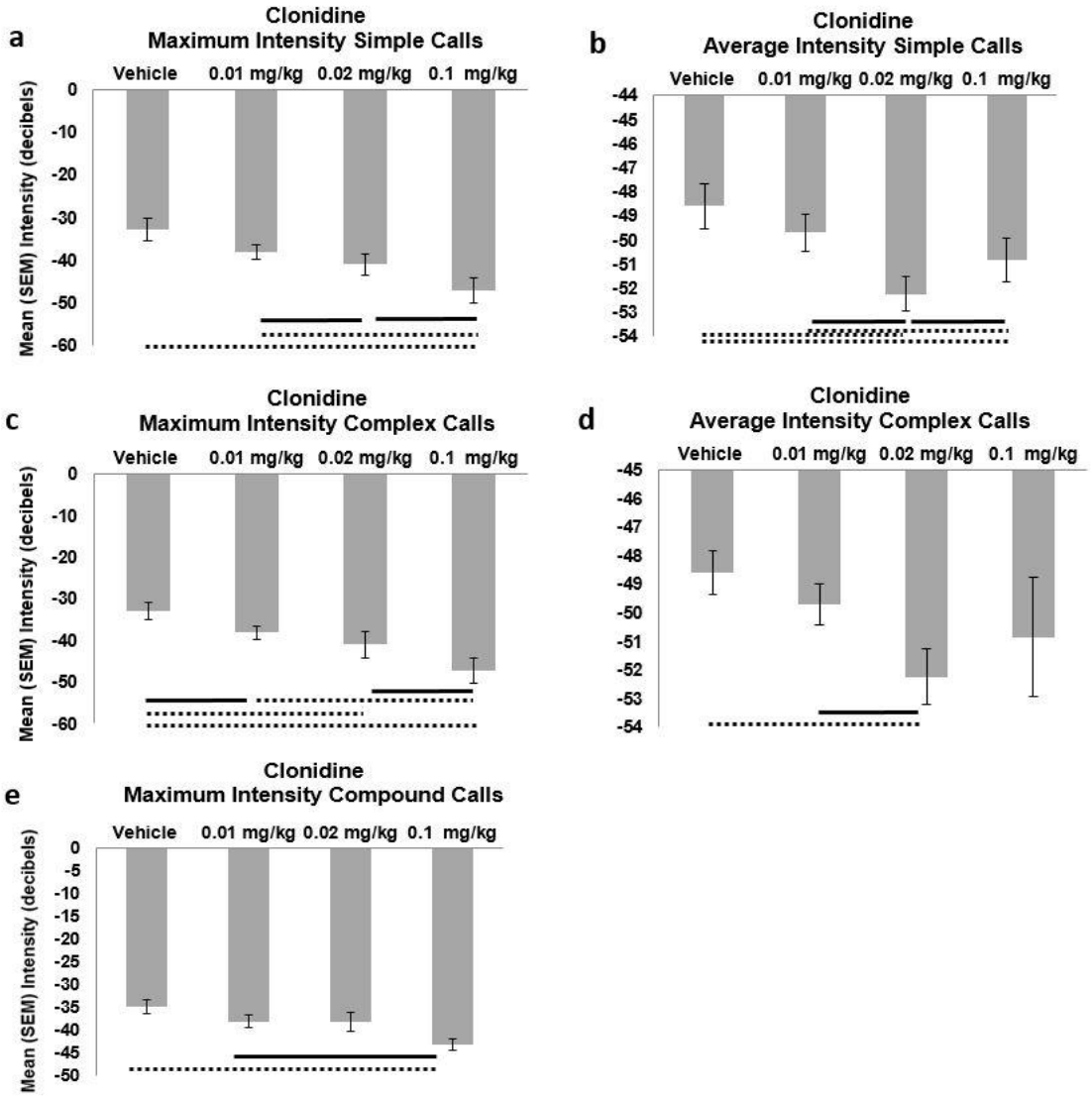

Figure 3.9: Effects of Clonidine on Intensity of Ultrasonic Vocalizations. For simple calls, maximum (a) intensity was reduced at the highest dose and average (b) intensity was reduced at the two highest doses of clonidine. Maximum (c) intensity of complex calls was reduced at all doses of clonidine while average (d) intensity was reduced at the 0.2 mg/kg dose of clonidine. Maximum intensity of compound calls was significantly reduced at the highest dose of clonidine (e). Solid line: $p<0.05$, dashed line: $p<0.01$.

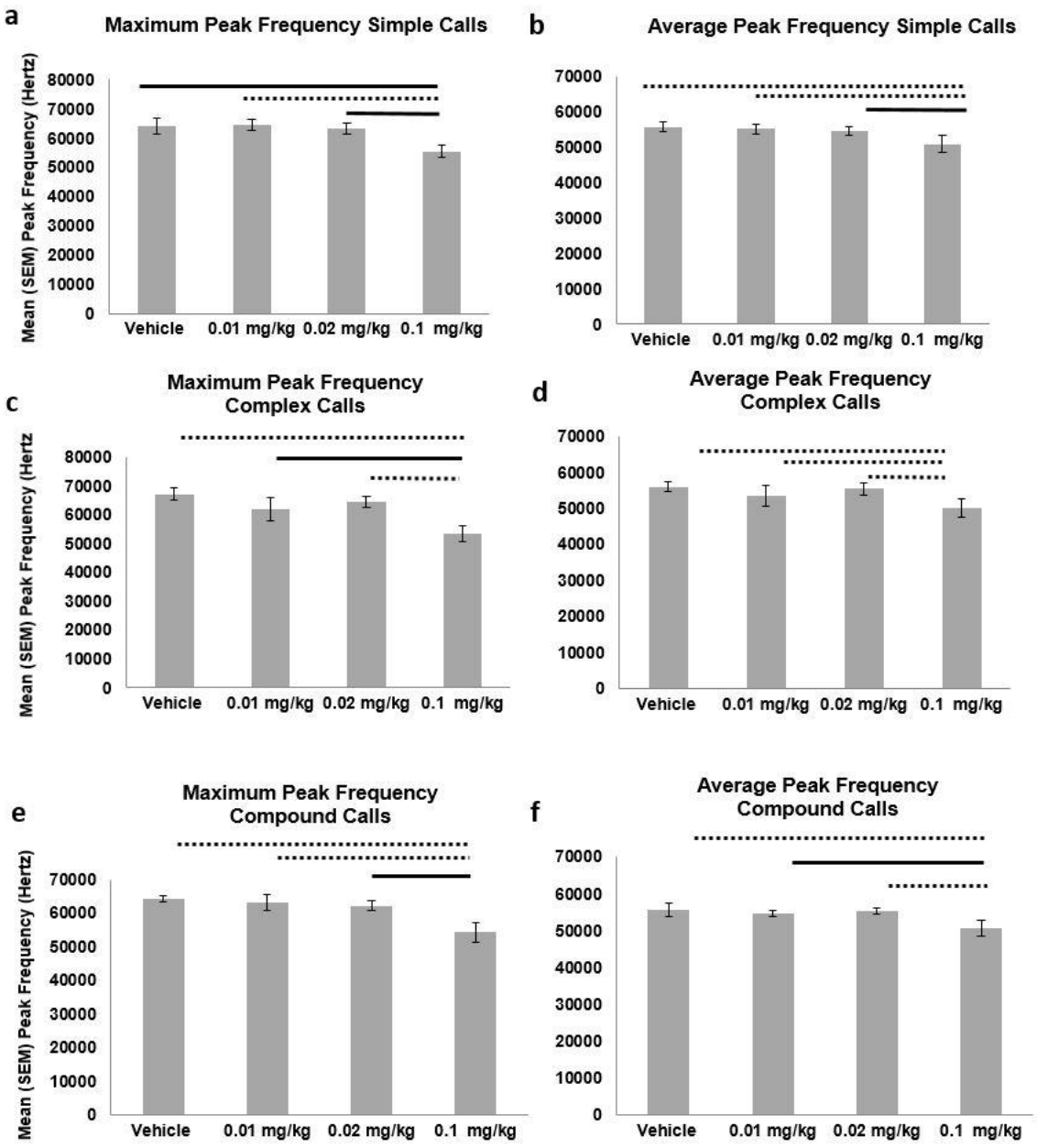

Figure 3.10: Effects of Clonidine on Peak Frequency of Ultrasonic Vocalizations.

Maximum and average peak frequency of simple (a, b), complex (c, d), and compound (e, f) calls were significantly reduced at the highest dose of clonidine Solid line: $p<0.05$, dashed line: $p<0.01$.

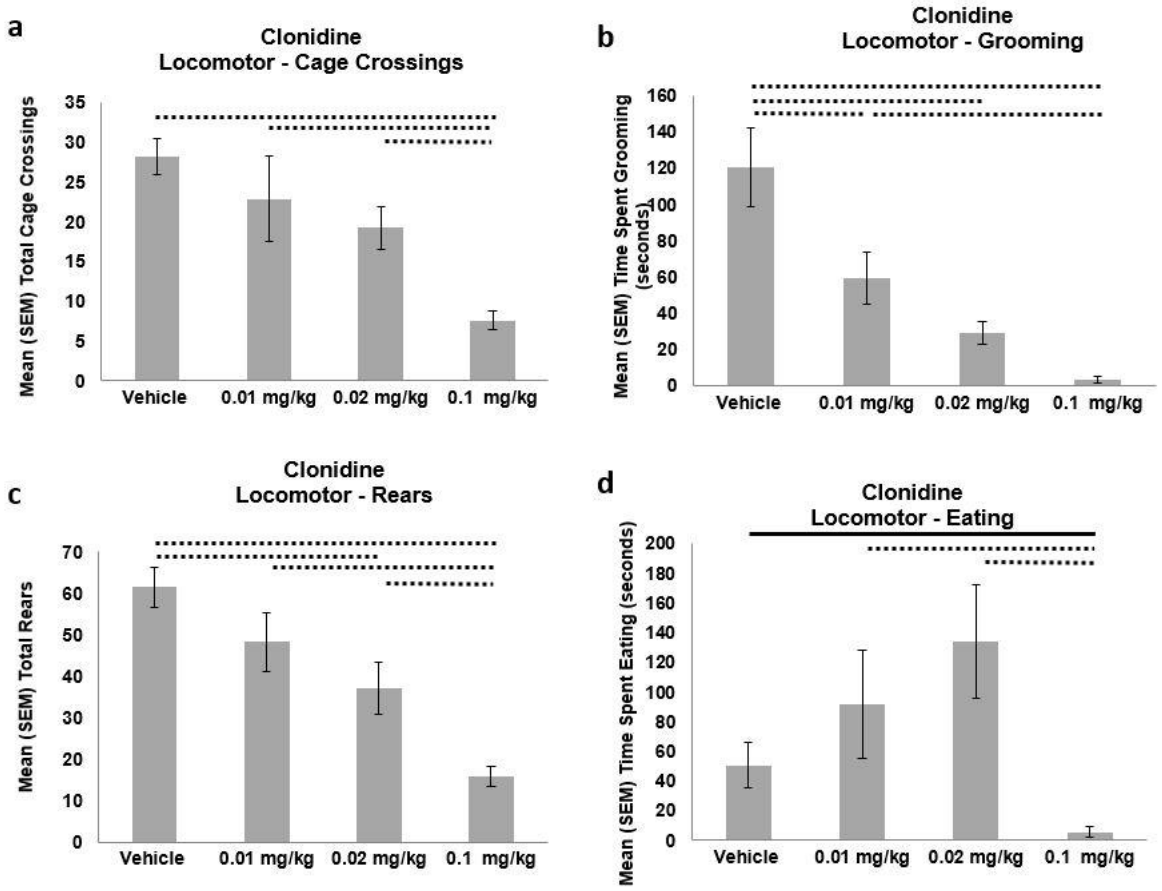

Figure 3.11: Effects of Clonidine on Locomotor Behavior. a) the number of cage crossings was significantly reduced with the highest dose of clonidine, 0.1 mg/kg. b) Total time spent grooming was significantly reduced at all doses of clonidine. c) The total number of rears was significantly reduced at the two highest doses of clonidine. d) Total time spent eating was significantly reduced at the highest dose of clonidine. Solid line: $p<0.05$, dashed line: $p<0.01$.

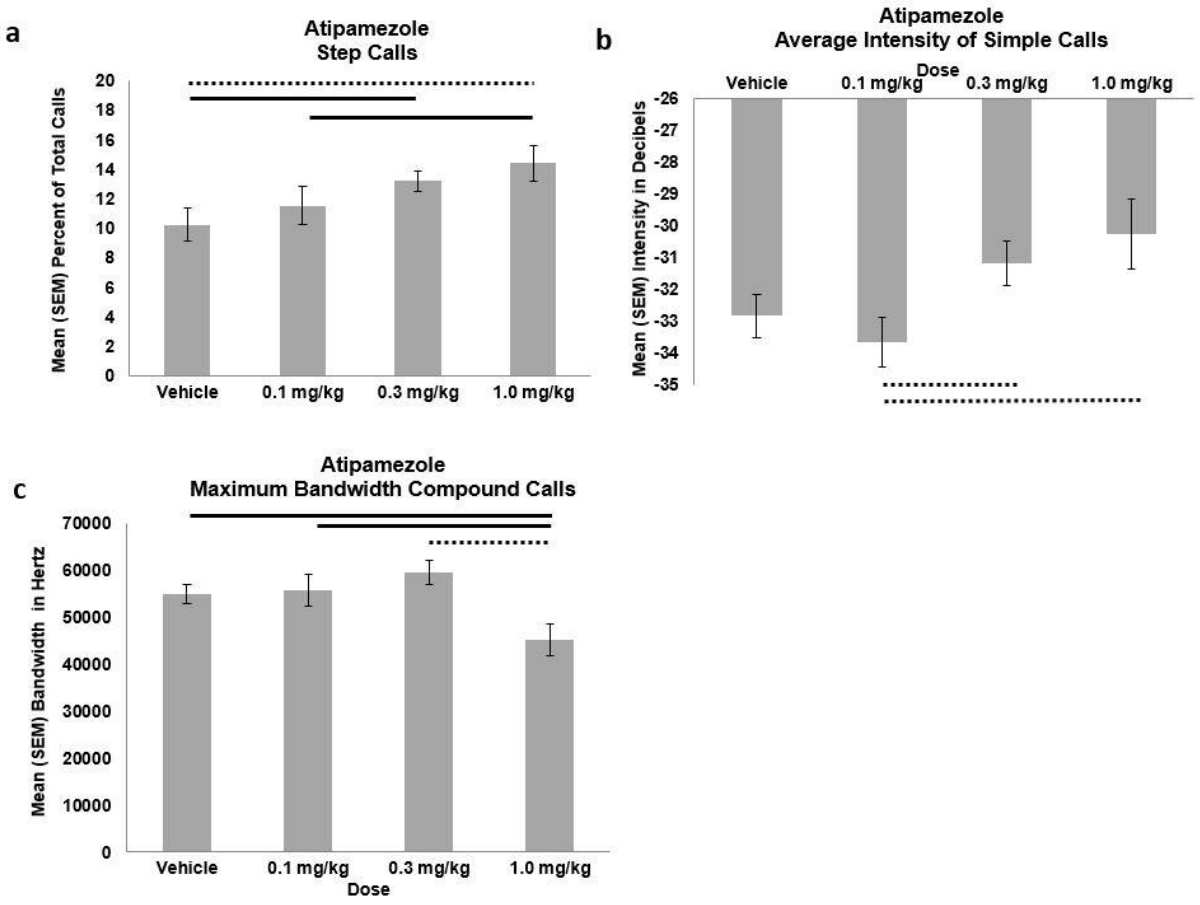

Figure 3.12: Effects of Atipamezole on Ultrasonic Vocalizations. a) The percent of step calls was significantly increased following 1.0 mg/kg of atipamezole compared to the 0.1 mg/kg dose and vehicle treatment while the 0.3 mg/kg dose was significantly increased compared vehicle. b) Average Intensity of simple calls was increased with the 0.3 mg/kg and 1.0 mg/kg doses of atipamezole compared to 0.1 mg/kg. c) Maximum bandwidth of compound calls was significantly reduced with 1.0 mg/kg of atipamezole compared to all other doses. Solid line: $p<0.05$, dashed line: $p<0.01$.

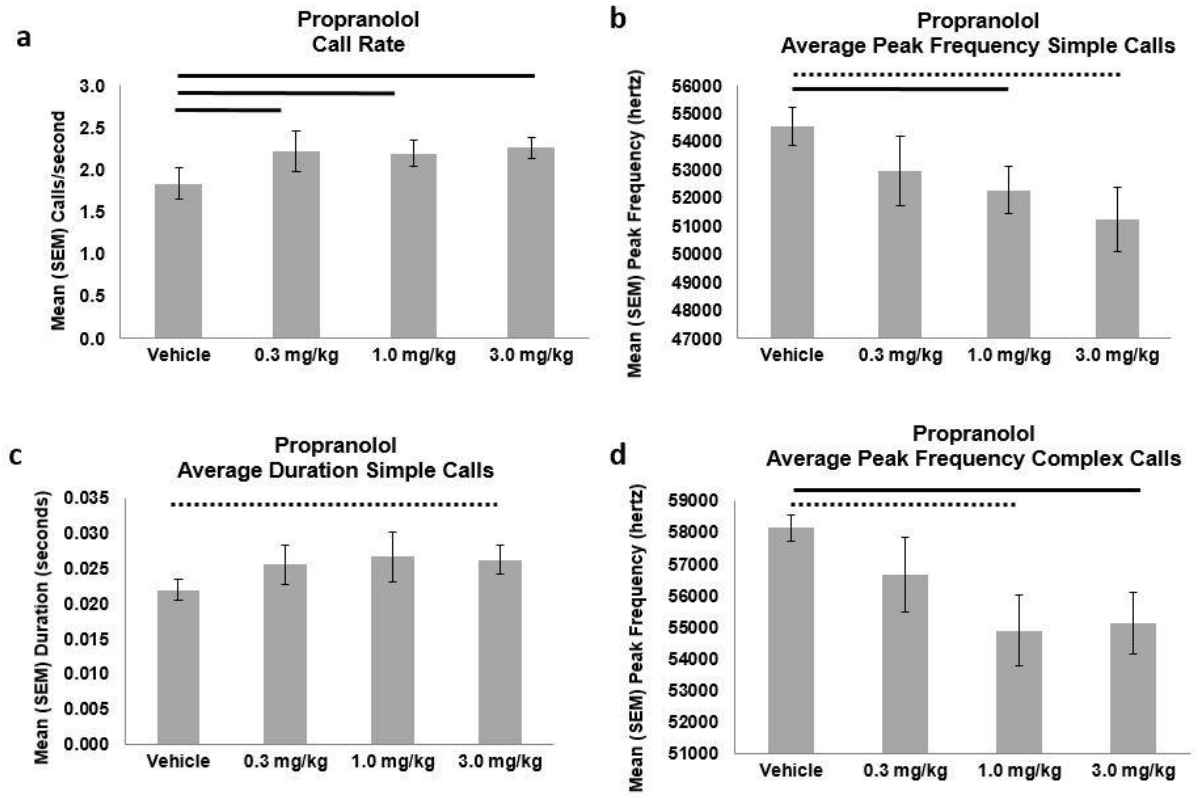

Figure 3.13: Effects of Propranolol on Ultrasonic Vocalizations. a) Call rate was significantly increased with all doses of propranolol. Average peak frequency of simple (b) and complex calls was increased at the highest dose. Average duration of simple calls (d) was increased 0.3 mg/kg of propranolol. Solid line: $p<0.05$, dashed line: $p<0.01$.

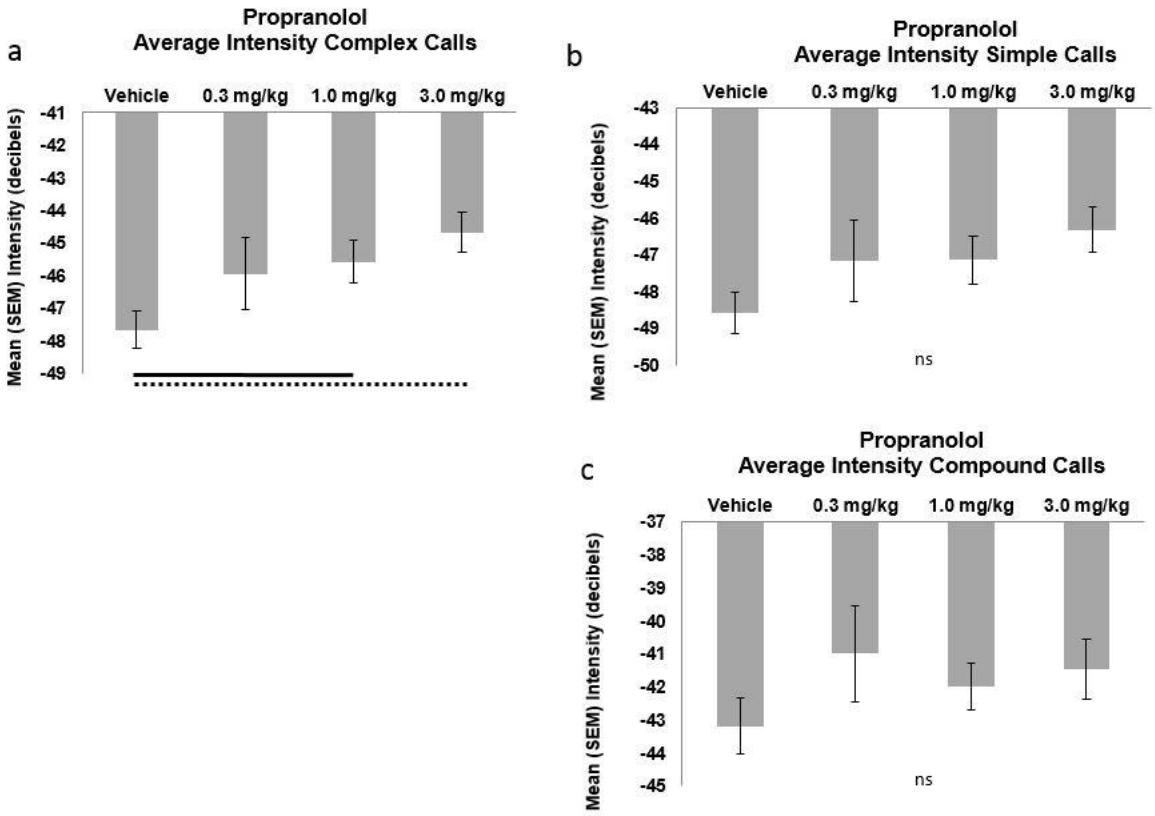

Figure 3.14: Effects of Propranolol on Intensity of Ultrasonic Vocalizations. a) Average intensity of complex calls was significantly increased at the 1.0 mg/kg and 3.0 mg/kg doses of propranolol. Average intensity of simple (b) and compound (c) calls was increased, though not significantly, with propranolol. Solid line: $p<0.05$, dashed line: $p<0.01$, ns: not significant.

Chapter 4 General Discussion

Overview

The mechanisms underlying voice deficits in Parkinson disease (PD) are unknown, limiting treatment and ultimately negatively impacting quality of life.[1,2] Noradrenergic mechanisms have been implicated in a number of refractory PD-deficits, including depression, postural instability, freezing of gait, cognitive difficulties, apathy and akathisia,[30,186,282] as well as in the manifestation and attenuation of levodopa induced dyskinesias.[190,191,262,282,283] However, the contribution of norepinephrine (NE) depletion to vocal deficits in PD has not been established, although recent evidence points to this as a plausible mechanism.[36,165] The purpose of this work was twofold. First, we aimed to establish if NE depletion was sufficient to disrupt pertinent ultrasonic vocalization (USV) parameters such as intensity, bandwidth, duration and peak frequency, that have been observed in animal models of PD [31,36,125,150-152] and are analogous to vocal deficits in humans with PD.[2,116,208] The second purpose of this work was to characterize the contribution of distinct NE receptors to these pertinent acoustic properties of USV. The results of the present work establishes that NE loss is sufficient to disrupt acoustic properties of USV such as intensity, peak frequency and bandwidth, independent of dopamine loss. We also found that modulation of distinct NE adrenoceptors (AR) resulted in significant dose dependent alterations in intensity, bandwidth, peak frequency, and duration as well as call rate and to a lesser extent, locomotor behaviors. Interestingly, antagonists for both the α_2-AR and β-AR resulted in louder (increased intensity) USV, suggesting that these receptors may be important targets for treating PD-related voice deficits.

Establishing Sufficiency

One way to establish the actions of a particular ligand, such as NE, is to determine whether alterations in that particular ligand are sufficient to disrupt the behavior of interest. The first study in this dissertation was aimed at determining whether depletions in NE were indeed sufficient to disrupt specific acoustic properties of rat USV. Specifically, we evaluated the effects of N-ethyl-2-bromobenzylamine (DSP-4), a noradrenergic neurotoxin, and saline control on call profile and call rate as well as the duration, bandwidth, intensity, and peak frequency of USV at baseline (prior to the injection), day 1, and day 6 post-injection. We found that DSP-4 treated rats had reduced intensity, bandwidth, and peak frequency as well as reduced call rate at day 1 post injection compared to saline treated controls. These effects were acute, almost universally transient, as USV parameters returned to baseline at day 6. These results establish that reductions in NE are sufficient to disrupt USV acoustic parameters that are pertinent to PD-related voice deficits.

While it has been demonstrated that modeling the primary disease pathology in PD (dopamine depletion) is sufficient to disrupt USV,[150,151] the present findings (Chapter 2) demonstrate that NE depletion results in similar effects. Following the administration of the neurotoxin 6-OHDA, rat USV have reduced bandwidth, intensity, and complexity.[125,150,151] Similarly, dopamine antagonists also result in reductions in intensity, bandwidth, and peak frequency.[149] Effect sizes indicate that the magnitude of change to intensity and bandwidth following DSP-4 is similar to those following dopamine depletion, making NE loss in PD an equally viable neural substrate in the manifestation of voice deficits. As voice deficits are not amenable to dopaminergic

interventions, identifying alternative neural substrates will be critical to developing supplemental therapies for treating vocal dysfunction in PD. The findings of this study were significant, however, as they establish that NE depletion is sufficient to disrupt pertinent USV parameters, providing the preliminary evidence necessary to warrant a more thorough examination of the contribution of NE to vocal communication.

Contribution of Noradrenergic Receptors to Vocalizations

In Chapter 2 we established that NE depletion is sufficient to disrupt pertinent USV parameters. However, NE is widely distributed throughout the central and peripheral nervous systems, acting broadly as a neuromodulator via the effects of 3 primary receptors. The contribution of specific NE receptors in mediating USV parameters pertinent to voice deficits in PD has not been evaluated. To determine how specific noradrenergic adrenoceptors (AR) contribute to specific vocal parameters, we selectively agonized and antagonized NE-ARs using drugs. Specifically, we evaluated USV in wild-type rats following the administration of cirazoline (α_1- AR agonist, increases NE signaling post-synaptically), prazosin (α_1- AR antagonist, decreases NE signaling post-synaptically), atipamezole (α_2- AR antagonist, increases NE signaling at the pre-synaptic autoreceptor), clonidine (α_2- AR agonist, decreases NE signaling at the pre-synaptic autoreceptor), and propranolol (β- AR antagonist, decreases NE signaling at the post-synaptic receptor).

We found that NE-AR agonists and antagonists did in fact differentially modulate multiple USV parameters. Specifically, clonidine (α_2- AR agonist, acts at the pre-synaptic auto receptor to reduce NE release) decreased call rate, intensity, and

bandwidth at all doses and peak frequency (at the highest dose) and increased duration at the highest dose. Similarly, prazosin (α_1- AR antagonist) which also decreases NE transmission, but post-synaptically, also reduced intensity and call rate, but only decreased bandwidth at the highest dose, peak frequency at the middle dose, and increased standard deviation of intensity at the lowest and highest doses only. Interestingly, atipamezole (α_2- AR antagonist, increases NE release at pre-synaptic autoreceptor) and cirazoline (α_1- AR agonist) which also increases NE transmission (post-synaptically) resulted in decreased bandwidth, as was the case for the agonists for the same receptor. So it appears that drugs that generally "decrease" the availability or transmission of NE, negatively impact USV. This was not universal, as we found similar effects with drugs that have the opposite effect at a given receptor (e.g., the agonist and antagonist for the same receptor induced the same behavioral effect – discussed more below). Most significantly, we found that two drugs, atipamezole (α_2- AR antagonist, promotes NE release at pre-synaptic autoreceptor) and propranolol (β- AR antagonist, decreases NE transmission post-synaptically) increased vocal intensity, suggesting that antagonizing the α_2- and β- AR's have therapeutic potential for treating PD-related decreases in vocal loudness. While the mechanisms underlying these unexpected findings are not apparent, some potential explanations are discussed below.

Noradrenergic Modulation of USV and Locomotion via Specific Receptors

We hypothesized that agonizing and antagonizing specific NE-AR's would result in a differential effect on USV parameters such as intensity, bandwidth, peak frequency

and duration. While our hypothesis was partially confirmed, we found that in several instances, the antagonist and agonist for a given receptor resulted in similar effects on the same acoustic parameter. There are several potential explanations that might account for these seemingly paradoxical results.

NE is widely distributed throughout the brainstem and cortical regions where it acts as a neuromodulator, influencing behavioral functions such as attention, arousal, cognitive functions, and sensorimotor gating,[257] though NE has been implicated in motor function as well.[190,259,262,284] NE is also directly involved modulating the signal-to-noise ratio with respect to cognitive tasks and integrating sensory information, a broad responsibility that puts NE in a prime position to affect multiple systems.[199] In this neuromodulatory capacity, disruptions to NE signaling via agonists/antagonists has the potential to impact many other systems,[30,199,257,277,285] such that both increases and decreases in NE transmission result in a similar disruption of a given behavior/function (discussed in more detail in the following paragraph). Given that depletions in both dopamine[125,149-151] and NE (Chapter 2 & 3) result in similar changes to USV parameters (decreased intensity, bandwidth, and peak frequency), it is likely that NE-mediated changes in USV may be the result of neuromodulation of systems rather than distinct effects of a particular neurotransmitter on a specific vocalization parameter.

This explanation is consistent with the Inverted-U (Yerkes-Dodoson) theory.[257] The Inverted-U hypothesis proposes that for a given behavioral function, there exists an optimal performance level (pinnacle of the inverted-U) that is associated with a particular level of endogenous activity, such that shifts away from this “optimal set-point” via injury or drugs results in impairments to performance.[257] In this case, we observed

similar effects on bandwidth with both the agonist and antagonist for the α_2-AR (atipamezole and clonidine) and on bandwidth and peak frequency with the agonist and antagonist for the α_1-AR (cirazoline and prazosin). Thus it appears that it may be a shift away from an "optimal" set-point, rather than an absolute change of NE availability (in one direction or another) that impacts these vocalization parameters.

In the same vein, multiple and varied mechanisms are capable of disrupting the same USV parameters (eg, 6-OHDA, *PINK1* knock-out, Thy1-aSyn, dopamine antagonists, and NE-AR agonists and antagonists are all associated with reduced intensity. This suggests that vocal sensorimotor control is a complex, dynamic system, dependent on multiple systems and neurotransmitters working in concert to function optimally. Given this, alterations in one or more of these systems or neurotransmitters may be sufficient to disrupt behavior, though it is unlikely that any one structure or system is solely responsible.

However, it should be acknowledged that there are direct projections from the LC to the periaqueductal gray,[162] a region of the brainstem important for the integration of vocal control.[160-162,178] Given this, the effects of NE-AR agonists and antagonists on USV parameters may also be due to direct modulation of the LC on the periaqueductal gray, or some combination of both neuromodulation and direct connections to vocal control centers.

Another consideration is the distribution and variable affinities of the drugs for the primary NE-ARs and their subtypes. The noradrenergic system has three primary receptor subtypes (α_1-, α_2-, and β-) distributed throughout the central and peripheral nervous system. The α_1- and β- AR are typically found post-synaptically, while α_2-ARs

are found post-synaptically and pre-synaptically as autoreceptors.[253] However, these primary AR's also have subtypes that are differentially distributed throughout the central nervous system. For example, α_2-ARs are further subdivided into α_2-AAR, α_2-BAR, α_2-CAR (A, B and C subtypes),[286] with the α_2-AAR being the primary presynaptic autoreceptor which is located in the brainstem, cortex, hippocampus, cortex, and spinal cord [287,288] while the α_2-CAR is highly concentrated in regions of the brain such as the striatum, LC, substantia nigra, and cortex.[289] In the present study, we did not examine each of these sub-types directly, but it is possible that the effects we observed are due to variable affinities of the drugs on different receptor sub-subtypes, and thus alterations of vocal parameters via different pathways.

Effects of Atipamezole and Propranolol on Intensity

Both atipamezole (α_2-AR antagonist, promotes NE release via pre-synaptic autoreceptor) and propranolol (β-AR antagonist, reduces NE transmission via post-synaptic receptor) significantly increased the intensity (loudness) of USV. Decreased loudness is a hallmark feature of dysphonia in PD. Likewise, vocal intensity is almost universally affected in Parkinsonian animal models (6-OHDA and *PINK1* knock-out in rats, Thy1-aSyn in mice).[31,125,150-152] Specifically, unilateral injections of 6-OHDA to the medial forebrain bundle (as a model of the primary disease pathology – dopamine depletion) results in significant reductions in loudness as well as reduced complexity and bandwidth. Similarly, mice overexpressing alpha-synuclein (aSyn) as a model of pre-manifest PD demonstrate early reductions in USV intensity range and aSyn aggregation in the LC.[31] Finally, homozygous *PINK1* knock-out rats also have reduced

vocal intensity beginning at 2 months of age as well as aSyn aggregations and reduced tyrosine hydroxylase- immunoreactivity in the LC (indicative of a reduced amount of NE) that is significantly correlated to intensity deficits.[36] The current findings with regard to intensity are highly significant and exciting, as these drugs (or at least these receptors) are viable pharmacological targets for improving PD-related voice deficits, which are currently undertreated. Future studies to evaluate the effectiveness of noradrenergic drugs (such as atipamezole and propranolol) in each of these models would help determine if NE mechanisms have therapeutic potential and if NE is really necessary for appropriate vocalizations. Specifically, determining whether atipamezole (α_2-AR antagonist, increases NE via pre-synaptic autoreceptor) and/or propranolol (β-AR antagonist, reduces NE transmission via post-synaptic receptor) rescue vocal intensity deficits in *PINK1* knock-out rats is most prudent, and offers the most construct validity as these rats experience intensity deficits that correlate with the amount of tyrosine hydroxylase – immunoreactivity in the locus coeruleus (LC). Atipamezole is an α_2-AR antagonist that primarily results in NE release via pre-synaptic autoreceptors. The α_2-AR is widely dispersed throughout the prefrontal cortex where it is thought to be important for setting the signal-to-noise ratio by attenuating or enhancing NE signaling.[199,257] Exactly how antagonism of the α_2-AR effects this change is not clear. One common side effect of chronic levodopa treatment is the development of levodopa induced dyskinesias which can be just as debilitating to motor function and quality of life as PD-deficits themselves. Interestingly, α_2-AR antagonists (such as atipamezole) are effective at attenuating levodopa induced dyskinesias[190,191,262], without diminishing the effectiveness of levodopa. While other NE-AR agonists (clonidine) and antagonists

(propranolol) have also been shown to improve dyskinesias, it is at the expense of the therapeutic effect of levodopa, meaning PD-deficits return.[283] Although the mechanisms underlying this improvement are not known, one of the proposed mechanisms by which α_2-AR antagonists mediate dyskinesia is by blocking presynaptic α_2-ARs, thus facilitating release of NE which in turn facilitates dopamine release.[191,205,290] Interestingly, one group found that antagonism of the α_2-CAR resulted in improvement of MPTP (1-methly-4-pheynl-1,2,3,6-tetrahydopyridine, neurotoxin model of PD) - induced locomotor deficits, suggesting that NE-targeted treatments have potential as a monotherapy, as well.[291]

The β-AR antagonist propranolol, which results in inhibitory post-synaptic effects, also increased the loudness of USV. Although somewhat confusing, taken together, these results are consistent with the Inverted-U hypothesis or neuromodulation.[257] Noradrenergic projections modulate various behaviors, such as vocal communication, within a specified window of NE activity and deviations away from this disrupt performance in a consistent way - regardless of whether NE transmission was increased or decreased. Interestingly, propranolol has also been shown to be effective at attenuating levodopa induced dyskinesias by mediating presynaptic dopamine efflux.[292] We did not measure changes in other neurotransmitters such as dopamine in conjunction with the agonists and antagonists evaluated in Chapter 3, so it is impossible to say if similar mechanisms are responsible for any of the effects we observed. However, these results are still promising, particularly given what is already known about the potential of noradrenergic drugs to mediate PD-related deficits.[61,71,72,186,191,259,292-294]

The Role of Noradrenergic Mechanisms in Vocal Communication

The contribution of NE to vocal behaviors is not well established, particularly with respect to pertinent acoustic parameters such as intensity, bandwidth, and peak frequency. Previous work has demonstrated that general USV parameters such as the amount of vocalizing (call rate) and the types of calls produced (call profile) are differentially altered following the administration of NE-AR agonists and antagonists.[165] The results of the present work confirm and validate this work (call rate and profile were also altered) and add to what is known regarding the contribution of NE to vocal control. Specifically, we found that DSP-4 and NE-AR agonists and antagonists alter the intensity, bandwidth, peak frequency and duration of USV. While work in songbirds implicates NE involvement multiple aspects vocal behaviors such as song learning and motor control,[238,295,296] little work has been done in the rat to characterize the contribution of NE to vocal communication. The results of the present work, along with Wright et al (2012)[165] highlight the significance of exploring the contribution of NE to vocal behaviors, particularly in the context of diseases such as PD, which include both a substantial loss of NE[21,22,26,297] and refractory voice deficits. Given the contribution of NE to both the sensory and motor aspects of song learning in birds, further studies looking at the contribution of NE to other aspects of communication and not just acoustic properties of calls in rats, particularly with respect to PD-related communication deficits, are warranted and may help us to develop effective treatments for these deficits.

Contribution of Norepinephrine to Voice Deficits in Parkinson Disease

Compromise of the LC and NE begins relatively early in PD, possibly prior to the onset of dopamine depletion and the cardinal signs typical of PD. Voice deficits also manifest in the early, prodromal stages of the neurodegeneration in PD and remain refractory to standard dopaminergic treatments, underscoring the likely involvement of alternative pathologies. While the results of the present work did not directly test the contribution of NE to the vocal deficits in a PD, there is ample evidence that NE loss or degeneration of the LC plays a role. As described in Chapter 1, several recent studies from our lab have demonstrated vocalization deficits in conjunction with neuropathological signs in the LC in rodent models of PD. Germane to the current discussion of NE in vocal control, Thy1-aSyn mice overexpress aSyn as a model of pre-manifest PD, and show early USV deficits in call type and intensity range as well as aSyn aggregation it the LC.[31] Likewise, in a genetic knock-out model of PD, homozygous *PINK1* rats demonstrate early and progressive USV deficits including reductions in bandwidth, peak frequency, and intensity along with a reduction in tyrosine hydroxylase-immunoreactive cells and aSyn aggregation in the LC.[36] While LC/NE pathologies are not the only significant neurodegenerative processes identified in these models, reduction in tyrosine hydroxylase-immunoreactivity in the LC of *PINK1* knock-out rats was significantly correlated with vocal intensity, indicating that reductions in NE in the LC may underlie reduced loudness in this model.

The present findings are consistent with this, as we observed alterations in multiple USV parameters with NE-AR modulation. Vocal intensity, in particular, was reduced with both clonidine (α_2-AR agonist, reduces functional release of NE) and

prazosin (α_1-AR antagonist, reduces NE signaling) and increased with both atipamezole (α_2-AR antagonist, increases functional release of NE) and propranolol (β-AR antagonist, reduces NE signaling). As this parameter is compromised in both genetic and transgenic models of PD (as well as 6-OHDA neurotoxin model), this strongly suggests that noradrenergic mechanisms may be underlie this vocalization deficit.

Relationship between Ultrasonic Vocalizations and Gross Locomotor Function

The neuropathology in PD in the central nervous system is broad, affecting multiple brainstem regions, cortical areas, and neurotransmitter systems. PD-related sensorimotor deficits reflect this widespread degeneration and include not only the cardinal motor deficits in the limb (appendicular deficits), but also deficits in the head and trunk such as voice and swallowing dysfunction, freezing of gait, and postural instability (axial deficits).[1,2,84,111,115,116,208,298,299] Many axial deficits, such as vocal dysfunction, are refractory to standard treatments aimed at treating the primary disease pathology of nigrostriatal dopamine depletion such as levodopa and deep brain stimulation [5,43,47,124,129-133,300,301] indicating that other PD-related pathologies underlie these deficits. Consistent with this, some axial deficits, such as freezing of gait (and possibly dysarthria), are responsive to alternative treatments aimed at restoring NE (See below),[258-260] further dissociating these deficits from the cardinal motor signs in PD that are responsive to dopamine replacement therapies. In the present work, we evaluated the contribution of NE to USV and gross locomotor function. With the exception of clonidine, which has known sedative effects,[274] we found that while NE loss or modulation of NE-AR's is sufficient to disrupt multiple, pertinent USV parameters,

without abolishing calling and gross locomotor function was generally intact (catalepsy and spontaneous activity in Chapter 2; locomotor in Chapter 3). While we did not directly relate USV function to gross limb function, these data indicate that vocalizations are dependent on NE neurotransmission, independent of gross limb function.

Implications for Treating Voice Deficits in Parkinson Disease

The contribution of NE to voice deficits in humans has not been directly evaluated. However, there is some evidence that L-threo-DOPS (synthetic NE precursor) improves dysarthria along with freezing of gait (also refractory to standard treatments).[258,259] There is also evidence suggesting that pharmacological treatments aimed at NE receptors is effective at alleviating not only levodopa induced dyskinesias,[190,191,197,262,282,283,290] but also depression,[302,303] freezing of gait,[258-260] and executive dysfunction.[304] While the efficacy of NE-treatments likely reflects the integrity of remaining LC-NE fibers,[282] these findings are promising for other refractory deficits such as voice and swallowing which share important neuroanatomical features (axial control) and functional similarities (dependent on attention and cognition). Depending on what is found in future studies (discussed below) regarding the ability of AR antagonists to attenuated deficits in rodent models of PD, repurposing NE-modifying drugs already in use would mean a fast bench-to-bedside transition, which is exciting.

Currently, the only potentially effective treatment for voice and communication deficits in PD is intensive behavioral speech therapy, such as the Lee Silverman Voice Treatment (LSVT).[12,16,17] However, the mechanisms underlying improvements observed with therapy are not known, though they do not appear to be dopamine-dependent.[20]

While the present work does not address the role of NE in behavioral therapy, given the neuromodulatory nature of NE, exploring its role in speech therapy related improvement in PD seems prudent, particularly in light of the results of that NE contributes to multiple, pertinent vocal parameters. Given the relationship of behavioral outcomes with voice therapy in humans to regions such as the prefrontal cortex[19] and the critical role of noradrenergic mechanisms in attention and goal-directed behavior, evaluating the contribution of NE to successful voice therapy may be essential in understanding the neural substrates contributing to effective therapy and thus optimizing treatment outcomes for individuals with voice deficits in PD.

Limitations and Future Directions

Several limitations in the present work exist and necessitate future studies to validate and expand these findings regarding the contribution of NE to PD-related voice deficits. First of all, although the administration of the NE-neurotoxin DSP-4 resulted in USV deficits, a reduction of NE in the brain at either the synaptic terminals or LC was not confirmed with IHC findings. While our findings are consistent with studies demonstrating that single doses of DSP-4 result in transient changes to NE levels without impacting NE neuron cell bodies,[225,239] it is possible the acoustic changes we saw were not solely due to a NE depletion in the LC. Future studies employing a repeated DSP-4 regimen along with more sophisticated neurochemical analysis techniques such as HPLC, ELISA, or radio-ligand binding to look at receptor changes would allow us to directly link changes in NE, dopamine β-hydroxylase – the enzyme that converts dopamine to NE - and/or NE metabolites to specific changes in USV. In

this way, we might be able to correlate and quantify dose response effects of DSP-4 induced NE depletion on vocalization parameters.

In addition, while we evaluated the effects of NE-AR agonists and antagonists on pertinent USV parameters, we did not delineate sub-subtypes of each receptor. As the distribution of receptor sub-sub-types varies in location throughout cortical and brainstem areas, this could be an important factor mediating the effects we observed. As agonists and antagonists for many of these receptor sub-types already exist, validating and expanding the results of this work will be feasible and, in the case of α2-AR and β-AR receptors, important for determining how antagonism of these receptors increases vocal loudness.

Likewise, we recognize that it is important to evaluate the effects of neurotransmitters such as NE in isolation in order to appreciate the contribution an individual ligand to a given behavior. However, it is likely that multiple systems, and neurotransmitters, are working in concert to affect that particular behavior. Given this, future studies looking at the combined effects of pertinent neurotransmitter systems (dopamine, NE, acetylcholine, serotonin) are necessary to fully appreciate the neural mechanisms underlying vocal function in normal and diseased states. Interestingly, it has been suggested that it is the combined loss of dopamine, NE, and serotonin, rather than the loss of any of these neurotransmitters alone, that results in the refractory nature of PD-induced depression to deep brain stimulation.[305] This is likely the case, at least to some degree, for other refractory deficits in PD, though this has not been established.

Finally, we did not evaluate the contribution of NE to USV in a PD animal model. Vocalization parameters such as intensity, bandwidth, and peak frequency are all reduced in several rodent models of PD,[31,125,150-152] however, wild-type animals may already be vocalizing at the top of their range for those parameters. This ceiling effect could be why we did not observe as many effects with NE enhancing drugs (atipamezole and cirazoline) as we did with drugs that depress NE transmission (clonidine, prazosin, and propranolol). Testing these drugs (particularly atipamezole and propranolol) in an established rodent model of PD (such as the *PINK1* knock-out line) would establish if this was the case and also help establish if NE is necessary for vocal function (as homozygous *PINK1* knock-out rats have reduced tyrosine hydroxylase-immunoreactivity in the LC, indicating NE depletion).

Conclusion

Characterizing the contribution of noradrenergic mechanisms to vocal communication has important clinical implications for treating refractory voice deficits in PD. The work presented here establishes that NE depletion is sufficient to alter vocalizations, and identifies two pertinent NE receptors as viable targets for attenuating PD-related deficits in loudness. Fully delineating the contribution of specific receptor subtypes in a rodent model of PD will be essential towards fully recognizing the contribution of NE to vocal control and potentially repurposing drugs to treat voice and communication deficits in PD. The present findings, however, are an important first step towards bridging the treatment gap for voice deficits in PD, which remain undertreated and negatively impact quality of life.

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