The Role of Norepinephrine in Vocal Communication and Anxiety in Parkinson Disease

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Dedication:

To those whose voices do not express what they wish them to express.

To my husband, Michael Robert Hoffmeister, whose kindness, patience, and brilliance inspire me, and without whose constant support, this work could not have been completed.

To my parents, Daniel and Laura Hoffmeister, for their endless love and support, and for their willingness to help me to externally process research questions when my radio is broken.

I have been incredibly privileged in many areas of my life. Among the greatest of these has been the opportunity to be mentored, trained, and guided by intelligent and wise women, including the members of my doctoral dissertation committee, Michelle R. Ciucci, Nadine P. Connor, Stephanie Misono, Nicole Rogus-Pulia, and Susan Thibeault, as well as Deirdre Michael, Laura Hoffmeister, and Georgian Resch, among many, many others. This work is dedicated to them.

To my mentor, Michelle R. Ciucci, a wise woman if ever there was one.

Abstract: Vocal communication impairment and anxiety are co-occurring and interacting signs of Parkinson Disease (PD) that are common, poorly understood, and under-treated. Both vocal communication and anxiety are influenced by the locus coeruleus-noradrenergic system. In light of this shared neural substrate and considering that noradrenergic dysfunction is a defining characteristic of PD, tandem investigation of vocal impairment and anxiety in PD relative to noradrenergic mechanisms is likely to yield insights into the underlying disease-specific causes of these impairments. In order to address this gap in knowledge, we assessed vocal impairment and anxiety behavior relative to norepinephrine in a genetic rat model of early-onset PD (*Pink1*-/-) that demonstrates vocal deficits.

In Study 1, *Pink1*-/- rats and wild type controls (WT) underwent testing of ultrasonic vocalization and anxiety (elevated plus maze) at 4, 8, and 12 months of age. At 12 months of age, brainstem norepinephrine markers were analyzed with immunohistochemistry. We hypothesized that 1) anxiety would be increased in *Pink1*-/- rats, 2) vocal deficits and anxiety would be correlated to one another, 3) noradrenergic markers in the locus coeruleus, nucleus ambiguus, dorsal motor nucleus of the vagus nerve, and the nucleus of the solitary tract would be disrupted in *Pink1*-/-, and 4) brainstem noradrenergic markers would be associated with vocal acoustic changes and anxiety level. Results demonstrated that vocal impairment and anxiety were increased in *Pink1*-/- rats, and increased anxiety was associated with greater vocal deficit in this model of PD. Further, brainstem noradrenergic markers including tyrosine hydroxylase and α1 adrenoreceptor immunoreactivity in the locus coeruleus, and β1 adrenoreceptor immunoreactivity in vagal nuclei differed by genotype, and were associated with vocalization and anxiety behavior.

In Study 2, we assessed the influence of pharmacologic increases in the activation of noradrenergic systems on vocal impairment and anxiety in the *Pink1-/-* rat model of PD. Anxiety behavior on the elevated plus maze and ultrasonic vocalizations were tested twice for each rat: once after injection of saline and once after administration of one of the three drugs. We hypothesized that norepinephrine reuptake inhibitors (atomoxetine and reboxetine) and a β receptor antagonist (propranolol) would decrease vocal impairment and anxiety compared to saline. Our results demonstrated that both atomoxetine and reboxetine decreased anxiety.

Atomoxetine also modulated several acoustic parameters of ultrasonic vocalization, including increases in call intensity, a ubiquitous characteristic of vocal deficits in PD. Propranolol influenced neither anxiety nor vocalization. Collectively, these studies demonstrate significant relationships among vocal impairment, anxiety and central noradrenergic systems in the *Pink1*-/- rat model of PD.

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Chapter 1: Review of the Literature

1.1 Introduction

Parkinson Disease (PD) is a common degenerative disorder, affecting more than 10 million people worldwide (de Lau et al., 2004) and approximately 2% of individuals over the age of 65 (Forsaa et al., 2008). Over the next 20 years, prevalence of PD in the United States is projected to double in tandem with increasing average age of the population (Kowal et al., 2013).

PD is traditionally associated with nigro-striatal dopaminergic cell death and accompanying hallmark gross motor signs of bradykinesia, tremor, rigidity, instability and gait disturbance (Berardelli et al., 2001; Bernheimer et al., 1973; Sprenger & Poewe, 2013). Because they lead to falls, reduced independence, and impairment in activities of daily living, it is the hallmark gross motor signs of PD that typically trigger initial diagnosis (Hariz & Forsgren, 2011; Yousefi et al., 2009). In addition, PD is characterized by cranial sensorimotor impairments such as swallowing and speech disorders, affective disorders such as anxiety and depression, and autonomic dysfunction. Cranial sensorimotor deficits lead to communication impairment and social isolation as a result of deficits in speech function (Barone et al., 2009; Lirani-Silva et al., 2015; Martinez-Martin et al., 2011; Miller et al., 2006), as well as pneumonia resulting from aspiration associated with swallowing impairment, whose presence has the highest mortality risk among all comorbidities in PD (Fernandez & Lapane, 2002; Gorell et al., 1994).

PD has a substantial impact on well-being and economic factors at individual and societal levels. At the level of the individual, gross motor, cranial sensorimotor, affective and autonomic disorders negatively impact quality of life and disease burden (Carod-artal et al.,

2008; Hariz & Forsgren, 2011; Lirani-Silva et al., 2015; Opara et al., 2012; Plowman-Prine et al., 2009), and result in a substantial increase in healthcare costs for elderly individuals with PD compared to elderly individuals without PD (Noyes et al., 2006). Degree of impairment in individuals with PD also predicts degree of caregiver burden, and mental well-being (Martínez-Martín et al., 2007). In broader economic terms, direct medical costs associated with PD have been estimated at nearly $14 billion in the United States alone (Kowal et al., 2013), with disease-related indirect costs likely to be even greater (Bovolenta et al., 2017).

The negative impacts of PD on individuals and on society are compounded when considering the fact that the disease is progressive and uncurable (Beitz, 2014; Braak et al., 2004). While some treatments for symptoms are available, these treatments primarily target gross motor deficits via pharmacologic dopamine replacement and neurosurgical procedures. Unfortunately, these interventions leave most features of PD untreated (Calleo et al., 2015; Pinho et al., 2018; Ramig et al., 2018; Renfroe et al., 2016). The lack of adequate treatment for non-hallmark signs of PD, including cranial sensorimotor deficits such as dysphagia and hypokinetic dysarthria, and affective disorders such as depression and anxiety, largely stems from a limited understanding of the pathophysiology of these deficits. In order to develop adequate treatment, and to better-understand the disease, the limitations of studying only hallmark motor deficits and hallmark nigrostriatal dopaminergic pathology must be recognized.

1.2 The limitations of considering only hallmark motor deficits of Parkinson Disease

Historically, the primary pathophysiology of PD has been considered to be the loss of dopaminergic neurons in the nigrostriatal pathway (Bernheimer et al., 1973; Braak et al., 2004).

The death of nigrostriatal dopaminergic neurons is closely associated with the hallmark gross motor signs of PD, including rigidity, tremor, bradykinesia, instability, and gait abnormalities (Ehringer & Hornykiewicz, 1960; Hornykiewicz, 2006). The primary pathological finding linked to neural degeneration and death in PD is the presence of Lewy bodies in dopaminergic neurons (Forno, 1996). Lewy Bodies are composed of collections of misfolded proteins, of which α-synuclein is the primary building block; see Henderson et al, 2019 for recent review (Baba et al., 1998; Henderson, Trojanowski, et al., 2019). While all of the functions of α-synuclein have not yet been completely defined, its location in pre-synaptic terminals in the brain and importance in the regulation of neurotransmission via vesicular release have been well-established (Sun et al., 2019). Misfolding and subsequent aggregation of α-synuclein is a major component of the formation of Lewy bodies that are observed in early PD in the substantia nigra pars compacta; this pathology progresses rostrally over time to midbrain and neocortical structures (Braak et al., 2003; Galvin et al., 1999; Henderson, Cornblath, et al., 2019; Henderson, Trojanowski, et al., 2019; Lin et al., 2012). The immediate cause of misfolding of α-synuclein is unclear at this time. However, emerging evidence suggests that genetic factors may lead to mitochondrial dysfunction, triggering diverse inflammatory processes that are associated with the pathological cascade caused by misfolding of α-synuclein (Chung et al., 2016; Creed & Goldberg, 2019; Emmanouilidou et al., 2020; Gilmozzi et al., 2020; Hauser & Hastings, 2013; Liu et al., 2009; Minakaki et al., 2020). As this classical disease progression continues, it leads to emergence of hallmark gross motor signs of PD, which generally triggers diagnosis by a neurologist (Berardelli et al., 2013); by the time of diagnosis, up to 70% of dopaminergic neurons in the substantia nigra pars compacta have already died (Dauer & Przedborski, 2003).

The hallmark, diagnosis-triggering signs of PD are clearly visible and correlate closely with the above-described nigrostriatal dopaminergic cell death, which can be seen unambiguously with the naked eye in post-mortem neural tissue. Not unexpectedly, dopamine replacement therapies like levodopa result in dramatic improvements in hallmark deficits. While understanding hallmark deficits is important, it considers only a small proportion of the neuropathology and deficits associated with the disease. Deficits in more complex behaviors and aspects of human function are also present in PD. These "non-hallmark" deficits affect vocal communication, swallowing, cognition, and psychological regulation. In non-pathological states, the complexity of these behaviors is mirrored by the complexity of their neural underpinnings, which require widespread integration of multiple brain systems. Consequently, "non-hallmark" signs of PD do not correlate to nigrostriatal dopaminergic cell death in as linear a fashion as do the hallmark signs, nor do they respond as well to dopamine replacement therapies.

Several years prior to emergence of nigro-striatal dopaminergic cell death and diagnosis-triggering hallmark signs of PD, pathological changes are observed in brainstem structures including the locus coeruleus (LC) and vagal nuclei; these changes appear to progress rostrally during the pre-symptomatic, or prodromal phase of the disease (Figure 1) (Braak et al., 2004). The LC is a noradrenergic nucleus in the pons that has widespread connections throughout the neocortex and brainstem. Noradrenergic cell death in the LC occurs earlier and is greater in magnitude than dopaminergic cell death in the nigrostriatal pathway (Zarow et al., 2003), making it a likely target for investigation of complex, non-hallmark deficits in speech and swallowing behaviors, cognition, and psychological regulation (Faivre et al., 2019; Ma et al.,

2020). The hypothesis that non-hallmark deficits are related to brainstem noradrenergic dysfunction is further strengthened by the fact that, like brainstem noradrenergic dysfunction, non-hallmark deficits are often observed prior to the onset of hallmark gross motor deficits (Bower et al., 2010; Harel et al., 2004; Jones et al., 2018; Postuma et al., 2012; Shiba et al., 2000; Weisskopf et al., 2003). The integrated study of noradrenergic pathology with multiple non-hallmark deficits in PD is likely to yield a deeper understanding of the disease process. The following sections review clinical presentation, standard interventions, and putative neural abnormalities of two common, devastating, and likely pathophysiologically-related non-hallmark signs of PD: vocal communication deficits and anxiety.

Figure 1

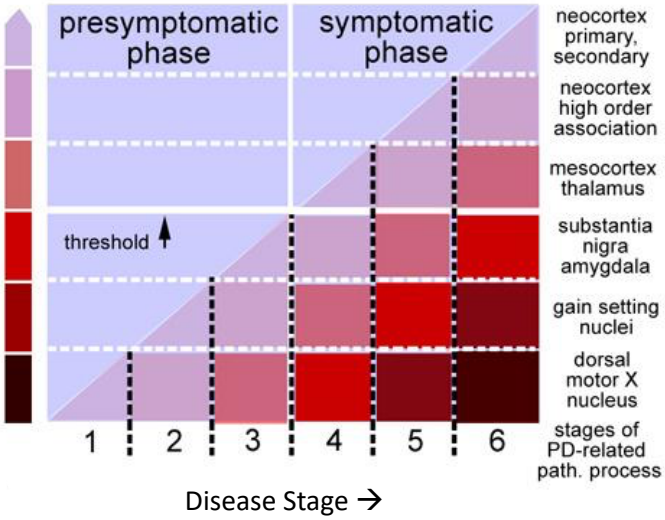

Figure 1: Staging of PD with consideration of symptoms and progression of neural pathology. Darker color indicates greater degree of neural degeneration. Adapted with permission from Braak et al, 2004.

1.3 The impact of vocal communication impairment in Parkinson disease

Approximately 90% of individuals with PD exhibit hypokinetic dysarthria, a vocal communication impairment that includes dysfunctions in respiration, vocalization, and speech

production (Anand & Stepp, 2015; Fox & Ramig, 1997; Ho et al., 1998; Huber & Darling, 2011; Logemann et al., 1978; Matheron et al., 2017; Sapir, 2014; Sapir et al., 2008; Stepp, 2013). The timeline and onset of hypokinetic dysarthria differs from that of gross motor signs in several ways, including the fact that changes to cranial sensorimotor function affecting speech and voice can be detected over 9 years prior to onset of hallmark motor signs (Harel et al., 2004; Postuma et al., 2012). The most-salient characteristics of hypokinetic dysarthria include imprecise articulation, reduced loudness (hypophonia), and reduced prosodic variation (monopitch and monoloudness) (Bowen et al., 2013; Duffy, 2013; Sapir, 2014), leading to reduced intelligibility and breakdown of communication. Hypokinetic dysarthria thus results in degradation of quality of life and increased disease burden (Barone et al., 2009; Lirani-Silva et al., 2015; Martinez-Martin et al., 2011; Miller et al., 2006). Several aspects of hypokinetic dysarthria worsen with disease progression (Klawans, 1986; Mutch et al., 1986; Skodda et al., 2009, 2011, 2012, 2013), compounding the impact of this disorder on quality of life over time.

1.3.1 Dopamine-centered drugs and deep brain stimulation have minimal impact on vocal communication deficits in PD.

The standard treatment for gross motor signs of PD is dopamine replacement therapy through use of dopamine-synthesizing drugs such as levodopa/carbidopa (Markham et al., 1974; Titova et al., 2018). These drugs markedly improve gross motor signs, but have resulted in mixed changes to cranial sensorimotor deficits, such as hypokinetic dysarthria (Brabenec et al., 2017; Pinho et al., 2018; Sanabria et al., 2001; Wolfe et al., 1975). While some studies have shown improvements in certain aspects of speech, such as stop consonant production and labial tone, overall improvements to speech intelligibility are limited (Pinho et al., 2018; Rusz et

al., 2016; Sanabria et al., 2001; Wolfe et al., 1975). This lack of change to vocal communication with medications in humans has been recapitulated in rodent models of vocal communication deficits in PD (Kelm-Nelson, Brauer, et al., 2016). The limited impact of dopamine replacement therapies on cranial sensorimotor deficits in PD is not unexpected, given the mounting evidence suggesting that the pathophysiology of these deficits exists at least partially outside of the canonical nigro-striatal dopaminergic pathway (Brabenec et al., 2017; Kompoliti et al., 2000; Plowman & Kleim, 2011).

Deep brain stimulation (DBS) is a surgical intervention that involves placement of electrodes in the brain, typically in the internal segment of the globus palidus or the subthalamic nucleus for high frequency stimulation to treat tremor, or in the peduncular pontine nucleus for low frequency stimulation to treat freezing of gait. DBS has been shown to improve hallmark motor impairments associated with PD (see Fang and Tolleson, 2017 for recent review) (Fang & Tolleson, 2017), although the exact mechanisms by which the improvements occur are not completely understood. Similar to dopamine-centered pharmacological therapies, however, DBS results in limited improvement to speech and voice, and can even be detrimental to intelligibility (Aldridge et al., 2016; Chiu et al., 2020; Skodda, 2012). Further, in 6-hydroxydopamine lesion models of PD in rats, DBS has been shown to exacerbate vocal deficits (King et al., 2016).

1.3.2 Behavioral therapy for vocal deficits in PD has mixed efficacy and is time-intensive

Due to the limited efficacy of pharmacological and surgical interventions, the primary treatment for hypokinetic dysarthria in PD is behavioral therapy. One of the most common behavioral interventions, Lee Silverman Voice Treatment (LSVT LOUD®), has been used since

the 1990s (Dromey & Ramig, 1998; Ramig et al., 1994). LSVT LOUD® involves an intensive course of voice therapy (16 sessions over the course of a month in addition to home practice) directed at improving speech by focusing on increasing vocal intensity by targeting laryngeal and respiratory subsystems of phonation. While improvements in vocal intensity have been observed for most individuals at 7 and 24 months following LSVT LOUD® (Ramig et al., 2001; Ramig et al., 2018), the effect size of the increase in intensity diminishes over time. Additionally, a number of individuals demonstrate no improvement or worsening of speech outcomes with treatment (Cannito et al., 2012; Ramig et al., 2018). This is particularly problematic in light of the fact that the neural changes associated with vocal improvement following LSVT LOUD® do not appear to be directly related to neural mechanisms of the disease process (Narayana et al., 2010; Sapir, 2014). Because of the limitations to pharmacologic, surgical and behavioral interventions, vocal deficits remain untreated in many individuals with PD. Unfortunately, the lack of treatment options for vocal deficits in PD is also present in other non-hallmark signs, such as anxiety.

In sum, clinical research, pharmacological and surgical intervention data, and behavioral therapy outcomes all suggest that that pathophysiology of vocal deficits in PD, and subsequently their treatment, lie outside of the standard nigrostriatal dopamine-centered framework.

1.3.3 Neural pathophysiology of vocal communication impairment in Parkinson Disease: emerging evidence for the role of norepinephrine

While the exact physiologic mechanisms of hypokinetic dysarthria in PD remain uncertain, it is becoming increasingly clear that they are at least partially independent of

classical dopaminergic degeneration (Brabenec et al., 2017; Plowman et al., 2009; Schulz & Grant, 2000). Aside from dopamine-mediated deficits, other neural substrates, including those governed by norepinephrine (NE), are disrupted in PD (Buddhala et al., 2015; Espay et al., 2014; Lewitt, 2012; Marien et al., 2004; Rommelfanger & Weinshenker, 2007; Tredici & Braak, 2013; Vazey & Aston-Jones, 2012). It has thus been suggested that non-hallmark signs of PD may be related to NE processes (Cash et al., 1987; Espay et al., 2014; Kreiner et al., 2019).

Norepinephrine is a catecholamine with varied functions in the central nervous system, including activation of the autonomic nervous system and activation of multiple brainstem nuclei responsible for laryngeal, pharyngeal, and esophageal movements. NE is produced primarily in the locus coeruleus in the pons. Noradrenergic cell death and impairment of NE processes have been observed in both humans with PD and in animal models (Dave et al., 2014; Espay et al., 2014; Kelm-Nelson et al., 2018; Sommerauer et al., 2018; Vermeiren & De Deyn, 2017; Weinshenker, 2018). In humans, reduced concentration of NE transporter (NET) has been suggested to be influential in networks associated with higher-level processes including cognition and attention (Berridge & Deveilbliss, 2011; Berridge & Waterhouse, 2003). In the *Pink1*-/- model of PD in rats, concentrations of NE are reduced in the locus coeruleus (Kelm-Nelson et al., 2018). In other animal models of PD, NE cell death and reduced concentrations of NE in the locus coeruleus have increased the effects of dopaminergic cell death and modulated L-DOPA-induced dyskinesias (Barnum et al., 2012; Bing et al., 1994; Fornai et al., 1995; Mavridis et al., 1991; Shin et al., 2014; Szot et al., 2010).

While midbrain and neocortical NE deficits are more consistently addressed in PD,

pontine and medullary noradrenergic systems remain relatively uninvestigated. One such system, the locus coeruleus-vagal system appears to be simultaneously responsible for modulation of vocalization and activation of autonomic responses to anxiety (George et al., 2008; Kalia et al., 1984; Mather et al., 2017; Samuels & Szabadi, 2008; Wang et al., 2014). NE neurons in the locus coeruleus project to the dorsal motor nucleus of the vagus nerve and the nucleus 10ambiguous, where they release NE. NE then binds to $\alpha 1$ and β noradrenergic receptors on the post-synaptic target neuron (Wang et al., 2014). Excess NE in the synaptic cleft is tightly regulated by the presynaptic NET. The nucleus 10ambiguous, in turn, houses the alpha motoneurons of the larynx, as well as neurons that innervate autonomic preganglionic cells that trigger increases in heart rate, while the dorsal motor nucleus of the vagus houses preganglionic cells important for regulation of gastric digestive processes, and secretion of sweat (see Benarroch, 2018 for recent review) (Benarroch, 2018). Sensory receptors in the periphery then project to the nucleus of the solitary tract and the dorsal motor nucleus of the vagus (for the larynx) and to the spinal sensory nucleus, which project back to the nucleus ambiguus and the dorsal motor nucleus of the vagus nerve in order to modulate subsequent laryngeal (Ludlow, 2015), gastric, and cardiopulmonary motor functions. Concentrations of NE, NET, and density of both $\alpha 1$, $\beta 1$ and $\alpha 2$ noradrenergic receptors in the LC, dorsal motor nucleus of the vagus nerve, nucleus ambiguus, and nucleus of the solitary tract could potentially vary in tandem with degree of vocal impairment and level of anxiety in PD.

A relationship between vocal communication impairments in PD, such as hypokinetic dysarthria, and extra-dopaminergic mechanisms has been observed in several different

experimental approaches. When patients with PD and hypokinetic dysarthria are provided with central dopaminergic stimulation via apomorphine, minimal changes in laryngeal or articulatory function are observed (Kompoliti et al., 2000), although specific dopamine receptor modulation has been shown to alter acoustics of vocalization in rats (Ringel et al., 2013). Progression of gross motor deficits known to be governed by dopaminergic processes does not correlate with progression of speech deficits (Skodda et al., 2009, 2013), and improvements to speech intensity following behavioral therapy are correlated with higher cortical changes, rather than changes to dopaminergic nigrostriatal pathways (Narayana et al., 2010). Another non-hallmark sign of PD, anxiety, is linked to vocal deficits by shared onset and progression, lack of response to standard interventions, and putative pathological mechanism.

1.4 Anxiety disorders in Parkinson Disease

The affective disorder of anxiety is common in PD (Bernal-Pacheco et al., 2012). Prevalence has been estimated as high as 55%, with an average point prevalence of 31% (Broen et al., 2016; Yamanishi et al., 2013). While clinicians initially believed that anxiety was a behavioral response to the motor signs of PD, several lines of research have suggested that anxiety in PD often occurs as a consequence of disease-specific neural pathology (Dissanayaka et al., 2014; Menza et al., 1999; Thobois et al., 2017; Wee et al., 2016). This theory is supported by the fact that anxiety is often present prior to the onset of motor signs (Shiba et al., 2000), and that presence of anxiety increases risk of PD (Bower et al., 2010; Weisskopf et al., 2003).

1.4.1 Neural pathophysiology of anxiety in PD

As with vocal impairment, the exact, disease-specific mechanism of anxiety in PD are poorly understood. Because anxiety in PD does not consistently correlate with nigrostriatal dopaminergic deficits, other neurotransmitter systems, particularly serotonin and norepinephrine, have been suggested (Khatri et al., 2020; Maillet et al., 2016). The seminal staging studies of Braak and colleagues (Braak et al., 2003; Braak et al., 2004) have demonstrated that noradrenergic cell death in the LC precedes and is greater in magnitude than dopaminergic cell death in the substantia nigra. Disruptions to NE function are implicated in anxiety in general (Benarroch, 2009; Berridge & Waterhouse, 2003); as such, loss of NE neurons in the LC may be related to anxiety associated with PD (Dissanayaka et al., 2014). While empirical evidence remains limited, initial investigations have demonstrated correlations between norepinephrine transporter binding in the LC and anxiety levels in patients with PD (Remy et al., 2005).

1.4.2 Pharmacological, surgical, and behavioral interventions for anxiety in PD: non-specific and modest in effect

There is no disease-specific treatment for anxiety in PD (Seppi et al., 2019; Zesiewicz et al., 2010), and responsiveness of anxiety and other non-motor features of PD to dopamine replacement therapies is limited (Chaudhuri et al., 2006), with some evidence of exacerbation of anxiety during dopamine replacement therapy (Ganjavi & Macdonald, 2015). Thus, treatment of anxiety in PD typically mirrors standard anxiety treatments. Benzodiazepines, selective serotonin reuptake inhibitors (SSRIs), tricyclic antidepressants (TCAs) and serotonin-norepinephrine reuptake inhibitors (SNRIs) have all been shown to have some degree of anxiolytic effect in PD, but responsiveness is often mixed (Prediger et al., 2012). Anxiety

outcomes have also been assessed following deep brain stimulation. Similar to pharmacologic intervention, results from different studies vary widely, with some demonstrating improvement in anxiety and some demonstrating exacerbation (Couto et al., 2014). Investigation of behavioral interventions, such as cognitive-behavioral therapy, have shown improvement in symptoms with moderate effect sizes (Calleo et al., 2015; Reynolds et al., 2019; Troeung et al., 2014; Wuthrich & Rapee, 2019). In a recent review of non-pharmacological interventions for anxiety in PD, Chandler and colleagues (Chandler et al., 2019) observed statistically significant improvements in anxiety in just over 50% of studies, and noted that a common element across studies was the use of intentional modification of breathing. While initial findings of both pharmacologic and non-pharmacologic interventional studies for the treatment of anxiety in PD are promising, the relative number of non-responders or minimal-responders is particularly troubling in light of the significant impact that anxiety has on quality of life and disease burden in PD (Carod-artal et al., 2008; Carod-Artal et al., 2007; Renfroe et al., 2016).

As described above, several gaps in knowledge regarding the pathophysiology of vocal deficits and anxiety in PD remain. Accordingly, patient responses to pharmacologic, neurosurgical and behavioral interventions are difficult to predict, leaving these devastating symptoms of PD untreated in many individuals. In an effort to explore these gaps in knowledge with increased experimental control, translational research has begun to investigate animal models of vocal deficits and anxiety in PD.

1.5 Animal models of vocal communication impairment and anxiety in Parkinson Disease

Clinical research involving individuals with PD is often confounded by variables including age, degree of disease progression, environmental influences, pharmacologic factors, social condition, and personality factors, in addition to multiple Parkinsonian phenotypes (Fereshtehnejad et al., 2015; Foltynie et al., 2002). The use of animal models of certain features of the disease facilitates experimental control that is not possible in humans, allowing for investigation into neurobiological mechanisms of the disease process (Pankevich et al., 2013). Vocal communication impairment in PD has been modeled extensively in rats through lesioning of dopaminergic neurons in the nigrostriatal pathway with neurotoxins, such as 6-hydroxydopamine (Ciucci et al., 2007; Ciucci et al., 2009; Fleming et al., 2012; Plowman & Kleim, 2011; Ringel et al., 2013). While this neurotoxin model has advanced our understanding of relationships among dopaminergic denervation, cranial sensorimotor dysfunction, and limb impairment, it is an acute, unilateral injury that most-closely models late-stage PD.

More recently, a genetic rat model of PD, the *Pink1* knockout (*Pink1-/-*) has been developed. The *Pink1* gene is implicated in normal mitochondrial function, contributing to regulation of mitochondrial proteins, including Parkin (Kim et al., 2008). As recently reviewed by Hauser & Hastings in 2013 (Hauser & Hastings, 2013), when there is build-up of the PINK-1 protein on mitochondrial membranes that are depolarized, translocation of the protein Parkin to the mitochondria is triggered, which ultimately leads to these mitochondria being tagged as targets for mitophagy . Disruption of this process may contribute to deficiencies in cellular respiration that have been observed in the absence of the Pink1 gene in mouse models (Gautier et al., 2008). Importantly, mutations to *Pink1* have been associated with an early-onset form of familial PD (PARK6) (Guo et al., 2011). A body of work by Ciucci and colleagues has

demonstrated that rats with a knockout of the *Pink1* gene demonstrate early and progressive cranial and limb sensorimotor deficits, analogous to those observed in human PD (Cullen et al., 2018; Grant et al., 2015; Kelm-Nelson et al., 2015). This model has been useful for assessing treatments of vocal deficits (Kelm-Nelson et al., 2015), and for understanding neurobiological factors that drive behavioral deficits (Grant et al., 2015; Kelm-Nelson et al., 2018; Kelm-Nelson, Stevenson, et al., 2016). The validation of this progressive, early-onset model of PD is particularly valuable for investigation of cranial sensorimotor deficits, including vocal communication impairment and dysphagia, because subtle cranial sensorimotor deficits manifest early in the disease process, often prior to PD diagnosis (Harel et al., 2004; Hlavnika et al., 2017; Postuma et al., 2012).

Rodent models of anxiety in PD have been less robust. While most models demonstrate an increase in anxiety behavior in PD models compared to controls, several studies have demonstrated no effect, or even decreases in anxiety behavior (Faivre et al., 2019; Prediger et al., 2012). This is likely due in part to variations in methods of testing anxiety, rat strain, the type of model used (neurotoxin, genetic etcetera), and interactions among these factors (Pankevich et al., 2013). To date, anxiety in the *Pink1-/-* rat model of PD has been investigated in three studies, with two demonstrating increases in anxiety behavior in *Pink1-/-* rats compared to WT controls (Dave et al., 2014; Marquis et al., 2020), and one that showed no difference by genotype (Grigoruta et al., 2019). It should be noted, however, that each of these studies used a different method for assessing anxiety (elevated plus maze, open field test, and cat urine avoidance, respectively), and each study assessed rats at different ages. When no differences in anxiety were present, rats were tested between 2 and 3 months of age (Grigoruta

et al., 2019), versus testing at 4,6 and 8 months (Dave et al., 2014; Marquis et al., 2020) in studies that did report differences in anxiety.

1.6 Vocal communication and psychological state influence one another in the absence of Parkinson Disease

There is a complex, yet poorly understood relationship between psychological factors and successful skilled movements, such as voice and swallowing performance (Cardoso et al., 2019; Verdonschot et al., 2017). This relationship has been frequently commented upon in clinical textbooks and expert opinion in voice and voice disorders research since the mid 20th century (Diehl, 1960; Green, 1988; Roy & Bless, 2000). In the field of psychology, a large body of work has demonstrated relationships between psychological diagnoses, such as depression, and acoustic aspects of vocalization, such as fundamental frequency and fundamental frequency variation, since the 1930s. Data-driven research into the relationship between psychological factors and voice/voice disorders from the perspective of voice scientists began in earnest in 2000, when Roy and Bless established the personality-trait theory of voice disorders (Roy & Bless, 2000); this was supported by findings that certain personality traits co-occurred more-frequently with some types of voice disorders than others (Roy et al., 2000b). Additional investigations into the relationship raised questions about whether presence of voice disorders resulted in psychological consequences (referred to as a "disability" model), or whether presence of certain psychological factors resulted in a predisposition to development of a voice disorder (referred to as a "vulnerability" model), with initial findings suggesting that the later may be the more-likely case (Roy et al., 2000a). Experimental manipulation of psychological

state has further-confirmed initial findings of strong personality trait to dysphonia relationships. van Mersbergen and colleagues (2008) found that individuals with functional dysphonia had different psychological and psychophysiological responses to emotional stimuli compared to both healthy controls and individuals with social anxiety without dysphonia (van Mersbergen et al., 2008).

A body of work by Misono and colleagues has demonstrated a high incidence of psychosocial distress in individuals with voice disorders regardless of etiology (Misono et al., 2014). Subsequent studies specifically reported correlations between degree of psychological distress and voice handicap, which were moderated by the psychological factor of perceived control (Misono et al., 2016). In response to these findings, perceived control was targeted in a pilot study as an intervention to reduce voice handicap, resulting in a significant improvement in voice handicap with large effect size (Nguyen-Feng et al., 2018). Collectively, this series of studies support previous evidence for relationships between psychological factors and voice, and cements the theory that manipulation of psychological factors can positively influence voice outcomes.

Several lines of inquiry seek to provide physiologic quantification of the effect of psychological factors on voice production. Increases in arousal and in negative emotional states result in increased closed-phase quotient during phonation as measured by electroglottography (EGG) (van Mersbergen et al., 2015; van Mersbergen & Delany, 2014). Individuals who have greater levels of submental and infrahyoid muscle activation measured by surface electromyography (EMG) are more-likely to exhibit reduced extroversion (Dietrich & Verdolini Abbot, 2014). When measuring intrinsic laryngeal muscle activity with bipolar electrodes, an

increase in muscular activity has been observed during autonomic arousal (a common consequence of stress or anxiety) induced by a cold pressor task compared to baseline levels, suggesting a possible physiologic explanation for the relationship between psychological state and voice (Helou et al., 2013). Changes to vocal acoustic outcomes, such as relative fundamental frequency and cepstral peak prominence have additionally been reported following experimental manipulation of transient emotional state and autonomic arousal induced through cognitive loading tasks, respectively (Macpherson et al., 2017; van Mersbergen & Lanza, 2018). Because of similarities between vocal acoustic outcomes in individuals with PD and those with major depression (Cannizzaro et al., 2004; Harel et al., 2004), it has been suggested that a shared neural substrate may be implicated (Darby et al., 1984; Sapir, 2014).

Alterations in psychological state, such as anxiety, clearly interact with vocal communication. Deficits in vocal communication and psychological disorders in the context of PD are similar in many ways. For example, they are widely prevalent in PD, similar in their onset and progression, are likely mediated by extra-dopaminergic and noradrenergic mechanisms, and show suboptimal treatment outcomes in the context of the disease. Thus the simultaneous study of brainstem noradrenergic systems with vocal deficits, anxiety, and their interactions in the context of PD will increase our understanding of how these phenomena relate to one another on a systems level and with regard to disease-specific pathophysiology. Results from such a study would also help to inform optimization of interventions.

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Chapter 2: Statement of the Problem

2.1: Introduction

As described in Chapter 1, Parkinson disease (PD) is a progressive neurologic condition that causes sensorimotor, affective, cognitive, and autonomic deficits. Vocal communication impairment and anxiety are among the most-common and earliest of these deficits (Bezard & Fernagut, 2014; Braak et al., 2004; Broen et al., 2016; Dissanayaka et al., 2010; Hartelius & Svensson, 1994; Midi et al., 2008; Pont-sunyer et al., 2015). Standard pharmacologic interventions (levodopa) do not yield satisfactory results for vocal impairments or anxiety; additionally, behavioral treatments such as Lee-Silverman Voice Treatment and cognitive-behavioral therapy for anxiety result in incomplete and transient improvement (Calleo et al., 2015; Pinho et al., 2018; Ramig et al., 2018; Renfroe et al., 2016). Effective treatment of vocal impairment and anxiety in PD has been stymied by a lack of understanding of their disease-specific causes. Because vocal impairment and anxiety share neural substrates, as well as chronologic and symptomologic similarities, the tandem study of vocal impairment and anxiety in PD may yield insights into underlying neurobiological mechanisms.

Preliminary investigations into vocal impairment and anxiety in PD suggest that they are associated with disease-specific pathology related to brainstem norepinephrine. Vocal and affective behaviors share neural substrates and are strongly influenced by noradrenergic mechanisms (Berridge & Waterhouse, 2003; Goddard et al., 2010; Kano et al., 2011; Kelm-Nelson et al., 2018). Brainstem nuclei, including the locus coeruleus, nucleus ambiguus, and nucleus solitarius are involved in vocal communication ***and*** activation of the autonomic nervous system (a response to anxiety). These nuclei are connected by complex interplays of excitation and inhibition driven by noradrenergic processes (Benarroch, 2018; Berridge & Waterhouse,

2003; Mather et al., 2017; Samuels & Szabadi, 2008). Additionally, research has demonstrated impairment of noradrenergic functions in PD (Buddhala et al., 2015; Espay et al., 2014; Lewitt, 2012; Marien et al., 2004; Rommelfanger & Weinshenker, 2007; Tredici & Braak, 2013; Vazey & Aston-Jones, 2012). Further, vocal impairment and anxiety often manifest in prodromal and early stages of the disease, compared to later-occurring motor impairments. Likewise, noradrenergic cell death in the LC precedes dopaminergic cell death in the nigrostriatal pathways (Zarow et al., 2003). Thus, the shared phenomenology of vocal impairment and anxiety in PD warrants tandem investigation relative to noradrenergic disruption.

There is incomplete understanding of how impairments in noradrenergic modulation of brainstem cranial nuclei influence vocal impairment and anxiety in PD, and a concurrent lack of adequate treatment for vocal impairment and anxiety in PD. Therefore, the purpose of this dissertation was to test the central hypothesis that disruption of brainstem noradrenergic functions results in vocal dysfunction and anxiety. To address this problem with increased experimental control and the ability to study underlying neural mechanisms, a translational model of PD in rats with a knockout of the *Pink1* gene and wild type (WT) control rats were used. Specifically, we hypothesized that 1) anxiety would be increased in Pink1-/- rats, 2) vocal deficits and anxiety would be correlated to one another, 3) noradrenergic markers in the locus coeruleus, nucleus ambiguus, dorsal motor nucleus of the vagus nerve, and the nucleus of the solitary tract would be disrupted in *Pink1*-/-, 4) brainstem noradrenergic markers would be associated with vocal acoustic changes and anxiety level, and 5) administration of drugs that modulate noradrenergic function will modulate both vocal impairment and anxiety.

2.2 Aims

2.2.1 Study 1

As described in Chapter 1, associations between affective states (such as anxiety) and cranial sensorimotor functions (such as vocal communication) have been identified in humans. The underpinnings of this relationship, however, have been minimally investigated and remain poorly understood (Helou et al., 2013; Manor et al., 2009; Misono et al., 2014; van Mersbergen et al., 2015). Vocal communication requires activation and fine control of the larynx. Affective states (*i.e.* anxiety) influence the autonomic nervous system. Both vocal communication and the autonomic nervous system are activated and modulated through shared neural substrates including the nucleus ambiguus, the nucleus of the solitary tract, the dorsal motor nucleus of the vagus nerve, and the locus coeruleus (Kalia et al., 1984; Samuels & Szabadi, 2008).

Interestingly, vocal communication impairment and anxiety are both common features of PD whose disease-specific neurobiological mechanisms are poorly understood. Neither of these features respond adequately to pharmacologic or behavioral interventions. Unfortunately, treatment of vocal communication impairment and anxiety in PD is unlikely to advance without a deeper understanding of the neurobiological processes that lead to their manifestation, and investigation into neurobiological mechanisms of PD in humans is impeded by ethical and experimental challenges.

The *Pink1-/-* rat allows for circumvention of some of these challenges, has been shown to have strong construct validity as a model for vocal communication impairment in PD (Grant et al., 2015), and has provided insight into possible mechanisms for vocal communication impairment (Kelm-Nelson et al., 2018). Previous work has demonstrated that a relationship

exists between norepinephrine in the central nervous system and vocal impairment in the *Pink1-/-* rat (Kelm-Nelson et al., 2018). However, the *Pink1-/-* rat has been minimally explored as a model for anxiety in PD. Further, prior to Study 1, relationships between vocal communication impairment and anxiety in had been explored in neither the *Pink1-/-* rat nor in wild type (WT) animals. Thus, the purpose of this study was to explore the relationships among vocal communication, anxiety, and brainstem norepinephrine in the *Pink1-/-* rat model of PD and in wild type controls.

Specific Aim 1: To quantify brainstem norepinephrine relative to vocal impairment and anxiety in the *Pink1-/-* model of PD compared to WT controls. 16 *Pink1-/-* and 16 WT control rats underwent assessment of anxiety and vocal communication at 4, 8, and 12 months of age. We compared anxiety levels between *Pink1-/-* rats and WT controls, and assessed relationships between anxiety level and vocal acoustic outcomes. We then quantified the relative optical density of norepinephrine transporter in the locus coeruleus, dorsal motor nucleus of the vagus nerve, the nucleus of the solitary tract, and the nucleus ambiguous, as well as the relative optical density of noradrenergic receptors in the nucleus of the solitary tract, and the number of cells positively labeled for tyrosine hydroxylase and noradrenergic receptors in the locus coeruleus and number of cells positively labeled for noradrenergic receptors in the dorsal motor nucleus of the vagus and the nucleus ambiguus. Noradrenergic markers were compared between genotypes, and were then correlated with changes to anxiety and vocalization behaviors. **Hypotheses: H1a:** *Pink1-/-* rats would show increased anxiety compared to WT controls. **H1b:** Anxiety level would be associated with vocal acoustic outcomes. **H1c:** Number of cells labeled for tyrosine hydroxylase would be reduced in the locus coeruleus, and

norepinephrine transporter and noradrenergic receptors would be altered in brainstem nuclei of *Pink1*-/- versus WT rats. **H1d**: There would be a negative correlation between noradrenergic markers and both vocal impairment and anxiety. **Significance:** Data from this aim are essential for understanding vocal impairment and anxiety in PD. Results increase foundational knowledge about non-motor signs of PD, and help to guide potential pharmacologic treatments for vocal impairment.

2.2.2 Study 2

Studies of pharmacologic increases in norepinephrine (NE) have resulted in improvements to both motor and non-motor aspects of PD (Espay et al., 2014; Lewitt, 2012). These include modification of attention, hallucination, cognitive impairments, freezing of gait, and response inhibition (Jankovic, 2009; Vazey & Aston-Jones, 2012). Research on the effects of NE manipulation in PD on anxiety and vocal impairment is sparse, and research on interactions among NE, vocal impairment, and anxiety is absent from the literature. Therefore, the purpose of this study was to assess the influence of pharmacologic manipulation of NE systems on vocal impairment and anxiety in the *Pink1-/-* rat model of PD. Atomoxetine, reboxetine and propranolol were chosen for investigation in this study because they have been used to treat anxiety through NE mechanisms. Other anxiolytic drugs (*i.e.* valium, lorazepam) were not chosen because they either do not target NE mechanisms, and/or are associated with motor impairment.

Specific Aim 2: To assess how modulation of norepinephrine affects vocal impairment and anxiety in the *Pink1-/-* model of PD. We administered three different drugs that modulate noradrenergic functions to *Pink1*-/- rats to determine effects on vocal communication and

anxiety. **Hypotheses:** Norepinephrine reuptake inhibitors (atomoxetine and reboxetine) and a β receptor antagonist (propranolol) would decrease vocal impairment and anxiety. **Significance:** Data from this aim increase understanding of the influence of brainstem norepinephrine on vocal communication and anxiety and provide support for exploration of NET inhibitors as potential pharmacologic targets for treatment of both of these deficits in PD.

2.3: Appendices

In addition to the studies proposed above, I have completed a retrospective observational cohort study on post-extubation dysphagia in pediatric populations. The published manuscript is found in appendix A as evidence for other scholarly endeavors.

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Chapter 3

Quantification of Brainstem Norepinephrine Relative to Vocal Impairment and Anxiety in the Pink1-/- Rat Model of Parkinson Disease

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Key Words: Parkinson Disease; Rat; Pink1; Ultrasonic Vocalization; Anxiety; Norepinephrine

Highlights:

- *Pink1*-/- rats demonstrate more anxiety behaviors than wild type controls, mirroring human Parkinson disease.
- Anxiety is correlated with vocal deficits in *Pink1*-/- rats.
- Brainstem noradrenergic markers differ between *Pink1*-/- rats and wild type controls.
- Brainstem noradrenergic markers are correlated with vocal deficits and anxiety.

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Abbreviations: 10N: Dorsal Motor Nucleus of the Vagus; AMB: nucleus ambiguous; CI: Confidence interval LC: locus coeruleus; NET: norepinephrine transporter; NTS: Nucleus of the solitary tract; PD: Parkinson Disease; *Pink1*-/-: *PTEN induced putative kinase 1* gene knockout; ROD: relative optical density; US: unbiased stereology; USV: ultrasonic vocalization; WT: wild type

Abstract:

Vocal communication impairment and anxiety are co-occurring and interacting signs of Parkinson Disease (PD) that are common, poorly understood, and under-treated. Both vocal communication and anxiety are influenced by the noradrenergic system. In light of this shared neural substrate and considering that noradrenergic dysfunction is a defining characteristic of PD, tandem investigation of vocal impairment and anxiety in PD relative to noradrenergic mechanisms is likely to yield insights into the underlying disease-specific causes of these impairments. In order to address this gap in knowledge, we assessed vocal impairment and anxiety behavior relative to brainstem noradrenergic markers in a genetic rat model of early-onset PD (*Pink1*-/-) and wild type controls (WT). We hypothesized that 1) brainstem noradrenergic markers would be disrupted in *Pink1*-/-, and 2) brainstem noradrenergic markers would be associated with vocal acoustic changes and anxiety level. Rats underwent testing of ultrasonic vocalization and anxiety (elevated plus maze) at 4, 8, and 12 months of age. At 12 months, brainstem norepinephrine markers were quantified with immunohistochemistry. Results demonstrated that vocal impairment and anxiety were increased in *Pink1*-/- rats, and increased anxiety was associated with greater vocal deficit in this model of PD. Further, brainstem noradrenergic markers including TH and α1 adrenoreceptor immunoreactivity in the locus coeruleus, and β1 adrenoreceptor immunoreactivity in vagal nuclei differed by genotype, and were associated with vocalization and anxiety behavior. These findings demonstrate statistically significant relationships among vocal impairment, anxiety, and brainstem norepinephrine in the *Pink1*-/- rat model of PD.

3.1 Introduction

3.1.1 *Vocal deficits and anxiety in Parkinson Disease are linked by lack of response to pharmacologic intervention, shared neural substrates, and onset and progression.*

In addition to hallmark gross motor signs of Parkinson Disease (PD), 90% of individuals with PD exhibit vocal communication impairment that involves break-down of speech subsystems including respiration, phonation, and articulation (Anand & Stepp, 2015; Fox & Ramig, 1997; Huber & Darling, 2011; Logemann et al., 1978; Matheron et al., 2017; Sapir et al., 2008; Stepp, 2013). The impact of vocal impairment on quality of life and disease burden is substantial (Barone et al., 2009; Lirani-Silva et al., 2015; Martinez-Martin et al., 2011; Miller et al., 2006). Behavioral interventions for treating vocal impairment in PD, such as Lee Silverman Voice Treatment (LSVT), are time-intensive and result in incomplete and inconsistent improvement (Mahler et al., 2015; Ramig et al., 2018). Pharmacologic interventions such as levodopa change some acoustic features of voice production (loudness) and speech production (lip movement), however, the functional impact of these changes is limited, with minimal improvement in speech intelligibility (Pinho et al., 2018; Sanabria et al., 2001; Wolfe et al., 1975). As a result, the vocal impairment in PD remains untreated. A deeper understanding of the disease-specific mechanisms that cause vocal impairment in PD is essential to develop much needed efficient and effective treatment.

Another consequence of PD that frequently co-occurs with vocal deficits is anxiety. Incidence of anxiety in patients with PD is estimated between 30 and 50%, and is associated with significant negative impact on quality of life and level of disability (Broen et al., 2016; Carod-artal et al., 2008; Renfroe et al., 2016; Yamanishi et al., 2013). Anxiety often manifests

prior to the onset of motor signs of PD, suggesting that a *neurobiological* mechanism might link PD and anxiety (Faivre et al., 2019; Kano et al., 2011; Pont-sunyer et al., 2015; Shiba et al., 2000). Some studies have reported reduced anxiety with behavioral treatment and medication. However, similar to vocal impairment, the benefits are incomplete and inconsistent (Calleo et al., 2015; Renfroe et al., 2016; Weintraub et al., 2010). As with vocal impairment, the mechanisms underlying anxiety are not well-understood.

Relationships between affective states, such as anxiety versus calm, behaviors mediated by cranial nerves, such as vocalization, have been identified in humans; however, the neural mechanisms that drive this relationship remain unclear (Helou et al., 2013; Macpherson et al., 2017; Manor et al., 2009; Misono et al., 2014; van Mersbergen et al., 2008, 2015). A potential target mechanism is the locus coeruleus-vagal system. Vocal communication involves fine motor control of the larynx. Anxiety causes changes in degree of arousal of the autonomic nervous system. The Locus Coeruleus-Vagal system, largely driven by norepinephrine (NE), appears to be simultaneously responsible for modulation of vocalization and activation of autonomic responses to anxiety (George et al., 2008; Kalia et al., 1984; Mather et al., 2017; Samuels & Szabadi, 2008; Wang et al., 2014). NE neurons in the locus coeruleus project to the dorsal motor nucleus of the vagus nerve(10N) and the nucleus ambiguus. The nucleus ambiguus houses motoneurons responsible for laryngeal movement, as well as neuron groups responsible for cardiopulmonary modulation, while the 10N houses preganglionic cells important for regulation of gastric digestive processes, and secretion of sweat (see Benarroch, 2018 for recent review) (Benarroch, 2018). Sensory receptors in the periphery project to the nucleus of the solitary tract and vagal nuclei (specifically for the

larynx) and to the spinal sensory nucleus, which project back to the nucleus ambiguus and the 10N in order to modulate laryngeal (Ludlow, 2015), gastric, and cardiopulmonary motor functions (Petko & Tadi, 2020; Travagli et al., 2008). The fact that these neural substrates are tightly linked to both vocal communication and anxiety suggests that the two phenomena may influence one another (Figure 1). Further, it suggests that they may both be susceptible to pathology of this shared substrate. In particular, concentrations of NE, NET, and density of both α1, β1 and α2 noradrenergic receptors in the locus coeruleus, dorsal motor nucleus of the vagus nerve, nucleus ambiguus, nucleus of the solitary tract, and the spinal sensory nucleus could potentially vary depending upon degree of vocal impairment and level of anxiety in PD. Neuroimaging studies in humans have demonstrated upregulation of α1 and β1 adrenoreceptors in neocortical structures in humans with PD compared to controls (Roland Cash et al., 1984), and decreases in norepinephrine and number of noradrenergic cell bodies in the LC (Grant et al., 2015; Kelm-Nelson, Trevino, et al., 2018) have been demonstrated in the *Pink1*-/- rat model of PD. However, exploration of noradrenergic markers in lower brainstem structures, including those important for vocalization and anxiety have been minimal.

Figure 1

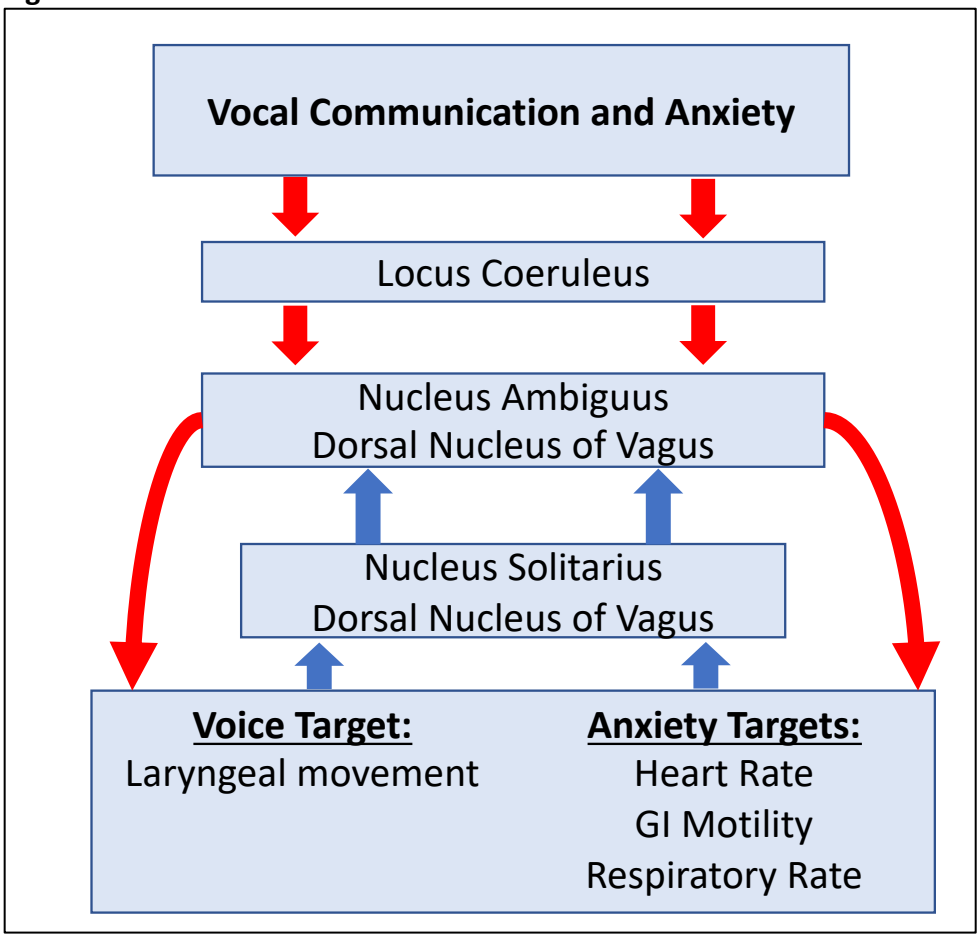

Figure 1: Pathways of both vocal communication and anxiety. Vocal communication and anxiety are both associated with the locus-coeruleus-vagal system, and travel the same efferent (red) and afferent (blue) pathways, often with NE as a primary neurotransmitter. The disruption of NE these pathways could simultaneously explain both vocal impairment and anxiety in PD.

3.1.2: *Brainstem noradrenergic disruption: a pathophysiological link between vocal deficits and anxiety in PD*

PD is traditionally characterized by nigrostriatal dopaminergic cell death, (Braak et al., 2004) leading to hallmark signs of tremor, bradykinesia, and postural instability (Bernheimer et al., 1973). While the exact physiologic mechanisms of vocal deficits in PD remain uncertain, it is becoming increasingly clear that they are at least partially independent of classical dopaminergic degeneration (Brabenec et al., 2017; Plowman-Prine et al., 2009;

Schulz & Grant, 2000). The likelihood of an extra-dopaminergic pathological process is further-supported by the fact that the most commonly prescribed medication, levodopa, which increases dopamine availability, markedly improves motor impairments in the limbs (The Parkinson Study Group, 2004), while non-hallmark signs, including vocal impairment and anxiety, have a minimal response to dopamine replacement therapies (Calleo et al., 2015; Pinho et al., 2018; Renfroe et al., 2016; Sanabria et al., 2001; Wolfe et al., 1975). Aside from dopamine-mediated deficits, other neural substrates, including those governed by NE, are disrupted in PD (Buddhala et al., 2015; Espay et al., 2014; Lewitt, 2012; Marien et al., 2004; Rommelfanger & Weinshenker, 2007; Tredici & Braak, 2013; Vazey & Aston-Jones, 2012). It has thus been suggested that non-hallmark signs of PD, including vocal deficits and anxiety, may be related to NE processes (Cash et al., 1987; Espay et al., 2014; Kreiner et al., 2019).

An additional feature shared by vocal deficits and anxiety in PD is their manifestation in prodromal and early stages of the disease, compared to the later-appearing motor signs (Harel et al., 2004). The possibility that vocal impairment and anxiety in PD are linked through NE mechanisms is further-supported by the fact that NE cells in the locus coeruleus die earlier in the disease process than dopaminergic cells in the nigrostriatal pathway (Braak et al., 2004; Tredici & Braak, 2013). Thus, the timelines of anxiety and vocal impairment and NE cell death are similar, and can be contrasted with the timelines of motor signs and dopaminergic cell death.

There is thus substantial overlap between vocal communication and anxiety in PD with regard to onset (prodromal), lack of pharmacologic treatment response, and neuroanatomical and neurochemical substrates. As such, the tandem study of vocal communication and anxiety

in PD at behavioral and histological levels is warranted and currently absent from the literature. In order to address this gap in knowledge with increased experimental control and the ability to analyze neural tissue, we assessed vocal impairment and anxiety relative to brainstem noradrenergic markers in a translational model of PD in rats with a knockout of the *Pink1* gene and wild type (WT) controls. The *Pink1*-/- rat is based on a genetic form of early and progressive PD (PARK6) that is nearly clinically identical to idiopathic PD (Dehay & Bezard, 2011); the *Pink1*-/- rat has been well-validated as a model of vocal communication impairment in PD (Grant et al., 2015; Kelm-Nelson et al., 2015; Kelm-Nelson, Trevino, et al., 2018). Additionally, noradrenergic differences in the LC have previously been identified in this model (Grant et al., 2015; Kelm-Nelson, Trevino, et al., 2018)and vocal acoustic outcomes have been correlated norepinephrine in the LC (Kelm-Nelson, Trevino, et al., 2018). Further, pharmacologic manipulation of norepinephrine in WT rats has been shown to modulate ultrasonic vocalization acoustics (Grant et al., 2018; Wright et al., 2012). However, LC target nuclei in the brainstem have not been investigated relative to noradrenergic markers, vocalization and anxiety. We hypothesized that 1) *Pink1*-/- rats would show increased anxiety compared to WT controls; 2) Anxiety level would be associated with vocal acoustic outcomes. 3): Number of cells labeled for tyrosine hydroxylase would be reduced in the locus coeruleus, and norepinephrine transporter and noradrenergic receptors would be altered in brainstem nuclei of *Pink1*-/- versus WT controls. 4) There would be a negative correlation between noradrenergic markers and both vocal impairment and anxiety.

3.2 Methods

3.2.1: Experimental Procedure

To assess the relationships among anxiety, vocalization and NE in the *Pink1-/-* rat model of PD, *Pink1-/-* rats and WT control rats underwent anxiety testing by performing the elevated plus maze and were immediately transferred to their home cage for ultrasonic vocalization recording (behavioral assays described below). Data were collected at 4, 8, and 12 months of age (Figure 1). Following data collection at the 12-month time point, rats were euthanized and neural tissue was collected and preserved for histological analysis. (**Figure 2)**

Figure 2

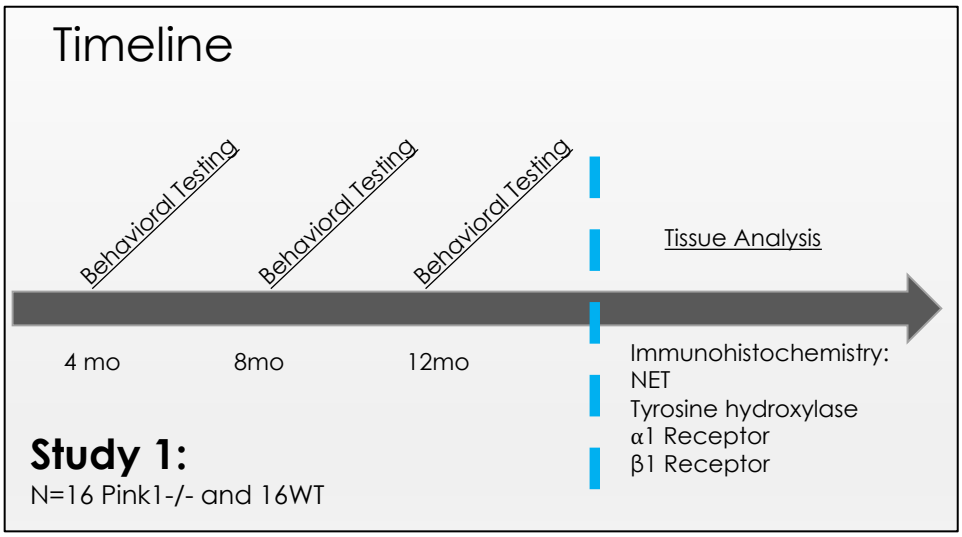

Figure 2: Experimental timeline. Behavioral testing includes analysis of ultrasonic vocalizations and measurement of anxiety behavior on the elevated plus maze. NET: norepinephrine transporter.

3.2.2: Animals

All procedures were approved by the University of Wisconsin-Madison School of Medicine and Public Health Institutional Animal Care and Use Committee (IACUC; protocol M005177-R01-A04) and were conducted in accordance with the NIH Guide for the Care and Use of Laboratory Animals, Eight Edition(National Research Council Committee for the Update of the Guide for

the Care and Use of Laboratory, 2011). Thirty-two (16 *Pink1-/-* and 16 Wild Type) rats were used for this study. Power analysis determined that a sample size of 13 rats per group (Pink1-/- and WT) should detect differences in vocalization (based on differences in USV intensity reported by Grant et al, 2015 (Grant et al., 2015)) and brainstem tyrosine hydroxylase concentrations (based on Kelm-Nelson et al, 2018 (Kelm-Nelson, Trevino, et al., 2018)) with an α level of 0.05 and 90% power. A rate of 20% attrition was also accounted for. Thus, the total sample size for each group was n=16 rats. In addition, 12 female Long-Evans rats were used to elicit ultrasonic vocalizations (see protocol below). These female rats were continually housed in the colony maintained by our laboratory for the purpose of ultrasonic vocalization elicitation in several ongoing studies but were not included in current data analyses. This number of female rats was chosen to ensure that at least one rat was in estrous on each day of acclimation and testing. All animals were obtained from SAGE Labs (Envigo, Boyertown, PA). Rats were housed in pairs the Biomedical Research Model Services facilities of the UW School of Medicine and Public Health, were 12-hour light-cycle reversed and underwent behavioral testing under red light during the dark period when the rats were most active. Rats were handled and weighed weekly until testing and throughout the duration of the study. Standard husbandry and handling practices and procedures were used in accordance with institutional guidelines regarding animal experimentation. Animals were tested at 4, 8, and 12 months of age. These timepoints were chosen because they represent prodromal, early and middle stages of disease progression (Dave et al., 2014; Grant et al., 2015; Marquis et al., 2020). Rats were then euthanized, and neural tissue collected and preserved in cryoprotectant.

3.2.3: Behavioral Testing

3.2.3.1: Ultrasonic Vocalization Recording

Rats produce ultrasonic vocalizations (USVs) in the 50-kiloHertz (kHz) range to initiate and maintain conspecific contact. These USVs demonstrate remarkable complexity and are produced in stereotyped patterns. Increases in features of USVs, including mean peak frequency, bandwidth, and complexity result in increased approach behavior of conspecifics (Pultorak et al., 2016; Willadsen et al., 2014). It is thus thought that these features are "preferred," and can be considered to be at least partially goal directed (Bialy et al., 2000; Blanchard et al., 1992; Brudzynski & Pniak, 2002; McGinnis & Vakulenko, 2003; Riede, 2014; Wöhr et al., 2008). Analysis of acoustic variables of rat USVs to study vocal impairment in PD and other neurologic diseases is well-established (Ahrens et al., 2009; Ciucci et al., 2007; Grant et al., 2018; Grant, Kelm-nelson, et al., 2015; A. Johnson et al., 2011; Kelm-Nelson et al., 2015, 2016). To assess vocal communication in the current study, USVs were elicited and recorded with the following mating paradigm, as reported in previous studies (Grant, Kelm-nelson, et al., 2015; Johnson et al., 2011; Johnson et al., 2013; Kelm-Nelson et al., 2016). Test rats were placed in their home cage under a microphone attached to an ultrasonic recording system (Avisoft, Germany). A female conspecific in estrus was then placed in the male rat's home cage. After the male rat showed interest in the female, the female was removed. The USVs from the male rat were then recorded for 90 seconds. USVs were analyzed offline using automated software (SASLab Pro, Avisoft, Germany). Waveforms generated by Avisoft were used to create spectrograms with the following parameters: Fast Fourier Transform (FFT) of 512 points, frame size of 100%, flat top window with temporal resolution set to display 75% overlap. Noise below 25 kilohertz(kHz) was removed via a high pass filter. Dependent variables for the purposes of

this study were the average bandwidth (kHz), mean peak frequency(kHz), duration(s), and intensity in decibels(dB) of calls.

3.2.3.2: Elevated Plus Maze: The elevated plus maze was used to assess anxiety behavior (Hogg, 1996; Hopkins & Bucci, 2010; Pellow et al., 1985; Walf & Frye, 2007). The maze consists of a plus-shaped platform with 4 equally sized arms. Two arms, opposite one another, are open with no walls (50x10cm), and the remaining two arms have walls with open tops on 3 of 4 sides (50x10x50cm). Each arm is accessible from a square area in the center of the platform. The rats were placed in the center of the maze under red light and video-recorded for 5 minutes. Movement was tracked and analyzed using EthoVision software (Noldus Ethovision XT (Wageningen, Netherlands)). Outcome variables were total entries into closed arms and total time spent in closed arms in seconds. Increased time and frequency of entry into closed arms represent increased anxiety. Because differences in overall movement between genotypes may be present, a closed arm ratio was also calculated using the formula:

$$\text{Closed Ratio} = \frac{\left(\frac{Closed\ Arm\ Entries}{Time\ in\ Closed\ Arms}\right)}{\left(\frac{Total\ Arm\ Entries}{Time\ in\ Any\ Arms}\right)}$$

A smaller ratio indicates increased preference for closed arms of the maze, indicating an increase in anxiety (Walf & Frye, 2007).

3.2.4: Neural Tissue Processing

After testing at the 12-month timepoint, rats were anesthetized under 5% isoflurane. Transcardial perfusion with cold saline was followed by 4% paraformaldehyde in 1% phosphate-buffered saline. Brains were post-fixed for 24 hours in 4% paraformaldehyde, then stored in

0.02% sodium azide at 4°C. Prior to tissue sectioning, brains were placed in a 30% sucrose for 48 hours. Brains were then mounted on a cryostat, sliced coronally at 50 microns from the cortex through the brainstem, placed in 30% sucrose cryoprotectant and stored at -20°C until they were stained for immunoreactivity over every 6th section.

3.2.5: Immunohistochemistry

Four separate immunohistochemistry assays were performed to compare noradrenergic markers between genotypes and assess the relationships between these markers and USVs and anxiety. Assays for noradrenergic markers included Tyrosine Hydroxylase(TH) (a norepinephrine precursor) α1 adrenoreceptors (α1AR), β1 adrenoreceptors (β1AR), and norepinephrine transporter (NET). Each assay was completed across three runs for each assay, with each genotype equally represented in each run. For each assay, incubation in the absence of primary antibody was used for control sections, which resulted in absence of immunoreactivity. Confirmation of antibody specificity was demonstrated by manufactures. Western immunoblotting appropriately detected bands at molecular weights of 62kDA TH (EMD Millipore, Temecula, CA), 50kDA for β1AR (abcam, Cambridge, MA), 60kDA for α1AR (Thermo Fisher Scientific, Waltham, MA), and 80kDA for NET (Thermo Fisher Scientific, Waltham, MA). All antibodies stained appropriate patterns of distribution, as demonstrated previously (Ghosh et al., 2019; Grant et al., 2015; Smith et al., 2006; Wee et al., 2008).

For each assay, tissue sections were blocked in 20% normal goat serum and incubated overnight in a primary antibody solution (see Table 1 for primary antibody product information and concentrations), as recently described (Grant et al., 2015). Samples were then incubated in conjugated biotinylated secondary solution at 1:500 (Millipore, BA-1000) for 2-hr and incubated

in an avidin– biotin solution (Vector Laboratories, Burlingame, CA) for 1-hr, and the complex was visualized by using SIGMAFAST 3,3-diamino- benzidine with metal enhancer (DAB; Sigma Aldrich, St. Louis, MO, D0426). All sections were float mounted onto gelatin-coated slides, dehydrated in a graded series of alcohols and xylenes, and coverslipped with Cytosol 60 mounting medium (Richard-Allen Scientific, Kalamazoo, MI)

Table 1: List of antibodies and quantification method for immunohistochemistry

Primary Antibody	Immunogen Target	Manufacturer (product number); RRID; Lot Number	Host/Concentration:	Type	Brain Region Quantification method			
					LC	NTS	10N	AMB
Anti-β1AR	Synthetic peptide corresponding to mouse β1AR aa 394-408	abcam (ab3442); AB_10890808; GR3295387-4	Rabbit/1:2000	Polyclonal	US	ROD	US	US
Anti-α1AR	Synthetic peptide corresponding to residues K(339) F S R E K K A A K T(349) of the 3rd intracellular loop of human α1AR	Thermo Fisher Scientific/Invitrogen (PA1-047); AB_2273801; UG277737	Rabbit/1:2000	Polyclonal	US	ROD	US	US
Anti-NET	Peptide (C)KLLNASVLGDHTKYS K, corresponding to amino acid residues 189-204 of mouse NET	Thermo Fisher Scientific/Invitrogen (PA5-77494); AB_2736247; VB2931552	Rabbit/1:5000	Polyclonal	ROD	ROD	ROD	ROD
Anti-TH	TH (NCBI gene ID: 25085	EMD Millipore (AB152); AB_390204; 3328928	Rabbit/1:2000	Polyclonal	US	---	---	---

Table 1: List of antibodies and quantification method for immunohistochemistry *AR* adrenoreceptor, *NET* norepinephrine transporter, *TH* tyrosine hydroxylase, *LC* locus coeruleus, *NTS* nucleus of the solitary tract, *10N* dorsal motor nucleus of the vagus, *AMB* nucleus ambiguus, *US* unbiased stereology, *ROD* relative optical density, *RRID* research reference ID

3.2.6: Unbiased Stereology and Relative Optical Density Measurement

Unbiased cell count estimation was completed in the locus coeruleus (for TH, α1AR, β1AR), 10N (for α1AR, β1AR), and nucleus ambiguus (for α1AR, β1AR) using the optical fractionator method as described by West et al, 1991 and adapted from Kelm-Nelson et al, 2018 (Kelm-Nelson, Brauer, et al., 2018; West et al., 1991). Cell number was estimated in the right or left hemisphere only for each rat. Stereological analyses were completed using Stereo Investigator® (MBF Bioscience, Williston, VT) and the Optical Fractionator Probe. An Olympus BX53 microscope was fitted with a QImaging Retiga 1300c monochrome camera and a Prior XYZ Proscan III motorized stage kit, with images displayed on a plasma screen monitor. Brain regions were outlined based on an atlas of stereotaxic coordinates of the rat brain (Paxinos & Watson, 2005) using a 4x magnification. Three sections between bregma -9.48 and -10.2 were counted in the locus coeruleus. Eight to 9 sections were counted between bregma -12.36 and 14.76 in the 10N, and 7 sections between bregma -12.00 and -14.16 were counted in the nucleus ambiguus. For each nucleus, every 6th section was counted (250μm between sections). Random sampling of the outlined regions was completed at 40x magnification using a sampling grid of 100 x 100μm and a counting frame of 75 x 75μm with a dissector height of 12μm and guard zones of 2μm. Section thickness was measured at each counting site (average thickness of 20.4μm, 17.1μm and 19.5μm for locus coeruleus, 10N and nucleus ambiguus respectively). . This combination of sampling parameters was established to achieve a Gunderson coefficient of error (m=1) of less than 0.10 for each region, indicating that parameters used were accurate for stereological investigation. Cell bodies stained for TH, α1AR, and β1AR were counted if the top of the leading edge came into focus within the inclusion lines of the dissector and outside of the

2μm guard zones. Estimated cell counts were averaged for individual rats and combined to produce genotype averages.

Optical density measurements were taken in the locus coeruleus, 10N and nucleus ambiguus (for NET), and in the NTS (for NET, α1AR and β1AR). Sections containing nuclei of interest were outlined at 4x magnification using the slide-scanning workflow in Stereo Investigator®, and the equipment set-up described for stereology. Following background correction, images were obtained at 10x magnification to ensure similar focus between hemispheres. For locus coeruleus, 10N and nucleus ambiguus, the same number of sections were analyzed and at same bregma coordinates as above; in addition, 3 sections between bregma -12.96 and -13.8 were analyzed in the NTS (NET, α1AR, and β1AR). For each nucleus, both hemispheres of every 5th section were analyzed (250μm between sections). Analysis was completed using *ImageJ* (US National Institutes of Health, Bethesda, MD). ImageJ was then used to place uniformly-sized boxes inside of the nucleus of interest in each hemisphere (250 x 500 pixels in the locus coeruleus, 500 x 500 pixels in the NTS, 300 x 300 pixels in the nucleus ambiguus, and 250 x 500 pixels in the 10N), as well as a region devoid of staining (50 x 50 pixels region in the spinal tract of 5) on each slice. Images were converted from RGB color to 8-bit, and pixels in the images were calibrated on a provided gray scale (ImageJ). An optical density reading of the background and bilateral regions of interest were then taken. The relative optical density for each section was calculated as relative optical density = (average optical density of the nucleus of interest) – optical density of the background image. Relative optical density was then averaged for individual rats, and then combined to produce genotype averages.

3.2.7: Statistical Analysis

Mixed effect models were used to assess the influence of time point (3 levels), genotype (two levels), as well as interaction between time and genotype on USV and anxiety parameters. Multiple regressions were fitted separately to assess USV outcomes of call intensity, bandwidth, duration, and average peak frequency with anxiety, genotype, and the interaction between anxiety and genotype as independent variables at the 12-month timepoint. Student's t-tests were used to compare brainstem NE markers between genotypes. Univariate linear regression analysis was conducted to assess relationships between brainstem NE and USV parameters, as well as between NE and anxiety at the 12-month timepoint. Sample sizes and degrees of freedom reflect incidental tissue loss (n=1 Pink1-/- rat for NET in the NTS and 10N), absent vocalization (n=1 WT rat at 4months, n=1 Pink1-/- rat at 8 months, and n=1 WT rat at 12 months), and a rat removing itself from the maze during anxiety testing (n=1 Pink1-/- rat at 4 months).

The outcome variables were USV measures of bandwidth, mean peak frequency, intensity, and call duration, as well as time spent in closed arms of the plus maze, and frequency of entries into closed arms of the plus maze. Independent variables included relative optical density of NET in the locus coeruleus, NTS, 10N, and nucleus ambiguus, relative optical density of α1AR and β1AR in the NTS, and estimated cell counts of cell bodies stained for TH in the locus coeruleus, and α1AR and β1AR separately in the locus coeruleus, 10N and nucleus ambiguus. For USV and plus maze measurements, inter-rater reliability was determined by calculating intra-class correlation coefficients (ICC) on 5% of data files. This methodology was chosen based on our hypothesis that observations from behavioral testing are the result of

long-term changes to NE involving these brain regions. Statistical analyses were performed with a significance level of 0.05 using software R (version 3.6.0) and SAS (version 9.4). Due to the exploratory nature of this work and associated risk of type II statistical error, no corrections for multiple comparisons were made.

3.3 Results

3.3.1: Ultrasonic Vocalizations

Inter-rater reliability was greater than 0.90 for USV measurements. There was no interaction between genotype and timepoint on bandwidth ($F(2,57)=0.21$, $p>0.05$). There was no main effect of genotype ($F(1,30)=0.31$, $p=0.5796$). There was a significant main effect of timepoint ($F(2,57)=7.01$,$p=0.0019$) on call bandwidth. Post-hoc analysis comparing least squares means demonstrated that bandwidth decreased after the 4-month timepoint. At 4 months (18803Hz), bandwidth was greater than 8months (16322Hz) and 12 months (15843hz) ($t(57)=2.91$, $p=0.014$ and $t(57)=3.49$, $p=0.0027$, respectively), but 8- and 12-month timepoints were not significantly different from one another($t(57)=0.56$, $p=0.89$).

There was no interaction between genotype and timepoint on call intensity ($F(2,57)=0.63$, $p=0.54$). There was a significant main effect of genotype on call intensity ($F(1,30)=15.79$, $p=0.0004$). In post-hoc analysis, the Pink1-/- rat group (-50.02dB) was quieter than the WT rat group (-48.51dB) ($t(30)=3.97$, $p=0.004$). There was also a significant main effect of timepoint on call intensity ($F(2,57)=34.99$, $p<0.0001$) (4mo=-47.35, 8mo=-50.87, 12mo=-51.07). Rats were quieter at 4mo than 8mo ($t(57)=7.03$,$p<0.0001$) and 12mo ($t(57)=7.43$, $p<0.0001$). There was no difference in intensity between 8mo and 12mo ($t(57)=0.37$, $p=0.93$).

There was no interaction between genotype and timepoint on average peak frequency ($F(2,57)=2.14$, $p=0.13$). There were significant main effects of timepoint ($F(2,57)=4.08$, $p=0.02$) and genotype($F(1,30)=9.11$,$p=0.005$) on average peak frequency of calls. The Pink1-/- rat group (54988hz) had a lower average peak frequency that the WT rat group (57653hz) ($t(30)=3.02$, $p=0.005$). Additionally, Average peak frequency at 4mo (55483hz) was lower than 8mo (57168hz) ($t(57)=2.85$,$p=0.02$), but was not significantly different from 12mo (56312) ($t(57)=0.16$, $p=0.34$). Average peak frequency did not differ from 8 to 12 months ($t(57)=0.15$, $p=0.32$).

There was a significant interaction between genotype and timepoint call duration ($F2,57)=3.38$, $p=0.41$). The Pink1-/- rat group and WT rat group did not differ in call duration at 4 months (Pink1-/- mean=0.036s, WT mean= 0.0321s, $t(57)=1.18$, $p=0.85$). By 8 months, duration was longer for The Pink1-/- group than for WT group (mean pink= 0.043s, mean WT= 0.030s, ($t(57)=4.12$,$p=0.002$). By 12 months, however, call duration was no longer different between genotypes (mean Pink1-/-=0.36s, mean WT= 0.030 ($t(57)=1.65$, $p=0.57$).

Figure 3

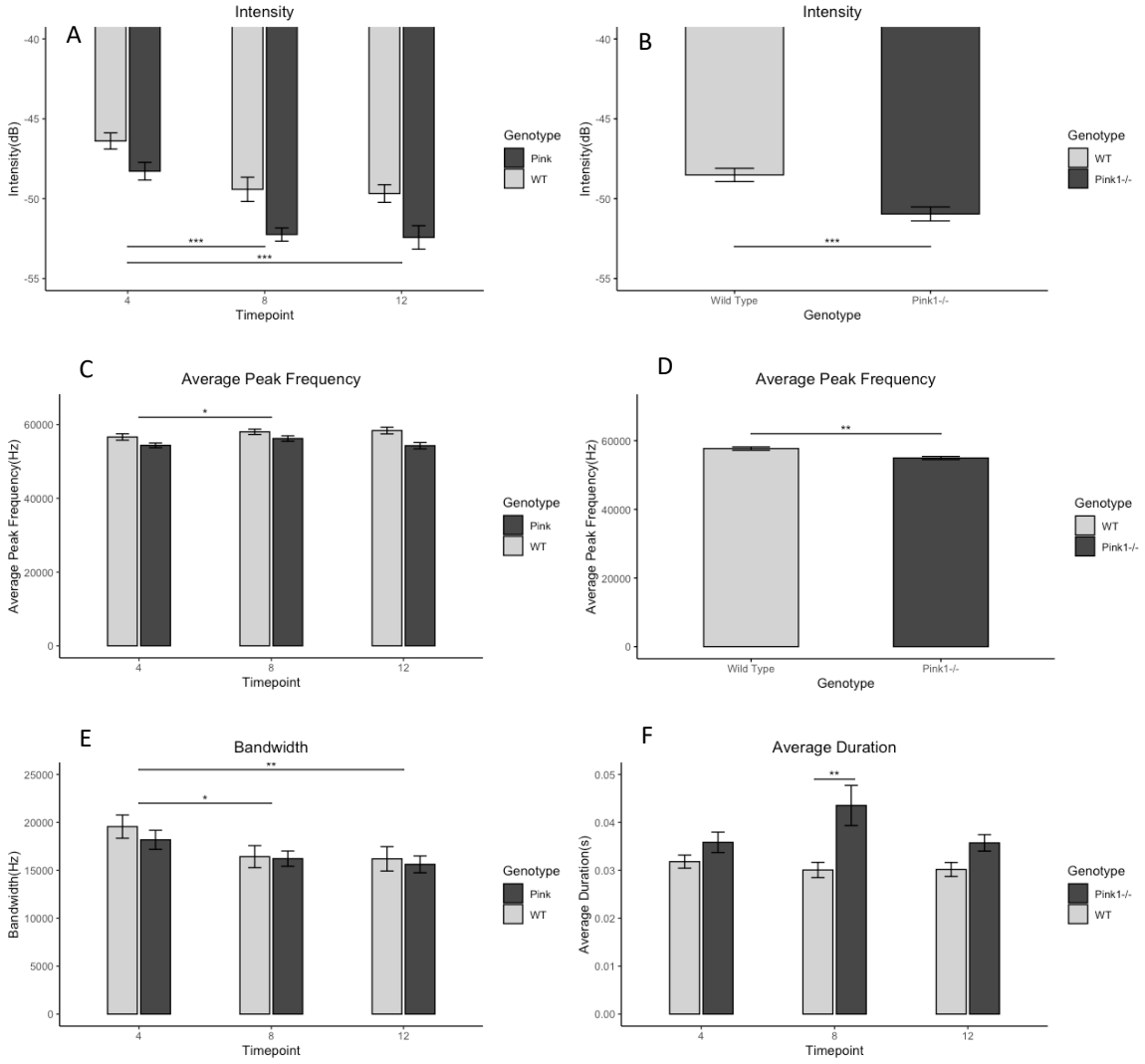

Figure 3. Ultrasonic Vocalization. **A**: Average intensity of calls by genotype and timepoint; **B:** Average intensity with data collapsed across time to show main effect for genotype. Less-negative value indicates louder call. **C:** Average peak frequency; **D:** Average peak frequency data collapsed across time to show main effect for genotype. *Pink1-/-* calls were significantly lower than WT across timepoints; average peak frequency at 8 months was greater than at 4 months, regardless of genotype. **E:** Average bandwidth. Bandwidth was significantly smaller at 8 and 12 months than 4 months for both genotypes. **F:** Duration. *Pink1-/-* calls were significantly longer than WT calls at the 8-month timepoint only. dB: decibels. Hz: Hertz . * p<0.05. **p<0.01. ***p<0.001. Error bars indicate +/- SEM.

3.3.2: Anxiety

Inter-rater reliability was greater than 0.90 for plus maze measurements. Because a number of rats of both genotypes (n=8 to 17 per timepoint) did not enter the open arms of the

maze at some timepoints, entries into and duration in open arms were not used as outcome measures.

There was no interaction effect between genotype and timepoint on duration in closed arms of the maze ($F(2,59)=1.02, p=0.37$). There was a significant main effect of timepoint ($F(2,59)=5.02, p=0.01$) on duration in closed arms of the maze. Rats spent the least amount of time in closed arms of the maze at 8 months (mean 4=105.83, mean 8=86.36, mean 12=122.01). Rats spent less time in closed arms at 8 months than 4 months, but this did not reach statistical significance in post-hoc testing ($t(59)=1.73$, $p=0.08$). Rats spent more time in closed arms at 12 months than 8 months ($t(59=-3.16$, $p=0.003$). Four-month and 12-month timepoints did not differ in this measure ($t(59)=-1.45, p=0.15$). There was no main effect of genotype ($f(1,30)=0.84, p=0.37$).

There was no interaction between genotype and timepoint on number of closed arm entries ($F(2,59)=1.26$, $p=0.29$). There were significant main effects of Timepoint ($F2,59)=7.18$, $p=0.0016$) and Genotype ($F(1,30)=18.29$, $p=0.0002$) on number of entries into closed arms of the maze. Pink1-/- rats entered closed arms more often than WT rats (mean of 11.8 vs 8.17, respectively) ($t(30)=4.28$, $p=0.0002$). Averaged across genotypes (mean 4mo= 11.3 entries, mean 8mo= 8.9 entries, mean 12mo=9.7 entries), there were significant differences between 4 and 8 months ($t(59)=3.7, p=0.001$), and 4 and 12 months ($t(59)=2.53$, $p=0.03$), but not between 8 and 12 months ($t(59)=-1.2$, $p=0.46$).

Because overall differences in movement could potentially influence performance on the elevated plus maze, a ratio of the number of closed arm entries divided by closed arm time to total number of entries into any arm divided by total time in any arm was calculated. Lower

numbers indicate increased preference for closed arms, and are thus though to be indicative of anxiety. There was no interaction between timepoint and genotype ($F(2,59)=2.32$, $p=0.11$) on this ratio. There was a main effect of genotype ($F(1,30)=4.24$, $p=0.048$). The closed ratio for Pink rats (mean=1.03) was smaller than for WT rats (mean=1.15) ($t(59)=-2.06$, $p=0.048$), indicating greater preference for closed arms. There was no main effect of timepoint ($F(2,59)=3.0$, $p=0.058$).

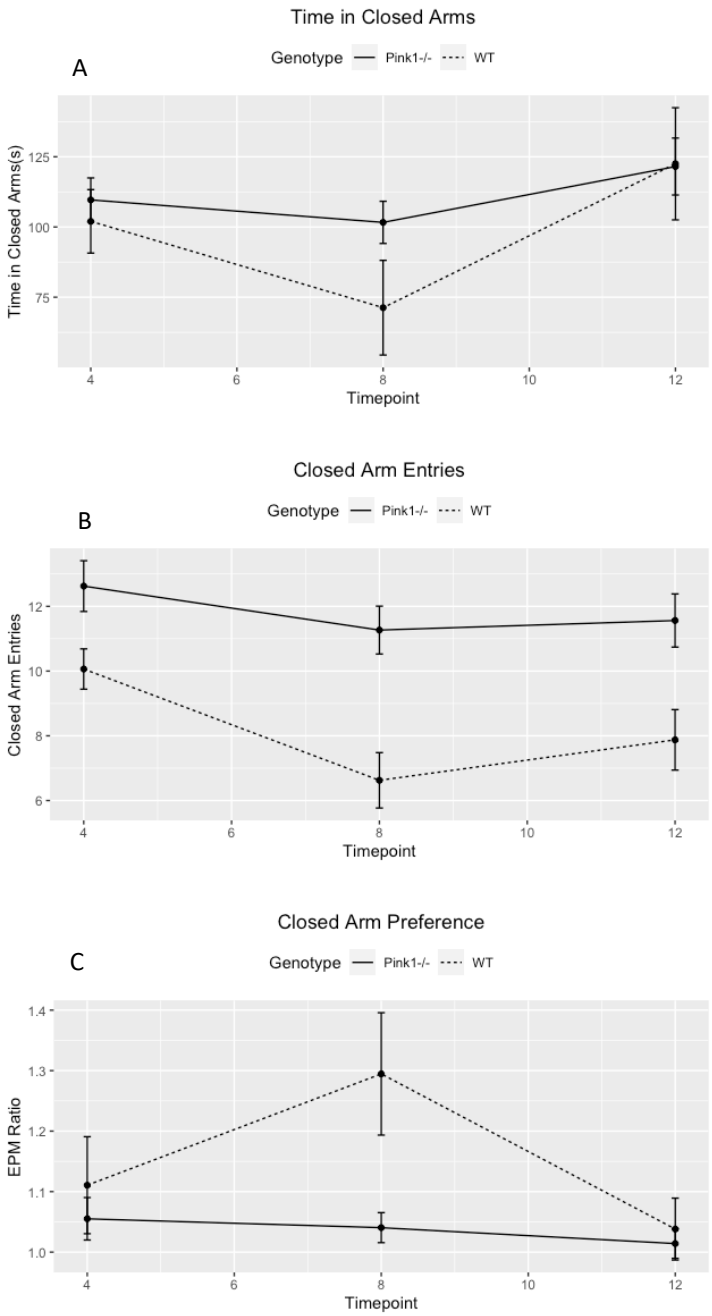

Figure 4. Anxiety Behavior. **A:** Time spent in closed arms. **B:** Entries into closed arms. **C:** Ratio of closed arm entries/time in closed arms to total entries into any arm/total time spent in any arm. *Pink1-/-* rats spent more time in closed arms than WT rats (B), and demonstrated a greater preference for closed arms(C). Number of entries into closed arms did not differ by genotype. Across genotypes, rats made more entries into closed arms at 4 months than 8 or 12 months, and spent the least amount of time in closed arms at 8 months. Error bars indicate +/- SEM.

3.3.3: Relationships Among Anxiety, Ultrasonic Vocalization, and Genotype

Four multiple linear regressions were fitted separately to assess USV outcomes of call intensity, bandwidth, duration, and average peak frequency with anxiety, genotype, and the interaction between anxiety and genotype as independent variables at the 12-month timepoint. A significant regression model was found for call intensity ($F(3,27) = 9.41$, $p=0.0002$), with an R^2 of 0.51. There was an interaction between genotype and time in the closed arms of the maze. For every one-second increase in closed arm time, USV Intensity decreased by 0.052dB in Pink1 -/- rats ($\beta=-0.052$, $t(30)=-3.79$, $p=0.0008$). Additionally, USV intensity was 4.39dB softer on average for WT-/- rats than for Pink1-/-s ($\beta=-4.39$, $t(30)= -2.19$, $p=0.0007$) when time in the closed arms of the maze was 0 seconds.

A significant regression was found for call duration ($F(3,27) = 3.57$, $p=0.027$), with an R^2 of 0.28. There was a non-significant interaction between genotype and time in the closed arms of the maze. For every one-second increase in closed arm time, USV duration decreased by 0.000069 seconds in Pink1 -/- rats ($\beta=-0.000069$, $t(30)=-1.73$, $p=0.095$). Additionally, USV duration was 0.016 seconds shorter on average for WT rats than for Pink1-/-s ($\beta=-0.016$, $t(30)= -2.86$, $p=0.008$) when time in the closed arms of the maze was 0 seconds.

A significant regression was also found for average peak frequency ($F(3,27) = 3.78$, $p=0.022$), with an $R^2$of 0.30. However, individual β-estimates were not able to significantly determine average peak frequency independently in this model, likely due to a high degree of collinearity.

A regression model to assess call bandwidth based on anxiety, genotype, and the interaction between anxiety and genotype was not significant ($F(3,27) = 1.16$, $p=0.34$), with an R^2 of 0.11.

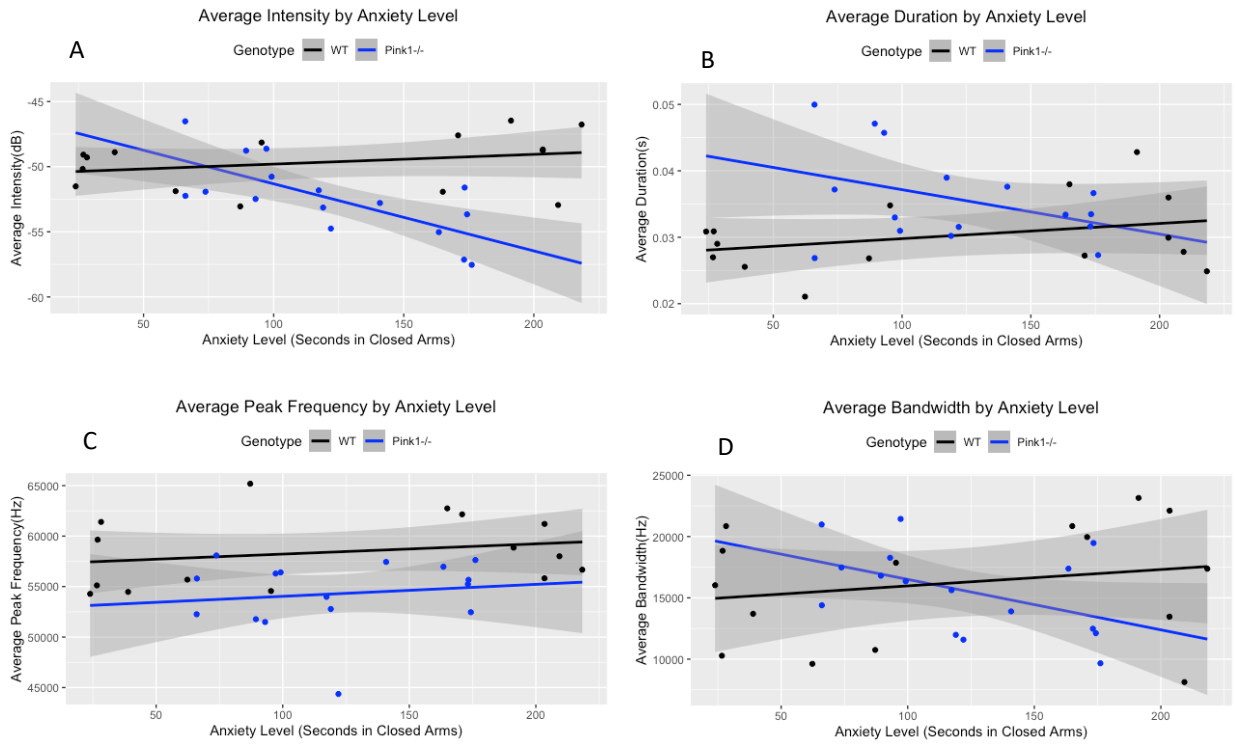

Figure 5. Relationships between anxiety behavior and ultrasonic vocalization. **A:** Average intensity by time spent in closed arms. **B:** Average duration by time spent in closed arms. **C:** Average peak frequency by time spent in closed arms. **D:** Average bandwidth by time spent in closed arms. Intensity and duration of *Pink1-/-* calls were associated with anxiety level, with increases in anxiety correlating to decreases in duration and intensity. Calls of WT rats were less strongly associated with anxiety. dB: decibels. Hz: Hertz. Gray shading indicates 95% confidence interval.

3.3.4: Immunohistochemistry

3.3.4.1: Tyrosine Hydroxylase

There were significantly fewer cells labeled for TH in the locus coeruleus for Pink1-/- rats (mean=2802, SD=537) than for WT rats (mean=3730, SD=1334) ($t(19.75)=-2.58$, $p=0.018$).

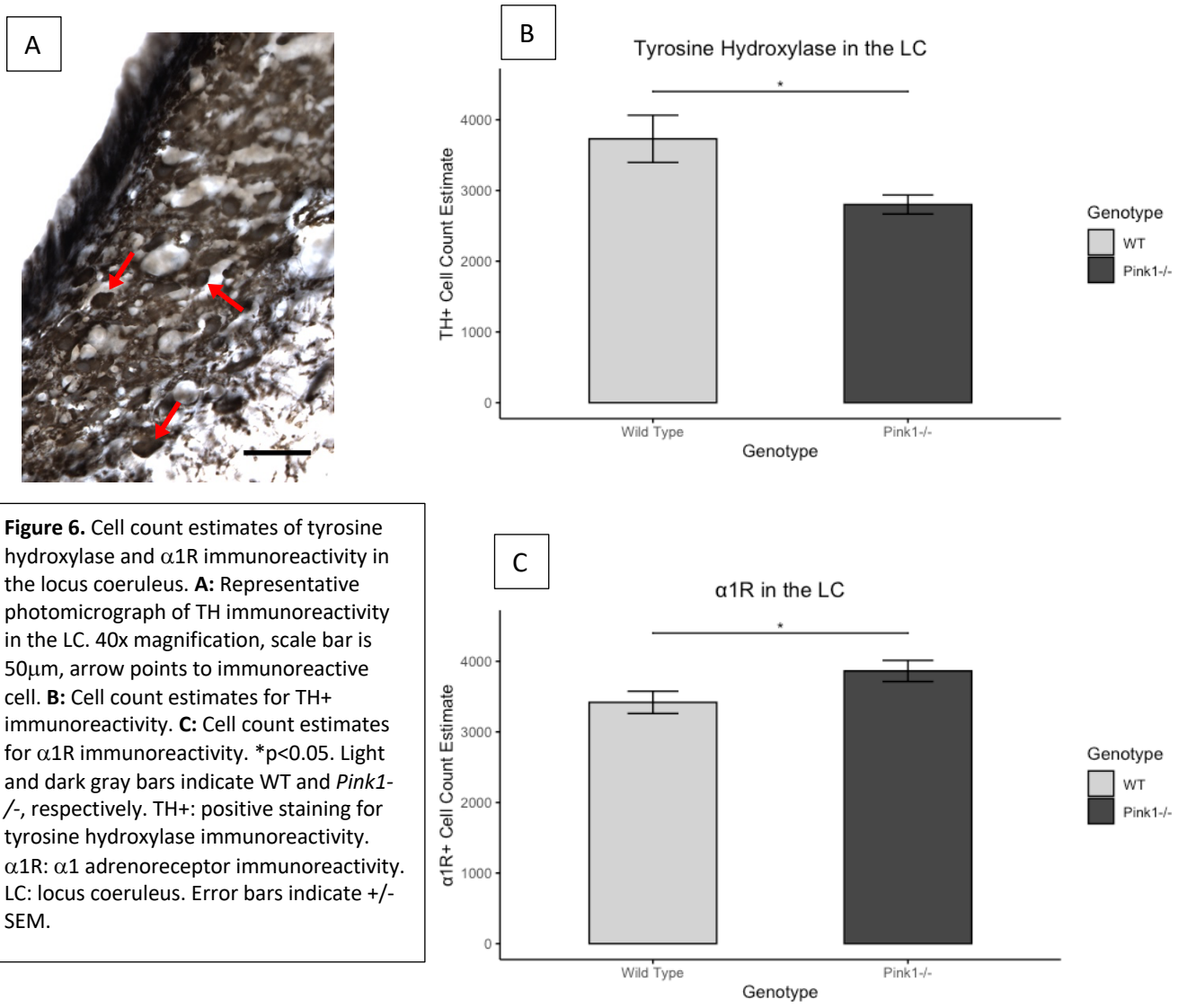

Figure 6. Cell count estimates of tyrosine hydroxylase and α1R immunoreactivity in the locus coeruleus. **A:** Representative photomicrograph of TH immunoreactivity in the LC. 40x magnification, scale bar is 50μm, arrow points to immunoreactive cell. **B:** Cell count estimates for TH+ immunoreactivity. **C:** Cell count estimates for α1R immunoreactivity. *p<0.05. Light and dark gray bars indicate WT and *Pink1-/-*, respectively. TH+: positive staining for tyrosine hydroxylase immunoreactivity. α1R: α1 adrenoreceptor immunoreactivity. LC: locus coeruleus. Error bars indicate +/- SEM.

3.3.4.2: Norepinephrine Transporter

There were no differences between genotypes in relative optical density of NET in the locus coeruleus (t(28.51)=-0.517, p=0.61), the NTS, (t(28.56)=-1.4, p=0.17), the 10N (t(28.65)=-1.45, p=0.16), or the nucleus ambiguus (t(28.41)=-1.54, p=0.14).

3.3.4.3: α1 Adrenoreceptors

There were more cells labeled for α1R in the in the locus coeruleus for Pink1-/- rats (mean=3863, SD=598) than for WT rats (mean=3418, SD=628) (t(29.93)=2.05, p=0.0495). There

were no differences between genotypes in the relative optical density of α1R in the NTS (t(29.48)=0.53, p=0.6). The number of cells labeled for α1R did not differ between genotypes in the 10N (t(29.82)=0.89, p=0.38) or in the nucleus ambiguus (t(29.71)=0.69, p=0.50)

3.3.4.4: β1 Adrenoreceptors

There was no difference in number of cells labeled for β1R in the locus coeruleus between genotypes (t(27.12)=1.07, p=0.29). The relative optical density of β1R in the NTS was significantly lower for Pink1-/- rats (mean=0.41, SD=0.076) than WT rats (mean=0.49, SD=0.075) (t(29.98)= -3.08, p=0.0044). There were more cells labeled for β1R in the in the 10N for Pink1-/- rats (mean=5872, SD=977) than for WT rats (mean=4883, SD=1124) (t(29.43)=2.65, p=0.013). In the nucleus ambiguus, Pink1-/- rats also had a greater number of cells labeled for β1R (mean=2974, SD=780) than WT rats (mean=2446,SD=521) (t(26.16)=2.25, p=0.033).

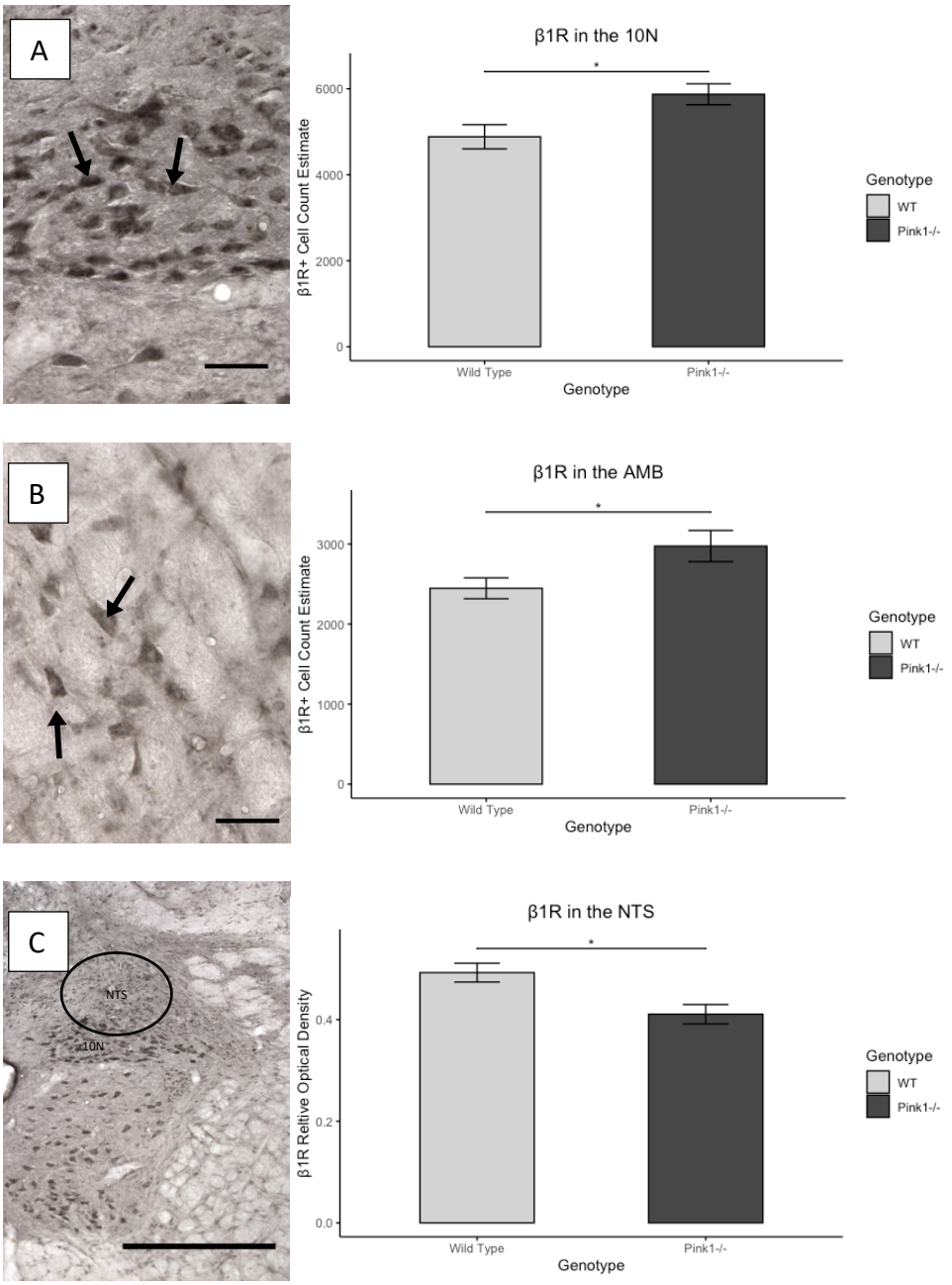

Figure 7: β1R immunoreactivity. **A:** Left: Representative photomicrograph of β1R immunoreactivity in the. Right: Cell count estimates in the 10N. **B:** Left: Representative photomicrograph of β1R in the AMB. Right: Cell count estimates in the AMB. **C:** Representative photomicrograph of β1R in the NTS. Relative optical density in the NTS. β1R cell count estimates in the 10N and AMB were greater for *Pink1-/-* rats than for WTs. Relative optical density in the NTS was lower for *Pink1-/-* rats than for WTs. A and B images at 40x magnification, scale bars are 50μm; C image taken at 10x magnification, scale bar is 500μm. Arrows in A and B point to immunoreactive cells. β1R: β1 adrenoreceptor immunoreactivity. 10N: dorsal motor nucleus of the vagus. NTS: Nucleus of the solitary tract. *p<0.05. Light and dark gray bars indicate WT and *Pink1-/-*, respectively. Error bars indicate +/- SEM.

3.3.5: Relationships Between Ultrasonic Vocalization and Noradrenergic Markers

3.3.5.1: Tyrosine Hydroxylase

Linear regressions were fitted to determine associations between USV outcomes and the number of cells labeled for TH in the locus coeruleus. Number of cells labeled for TH in the locus coeruleus did not determine USV bandwidth ($F(1,29)=0.20$, $p=0.66$, R^2 of 0.01), intensity ($F(1,29)=2.56$, $p=0.12$, R^2 of 0.08), average peak frequency ($F(1,29)=0.7$, $p=0.41$, R^2 of 0.02), or duration ($F(1,29)=1.14$, $p=0.29$, R^2 of 0.04).

3.3.5.2: Norepinephrine Transporter

Linear regressions were fitted to determine associations between USV outcomes and the relative optical density of NET in the locus coeruleus, NTS, 10N and nucleus ambiguus. No significant regressions were found (Table 2).

3.3.5.3: α1 Adrenoreceptors

Linear regressions were fitted to determine associations between USV outcomes and number of cells labeled for α1 receptors in the locus coeruleus, nucleus ambiguus, and 10N, and relative optical density of α1 receptors in the NTS. No significant regressions were found (Table 2).

3.3.5.4: β1 Adrenoreceptors

Linear regressions were fitted to determine associations between USV outcomes and number of cells labeled for β1 receptors in the locus coeruleus, nucleus ambiguus, and 10N, and relative optical density of β1 receptors in the NTS. No significant correlations were found for duration or bandwidth. Three independent variables were found to be significantly

associated with intensity. In the NTS, ($F(1,29) = 4.74$, $\beta = 14.59$, 95% confidence interval=1.46 to 27.73 p=0.04), $R^2 = 0.14$) for every unit increase in relative optical density, intensity increased by 14.59dB. In the 10n ($F(1,29) = 13.57$, $\beta = -0.002$, 95%CI= -0.002 to -0.0007 p=0.001, $R^2 = 0.320$), for every additional cell labeled for β1R, intensity decreased by 0.002dB. In the nucleus ambiguus ($F(1,29) = 9.43$, $\beta = -0.002$, 95%CI=-0.003 to -0.0006. p=0.005, $R^2 = 0.25$), for every additional cell labeled for β1R, intensity decreased by 0.002dB.

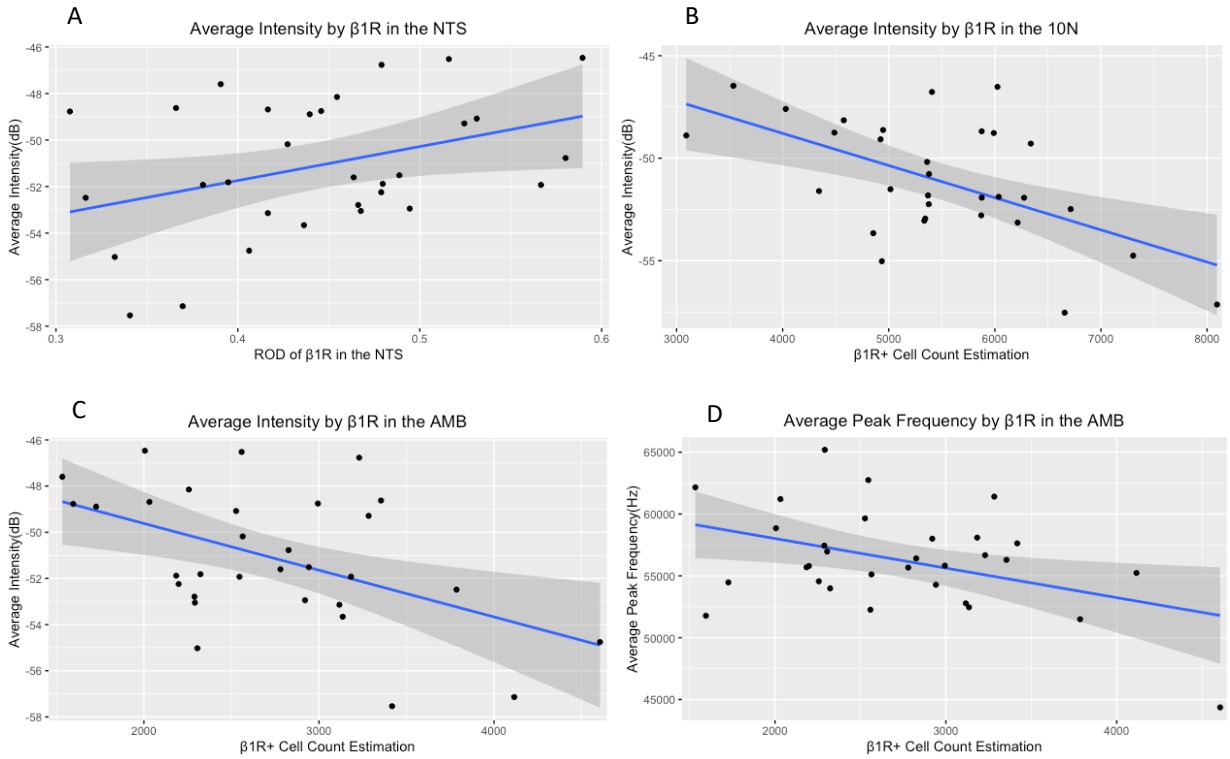

Figure 8: Relationships between β1R immunoreactivity and ultrasonic vocalization intensity and average peak frequency. **A:** Average intensity by ROD of β1R in the NTS. **B:** Average intensity by β1R+ cell count estimates in the 10N **C:** Average intensity by β1R cell count estimates in the AMB. **D:** Average peak frequency by β1R cell count estimates in the AMB. Increases in cell count estimates in the AMB and 10N were associated with decreases in average intensity, and decreases in ROD in the NTS were associated with increases in intensity. Decreases in cell count estimation in the AMB were associated with decreases in Average peak Frequency. β1R: β1 adrenoreceptor immunoreactivity. 10N: dorsal motor nucleus of the vagus. AMB: nucleus ambiguus. NTS: Nucleus of the solitary tract. dB: decibels. Hz: Hertz. ROD: relative optical density. Gray shading indicates 95% confidence interval.

In addition, cell count estimations for β1R-labeled cells in the nucleus ambiguus were significantly associated with average peak frequency ($F(1,29) = 6.3$, $\beta = -2.39$, 95%CI=-4.26 to -

0.52, p=0.02), with an R^2 of 0.18). For every additional cell labeled for β1R in the nucleus ambiguus, average peak frequency decreased by 2.39Hz.

Table 2

Noradrenergic Marker	**Outcome Measure(s)**	**Independent Variables**	**Results**
Tyrosine Hydroxylase	Average Bandwidth	US in the LC	F(1,29)=0.20, p=0.66), R^2=0.01.
	Average Intensity	US in the LC	F(1,29)=2.56, p=0.12), R^2=0.08.
	Average Peak Frequency	US in the LC	F(1,29)=0.7, p=0.41), R^2=0.02.
	Average Duration	US in the LC	F(1,29)=1.14, p=0.29), R^2=0.04.
Norepinephrine Transporter	Average Bandwidth	ROD in the LC	F(1,29)=1.47, p=0.24, R^2=0.05.
		ROD in the NTS	F(1,28)=0.28, p=0.6, R^2=0.01.
		ROD in the 10N	F(1,28)=0.32, p=0.58, R^2=0.01.
		ROD in the AMB	F(1,29)=0.05, p=0.82, R^2=0.002.
	Average Intensity	ROD in the LC	F(1,29)=0.29, p=0.6, R^2=0.01.
		ROD in the NTS	F(1,28)=0.69, p=0.41, R^2=0.02.
		ROD in the 10N	F(1,28)=0.62, p=0.44, R^2=0.02.
		ROD in the AMB	F(1,29)=0.17, p=0.68, R^2=0.01.
	Average Peak Frequency	ROD in the LC	F(1,29)=0.31, p=0.58), R^2=0.01.
		ROD in the NTS	F(1,28)=2.46, p=0.13 R^2=0.08.
		ROD in the 10N	F(1,28)=1.25, p=0.27, R^2=0.04.
		ROD in the AMB	F(1,29)=0.96, p=0.34, R^2=0.03.
	Average Duration	ROD in the LC	F(1,29)=0.61, p=0.44), R^2=0.02.
		ROD in the NTS	F(1,28)=0.18, p=0.67), R^2=0.006.
		ROD in the 10N	F(1,28)=0.1, p=0.76), R^2=0.003.
		ROD in the AMB	F(1,29)=0.03, p=0.86), R^2=0.001.

Table 2 continued

Noradrenergic Marker	Outcome Measure(s)	Independent Variables	Results
β1 Receptor	Average Bandwidth	US in the LC	F(1,29)=0.36, p=0.55), R^2=0.01.
		ROD in the NTS	F(1,29)=1.19, p=0.28), R^2=0.04.
		US in the 10N	F(1,29)=1.92, p=0.17), R^2=0.06.
		US in the AMB	F(1,29)=1.65, p=0.21), R^2=0.05.
	Average Intensity	US in the LC	F(1,29)=0.84, p=0.37), R^2=0.03.
		ROD in the NTS	F(1,29) =4.74, p=0.04), $R^2$0.14.
		US in the 10N	F(1,29) =13.57, p=0.001), R^2=0.32.
		US in the AMB	F(1,29) =9.43, p=0.005), R^2=0.25.
	Average Peak Frequency	US in the LC	F(1,29)=0.15, p=0.70), R^2=0.01.
		ROD in the NTS	F(1,29)=3.73, p=0.06), R^2=0.11
		US in the 10N	F(1,29)=1.77, p=0.19), R^2=0.06.
		US in the AMB	F(1,29) =6.3, p=0.02), R^2=0.18.
	Average Duration	US in the LC	F(1,29)=0.57, p=0.45), R^2=0.001.
		ROD in the NTS	F(1,29)=0.42, p=0.52), R^2=0.01.
		US in the 10N	F(1,29)=0.46, p=0.51), R^2=0.02.
		US in the AMB	F(1,29)=0.08, p=0.78), R^2=0.003.
α 1 Receptor	Average Bandwidth	US in the LC	F(1,29)=0.03, p=0.87), R^2=0.001.
		ROD in the NTS	F(1,29)=0.17, p=0.69), R^2=0.006.
		US in the 10N	F(1,29)=0.63, p=0.44), R^2=0.02.
		US in the AMB	F(1,29)=0.35, p=0.56), R^2=0.01.
	Average Intensity	US in the LC	F(1,29)=0.49, p=0.48), R^2=0.02.
		ROD in the NTS	F(1,29)=0.23, p=0.64), R^2=0.01.
		US in the 10N	F(1,29)=2.96, p=0.10), R^2=0.09.
		US in the AMB	F(1,29)=1.04, p=0.32), R^2=0.04.
	Average Peak Frequency	US in the LC	F(1,29)=0.24, p=0.63), R^2=0.008.
		ROD in the NTS	F(1,29)=0.69, p=0.41), R^2=0.02.
		US in the 10N	F(1,29)=0.12, p=0.73), R^2=0.004.
		US in the AMB	F(1,29)=0.28, p=0.60), R^2=0.009.
	Average Duration	US in the LC	F(1,29)=0.67, p=0.42), R^2=0.02.
		ROD in the NTS	F(1,29)=1.16, p=0.29), R^2=0.04.
		US in the 10N	F(1,29)=0.31, p=0.58), R^2=0.01.
		US in the AMB	F(1,29)=2.57, p=0.12), R^2=0.08.

Table 2 Linear regression result assessing relationships between brainstem noradrenergic markers and vocal acoustic outcomes. US: unbiased stereology. ROD: relative optical density. LC: locus coeruleus. NTS: nucleus of the solitary tract. 10N: dorsal motor nucleus of the vagus. AMB: nucleus ambiguus.

3.3.6: Relationships Between Anxiety and Noradrenergic Markers

3.3.6.1: Tyrosine Hydroxylase

A linear regression was fitted to determine associations between duration in closed arms of the plus maze and the number of cells labeled for TH in the locus coeruleus. A significant association was found ($F(1,30 = 4.6$, β=-0.02, 95%CI= -0.04to -0.001 p=0.04), with an R^2 of 0.13. For every additional cell labeled for TH in the locus coeruleus, duration in closed arms of the plus maze decreased by 0.02 seconds.

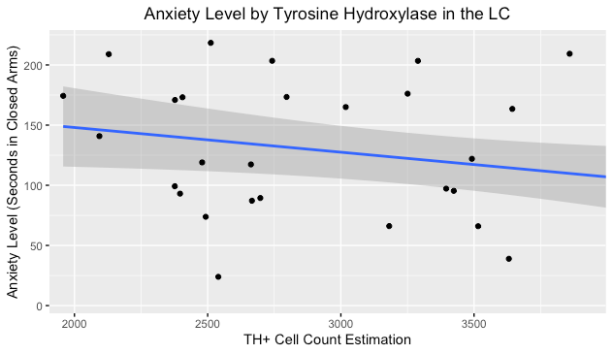

Figure 9: Relationship between anxiety behavior and cell count estimation for TH immunoreactivity in the LC. Greater estimate of TH+ cells in the LC was associated with decreased number of seconds in closed arms of the plus maze, indicating decreased anxiety. TH+: tyrosine hydroxylase immunoreactivity. LC: locus coeruleus. Gray shading indicates 95% confidence interval.

3.3.6.2: Norepinephrine Transporter

Linear regressions were fitted to determine associations between duration in closed arms of the plus maze and OD of NET in the locus coeruleus, NTS, 10N and nucleus ambiguus. Regressions using the locus coeruleus (F(1,30)=0.26, p=0.61, R^2=0.009), NTS (F(1,29)=0.55, p=0.47, R^2=0.02), 10N (F(1,29)=0.35, p=0.56, R^2=0.01), and nucleus ambiguus (F(1,30)=0.61, p=0.44, R^2=0.02) were not significant.

3.3.6.3: α1 Adrenoreceptors

Linear regressions were fitted to determine associations between duration in closed arms of the plus maze and the number of cells labeled for α1R in the locus coeruleus, 10N and

nucleus ambiguus, and on the relative optical density of α1R in the NTS. Regressions using the locus coeruleus (F(1,30)=1.62, p=0.21, R^2=0.05), NTS (F(1,30)=0.06, p=0.8, R^2=0.002), 10N (F(1,30)=0.03, p=0.87, R^2=0.001) and nucleus ambiguus (F(1,30)=.69, p=0.41, R^2=0.02) were not significant.

3.3.6.4: β1 Adrenoreceptors

Linear regressions were fitted to determine associations between duration in closed arms of the plus maze and the number of cells labeled for β1R in the locus coeruleus, 10N and nucleus ambiguus, and on the relative optical density of β1R in the NTS. Regressions using the locus coeruleus (F(1,30)=3.51, p=0.07, R^2=0.10), NTS (F(1,30)=0.1,p=0.76, R^2=0.003), 10N (F(1,30)=0.43, p=0.51, R^2=0.01) and nucleus ambiguus (F(1,30)=0.1, p=0.75, R^2=0.003) were not significant.

3.4: Discussion

The current study demonstrates that several aspects of the noradrenergic system are disrupted in the brainstem of the *Pink1-/-* rat model of PD. Further, we have demonstrated that these neural differences are not only associated with anxiety and vocal impairment, but that anxiety and genotype interact to influence changes to vocalization that mimics some aspects of hypokinetic dysarthria in humans with PD. These findings are relevant to a growing body of research that suggests noradrenergic dysfunction is a primary contributing factor to early and non-hallmark signs of PD (Cash et al., 1987; Espay et al., 2014; Kreiner et al., 2019; Lewitt, 2012; Marien et al., 2004; Rommelfanger & Weinshenker, 2007; Tredici & Braak, 2013; Vazey & Aston-Jones, 2012).

While hallmark gross motor deficits of PD associated with nigro-striatal dopaminergic impairment respond strongly to dopamine replacement therapies such as Levodopa, interventions for vocal impairment and anxiety remain inadequately treated (Calleo et al., 2015; Pinho et al., 2018; Ramig et al., 2018; Renfroe et al., 2016). Although it is clear that neural mechanisms of vocal impairment and anxiety lie at least partially outside of the classic nigro-striatal dopaminergic impairment, details of these mechanisms remain poorly understood. The relative lack of adequate treatments for these common aspects of PD is a direct consequence of our lack of understanding of their disease-specific pathophysiology.

Anxiety-Like Behavior

The current findings are relevant to future translational work assessing anxiety in PD. Consistent with previous investigations of anxiety in the *Pink1-/-* rat model of PD, (Dave et al., 2014; Grigoruta et al., 2019; Marquis et al., 2020), we identified an increase in anxiety-like behavior in *Pink1-/-* rats compared to WT controls. The current study followed rats 50% further (12 months vs 8 months and earlier) than in previous work with this progressive model, allowing for a more-nuanced interpretation. This longitudinal data revealed a U-shaped curve in anxiety-like behaviors for WT rats, with an increase in exploratory activity (e.g., decreased anxiety-like behavior) at 8 months compared to 4 and 12 months. This U-shaped curve is expected in WT rodents (Lafaille & Féron, 2014) , but was absent in the *Pink1-/-* rat (Figure 4). While *Pink1-/-* rats demonstrated increased anxiety-like behavior across timepoints, the spike at the 8 month timepoint is important in that it is thought to represent early-to-mid-stage progression in human PD, a time when prevalence of anxiety also increases (Pontone et al., 2009). While the exact etiology of anxiety in PD is not yet well understood, it is likely a

combination of disease-specific biological factors and psychological responses to disease progression and motor manifestation (Dissanayaka et al., 2010). Future work that longitudinally controls for increased stressors in the *Pink1-/-* rat (Grigoruta et al., 2019) may help to disentangle biological and psychological contributors to anxiety in PD.

Relationships between anxiety and vocalization

Consistent with previous studies (Grant et al., 2018; Grant, Kelm-nelson, et al., 2015; Johnson et al., 2020; Kelm-nelson et al., 2018; Kelm-Nelson et al., 2015), vocalizations of Pink1-/- rats were reduced in intensity and had a lower average peak frequency compared to WT controls. A novel finding of the study was the genotype-dependent relationship between anxiety and vocalization. Specifically, the vocalizations of *Pink1-/-* rats were associated with anxiety level, whereas those of WT controls were not. Relationships between voice and speech, and changes to cognitive/emotional state or anxiety level in humans have been well-documented (Dietrich et al., 2019; Helou et al., 2013; van Mersbergen et al., 2008, 2015; van Mersbergen & Delany, 2014; van Mersbergen & Lanza, 2018). This is intuitively understood when observing degradation of voice quality and speaking performance in the setting of anxiety during public speaking. Interestingly, this phenomenon of degraded voice quality and speaking performance during anxiety can be reduced through various methods of cognitive-behavioral training (Bodie, 2010; Glassman et al., 2016; Shaw et al., 2020). Our understanding of the neurobiological underpinnings of the phenomena are generally limited to physical measurements of autonomic arousal (Bodie, 2010). Interestingly, normalization of these measures of arousal occurs during targeted cognitive-behavioral intervention, and have been

simultaneously correlated with improved performance and with activation of higher cortical structures (Glassman et al., 2016), suggesting that the process by which degradation of voice and speech performance in the setting of anxiety can be overcome is "top-down" in nature. In this context, a possible explanation for the influence of genotype on the interaction between anxiety level and vocal acoustic outcomes is a reduced capacity to "over-ride" the relationship in *Pink1-/-* model. This might explain our observation that anxiety-based disruption to vocalization is present for *Pink1-/-* rats but not for WT controls, and may be relevant to human clinical translation, in which some patients with PD undergoing voice therapy do not respond to treatment (Cannito et al., 2012).

In order to explore these correlative findings further future research should experimentally control the "dose" of anxiety given to each rat, assess how anxiety "dose" influences vocal outcomes, and then assess how genotype interacts with the relationship between anxiety "dose" and vocal outcomes. Because continuous assessment of anxiety state and controlled modulation of anxiety dose is challenging in animal models, such a design may be more feasible in humans with PD than in rats.

An additional factor to consider regarding the clinical translation of these findings is the fact that modulation of vocalization and modulation of anxiety are related in their modulation of respiration. In humans, the use of respiratory modulation in the treatment of anxiety is well-established (Chen et al., 2017; Han et al., 1996; Jerath et al., 2015; Park et al., 2013; Zaccaro et al., 2018). Because of the process by which phonation occurs, any intentional change to vocalization involves a change to respiration and thus voice exercises used in the treatment of voice disorders necessarily modulate breathing. In our laboratory, we have demonstrated both

that targeted vocalization exercise changes USVs, and improves some aspects of calls in rat models of PD (Johnson et al., 2013; Kelm-Nelson et al., 2015, 2016; Russell et al., 2010), and that changes to respiration are present in the *Pink1-/-* rats (Johnson et al., 2020). In an effort further explore relationships among vocalization, anxiety and respiratory modulation, future experiments that involve targeted vocal exercise in rat models of PD should assess whether there is a cross-over effect of vocal exercise on anxiety level.

Brainstem Noradrenergic Markers

It has been well-established that noradrenergic degeneration in the locus coeruleus precedes and is more extensive than dopaminergic degeneration in the nigro-striatal pathway in humans with PD (Braak et al., 2003; Braak et al., 2004). The reduced number of cell bodies immunoreactive for TH in the locus coeruleus in *Pink1-/-* rats compared to WT controls in the current study mirrors human findings, and replicates previous work in our laboratory (Grant et al., 2015). Additionally, the number of cell bodies reactive for α1 receptors was *increased* in the locus coeruleus of *Pink1-/-* rats, compared to WT controls. Increased expression of α1 receptors has been demonstrated in humans with PD (Cash et al., 1984; Sharp et al., 2007), and over-expression of the α1B receptor subtype in murine models has resulted in central nervous system apoptosis and was associated with Parkinson-like hind-limb disorders (Zuscik et al., 2000). While the mechanism by which this increased expression occurs is not understood, it is thought to be a compensatory response to reduced bioavailability of norepinephrine.

There were no differences between genotype groups in the relative optical density of NET in any brain region assessed. It has been demonstrated that alterations in relative

expression of α-synuclein influences activity of NET by regulating its transportation to the cell membrane, which may provide a partial explanation for noradrenergic dysregulation in PD (Jeannotte & Sidhu, 2007). The absence of genotype differences in NET in the current study may have been due to the limited sensitivity of optical density measurements.

Differences in noradrenergic markers in the other brainstem structures investigated were minimal with the exception of β1 receptor, with an increase in number of positively-labeled cell bodies in the nucleus ambiguus and 10N, and a decrease in the relative optical density in the NTS. While the roles of the nucleus ambiguus and NTS in vocal communication are clear in that they house laryngeal motoneurons and are the primary target for laryngeal sensory information, respectively, it 10N is also important for laryngeal function in that, in addition to being a preganglionic sensory nucleus, it contains targets for sensory information carried by the superior laryngeal nerve, and thus could be implicated in regulation of laryngeal movement (Kobler et al., 1994). Reduced vocal intensity, an important translational aspect of this rat model of vocal deficit in PD, was significantly associated with number of cell bodies labeled for β1 receptor in the nucleus ambiguus and 10N and by the relative optical density of β1 receptor in the NTS. This finding is consistent with previous studies that have correlated vocalization with noradrenergic disruption in the central nervous system (Grant et al., 2015; Kelm-Nelson, Trevino, et al., 2018). In light of previous work that has shown increased vocal intensity in WT rats who were intraperitoneally injected with the β1 receptor antagonist, propranolol (Grant et al., 2018), our finding of a negative correlation between β1 immunoreactive cell bodies in laryngeal motor nuclei suggests that β1 receptors may be an important pharmacologic target in the treatment of vocal impairment in PD. Contrary to

findings reported by Grant et al, 2015, number of TH immunoreactive cell bodies in the locus coeruleus was not significantly associated any USV parameters. A possible explanation for this discrepancy is methodological differences implemented when conducting cell count estimation and the timepoint when data was collected (in the current study, rats were 12 months versus 8 months). Interestingly, the number of TH immunoreactive cell bodies in the locus coeruleus was weakly correlated with anxiety level. Thus, while anxiety and vocalization are associated with one another and are associated with some brainstem noradrenergic factors, the brainstem noradrenergic factors investigated do not appear to modulate the relationship between anxiety and vocalization in this model of PD. Future studies should investigate vocalization, anxiety, and noradrenergic factors at higher cortical levels to further inform whether other aspects of noradrenergic might contribute to the relationship between anxiety and vocalization. Finally, it is likely that more-complex interactions involving multiple catecholaminergic or other neurotransmitter systems that could explain the relationships between vocalization and anxiety in PD more-completely.

Limitations

In the study of anxiety, researchers and clinicians often differentiate between trait anxiety and state anxiety. Trait anxiety is thought to reflect an aspect of personality that predisposes one to increases in state anxiety, which has been defined as a transient emotional state associated with physiological arousal and conscious awareness of perceived negative feelings about future events (Endler & Kocovski, 2001; Spielberger, 1966). While these two aspects of anxiety, by definition, interact with one another, they are distinct and are

represented by different functional connectivity profiles in the human brain (Saviola et al., 2020). Assessment of state and trait anxiety in humans with PD demonstrates that both are increased relative to controls (Mondolo et al., 2007), reiterating the relationship between these aspects of anxiety. In animal models of anxiety, correlations between trait and state anxiety are less clear, and performance on measures that are thought to reflect trait anxiety, such as free exploration paradigms, are not consistently associated with measures that are thought to reflect state anxiety, such as the elevated plus maze (Goes et al., 2009). While *Pink1-/-* rats demonstrated greater anxiety-like behavior on the elevated plus maze than WT controls at each timepoint in the current study, a limitation is that these are simply multiple and repeated measures of state anxiety. Nevertheless, a consistency between anxiety-like behavior in *Pink1-/-* rats and humans with PD was observed.

An additional limitation of the study is the assessment of neural tissue at the 12-month timepoint only. It is possible that genotypic differences in brainstem noradrenergic markers may be evident at different timepoints relative either to further disease progression or to neuroplastic compensation in response to earlier neurobiological changes. It must also be acknowledged that, while unbiased stereology and measurement of relative optical density allow for a high degree of spatial resolution in assessment of neural tissue differences, they may be less sensitive to genotypic differences than more-direct methods of measurement such as high-performance liquid chromatography or western blot analysis. Future studies would benefit from additional quantification methods to solidify understanding of brainstem NE differences.

Future Directions and Translational Research Implications

The relationship between vocal outcomes and anxiety in humans is also present in animal models of PD, and appears to be influenced by central noradrenergic systems. Tandem assessment of voice and anxiety in both health and disease, especially in PD, is likely to improve management of these signs/symptoms and to increase understanding of their disease-specific etiologies. Given the relationships among anxiety, vocalization, and central noradrenergic systems demonstrated in this study, future research with the *Pink1-/-* model of PD should investigate vocalization and anxiety during pharmacologic manipulation of norepinephrine. In an effort to guide translation to human populations, assessment of voice and anxiety in convenience samples of patients with PD who are being treated with medications that modify the noradrenergic system should be conducted.

Conclusions

Vocal deficits and anxiety are related in the *Pink1-/-* model of PD, and are both influenced by norepinephrine. Future investigations with increased control of anxiety level and noradrenergic manipulation will help to further clarify the disease-specific nature of these relationships.

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Chapter 4

Manipulation of Vocal Communication and Anxiety through Pharmacologic Modulation of Norepinephrine in the *Pink1-/-* Rat Model of Parkinson Disease

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Key Words: Parkinson Disease; Rat; Pink1; Ultrasonic Vocalization; Anxiety; Norepinephrine

Highlights:

- Atomoxetine and reboxetine reduce anxiety behavior in the *Pink1*-/- rat
- Atomoxetine, but not reboxetine, modulates ultrasonic vocalizations.
- Relationships among anxiety, vocalization and norepinephrine are non-linear

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Abbreviations: NE: Norepinephrine; PD: Parkinson Disease; *Pink1*-/- *Pink*1 gene knockout; USV: ultrasonic vocalization; WT: wild type

Abstract:
Vocal deficits and anxiety are common, co-occurring, and interacting signs of Parkinson Disease (PD) that have a devastating impact on quality of life. Both manifest early in the disease process. Unlike hallmark motor signs of PD, neither respond adequately to dopamine replacement therapies, suggesting that their disease-specific mechanisms are at least partially extra-dopaminergic. Because noradrenergic dysfunction is also a defining feature of PD, especially early in the disease progression, drug therapies targeting norepinephrine are being trialed for treatment of motor and non-motor impairments in PD. Research assessing the effects of noradrenergic manipulation on anxiety and vocal impairment in PD, however, is sparse**.** In this pre-clinical study, we quantified the influence of pharmacologic manipulation of norepinephrine on vocal impairment and anxiety in *Pink1-/-* rats, a translational model of PD that demonstrates both vocal deficits and anxiety. Ultrasonic vocalization acoustics, anxiety behavior, and limb motor activity were tested twice for each rat: after injection of saline and after one of three drugs. We hypothesized that norepinephrine reuptake inhibitors (atomoxetine and reboxetine) and a β receptor antagonist (propranolol) would decrease vocal impairment and anxiety compared to saline, without affecting spontaneous motor activity. Our results demonstrated that atomoxetine and reboxetine decreased anxiety behavior. Atomoxetine also modulated ultrasonic vocalization acoustics, including an increase in vocal intensity, which is almost always reduced in animal models and patients with PD. Propranolol did not affect anxiety or vocalization. Drug condition did not influence spontaneous motor activity. These studies demonstrate relationships among vocal impairment, anxiety, and noradrenergic systems in the *Pink1-/-* rat model of PD.

4.1: Introduction

Deficits in vocal communication and anxiety are common signs of Parkinson Disease (PD) that share a disease-specific timeline, influence with one another, and are unfortunately poorly understood and chronically undertreated. The locus coeruleus-noradrenergic system strongly influences both anxiety and vocal communication. Because noradrenergic dysfunction is central to parkinsonian neural pathology, simultaneous exploration of noradrenergic dysfunction relative to both vocal deficits and anxiety may lead to more-nuanced understanding of the disease-specific neural mechanisms that drive these impairments.

In addition to experiencing hallmark motor signs of bradykinesia, tremor, and postural instability, vocal communication impairment is present in up to 90% of individuals with PD (Anand & Stepp, 2015; Fox & Ramig, 1997; Huber & Darling, 2011; Logemann et al., 1978; Matheron et al., 2017; Sapir et al., 2008; Stepp, 2013), and up to 55% are diagnosed with anxiety (Broen et al., 2016; Yamanishi et al., 2013). These two common, "non-hallmark" signs of PD are distinct from hallmark motor signs in that they occur early in the disease process, often prior to the onset of classical motor deficits such as tremor and bradykinesia (Bezard & Fernagut, 2014; Braak et al., 2004; Broen et al., 2016; Dissanayaka et al., 2010; Hartelius & Svensson, 1994; Midi et al., 2008; Pont-sunyer et al., 2015). In further contrast to classical motor deficits, vocal impairment and anxiety have limited responses to standard pharmacologic interventions, such as levodopa, suggesting that their disease-specific mechanisms are at least partially extra-dopaminergic. Additionally, behavioral interventions such as Lee-Silverman Voice Treatment (LSVT LOUD®), and cognitive-behavioral therapy for anxiety, result in incomplete and transient improvements (Calleo et al., 2015; Pinho et al., 2018; Ramig et al., 2018; Renfroe et

al., 2016). Unfortunately neurosurgical interventions, such as deep brain stimulation, also have a limited effect, and can even exacerbate vocal impairment and anxiety (Couto et al., 2014; Skodda, 2012). As a consequence, vocal impairment and anxiety in PD remain undertreated.

Multiple lines of research have begun to converge on the fact that dysfunction of norepinephrine (NE) in the central nervous system is associated with both vocal impairment (Buddhala et al., 2015; Cash et al., 1987; Espay et al., 2014; Kreiner et al., 2019; Lewitt, 2012; Marien et al., 2004; Rommelfanger & Weinshenker, 2007; Tredici & Braak, 2013; Vazey & Aston-Jones, 2012) and anxiety (Benarroch, 2009; Berridge & Waterhouse, 2003; Dissanayaka et al., 2014); additionally, preliminary evidence in rat models of PD from our laboratory has shown that anxiety behaviors and vocal communication are correlated (see study 1). These observations further distinguish vocal impairment and anxiety from the hallmark motor signs of PD. In response, investigations into pharmacologic manipulation of NE in PD have begun (Espay et al., 2014; Jankovic, 2009; Kreiner et al., 2019; Lewitt, 2012; Marsh et al., 2009).

Studies of pharmacologic modulation of NE have resulted in improvements to both motor and non-motor aspects of PD (Espay et al., 2014; Lewitt, 2012). These include modification of attention, hallucination, cognitive impairments, freezing of gait, and response inhibition in humans (Jankovic, 2009; Vazey & Aston-Jones, 2012). In addition, it has been shown that modulation of NE in non-parkinsonian rats influences acoustic features of vocalization (Grant et al., 2018; Wright et al., 2012). Two norepinephrine reuptake inhibitors that have shown particular promise for modulating symptoms of PD are atomoxetine and reboxetine, both of which block NE transporter. Treatment with atomoxetine has been associated with improved executive function (Marsh et al., 2009) and reduced indices of

depression (D Weintraub et al., 2010) in PD, suggesting modulation of multiple cortical systems. Further, atomoxetine has been found to significantly reduce levels of anxiety in certain populations (Snircova et al., 2016). Reboxetine also improves signs/symptoms of depression as well as motor performance in human and animal studies (Espay et al., 2014; Kreiner et al., 2019) of PD, and there is emerging evidence that reboxetine is effective in the treatment of panic disorder and anxiety (Dannon et al., 2002; Stahl et al., 2002).

Noradrenergic receptor modulation is another method of altering central NE functions that shows promise for addressing some parkinsonian deficits. The β-adrenoreceptor antagonist, propranolol, is most-commonly prescribed for the treatment of hypertension. Because of its sympatholytic properties, researchers have hypothesized that propranolol might be used to treat anxiety since the mid 20th century (Wheatley, 1969). Administration of propranolol has resulted in decreased anxiety behavior in rodents (Robinson et al., 2019; Schank et al., 2008; Wohleb et al., 2011), as well as decreased anxiety in some types of anxiety disorders in humans (Head et al., 1996; Mealy et al., 1996; Meibach et al., 1987; Steenen et al., 2016; Wheatley, 1969). In addition, the use of propranolol modifies vocal communication not only in wildtype (WT) control- rats (Grant et al., 2018; Wright et al., 2012) and in healthy humans(Giddens et al., 2010), but has also resulted in improvement in levodopa-induced limb and trunk dyskinesias in humans with PD (Carpentier et al., 1996), and levodopa-induced trunk, limb, and orolingual dyskinesias in rat models of the disease (Barnum et al., 2012). Research on the effects of NE manipulation *in PD* on *anxiety* and *vocal impairment*, however, is sparse, and research on interactions among NE, vocal impairment, and anxiety is absent from the literature.

In this pre-clinical study, we measured vocal communication and anxiety following administration of three different drugs that modulate NE in rats with a knockout of the *Pink1* gene, an established model of PD (Dave et al., 2014; Grant et al., 2015; Johnson et al., 2020; Kelm-Nelson et al., 2015; Marquis et al., 2020). The *Pink1*-/- rat is based on a genetic form of early and progressive PD (PARK6) that is nearly identical to idiopathic PD (Dehay & Bezard, 2011); the *Pink1/-* rat has been well-validated as a model of vocal communication impairment in PD (Grant et al., 2015; Kelm-Nelson et al., 2015, 2018), and preliminary research from our laboratory has demonstrated increased anxiety in this model compared to WT controls (Marquis et al., 2020). We hypothesized that NE reuptake inhibitors (atomoxetine and reboxetine) and a β-adrenoreceptor antagonist (Propranolol) would decrease vocal impairment and anxiety, but would not change spontaneous motor activity. Further, we hypothesized that the relationships between anxiety and vocal impairment would be altered by each drug. Atomoxetine, Reboxetine and Propranolol were chosen because they have been used to treat anxiety through NE mechanisms. Other anxiolytic drugs (*i.e.* benzodiazepines) were not chosen because they are often associated with motor impairment (Gagnon et al., 1977).

While these drugs have been used to investigate and to treat other non-motor signs of PD, their effect on vocal impairment and anxiety remains unstudied. Because these drugs target NE functions with a high degree of specificity and have been shown to have an influence on other non-motor signs of PD, the study of their effect on vocal impairment and anxiety would clarify the role of NE functions in vocal impairment and anxiety in PD. These drugs in particular were also chosen because they are FDA-approved, facilitating clinical translation, and because they are not associated with fatigue or reductions in motor coordination.

4.2: Methods

4.2.1: Experimental Procedure

Pink1-/- rats underwent testing of anxiety followed immediately by recording of ultrasonic vocalizations (USVs) for acoustic analysis. After USV recording, rats also underwent assessment of spontaneous motor activity with the cylinder test. All behavioral measures are described in detail below. Each rat was tested at two time points at 8-months of age: once following saline injection and once following injection of atomoxetine, reboxetine, or propranolol. Order of saline versus drug was randomized and counterbalanced for each drug cohort. Time points were separated by three weeks.

4.2.2: Animals

Thirty-six eight-month-old *Pink1-/-* rats were randomly assigned to one of three groups: atomoxetine, reboxetine, and propranolol (n=12 rats per group). Sample size was determined from power calculations based on vehicle-dose differences for atomoxetine reported by Robinson(2008) (Robinson et al., 2008). To account for potential attrition (e.g. not vocalizing) and to pair-house house the rats, sample size of 12 rats was predicted to detect differences at the 0.05 significance level with 90% power. The 8-month age was chosen to reflect the age at which anxiety in the *Pink1-/-* is most apparent (see chapter 3). Each rat underwent anxiety testing on the elevated plus maze followed immediately by recording of USVs after both saline injection and drug injection. Saline and drug testing conditions were separated by three weeks in order to reduce habituation to the testing apparatus (Walf & Frye, 2007), and to ensure adequate drug washout (Grant et al., 2018; E. S. J. Robinson et al., 2008; Vermoesen et al., 2012). The order of saline and drug condition was randomly assigned using a random number generator and counterbalanced in each drug group to account for potential order effects.

Behavioral testing following both saline and drug administration occurred at the same interval following injection, and was determined by the half-life of the drug (see below). In addition, 12 female Long-Evans rats were used to elicit USVs (protocol below). These female rats were continually housed in the colony maintained by our laboratory for the purpose of ultrasonic vocalization elicitation in several ongoing studies. All animals were obtained from SAGE Labs (Envigo, Boyertown, PA). Rats were housed in pairs the Biomedical Research Model Services facilities of the UW School of Medicine and Public Health on a 12-hour reversed light-cycle. All behavioral testing was under red light during the dark period when rats are most active. Rats were handled and weighed weekly until testing and throughout the duration of the study. Standard animal husbandry and other handling practices and procedures were implemented, related to animal health monitoring, diet, cage, environmental control, and general exercise in accordance with institutional guidelines regarding animal experimentation. All procedures were approved by the University of Wisconsin-Madison School of Medicine and Public Health Animal Care and Use Committee (IACUC; protocol M005177-R01-A04) and were conducted in accordance with the NIH Guide for the Care and Use of Laboratory Animals, Eight Edition (National Research Council Committee for the Update of the Guide for the Care and Use of Laboratory, 2011).

4.2.3: Drugs and Drug Administration

Atomoxetine (Sigma-Aldrich, St. Louis, MO, PubChem SID 329831496) was dissolved in sterile isotonic saline at 3mg/ml, for a dose of 1.5 mg/kg, and injected intraperitoneally. Behavioral testing was performed 30 minutes after the injection. Reboxetine (MedChem Express, Monmouth Junction, NJ, PubChem SID 210280742) was dissolved in sterile isotonic

saline at 30mg/ml for a dose 30 mg/kg and injected intraperitoneally, with behavioral testing performed 30 minutes after the injection. Propranolol (Sigma-Aldrich, St. Louis, MO, PubChem CID: 62882) was also dissolved in sterile isotonic saline at 3mg/ml, for a dose 3 mg/kg and injected intraperitoneally. Behavioral testing was performed 20 minutes after the injection. Saline vehicle was injected intraperitoneally at the same doses as paired drugs, and behavioral testing was completed at the same interval following injection. Order of drug and saline were randomized and counterbalanced such that half of the rats received saline first, and half received the drug first. Doses of all drugs were chosen based on clinically translatable doses in humans (Dannon et al., 2002; Jankovic, 2009; Marsh et al., 2009; Stahl et al., 2002; Weintraub et al., 2010) and, in the case of propranolol, on doses that have shown changes to vocalization in WT rats (Grant et al., 2018).

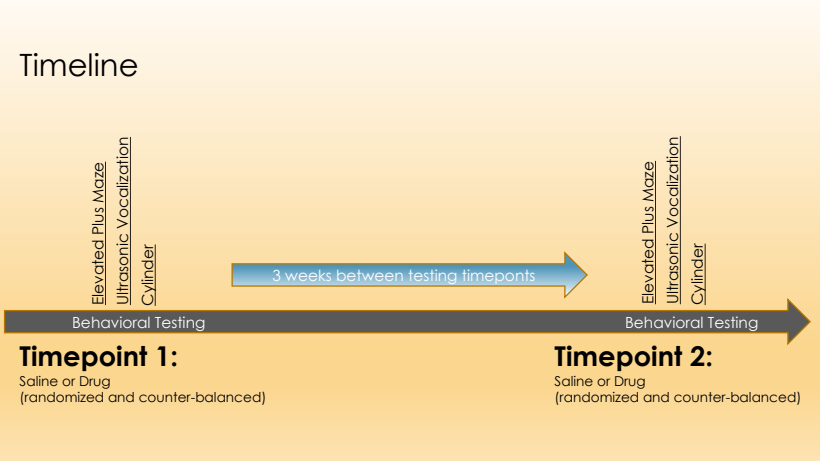

Figure 1: *Schematic of experimental timeline. Rats were divided into three experimental groups: propranolol (n at final analysis=12) atomoxetine (n at final analysis=11); reboxetine (n at final analysis=12.)Rats received either saline or drug and underwent behavioral testing at each timepoint. Order of drug versus saline was randomized and counterbalanced in each group.*

4.2.4: Behavioral Assays
4.2.4.1: Elevated Plus Maze:

At the designated time following injection, rats were placed on the elevated plus maze for the assessment of anxiety behavior (Hogg, 1996; Hopkins & Bucci, 2010; Pellow et al., 1985; Walf & Frye, 2007). The plus-shaped platform was constructed with 4 equally sized arms. Two arms are open with no walls (50x10cm), and the remaining two arms (opposite one another) have walls on 3 of 4 sides and an open top(50x10x50cm). Rats can enter each arm from a square in the center of the platform. Each arm is accessible from a square area in the center of the platform (Figure 1). The rats were placed in the center of the maze under red light facing an open arm, and were video-recorded for 5 minutes. Movement was tracked and analyzed using EthoVision software(Noldus Ethovision XT (Wageningen, Netherlands)). Outcome variables were total entries into open and closed arms and total time spent in open and closed arms in seconds. Increased time and frequency of entry into closed arms represent increased anxiety.

Figure 1: Elevated Plus Maze

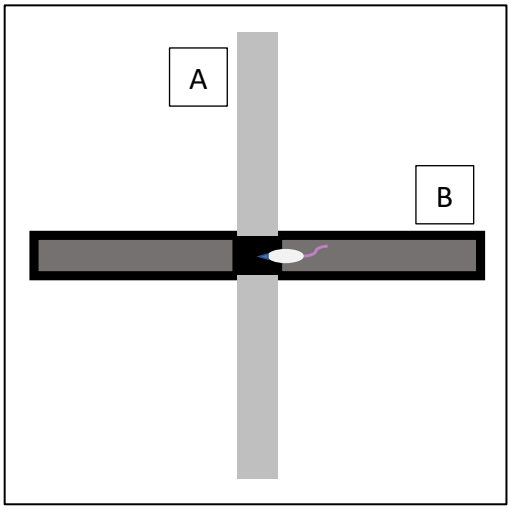

Figure 2: *Elevated Plus Maze. A: Open Arm; B: Closed Arm. Greater time in closed arms and greater number of entries into closed arms indicates increased anxiety behavior.*

4.2.4.2: Ultrasonic Vocalization Recording

Ultrasonic vocalizations (USVs) are produced in the 50-kiloHertz (kHz) range in a variety of conditions, especially to initiate and maintain conspecific contact. These USVs are complex and are produced in patterns. Modulation of some acoustic features of USVs, including mean peak frequency, bandwidth, and complexity, results in increased approach behavior of conspecifics (Pultorak et al., 2016), and can be considered to be at least partially goal directed(Bialy et al., 2000; Blanchard et al., 1992; Brudzynski & Pniak, 2002; McGinnis & Vakulenko, 2003; Riede, 2014; Wöhr et al., 2008). In rat models of PD and other neurologic diseases, acoustic analysis of USVs is commonly used to assay vocal deficits (Ahrens et al., 2009; Ciucci et al., 2007; Ciucci et al., 2009; Grant et al., 2015, 2018; Kelm-Nelson et al., 2015; Pultorak et al., 2016). To measure vocal communication in the current study, the following paradigm was used to elicit and record USVs: immediately following anxiety assessment on the elevated plus maze, test rats were placed in their home cage under a microphone attached to an ultrasonic recording system (CM16, Avisoft, Germany) with a 10-180kHz working frequency response range set to a 16-bit depth and 250kHz sampling rate. A female conspecific in estrus was then be placed in the male rat's home cage. After the male rat showed interest in the female, the female was removed. USVs from the male rat were recorded for 90 seconds and were analyzed offline using DeepSqueak 2.6 (Coffey et al., 2019) in MATLAB (version 9.5.0.944444[R2018b]; The MathWorks Inc., Natick, MA). DeepSqueak outputs a spectrogram and identifies calls using a pre-trained neural network (Coffey et al., 2019). Following automatic detection, calls were visually inspected and noise events misclassified as calls were rejected (Concha-Miranda et al., 2020; Lenell & Johnson, 2020) . Calls were categorized as either simple or complex based on visual inspection of the spectrogram. Simple calls were defined as having a

flat contour without repetitive frequency modulation (Ciucci et al., 2009); remaining calls were categorized as complex (Figure 2).

The following parameters were extracted: intensity as measured by power spectral density in decibels(dB)/kilohertz(kHz), duration in seconds, principal frequency (the median frequency of the call contour) in kHz, sinuosity (a measure reflective of call complexity defined as the ratio between the Euclidean distance of a straight line and curvilinear length along a curve; simple calls have sinuosity near 1, and complex calls have a greater sinuosity) , call bandwidth in kHz, and tonality (1 minus the geometric mean of the power spectrum divided by the arithmetic mean) as a measure of distinguishability of the signal of the contour from noise (higher numbers indicate less background noise present in the call). Data from individual calls were averaged for each animal at each recording timepoint for statistical analyses.

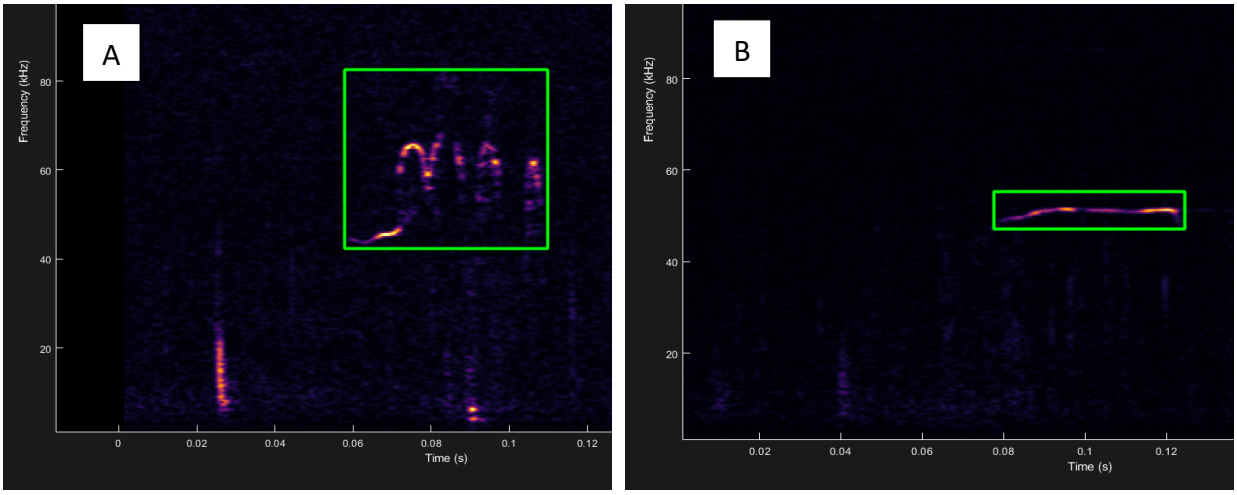

Figure 3: *Examples of spectrograms of ultrasonic vocalizations obtained from DeeqSqueak with time in seconds on the x-axis and frequency in kHz on the y-axis. A: Green box surrounding a complex call; note the frequency modulation between 40 and 65kHz. B: Green box surrounding a simple call; notice the flatness of contour with limited change in frequency around 50kHz.*

4.2.4.3: Cylinder Test

Spontaneous movement was assessed via the cylinder test (Fleming et al., 2004), as differences in spontaneous movement could potentially influence exploratory behavior on the elevated

plus maze. Immediately following USV testing, rats were placed in a 30cm by 20cm cylinder that was clear and positioned vertically to encourage rearing and vertical exploratory behavior for the assessment of general motor function. The cylinder was placed on top of a clear plastic box and rat behavior was videorecorded for two minutes. Videos were analyzed off-line to assess the number of forelimb movements, hindlimb movements, rears, and lands.

4.2.5: Statistical Analysis

For each drug, paired t-tests were used to compare spontaneous movement, anxiety, and USV acoustic outcome measures between drug group and saline group. USV analysis was performed with all calls for primary analysis, as well as with simple calls and complex calls separately in post-hoc secondary analysis. Linear mixed effects regression models were used in exploratory analysis to assess the interaction between drug condition and anxiety level on USV outcomes. Corrections for multiple comparisons were not performed due to the exploratory nature of this work and associated type II statistical error. Statistical analyses were performed with a significance level of 0.05 using software R (version 3.6.0) and SAS (version 9.4).

4.3: Results:

4.3.1: Cylinder

4.3.1.1: Propranolol

Cylinder outcomes of number of hindlimb movements ($t(10)=0.74$, $p=0.47$), forelimb elevations ($t(10)=1.03$, $p=0.33$), forelimb returns to the floor ($t(10)=0.73$, $p=0.48$), rears ($t(10)=0.41$, $p=0.69$), lands ($t(10)=0.18$, $p=0.86$), and rears plus lands ($t(10)=0.3$, $p=0.77$) all did

not differ by between saline and propranolol. One rat was not video-recorded.

4.3.1.2: Atomoxetine

Cylinder outcomes of number of hindlimb movements (t(10)=1.55, p=0.15), number of forelimb elevations (t(10)=1.07, p=0.31), number of forelimb returns to the floor (t(10)=0.41, p=0.69), number of rears (t(10)=0.98, p=0.35), number of lands (t(10)=0.74, p=0.48), and number of rears plus lands (t(10)=0.87, p=0.41) did not differ between saline and atomoxetine. One rat expired prior to study initiation.

4.3.1.3: Reboxetine

Cylinder outcomes for the number of hindlimb movements (t(11)=2.034, p=0.067), number of forelimb elevations (t(11)=1.29,p=0.22), number of forelimb returns to the floor (t(11)=0.51,p=0.607), number of rears (t(11)=1.95,p=0.08), number of lands (t(11)=-1.79, p=0.1), and number of rears plus lands (t(11)=1.88,p=0.09) did not differ by saline and reboxetine.

4.3.2: Anxiety

4.3.2.1: Propranolol

Anxiety outcomes of time spent in open arms of the plus maze (t(11)=0.13, p=0.9), time spent in closed arms of the plus maze (t(11)=0.66, p=0.52), number of entries into open arms of the plus maze (t(11)=0.57, p=0.58), and number of entries into closed arms of the plus maze (t(11)=1.6, p=0.14) did not differ between saline and propranolol conditions.

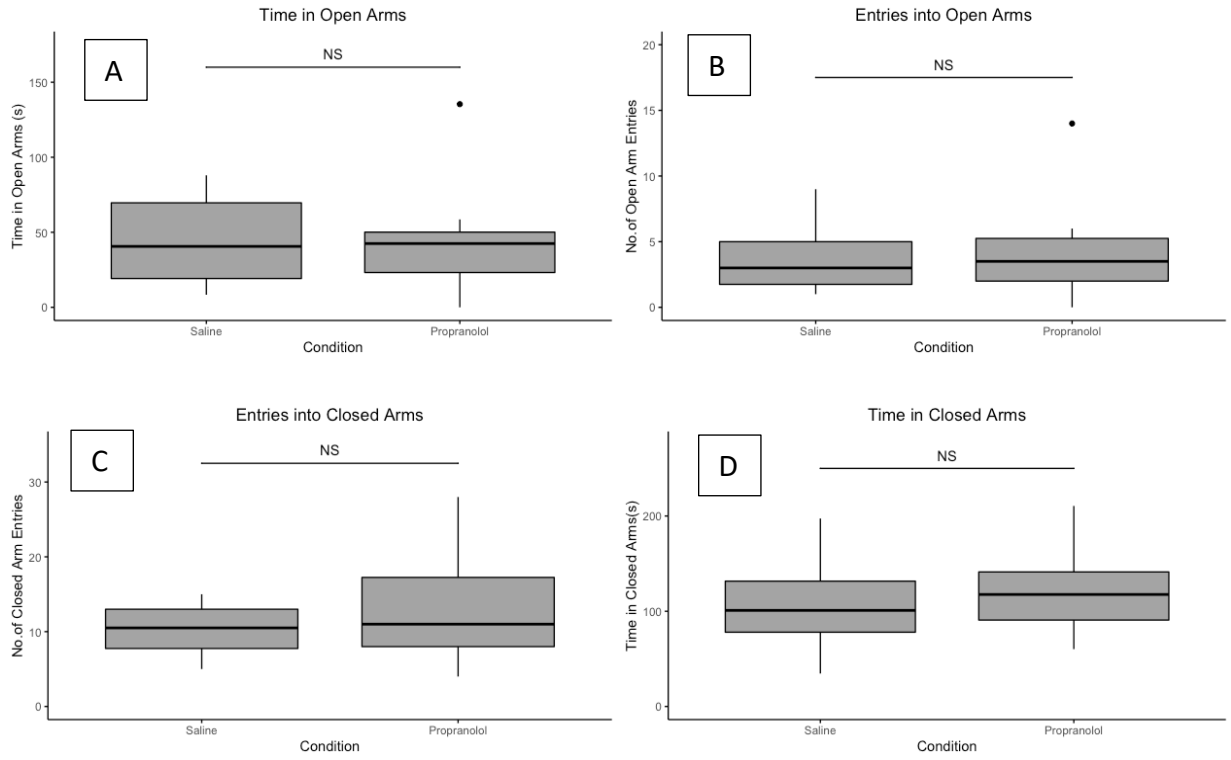

Figure 3: *Anxiety measures comparing propranolol to saline vehicle.* ***A:*** *Time spent in open arms of the elevated plus maze (more time in open arms reflects lower anxiety behavior).* ***B:*** *Number of entries into open arms (more entries into open arms reflects lower anxiety behavior).* ***C:*** *Time spent in closed arms of the elevated plus maze (more time in closed arms reflects higher anxiety behavior).* ***D:*** *Number of entries into closed arms (more entries into closed arms indicates higher anxiety behavior). NS: Non-significant; *: p<0.05; **: p<=0.01.*

4.3.2.2: Atomoxetine

There was a significant decrease in the amount of time spent in closed arms of the maze with atomoxetine compared to saline (mean difference=-48 seconds, t(9)=3.26, p=0.01). There was also a significant decrease in the number of entries into closed arms of the maze with atomoxetine compared to Saline (mean difference =2.9 entries, t(9)=2.69, p=0.025). These findings indicate decreased anxiety-like behavior with atomoxetine compared to saline. Time spent in open arms of the plus maze (t(9)=1.11, p=0.30), and entries into open arms of the plus maze (t(9)=1.17, p=0.27) did not differ between saline and atomoxetine conditions. Sample size for elevated plus maze measurements was 10 rats, as one rat fell from the maze during

the first testing timepoint.

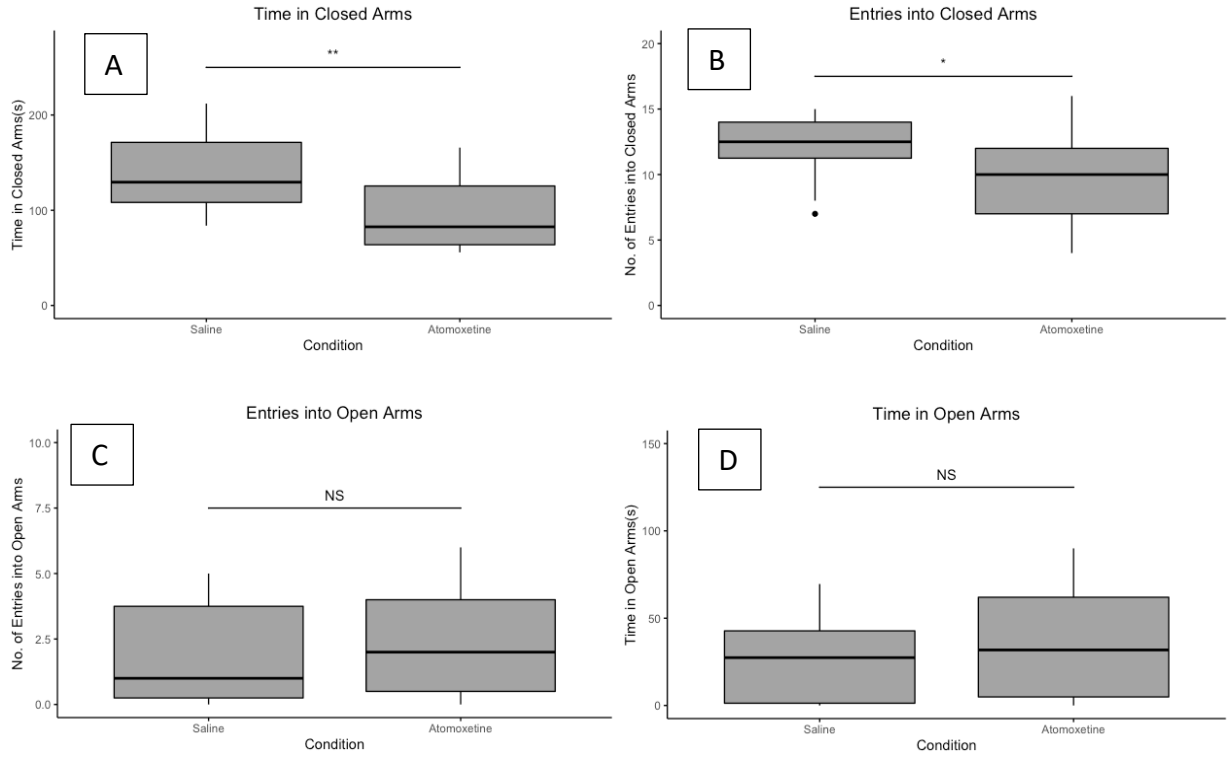

Figure 4 Anxiety measures comparing atomoxetine to saline vehicle. **A:** Time spent in open arms of the elevated plus maze (more time in open arms reflects lower anxiety behavior). **B:** Number of entries into open arms (more entries into open arms reflects lower anxiety behavior). **C:** Time spent in closed arms of the elevated plus maze (more time in closed arms reflects higher anxiety behavior). **D:** Number of entries into closed arms (more entries into closed arms indicates higher anxiety behavior). NS: Non-significant; *: p<0.05; **: p<=0.01

4.3.2.3: Reboxetine

There was a significant decrease in amount of time spent in closed arms of the plus maze with reboxetine compared to saline (mean difference=22.5 seconds, t(11)=2.33, p=0.04) There was also a significant decrease in the number of closed arm entries with reboxetine compared to saline (mean difference=4 entries, t(11)=3.63, p=0.004). There were not significant s in the number of open arm entries (t(11)=1.28, p=0.23), or time spent in open arms of the maze (t(11)=-1.33, p=0.21) between saline and reboxetine conditions.

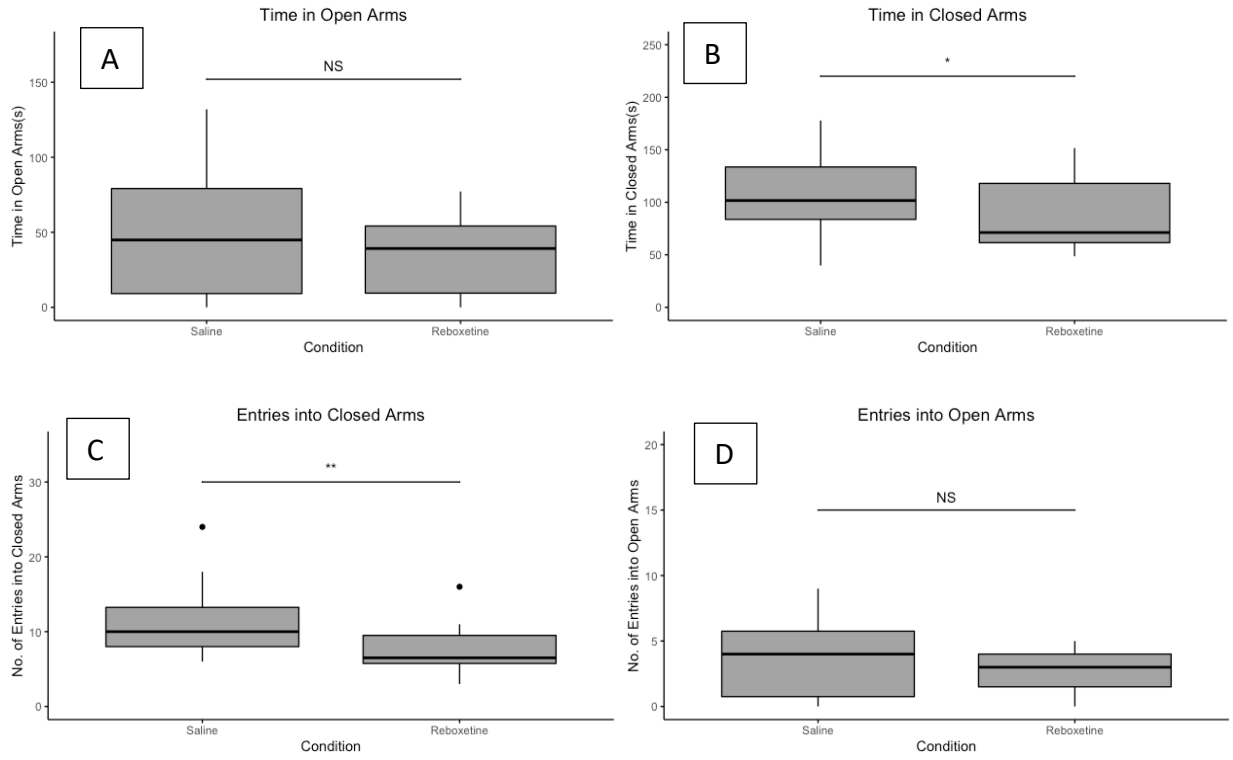

Figure 5: *Anxiety measures comparing reboxetine to saline vehicle.* ***A:*** *Time spent in open arms of the elevated plus maze (more time in open arms reflects lower anxiety behavior).* ***B:*** *Number of entries into open arms (more entries into open arms reflects lower anxiety behavior).* ***C:*** *Time spent in closed arms of the elevated plus maze (more time in closed arms reflects higher anxiety behavior).* ***D:*** *Number of entries into closed arms (more entries into closed arms indicates higher anxiety behavior). NS: Non-significant; *: p<0.05; **: p<=0.01.*

4.3.3: Ultrasonic Vocalization

The primary USV outcomes were measured on all calls for each rat. Exploratory analysis of sub-categories of complex and simple calls was then conducted. Simple calls were defined as calls that did not contain oscillatory pitch modulation or step-wise modulation. Complex calls represented all non-simple calls.

4.3.3.1: Propranolol

There were no significant changes in the USV parameters of call duration ($t(11)=-1.47$, $p=0.17$), principal frequency ($t(11)=-0.61$, $p=0.55$), tonality ($t(11)=0.28$, $p=0.78$), delta frequency ($t(11)=0.20$, $p=0.84$), mean power($t(11)=0.02$, $p=0.98$), or sinuosity ($t(11)=0.88$,

p=0.40) between saline and propranolol conditions.

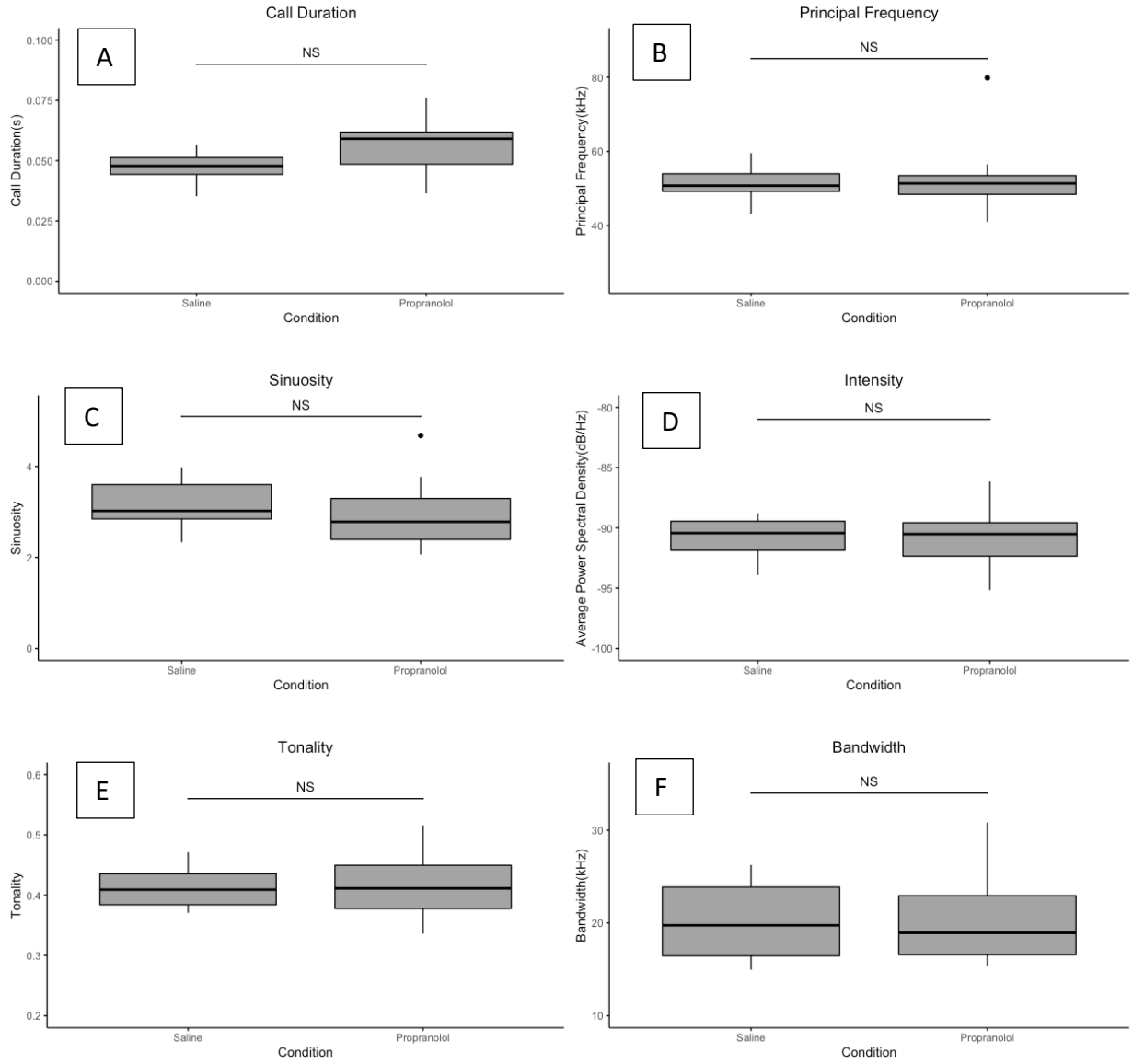

Figure 6: *Ultrasonic vocalization acoustic measures comparing propranolol to saline vehicle.* ***A:*** *Call duration.* ***B:*** *Principal frequency (the median frequency along the spectral contour of the call).* ***C:*** *Sinuosity (greater sinuosity indicates greater call complexity).* ***D:*** *Intensity as measured by average power spectral density in dB/Hz (more-negative indicates reduced loudness).* ***E:*** *Tonality as an indication of signal versus noise (greater tonality indicates greater prominence of the signal relative to noise).* ***F:*** *Bandwidth (difference between the highest frequency and lowest frequency of the call contour). NS: Non-significant; *: p<0.05; Dots indicate statistical outliers; dB: decibels; Hz: hertz.*

For simple calls, there were no significant changes in call duration (t(11)=1.28, p=0.23), principal frequency (t(11)=-0.75, p=0.47), tonality (t(11)=0.41, p=0.69), delta frequency

(t(11)=0.14, p=0.89), mean power (t(11)=0.89, p=0.39), or sinuosity (t(11)=0.74, p=0.48). For complex calls, there were also no significant changes in call duration (t(11)=-1.43, p=0.18), principal frequency (t(11)=-0.54, p=0.6), tonality (t(11)=0.44, p=0.67), delta frequency (t(11)=0.18, p=0.85), mean power (t(11)=0.3, p=0.77), or sinuosity (t(11)=1.28, p=0.23).

4.3.3.2: Atomoxetine

There was a significant decrease in the principal frequency with atomoxetine compared to saline (mean difference = 3.06 kHz, t(10)=2.85, p=0.017). Tonality increased significantly with atomoxetine compared to saline (mean difference=0.033, t(10)=2.47, p=0.03). There was a significant decrease in the delta frequency (bandwidth) with atomoxetine compared to saline (mean difference=2.43, t(10)=2.35, p=0.041), as well as a significant increase in mean power (mean difference=1.9, t(10)=2.92, p=0.015). Finally, there was a significant decrease in sinuosity for complex calls with atomoxetine compared to saline (mean difference=0.41, t(10)=2.35, p=0.04). There was no change in duration (t(10)=-0.91, p=0.39) between saline and atomoxetine conditions.

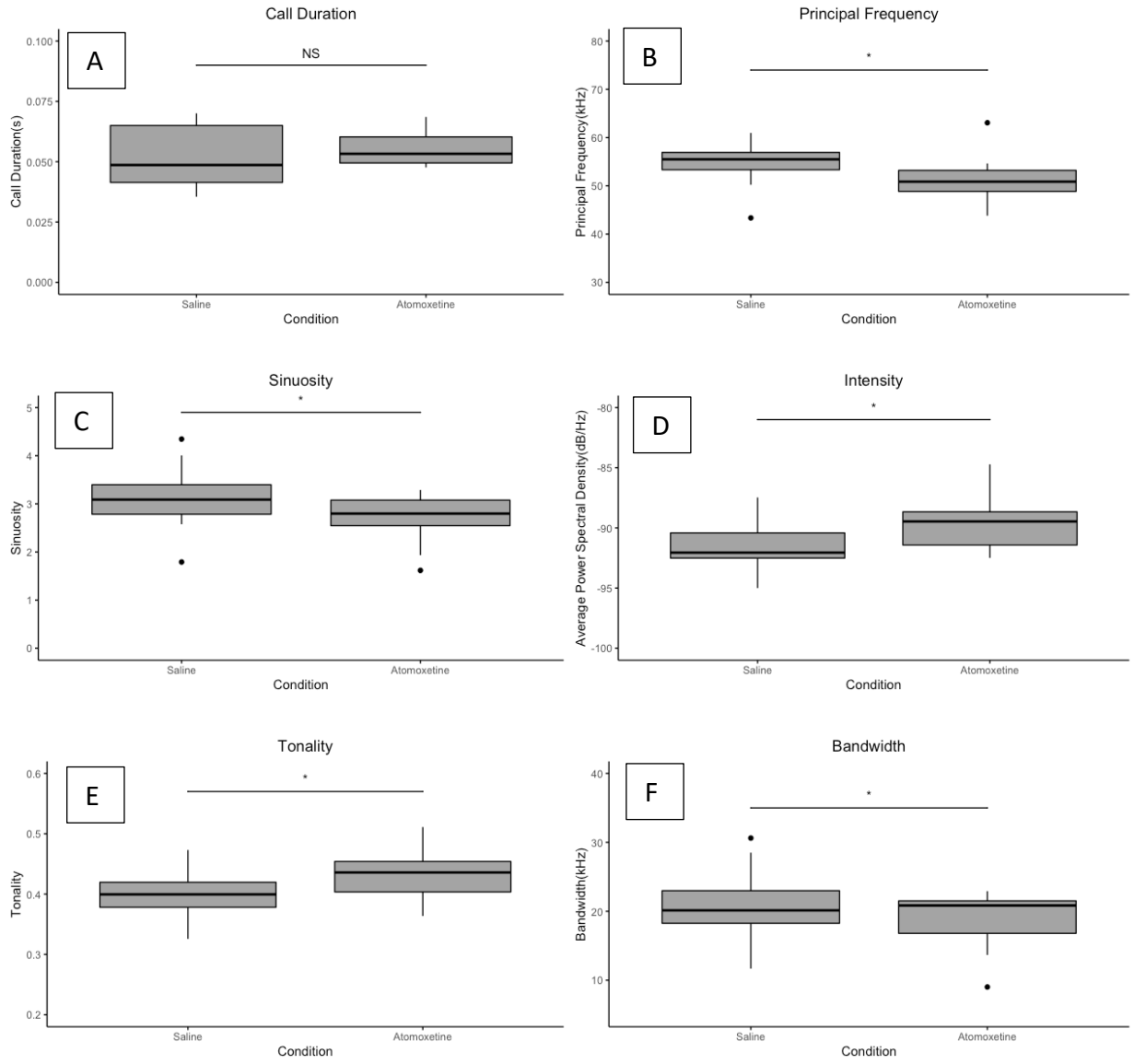

Figure 7: *Ultrasonic vocalization acoustic measures comparing atomoxetine to saline vehicle.* ***A:*** *Call duration.* ***B:*** *Principal frequency (the median frequency along the spectral contour of the call).* ***C:*** *Sinuosity (greater sinuosity indicates greater call complexity).* ***D:*** *Intensity as measured by average power spectral density in dB/Hz (more-negative indicates reduced loudness).* ***E:*** *Tonality as an indication of signal versus noise (greater tonality indicates greater prominence of the signal relative to noise).* ***F:*** *Bandwidth (difference between the highest frequency and lowest frequency of the call contour). NS: Non-significant; *: p<0.05; Dots indicate statistical outliers; dB: decibels; Hz: hertz.*

For complex calls, there was a there was a significant decrease in the principal frequency with atomoxetine compared to saline condition (mean difference = 2.51 kHz, t(10)=2.23, p=0.04975). There were no differences in duration (t(10)=0.95, p=0.37), tonality

(t(10)=1.45, p=0.18), delta frequency (t(10)=0.79, p=0.45), mean power (t(10)=2.05, p=0.07), or sinuosity (t(10)=1.47, p=0.17).

For simple calls, there were no significant differences in duration (t(9)=0.39, p=0.71), principal frequency (t(9)=1.94, p=0.08), tonality (t(9)=1.72, p=0.12), delta frequency (t(9)=1.43, p=0.19), mean power (t(9)=1.91, p=0.09) or sinuosity (t(9)=1.02, p=0.09).

4.3.3.3: Reboxetine

There were no significant differences between saline and reboxetine for USV outcomes of duration (t(11)=0.75, p=0.47), principal frequency (t(11)=1.85, p=0.09), tonality (t(11)=0.51, p=0.62), delta frequency (t(11)=1.79, p=0.10), mean power (t(11)=1.76, p=0.11), or sinuosity (t(11)=0.85, p=0.4).

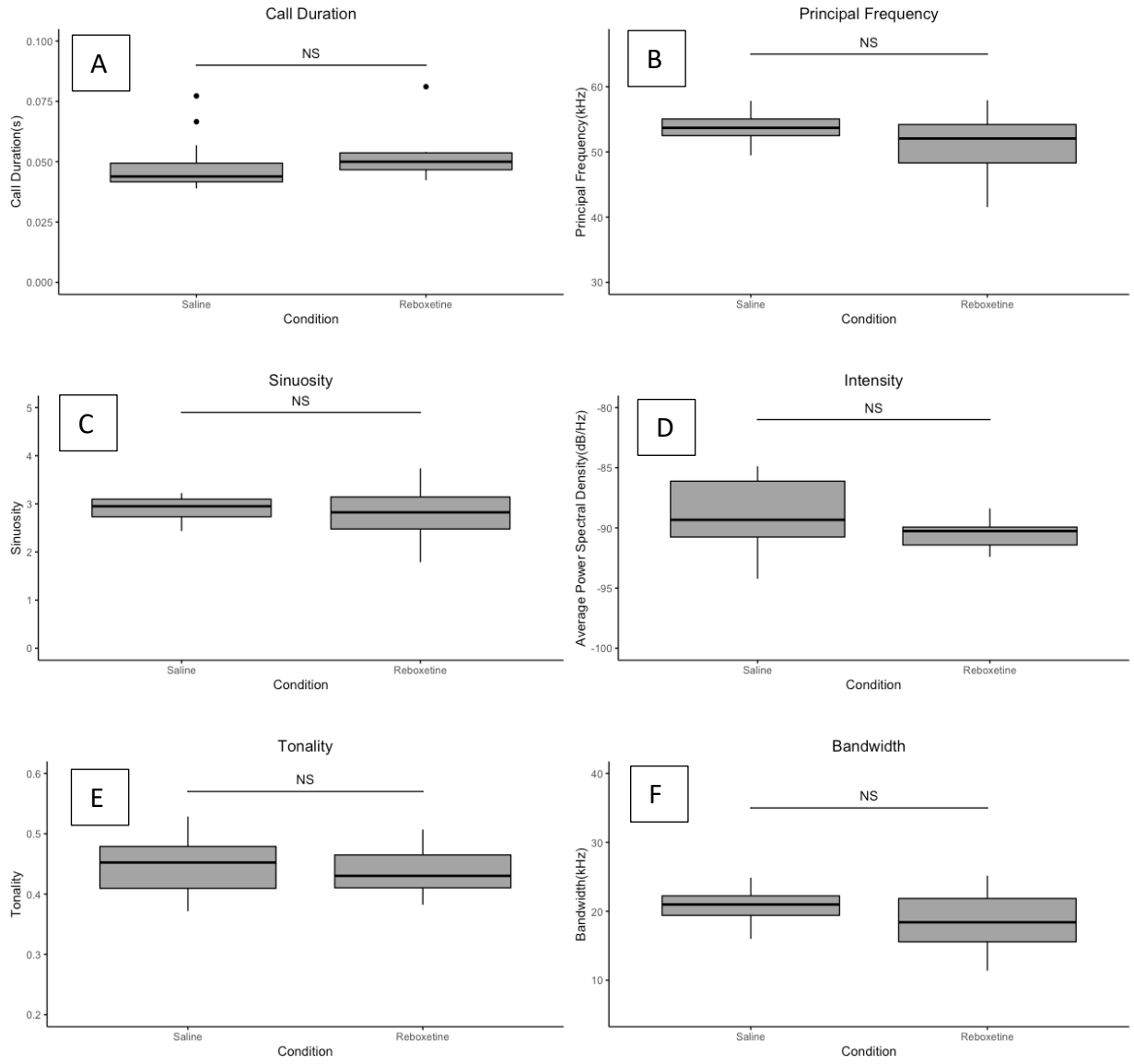

Figure 8: *Ultrasonic vocalization acoustic measures comparing reboxetine to saline vehicle.* ***A:*** *Call duration.* ***B:*** *Principal frequency (the median frequency along the spectral contour of the call).* ***C:*** *Sinuosity (greater sinuosity indicates greater call complexity).* ***D:*** *Intensity as measured by average power spectral density in dB/Hz (more-negative indicates reduced loudness).* ***E:*** *Tonality as an indication of signal versus noise (greater tonality indicates greater prominence of the signal relative to noise).* ***F:*** *Bandwidth (difference between the highest frequency and lowest frequency of the call contour). NS: Non-significant; *: p<0.05; Dots indicate statistical outliers; dB: decibels; Hz: hertz.4*

For complex calls, there was a significant decrease in the mean power with Reboxetine compared to saline (mean difference=1.79dB, t(11)=2.68, p=0.02). There was no difference in duration (t(11)=0.39, p=0.7), principal frequency (t(11)=1.73, p=0.11), tonality (t(11)=1.72, p=0.11), delta frequency (t(11)=1.99, p=0.07), or sinuosity (t(11)=0.68, p=0.51).

For simple calls, there was a significant decrease in the Principal Frequency of Simple calls with Reboxetine compared to saline (mean difference=3.56, t(11)=2.22, p=0.048). There were no differences in duration (t(11)=2.09, p=0.06), tonality (t(11)=0.6, p=0.56), delta frequency (t(11)=-0.87, p=-.4), mean power (t(11)=0.37, p=0.72), or sinuosity (t(11)=1.19, p=0.26).

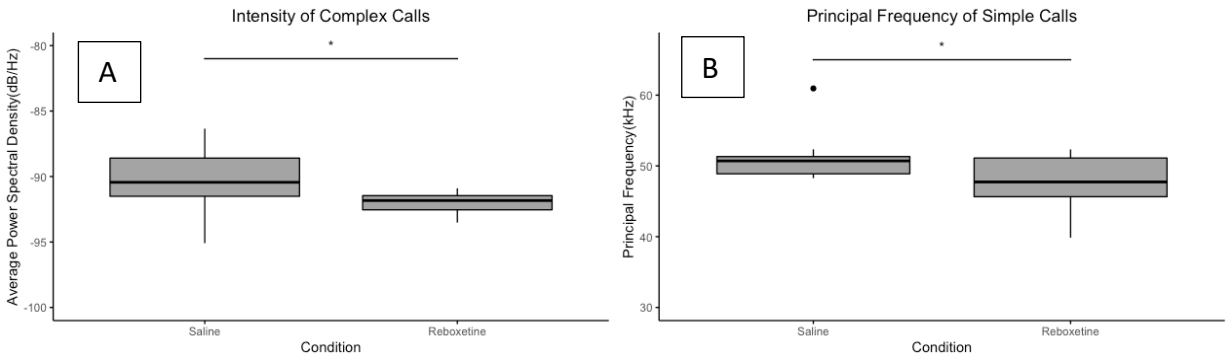

Figure 9: *Comparison of acoustic variables between reboxetine and saline vehicle in sub-categories of simple and complex calls* ***A:*** *Intensity of complex calls as measured by average power spectral density in dB/Hz (more-negative indicates reduced loudness).* ***B:*** *Principal frequency (the median frequency along the spectral contour of the call) of simple calls. *: p<0.05; Dots indicate statistical outliers; dB: decibels; Hz: hertz*

4.3.4: Interactions between Drug Condition and Anxiety on Ultrasonic Vocalization outcomes

4.3.4.1: Propranolol

The relationships between anxiety and duration (β=-0.0001223, t=0.35, p=0.66), anxiety and tonality (β=0.00027, t=-0.582, p=0.567), anxiety and principal frequency (β=0.021, t=0.33, p=0.75), anxiety and sinuosity (β=0.004, t=-0.76, p=0.46), anxiety and mean power (β=0.0009, t=-0.044, p=0.97), and anxiety and delta frequency (β=-0.024, t=-0.65, p=0.56) were not significantly influenced by drug condition.

4.3.4.2: Atomoxetine

The relationships between anxiety and call duration (β=-0.00013, t=-1.56, p=0.13), anxiety and tonality (β=-0.0002, t=0.48, p=0.64), anxiety and principal frequency (β=-0.039, t=-1.1, p=0.3), anxiety and sinuosity (β=-0.003, t=-0.59, p=0.57), anxiety and mean power (β=0.03, t=1.22, p=0.25), and anxiety and delta frequency (β=-0.01, t=-0.44, p=0.67) were not significantly influenced by atomoxetine.

4.3.4.3: Reboxetine

The relationships between anxiety and duration (β=-0.00006, t=-0.51, p=0.61), anxiety and tonality (β=-0.0005, t=-1.08, p=0.3), anxiety and principal frequency (β=0.011, t=0.27, p=0.79), anxiety and sinuosity (β=0.0029, t=0.61, p=0.55), anxiety and mean power(β=-0.02, t=-0.86, p=0.41), and anxiety and delta frequency (β=-0.001, t=-0.029, p=0.98) were not significantly influenced by reboxetine.

4.4: Discussion

Vocalization and anxiety interact with one another (see Chapter 3), and share several neural substrates. Activations of shared brainstem nuclei, such as the nucleus ambiguus, dorsal motor nucleus of the vagus and nucleus of the solitary tract, and higher cortical brain regions, such as the amygdala and cingulate cortex, are required for both vocalization and autonomic arousal (a physiologic consequence of anxiety). Further, these neural substrates are strongly influenced by monoaminergic systems, including NE, which are greatly disrupted in PD. Consistent with previous work that has demonstrated relationships between central noradrenergic histology, vocalization and anxiety in the *Pink1-/-* rat model of PD (see chapter

3), we demonstrate in the current study that systemic manipulation of norepinephrine results in changes to both vocalization and anxiety without changing spontaneous motor activity. The influence of noradrenergic manipulation on vocalization and anxiety, however, is non-uniform.

Atomoxetine increases the bioavailability of norepinephrine through inhibition of the norepinephrine transporter, and administration of atomoxetine resulted in increases in vocal intensity. This is relevant to the *Pink1-/-* rat model of PD, which demonstrate reduced call intensity compared to WT controls (Grant et al., 2015; Kelm-Nelson et al., 2018; Kelm-Nelson et al., 2015). Further, the increase in vocalization intensity is translationally important to clinical research focused on improving vocal deficits in human PD: vocal intensity is almost universally reduced in human PD, and contributes substantially to communication impairment (Darley et al., 1969; Ho et al., 1998; Plowman-Prine et al., 2009). An additional characteristic of *Pink1-/-* rat vocalization that is altered in comparison to WT controls is that of reduced average peak frequency (Grant et al., 2015; Kelm-Nelson et al., 2018; Kelm-Nelson et al., 2015). Whereas intensity was increased with atomoxetine in the current study (*i.e.* moved in the direction of WT intensity), principal frequency of calls decreased with atomoxetine (*i.e.* moved away from the direction of WT average peak frequency). While principal frequency (the mean frequency of the spectral contour of the call) and average peak frequency (the frequency at the point in the call with greatest intensity) are not identical measures, they both reflect the central tendency of the frequency of the call. Further, sinuosity of the calls (a measure of complexity) was reduced with atomoxetine. Ethologically, this change may not be beneficial, as calls with greater average peak frequency and greater complexity have been shown to be important factors in eliciting conspecific approach behavior (Pultorak et al.,

2016). Thus, administration of atomoxetine does not "normalize" all aspects of ultrasonic vocalization uniformly. The clinical relevance of changes to average peak frequency and sinuosity, however, is unclear.

While both atomoxetine and a similar norepinephrine reuptake inhibitor, reboxetine, reduced anxiety-like behavior compared to saline, only atomoxetine had an effect on vocalization. A possible explanation for this finding is that atomoxetine has a higher affinity for serotonin transporter and dopamine transporter (Roth et al., 2000; *The Psychoactive Drug Screening Program KI Database*, n.d.) compared to reboxetine. Dysfunction of several monoaminergic systems is present in PD (Buddhala et al., 2015; Kano et al., 2011; Menza et al., 1999), and monoaminergic disruption and manipulation are integral to our understanding and therapeutic management of affective disorders for both individuals with and without PD (Chaudhuri et al., 2006; Jakubovski et al., 2019; Raskin & Durst, 2010; Renfroe et al., 2016; Schildkraut, 1965; Slee et al., 2019; Weintraub et al., 2010; Williams et al., 2000). The direct role of these systems and their disruption is less clear in vocalization. Future work that assesses either selective manipulation of other monoamines, or diffuse monoamine manipulation (*i.e.* selective serotonin reuptake inhibitors and serotonin-norepinephrine reuptake inhibitors or serotonin-norepinephrine-dopamine reuptake inhibitors, respectively) would help to clarify this issue.

An unexpected finding of the current study was that administration of the β adrenoreceptor antagonist, propranolol, did not influence vocalization or anxiety in *Pink1-/-*. This contrasts with previous studies of WT rats, which have shown varied changes to ultrasonic vocalization with the same doses of propranolol (Grant et al., 2018; Wright et al., 2012). In

previous work (see study 1), β1 adrenoreceptors in the brainstem have been shown to be altered in *Pink1-/-* rats compared to WT controls. Specifically, the relative optical density of β1 adrenoreceptors was reduced in the nucleus of the solitary tract of *Pink1-/-* rats compared to WT controls, whereas cell count estimates of β1 adrenoreceptor immunoreactive cell bodies in the dorsal motor nucleus of the vagus and the nucleus ambiguus were increased. Further, relative optical density of β1 adrenoreceptors in the nucleus of the solitary tract was significantly positively correlated with call intensity, whereas cell count estimates of β1 adrenoreceptor immunoreactive cells in the dorsal motor nucleus of the vagus and nucleus ambiguus were significantly negatively correlated with call intensity. Thus, a possible explanation for the current findings is that changes to β1 adrenoreceptor expression in the nucleus of the solitary tract, a complex sensory brainstem nucleus, is a greater driver of vocal deficit than β1 adrenoreceptor disruption in other brain regions. If this were the case, antagonism of β1 adrenoreceptors would not increase vocal intensity, and might even reduce it in the *Pink1-/-* rat. While direct administration of β adrenoreceptor antagonists to lower brainstem nuclei would be beneficial for disentangling the correlational findings reported in this and earlier works, such direct administration is likely to be challenging due to the relatively small size of these nuclei and their co-localization with cardiac and respiratory neurons.

Because previous work has demonstrated a relationship between anxiety and vocalization in the *Pink1*-/- rat model of PD, we conducted exploratory analyses to determine whether this relationship was modulated by systemic manipulation of norepinephrine. None of the drugs administered in this study had an influence on the relationship between anxiety and

vocalization. This may have been due to the fact that the study was powered to detect differences in single behavioral measures, rather than behavioral interactions. As a consequence, the sample sizes used in the current study may have been too small to detect relationships between these two outcomes relative to drug condition. This is particularly likely given the variability inherent in animal behavior, which is exchanged for increased genetic and environmental control relative to the study of human patients with PD.

Limitations and Future directions:

A limitation of the current study is that it assessed only immediate changes to vocalization and anxiety following administration of drug doses. Chronic administration of monoamine reuptake inhibitors often results in down-regulation of pre-synaptic receptors, and it is through this longer-term plasticity that changes to behavior are observed (S. M. Stahl, 2013). Future studies using this model of PD would benefit from assessing vocalization and anxiety over a longer time course, and assessing changes to neural tissue that might accompany alterations in behavior.

An additional factor that must be considered when interpreting the current findings is that the drug doses used were based on human clinical doses that result in behavioral modification, and on doses that resulted in behavioral changes in WT rats in previous studies. More extensive dose-response curves for vocalization and anxiety may reveal different effects of the drugs in question, and inclusion of a wild type control group may identify differences in dose-response curves between genotypes. It will be important to complete these dose-response curves with very large samples, as anxiety tests using the plus maze should not be completed less than 3 weeks apart to avoid habituation to the apparatus (Walf & Frye, 2007).

The fact that the disease model is progressive complicates repeated testing of anxiety. For example, an animal tested 4 times (at saline, low, medium, high doses) would have 2 doses tested 9 weeks apart. In previous work, we have observed changes in anxiety and vocalization among 2, 4, 6, 8 and 12 month timepoints (see Study 1) (Grant et al., 2015; Marquis et al., 2020). Thus, very large animal numbers would likely be required for this type of investigation in order to allow for the large degree of variance that is present in animal behavior both by drug dose and by age/disease progression.

Translation of the results from the current study should be tempered by the fact that norepinephrine transporter is similar, but not identical across species in terms of pharmacological properties and distribution (Paczkowski et al., 1999; Smith et al., 2006). As a consequence, effects and lack of effects of the norepinephrine reuptake inhibitors atomoxetine and reboxetine on vocalization and anxiety observed in the current study may be altered in humans. Prior to completion of prospective, randomized studies assessing the effect of these drugs on vocalization and anxiety in humans with PD, it would be prudent to follow the bi-directional model of translational research suggested by Pankevich and colleagues (Pankevich et al., 2013) by completing exploratory investigations assessing vocal function and anxiety in individuals with PD who have and have not been prescribed noradrenergic modulators such as atomoxetine and reboxetine.

Conclusion

Systemic manipulation of norepinephrine results in non-uniform changes to vocalization and anxiety-like behavior in the *Pink1-/-* rat model of PD. Reductions in anxiety (with reboxetine and atomoxetine) and increases in vocal intensity (with atomoxetine) are

promising as potential interventions for addressing non-hallmark deficits in PD. A deeper understanding of normal and pathologic interactions among monoaminergic systems is necessary for successful translation of these findings to treatment of vocal communication deficits and anxiety in humans with PD.

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Chapter 5: Summative Discussion

Vocal deficits and anxiety are common in Parkinson Disease (PD). They also appear to influence one another: communication impairment resulting from vocal deficit is likely to induce a degree of anxiety, and anxiety is likely to have a negative impact on the complex sensorimotor act of vocalization. In addition to this apparent interaction, vocal deficits and anxiety share prodromal onset and patterns of progression in PD, and neither respond completely or consistently to standard treatments such as dopamine replacement therapies, deep brain stimulation, or behavioral therapy. There is thus an unmet need for improved efficiency, efficacy, and effectiveness of treatments for vocal deficits and anxiety in PD. Improved treatment of these deficits requires improved understanding of their disease-specific neural mechanisms. While recent research has begun to converge on the role of central noradrenergic system disruption as a possible contributor to both signs, barriers to deeper understanding include: challenges with identifying patients before PD is diagnosed (when vocal deficits and anxiety are manifesting and neural pathology has already begun), inability to directly assess neural tissue in humans, and a large degree of heterogeneity in PD presentation.

In order to investigate the role of noradrenergic disruption on vocal deficits and anxiety in PD with a high degree of experimental control and the ability to study neural tissue, the current series of studies used the *Pink1*-/- rat model of PD. In study 1, our findings partially supported the hypotheses that 1) anxiety would be increased in Pink1-/- rats, 2) vocal deficits and anxiety would be correlated to one another, 3) noradrenergic markers in the locus coeruleus, nucleus ambiguus, dorsal motor nucleus of the vagus nerve, and the nucleus of the solitary tract would be disrupted in *Pink1*-/-, and 4) brainstem noradrenergic markers would be

associated with vocal acoustic changes and anxiety level. In study 2, our findings supported the hypotheses that norepinephrine reuptake inhibitors (atomoxetine and reboxetine) would decrease vocal impairment and anxiety compared to saline, but did not support the hypotheses that a β receptor antagonist (propranolol) would decrease vocal impairment and anxiety compared to saline, or that the three drugs would influence the relationship between anxiety and vocalization.

In addition to demonstrating greater anxiety in *Pink1*-/- rats than WT rats, a primary finding of this work was that *Pink1*-/- rats had statistically significant relationships between anxiety and vocal communication that were not present in WT rats. That is, USV outcomes were more-strongly associated with anxiety level for *Pink1*-/- rats than for WT rats. A potential reason for this is that presence of anxiety has a negative influence on vocalization, and in a non-pathological state, rats might be more-able to over-ride the influence of anxiety on USV outcomes, particularly when considering the ethological perspective of the paradigm used (mating). That is, complex, high-intensity vocalization increases female conspecific approach behavior, making species propagation more likely (Bialy et al., 2000; Brudzynski, 2005; Riede, 2014). If anxiety decreases intensity and complexity of vocalization, it is reasonable that rats may be driven to over-ride the influence of anxiety on vocalization. Thus, the deficits in vocalization observed in *Pink1*-/- rats may in fact be deficits in the ability over-ride the influence of anxiety on vocalization. Additionally, both anxiety and vocal acoustic outcomes were correlated with brainstem noradrenergic markers, but not the same markers. Specifically, anxiety level was correlated with tyrosine hydroxylase immunoreactive cell count estimates in the locus coeruleus, whereas USV outcomes were associated with β1 adrenoreceptor

immunoreactive cell count estimates in the dorsal motor nucleus of the vagus and the nucleus ambiguus, and optical density of β1 adrenoreceptor immunoreactivity in the nucleus of the solitary tract. These findings are likely related to one another. Specifically, a decrease in the availability of NE due to reduced number of noradrenergic cell bodies in the LC could lead to compensatory upregulation of post-synaptic β1 adrenoreceptors. A more complex explanation that considers the impact of noradrenergic disruption in neocortical structures in addition to the brainstem, as well as other neurotransmitter systems, is likely to provide a more-nuanced and more-accurate picture, particularly in light of the possibility that vocal deficits may be the consequence of an inability to over-ride the influence of anxiety on vocalization. Further investigations in both patients with PD, and animal models of vocal deficits and anxiety in PD will be important for exploring these potential explanations.

In study 2, we assessed the influence of systemic noradrenergic manipulation on USV outcomes and anxiety. That is, we "zoomed out" from the granular assessment of specific NE markers in isolated brainstem nuclei that were assayed in study 1, and "shifted" to focus broadly on noradrenergic function across the central nervous system. We found that increasing the bioavailability of NE through systemic administration of norepinephrine reuptake inhibitors, atomoxetine and reboxetine, resulted in reduced anxiety behavior compared to administration of saline. Atomoxetine also altered ultrasonic vocalization acoustics. Specifically, intensity was increased, while sinuosity, principal frequency and bandwidth were decreased with atomoxetine compared to saline . The increase in intensity is promising in that it could plausibly address reductions in vocal intensity present in human PD; however, reductions in sinuosity, principal frequency and bandwidth could potentially be detrimental to intelligibility

when considering the fact that monopitch and monoloudness are also common features of vocal deficits in PD. Interestingly, reboxetine did not have a significant impact on vocalizations compared to saline. The difference in behavioral responses to the two drugs may be due to the fact that atomoxetine has a lower specificity for norepinephrine transporter than reboxetine, with some affinity for serotonin and dopamine transporters as well. This difference suggests that vocalization is influenced by a more-complex set of neurotransmitter systems, whereas anxiety behavior may be more specific to noradrenergic modulation in *Pink1*-/- rats. A greater degree of neurotransmitter and brain network complexity supports the possibility that vocal deficits may be the consequence of an inability to over-ride the influence of anxiety on vocalization, an action that would likely require coordination of diverse neural systems.

Limitations

There are limitations to the current studies that must be considered during interpretation of findings. While the current studies refer to “anxiety” and “anxiety behavior” as all-encompassing terms, the phenomenon of anxiety in humans is typically broken into categories of “state” and “trait” anxiety. State anxiety is a transient emotional state that is accompanied by increases in physiological arousal and consciousness awareness of the perception of negative feelings about future events, whereas trait anxiety reflects aspects of personality that predisposes an individual to increases in state anxiety (Endler & Kocovski, 2001; Spielberger, 1966). Certainly, state and trait anxiety interact with one another; however, they are distinct and are represented by different functional connectivity profiles in the human brain (Saviola et al., 2020). State and trait anxiety are both elevated in patients with PD

(Mondolo et al., 2007), underscoring the relationship between these aspects of anxiety. In animal models, however, measurement of trait anxiety can only be inferred from repeated measures of state anxiety (Goes et al., 2009). Unfortunately, repeated measurement of state anxiety can result in artificially elevated anxiety scores due to a lack of exploratory behavior following habituation to the testing apparatus (Walf & Frye, 2007). While *Pink1-/-* rats demonstrated greater anxiety-like behavior on the elevated plus maze than WT controls at each timepoint in the current study, a limitation is that these are simply multiple and repeated measures of state anxiety. Nevertheless, a consistency between anxiety-like behavior in *Pink1-/-* rats and humans with PD was observed.

The immunohistochemistry assays of the first study are limited by assessment of neural tissue at a single, 12-month timepoint. Brainstem noradrenergic markers that differ by genotype may not become fully apparent until later time-points, representing more-advanced disease progression. Conversely, it is possible that neuroplastic compensation in response to earlier neurobiological changes may have occurred by the 12-month timepoint, obfuscating genotypic differences that might be apparent at earlier timepoints. It must also be acknowledged that, while unbiased stereology and measurement of relative optical density allow for a high degree of spatial resolution in assessment of neural tissue differences, they may be less sensitive to genotypic differences than more-direct methods of measurement, such as high-performance liquid chromatography. Future studies would benefit from additional quantification methods to confirm current findings.

A factor that limits broad interpretation of the current findings is that the drug doses used in the second study were based on human clinical doses that result in behavioral

modification, and on doses that resulted in behavioral changes in WT rats in previous studies. More extensive dose-response curves for vocalization and anxiety may reveal different effects of the drugs in question. It will be important to complete these dose-response curves with very large samples, as anxiety tests using the plus maze should not be completed less than 3 weeks apart to avoid habituation to the apparatus (Walf & Frye, 2007). The fact that the disease model is progressive complicates repeated testing of anxiety. For example, an animal tested 4 times (at saline, low, medium, high doses) would have 2 doses tested 9 weeks apart. In previous work, we have observed changes in anxiety and vocalization among 2, 4, 6, 8 and 12 month timepoints (see Study 1) (Grant et al., 2015; Marquis et al., 2020). Thus, very large animal numbers would likely be required for this type of investigation in order to allow for the large degree of variance that is present in animal behavior both by drug dose and by age/disease progression.

An additional factor that underscores the challenges of studying the relationships between vocalization and anxiety, both in PD and in the absence of PD, is the fact that they are both complex behaviors that are influenced by a wide variety of physical and psychological factors. One example of this is the role of respiratory control in vocalization and anxiety. Respiratory control is integral to production of targeted vocalizations across species. In addition, respiration is altered in anxiety, and intentional respiratory modulation is a common method of reducing anxiety in PD (Chandler et al., 2019). When paired with the presence of altered respiration in animal models of PD (Johnson et al., 2020) and in patients with PD (Baille et al., 2016), disentangling presence or absence of causal relationships and their possible directionality becomes problematic, and future work that attempts to do so should

acknowledge the many possible confounding variables that can contribute to the relationship between vocalization and anxiety.

This series of studies demonstrates that relationships exist among vocal deficits, anxiety, and noradrenergic disruption in the *Pink1*-/- rat model of PD. However, the nature of these relationships is non-uniform. Findings from the current studies suggest that vocalization may be influenced by the interactions among multiple disrupted monoaminergic systems, rather than NE alone, although this cannot be confirmed from the current data set. Future investigations should consider simultaneous disruption of multiple monoaminergic neurotransmitter systems to further disrupt or modulate vocal acoustic outcomes. Given the complex nature of the relationships among vocal deficits, anxiety, and monoaminergic disruption identified in the current study, consideration should also be given to the possibility that the effectiveness of behavioral intervention could be modulated by manipulation of disrupted neurotransmitter system; that is, perhaps administration of a neuromodulating drug at the appropriate dose might facilitate behavioral change.

Future Directions: Broad investigation of vocalization and anxiety relationships

In addition to enhancing understanding of disease-specific mechanisms of vocal deficits and anxiety in PD, these studies introduce potential pathways of exploration for neural underpinnings of the relationship between anxiety and vocal communication in voice disorders outside of PD, and in non-pathological states. It can intuitively be understood that any complex, volitional motor act is likely to be altered in the setting of altered psychological states, such as anxiety. In limb motor systems, this has received a significant amount of research attention for

several decades (DeCaro et al., 2011; Hill et al., 2010), which has allowed for recent investigations into neural underpinnings that may be responsible for the relationship (Ganesh et al., 2019).

As an organ that sits at the junction of the respiratory and alimentary tracts, the biological purposes of the larynx include reflexive and semi-reflexive modulation of respiration, airway protection, and deglutition. While some patterns of emotional vocalization are activated through direct limbic pathways (Jurgens, 2002), the exquisitely fine-grained, volitional movements of the larynx necessary for normal vocal communication and speech are indirectly activated by higher cortical processes (Ludlow, 2005), are superimposed upon the biological purposes of the larynx. Because anxiety is, by definition, associated with limbic activation, and leads to functional changes in the aerodigestive tract, vocal communication may be uniquely susceptible to disruption by alterations in anxiety level. It follows that the ability to alter the influence of anxiety on voice (*i.e.* maintaining optimal phonatory functions despite the presence of high levels of anxiety) would be beneficial in the treatment of voice disorders. This is particularly relevant when considering the high rates of relapse following voice therapy (lack of efficiency and efficacy) (Roy et al., 1997).

Several lines of research have begun to describe the nature of this complex relationship. However, an understanding of effective methods of manipulating the relationship, and the neurobiological underpinnings that modulate the relationship, have been stymied for several reasons. One of the most challenging is a lack of consensus on methods for measuring vocal motor learning and control.

The are several preliminary steps to better understanding the relationships between vocal communication and anxiety, include the following. An appropriate testing battery for the assessment of vocal motor learning and control must be established; this battery can then be assessed for responsiveness to principles of motor learning established from research in limb motor function: accuracy, speed, response time (skill acquisition), and retention and transferability. Subsequently, the battery can be perturbed by manipulation of anxiety and other psychological factors in order to better-understand, phenomenologically, the relationships between vocal communication and anxiety. Neurostimulation and neuroimaging techniques can then be used to deepen understanding of the biological underpinnings of the relationship. Ultimately, this basic knowledge can then be leveraged to assess efficacy and efficiency of treatments that explicitly target the relationship between vocal communication and anxiety in order to improve outcomes for patients with voice disorders who are undergoing voice therapy.

Conclusion

The results of the current series of studies demonstrate that anxiety is greater in the *Pink1*-/- rat model of PD than in WT controls, and that anxiety is associated with vocal acoustic measures. Both anxiety and vocal deficits are associated with noradrenergic markers in the brainstem, and these markers differ by genotype. In addition, systemic manipulation of norepinephrine can reduce anxiety in the *Pink1*-/- rat. Manipulation of vocalization, however, may require more-complex neuromodulation. Future studies using the Pink1-/- rat model of

anxiety and vocal deficits in PD should consider higher cortical involvement, as well as the contribution of multiple interacting monoaminergic neurotransmitter systems.

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APPENDIX A

Post-Extubation Dysphagia in Pediatric Populations: Incidence, Risk Factors and Outcomes

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Abbreviations: Post-Extubation Dysphagia (PED); Complex Chronic Conditions (CCC); Adjusted Odds Ratio (AOR); Confidence Interval (CI); Flexible Endoscopic Evaluation of Swallowing (FEES); Videofluoroscopic Swallow Study (VFSS)

Short Title: Post-Extubation Dysphagia in Pediatric Populations

Abstract

Objective

To assess incidence, risk factors for, and impact on outcomes of Post-Extubation Dysphagia (PED) in pediatrics. We hypothesized incidence of PED in pediatrics would approximate or exceed that in adults, age and duration of intubation would increase odds for PED, and presence of PED would negatively impact patient outcomes.

Study Design

We performed a retrospective, observational cohort study of patients aged 0-16 admitted between 2011 and 2017. Patients were included if they were extubated in the ICU and fed orally within 72 hours. Records were reviewed to determine dysphagia status and assess the impact of patient factors on odds of PED. The impact of PED on patient outcomes was then assessed.

Results

Following application of inclusion and exclusion criteria, sample size was 372 patients. PED was observed in 29% of patients. For every day of intubation, odds of PED increased by approximately 50% ($p<.0001$). Age of <25 months increased odds of PED more than two-fold ($p<.05$). When controlling for age, diagnosis, number of complex chronic conditions and dysphagia status, patients with dysphagia had an increase in total length of stay of 10.95 days ($p<0.0001$). PED increased odds of gastrostomy or nasogastric tube at time of discharge ($p<.0001$).

Conclusion

This study is the first to systematically describe incidence, risk factors, and outcomes of PED in pediatrics. PED is associated with increased time between extubation and discharge and with odds of gastrostomy or nasogastric tube at time of discharge.

Introduction

Post-extubation dysphagia (PED) is a well-documented phenomenon in adults(1–23) with estimates ranging from 3%-62%(14). PED is associated with multiple negative outcomes, including pneumonia, in-hospital mortality, hospital length of stay, discharge status and need for alternative means of nutrition including nasogastric and gastrostomy tube placement(7,24). Intubation has been shown to result in laryngeal injury, oropharyngeal trauma, muscular weakness, loss of sensation, delirium/sedation, reflux, and disorganized breathing with swallowing(6). Each of these results of intubation have the potential to negatively impact swallowing. Factors associated with increased risk of PED in adults include duration of intubation, particularly intubation greater than 48 hours, age, and functional status/medical fragility(1–4,7,10,16,20)

There are considerable differences in swallowing anatomy and physiology between infants, children and adults, and these differences are likely to be relevant to intubation and its impact on swallow function(25). Further, because swallowing and feeding are not yet fully developed during infancy and early childhood, any interruption of swallowing and feeding at critical developmental stages can result in significant negative outcomes that include maladaptive oral motor learning and oral aversion(26).

Despite the potential for PED to significantly influence health, mortality and developmental outcomes in infants and children, its systematic investigation has been scarce.(27) Knowledge of the incidence of PED, characterization of PED, and association between PED and health

outcomes in pediatrics are not yet defined. To address these gaps in knowledge, we designed a retrospective, observational cohort study to describe the incidence of PED, determine factors that may contribute to PED, and to assess the impact of PED on patient outcomes in pediatrics. We hypothesized that the incidence of PED in pediatrics would approximate or exceed the incidence in adults, age and increased duration of intubation would have a strong association with diagnosis of dysphagia, and that the presence of PED would have a significant impact on outcomes including total length of stay and need for non-oral means of nutrition at discharge. To facilitate comparison to PED literature in adults, sub-groups of patients with only neurologic primary diagnoses and non-neurologic diagnoses were analyzed in addition to the full cohort.

Methods

Study Design and Case Ascertainment:

We performed a retrospective, observational cohort study of infants and children to assess incidence of PED, contributing factors and associations between PED and health outcomes. Three types of documentation were analyzed to define patient characteristics, determine presence of dysphagia, and to describe outcomes. First, clinical flow sheet reports in the electronic medical record of the first feeding following extubation were reviewed. These flow sheets were designed by pediatric nurses and speech-language pathologists and are completed by nursing staff or a speech-language pathologist for every oral intake event**.** All nursing staff are trained by a clinical nurse specialist or a speech-language pathologist on quality-based feeding assessments, and how to complete these flow-sheets. Data contained in flowsheets includes a quality-based feeding assessment with drop-down menus to describe aspects of

feeding, including changes in respiratory status, signs of aspiration (described above), oral motor coordination, volume of intake, and for nipple-fed infants organization with root and latch to the nipple. There are instances in which flow-sheet data were not completed. In these situations, the medical record was reviewed for clinical swallowing evaluations by the speech-language pathologist at the first feeding following extubation, including report of presence and description of signs of aspiration or absence of signs of aspiration. In the rare event that neither clinical flowsheets nor speech-language pathology swallow assessments were completed, nursing shift summaries and nursing notes were reviewed; if they contained documentation of overt signs of aspiration *during the first feeding following extubation* (explicitly stating that signs of aspiration were observed during the feeding, not separately from feeding), the patient was placed in the dysphagia group. If this was not documented, the patient was placed in the non-dysphagia group. This study was approved by the Institutional Review Board at the University of Wisconsin-Madison.

Sample:

Patients were included if they were admitted to the American Family Children's Hospital (AFCH) between 2011 and 2017, were aged 0-16, were intubated and mechanically ventilated, were subsequently extubated in an intensive care unit at AFCH, and attempted oral feeding within 72 hours of extubation. The inclusion of patients who attempted oral feeding within 72 hours of extubation was performed to isolate the impact of intubation on dysphagia, as those who were not able to attempt oral feeding in the first 72 hours may have developed dysphagia related to factors other than intubation. Patients with a documented history of dysphagia prior to

intubation, those with a history of head and/or neck cancer or head and/or neck surgery or radiation and those with a history of tracheostomy were excluded. Exclusion criteria were chosen in an attempt to separate dysphagia specific to intubation from pre-existing dysphagia and from dysphagia that may have resulted from non-intubation insults to the swallowing mechanism.

Outcome Measures

The primary outcome measure was presence versus absence of signs of dysphagia at the first feeding following extubation, including feeding-related coughing, choking, wet and gurgling voice and breathing quality and/or bradycardia with desaturation(25,28–31). Those demonstrating these common signs of dysphagia were included in the PED group. To more-precisely quantify the characteristics of patients with PED, secondary analysis assessed changing odds for PED among patient attributes including dichotomized age (0-24 months and 25 or more months), duration of intubation, weight, gender, number of complex chronic conditions(32,33), emergent versus planned intubation and primary diagnosis. Primary diagnosis groups included: traumatic brain injury, neurologic or neurosurgical, pulmonary (ie RSV bronchiolitis, status asthmaticus, and bronchopulmonary dysplasia), cardiac or cardiothoracic surgery (ie hypoplastic left heart, tetralogy of Fallot, cardiac arrest), trauma, and systemic or other medical complexity. Factors were chosen for analysis *a priori* because they have been implicated as risk factors in studies with adults[1-23]. Tertiary analysis examined the effect of PED on the following patient outcomes: total length of stay, need for gastrostomy or nasogastric tube at discharge, and time between extubation and discharge.

Associated Factors

The following factors and conditions were identified *a priori* and were analyzed for associations with PED: gender, age as a continuous variable in months, age as a binary variable (0-24 months and 25 or more months), primary diagnosis, duration of intubation, weight, gender, number of complex chronic conditions (CCC) and emergent versus planned intubation.

Subgroup Analysis

Data analysis was performed on all patients who met criteria, with subsequent analysis performed on subgroups identified *a priori*, including patients with a neurologic condition as primary diagnosis and patients with a non-neurologic condition as primary diagnosis. These subgroups were chosen to facilitate comparison with studies of PED in the adult literature, which sometimes consider these groups separately(7,9,14,24).

Statistical Analysis:

Categorical data (gender, primary diagnosis, age 0-24 versus 25 or more months, presence of PED, need for emergent intubation) are reported in terms of frequencies of occurrence and percentages, normally distributed data are presented as mean ± standard deviation notation, and non-normally distributed data are presented as median and interquartile range.

Bivariate analysis was performed using the chi-square test of independence for PED status and categorical factors, and two sample t-tests to identify numerical factors independently

associated with PED. When data were not normally distributed, Mann-Whitney Rank Sum tests were performed. Logistic regression analysis was used to identify factors associated with PED among gender, age, dichotomized age, weight, duration of intubation, primary diagnosis, and number of complex chronic conditions. Logistic regression analysis was also used to assess odds for need of alternative means of nutrition at discharge by PED status, controlling for number of CCCs and dichotomized age. Multiple linear regression was used to assess impact of PED status on total length of stay and time between extubation and discharge, adjusting for number of CCCs, dichotomized age and primary diagnosis.

Results:

Incidence of PED

After application of inclusion and exclusion criteria, 372 patients were included for analysis. One hundred eight of 372 (29%, 95% CI 24.5-33.9%) exhibited PED (Table 1). Of the subgroup of patients with a neurologic or neurosurgical primary diagnosis, 17 of 82 (20.7%, 95% CI 12.6-31.1%) exhibited PED. Of the subgroup of patients without a neurological or neurosurgical diagnosis, 89 of 285 (31%, 95% CI 25.9-37%) exhibited PED. Of note, 5 patients were excluded from subsequent analysis because no clearly defined primary diagnosis was listed in the electronic medical record.

Characteristics of patients with PED

Full Cohort

Bivariate analysis of patient characteristics (continuous and dichotomized age, weight, primary

diagnosis, duration of intubation, gender, and need for emergent intubation) with PED was completed for three patient cohorts: all patients, those patients with a neurologic or neurosurgical primary diagnosis, and those patients without a neurologic or neurosurgical diagnosis. In analysis of all patients, patients with dysphagia were significantly more likely to be 0-24 months versus 25+ months (65.7% of dysphagic patients versus 41.7% of non-dysphagic patients were aged 0-24 months, $p<0.0001$), and were intubated for a significantly greater amount of time than patients with no dysphagia (92.3. hours versus 19.3 hours, $p<0.001$). The categorical variable of primary diagnosis also differed between patients with and without PED ($p<0.01$), with pulmonary, cardiac and trauma diagnoses representing a greater percentage of patients with PED than those without. See Table 1 for additional characteristic outcomes.

Neurologic Cohort

Patients with a neurologic or neurosurgical primary diagnosis with dysphagia had significantly greater number of emergent intubations (47.1% versus 21.5%, $p=0.04$) than those without dysphagia, and were intubated for a significantly greater time (56.2 hours versus 17.6 hours, $p<0.001$). Those with dysphagia did not differ significantly from those without dysphagia in age (58.7 months versus 65.7 months, $p=0.73$), weight (22kg vs 24.8kg, $p =0.67$) or gender (47% female versus 49.1% female, $p=0.87$).

Non-neurologic Cohort

Patients without a neurological or neurosurgical diagnosis and dysphagia were significantly younger (3 months versus 59 months, $p<0.001$), had significantly lower weight (6kg versus

17.5kg, p=0.001), and were intubated for a longer period of time (94.4 hours versus 20.6 hours, p<0.001) than patients with no dysphagia. Primary diagnoses were also significantly different between groups (p<0.01), with pulmonary (34%), trauma (10%) and cardiac/cardiothoracic surgery (35%) diagnoses being more common in those with dysphagia than those without dysphagia. Those with dysphagia did not differ significantly from those without dysphagia in need for emergent intubation (71.9% versus 71.4%, p=0.93) or gender (35.8% female versus 39.1% female, p=0.47).

Risk factors for PED

Full Cohort

Multivariable logistic regression was used to assess the odds of patient characteristics for PED controlling for age, weight, number of complex chronic conditions and primary diagnosis, described in Table 2. When analyzing all patients, odds of dysphagia increased 2.63 times for patients aged 0-24 months versus patients aged 25 months or more (95% CI 1.2-6, p=0.02). Odds of dysphagia increased by 5.06 times for patients with a primary diagnosis of Trauma versus patients with a primary diagnosis of Systemic or Other Medical Complexity (95% CI 1.6-16.3, p<0.01). No other primary diagnosis resulted in a significant increase in odds of dysphagia. Every hour of intubation resulted in increased odds of dysphagia by 1.7% (p<0.0001). Thus, for each additional day of intubation, odds of dysphagia were observed to increase by approximately 50%. Weight and number of complex chronic conditions did not influence odds for PED. Figure 1 visualizes the increase in risk of PED as duration of intubation increases for three patient populations: patients with a trauma diagnosis aged 25 or

more months; patients with non-specific diagnosis aged 0-24 months, and patients with non-specific systematic diagnosis aged 25 or more months.

Neurologic Cohort

Of patients with a primary neurological or neurosurgical diagnosis, when controlling for age, weight, and number of complex chronic conditions, every hour of intubation was observed to increase odds of dysphagia by 4.7% ($p<0.01$). Weight, age and number of complex chronic conditions did not influence odds for PED.

Non-neurologic Cohort

Of patients without a primary neurological or neurosurgical diagnosis, age of 0-24 months increased odds of dysphagia 2.59 times compared to patients aged 25+ months ($p=0.04$). Primary diagnosis of trauma increased odds of dysphagia 4.87 times compared to primary diagnosis of systemic or other medical complexity ($p<0.01$). Every hour of intubation was observed to increase odds of dysphagia by 1.016% ($p<0.0001$). Weight and number of complex chronic conditions did not influence odds for PED.

Impact of PED on Patient Outcomes

Full Cohort

Multiple linear regression was used to assess the impact of PED on patient outcomes of total length of stay and time between extubation and discharge, and logistic regression was used to assess impact of PED on need for alternative means of nutrition at time of discharge. Analysis

of need for alternative means of nutrition for patients with a neurologic or neurosurgical primary diagnosis was not completed because the event count was insufficient for analysis. When controlling for age, primary diagnosis, number of complex chronic conditions and dysphagia status for all patients, PED was associated with an increase in total length of stay of 10.95 days ($p<0.0001$, 95% CI 8.7-13.2) as well as an increase in time between extubation and discharge of 8.65 days ($p<0.0001$, 95% CI 6.6-10.7) compared to patients without dysphagia. Age, primary diagnosis and number of complex chronic conditions did not significantly influence length of stay or time between extubation and discharge. Presence of dysphagia (OR 22.22, 95% CI 6.4-77.6, $p<0.0001$) and having 1 or more complex chronic conditions (OR 3.1, 95% CI 1.3-7.6, $p=0.012$) were found to be associated with an increase in odds of need for alternative means of nutrition at time of discharge. Age of 0-24 months versus 25 or more months (OR 2.84, 95% CI .981-8.19, $p>0.05$) did not significantly change odds of need for alternative means of nutrition. Table 4 (online only) describes frequency of need for alternative means of nutrition at time of discharge by dysphagia status.

Neurologic Cohort

Of patients with a primary neurological or neurosurgical diagnosis, when controlling for age, number of complex chronic conditions and dysphagia status, PED was associated with an increase in total length of stay of 16.7 days (95% CI 11.6-21.9, $p<0.0001$) as well as an increase in time between extubation and discharge of 14.6 days (95% CI 9.6-19.6, $p<0.0001$). In contrast, age of 0-24 months versus 25 or more months was associated with decreased length of stay by 4.8 days (95% CI 9.1-0.5, $p=0.028$) and decreased time between extubation and discharge by

4.9 days (95% CI 9--0.8, p=0.02). Number of complex chronic conditions did not significantly influence length of stay or time between extubation and discharge.

Non-neurologic cohort

Of patients without a primary neurological or neurosurgical diagnosis, when controlling for primary diagnosis, age, number of complex chronic conditions and dysphagia status, PED was associated with an increase in total length of stay of 9.6 days (95% CI 7.1-12, p<0.0001) as well as with an increase in time between extubation and discharge of 7.3 days (95% CI 5.1-9.4, P<0.0001). Additionally, having one or more complex chronic conditions was associated with increased length of stay by 4.3 days (95% CI 1.2-7.4, p<0.01) and with increased time between extubation and discharge by 3.3 days (95% CI .6-6.0, p=0.02). Age did not significantly influence length of stay or time between extubation and discharge. After adjusting for dysphagia status, dichotomized age and number of complex chronic conditions, presence of dysphagia (OR 13.7, CI 3.8-49, p<0.0001) and having 1 or more complex chronic conditions (OR 2.8, CI 1-7.9, p=.05) were found to be associated with increase in odds of need for alternative means of nutrition at time of discharge.

Discussion

Because of the significant negative impact of PED on patient outcomes, early identification and intervention are essential. A preliminary step to executing early identification and intervention is the scientific description of the phenomenon of PED in pediatric populations, which is distinct from the phenomenon in adult populations. Such systematic exploration has, to date, been

limited. A single study of a specific population (neurologically intact children with respiratory illness) found that children with moderate-severe dysphagia were significantly more likely to have been intubated than those with no dysphagia and of children who were intubated, those with no dysphagia were intubated for a significantly shorter duration than those with moderate-severe dysphagia(27). The current study is the first to systematically describe incidence of PED in a broader pediatric population, to describe factors that influence PED, and the first to report on outcomes associated with PED.

With an overall incidence of 29%, PED in pediatrics is common, and exceeds the 23% incidence in adults reported by Malandraki et al in 2016(9). Factors that increased odds for PED in this study, including age and duration of intubation generally agreed with reports in literature studying PED in adults, although age has a negative association with odds of PED in infants and children, as opposed to a positive association in adults(7,9,16,24). The increase in odds of PED for certain pediatric populations is striking: age of 0-24 months increases odds of dysphagia 2.63 times; every hour of intubation increases odds of dysphagia by 1.7%, or about 50% per day of intubation. For patients with a neurologic or neurosurgical diagnosis, every hour of intubation increases odds of dysphagia by 4.7%. Further, PED is associated with negative outcomes of increased total length of stay, increased length of stay after extubation, and increased odds of alternative means of nutrition. Patients with PED had an average total length of stay in the hospital of 16.3 days, versus 5.4 days for patients without PED even when controlling for primary diagnosis and number of CCCs. By way of comparison, children with high-impact conditions with moderate illness severity and extreme illness severity have an

average length of stay of 9.8 and 32.8 days respectively(34). The impact of these negative outcomes on pediatric health and wellbeing is significant, as need for nasogastric and gastrostomy tube feeding in children has been shown to increase food refusal and increase emergency room visits and hospital admissions related to tube feeding(35–38).

Interestingly, patients with a neurological or neurosurgical primary diagnosis demonstrated a lower incidence of PED than those without a primary neurological or neurosurgical diagnosis. This contrasts with the adult literature (24). It is possible that institutions that have different distributions of neurosurgical patients (for example a greater number of brainstem or posterior tumor excisions) may have different outcomes, particularly as they relate to structures responsible for respiration and deglutition. In addition, severity of neurologic injury across different neurologic diagnoses may directly impact duration of intubation and timing of initial oral feeding following extubation; these factors could conceivably have a significant impact on the results for this neurologic subgroup. Future studies would benefit from multi-center designs in order to ensure greater representation of pediatric neurosurgical subspecialties, mitigating the potential for institution-specific confounds.

An additional element that must be considered when discussing dysphagia in infants and children is the fact that swallowing and feeding change dramatically in physiology and behavior over the course of the first several years of life. Interruption of this development can lead not only to maladaptive swallowing function that compromises patient safety and efficiency or oral intake, but can also lead to food refusals and oral aversion(25,26,39). The psychosocial,

economic and healthcare burden of dysphagia and feeding difficulty in adults is tremendous(40,41). To help mitigate the potential negative impact of PED, routine screening for PED and referral to speech-language pathologists with specialization in pediatric dysphagia should be completed, allowing for early diagnosis and treatment through diet modification and behavioral intervention.

There are some differences between the current study and studies examining PED in adults. The current study defined PED as the presence of overt signs of dysphagia including feeding-associated coughing, choking, wet and gurgling voice and breathing quality and/or bradycardia with desaturation at the first feeding following extubation. These signs were reported by a speech-language pathologists, nursing staff, or a physician, which contrasts with several studies that use a diagnosis of dysphagia by a speech-language pathologist to define PED(7,9,24). While very specific, the use of a dysphagia diagnosis by a speech-language pathologist in this retrospective study would have produced a biased sample, as only patients that demonstrated sufficient swallowing or feeding difficulty as assessed by nursing or other providers would have received a referral to Speech-Language Pathology service, whereas those with minimal but clinically significant dysphagia could have been excluded. Our methods also differed from previous studies in adults, in that we only assessed subjects who attempted oral feeding within 72 hours of extubation, as opposed to studies who considered presence of dysphagia at any point between extubation and discharge from the hospital to constitute PED(7,9,24).

Limitations to this study include those inherent to its retrospective nature, and the fact that the

primary outcome measure required clearly observable signs of dysphagia without use of instrumented assessments of swallowing (i.e. fiberoptic endoscopic evaluation of swallowing (FEES) or videofluoroscopic swallow study). Studies in adults that have used FEES, which can detect passage of a bolus into the trachea without a cough or other airway protective response (silent aspiration), have shown that 38-44% of subjects who aspirate are silent aspirators(5,12). Further, in a 2010 systematic review, Skoretz et al reported studies that used FEES describe frequency of dysphagia ranging from 44-56%, much higher than those that used non-instrumented assessments of swallowing(14). This implies that the sensitivity of non-instrumented assessments of swallowing may be reduced, and that actual incidence of PED in pediatric populations may be higher. An additional limitation of this study is that it, as designed, it is not able to investigate the mechanisms PED, such as laryngeal injury, sedation, muscular weakness and disorganized breathing(6). Use of instrumented assessments of swallowing would help to clarify mechanisms of PED, and in doing so would either support or contrast with the current regression models. For example, absence of any obviously laryngeal or pharyngeal trauma in a patient with a neurologic primary diagnosis would imply that the mechanism of dysphagia was associated with neurologic status rather than with intubation, contrasting with our regression models. An additional limitation of this initial investigation into PED in pediatric populations is that the primary diagnosis categories are broad and do not account for severity of injury or illness. As such, the possibility remains that severity of injury or illness, and differences between more specific diagnoses may function as confounding variables. For example, a cardiac surgery that involves manipulation of the aortic arch and the recurrent laryngeal nerve may result in increased risk of dysphagia versus other cardiac surgeries. In

future studies, larger sample sizes from multiple institutions would allow for more-discrete categories and help to account for these factors.

Conclusion

This study is the first to systematically describe incidence of PED, to describe factors that influence PED, and to report on outcomes associated with PED. Our data support the hypotheses that incidence of PED in pediatrics exceeds the incidence in adults, age and increased duration of intubation and timing of initial oral feeding would have a strong association with diagnosis of dysphagia, with additional factors not reaching statistical significance; and presence of PED would have a significant impact on odds for outcomes including total length of stay and need for non-oral means of nutrition at discharge. Our data show that PED is common in pediatrics, and that age of 0-24 months, increased duration of intubation and primary diagnosis of trauma substantially increase odds of PED. Routine screening and early referral to speech-language pathologists and other providers specializing in dysphagia for evaluation and treatment are recommended.

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Table 1: Bivariate analysis of patient characteristics associated with PED for all patients

	Full Cohort (n=372)	Dysphagia (n=108)	No Dysphagia (n=264)	P-Value
Age in Months (Median (IQR))	29(3.3-129.8)	3.5 (0-128.5)	59.5 (9-132.3)	Mann-Whitney U p<.001*
Age (Categorical)				Chi Square <.0001*
0-24 months	181(48.7%)	71(65.7%)	110 (41.7%)	
25+ months	191(51.3%)	37(34.3%)	154(58.3%)	
Gender				Chi Square .3209
Female	149(40.1%)	39 (36.1%)	110 (41.7%)	
Male	223(59.9%)	69(63.9%)	154(58.3%)	
Emergent Intubation				Chi Square .3326
Yes	104(28%)	34(31.5%)	70(26.5%)	
No	268(72%)	74(68.5%)	194(73.5%)	
Primary Diagnosis (Dysphagia n=106, No Dysphagia n=261) (%)**				Chi Square .0016*
Traumatic Brain Injury	29(7.9%)	7(6.6%)	22(8.4%)	
Neurologic (Stroke, Degenerative, Other)	82(22.3%)	17(16%)	65(24.9%)	
Pulmonary	78(21.3%)	30(28.3%)	48(18.4%)	
Systemic or Other Medical Complexity	78(21.3%)	12(11.3%)	66(25.3%)	
Trauma	22(6%)	9(8.5%)	13(5%)	
Cardiac Diagnosis or Cardiothoracic Surgery	78(21.3%)	31(29.3%)	47(18%)	
Weight Kilograms (Median(IQR))	14(6.1-37.9)	6.2(4.2-32.2)	17.8(8.6-40.2)	Mann-Whitney U p<.001*
Duration of Intubation (Hours) (Median(IQR))	25.3(13.6-72.5)	92.3(41-129.3)	19.3(11-34.3)	Mann-Whitney U p<.001*

*=significant difference between dysphagia and no dysphagia(alpha=.05). **5 patients did not have a defined primary diagnosis

Table 2: Multivariable logistic regression to assess odds of patient characteristics for PED for all patients

	Adjusted Odds Ratio	p-value	95% Confidence Interval
Age (0-24mo versus 25+mo)	2.627	p=.0218*	1.151-5.997
Primary Diagnosis (compared to Systemic or Other Medical Complexity)			
Traumatic Brain Injury	1.549	p=0.4803	0.460-5.261
Neurologic (Stroke, Degenerative, Other)	1.317	p=0.5637	0.517-3.354
Pulmonary	0.858	p=0.7591	0.323-2.281
Trauma	5.058	p=.0067*	1.567-16.321
Cardiac Diagnosis or Cardiothoracic Surgery	2.235	p=0.0859	0.893-5.596
Weight (Kilograms)	1.004	p=.6703	0.987-1.021
Duration of Intubation (Hours)	1.017	p<.0001*	1.012-1.023
Complex Chronic Conditions (1 or more versus 0)	0.869	p=0.6791	0.447-1.690

*=significant (alpha=.05)

Table 3: Multiple linear regression was used to assess the impact of PED on patient outcomes for all patients

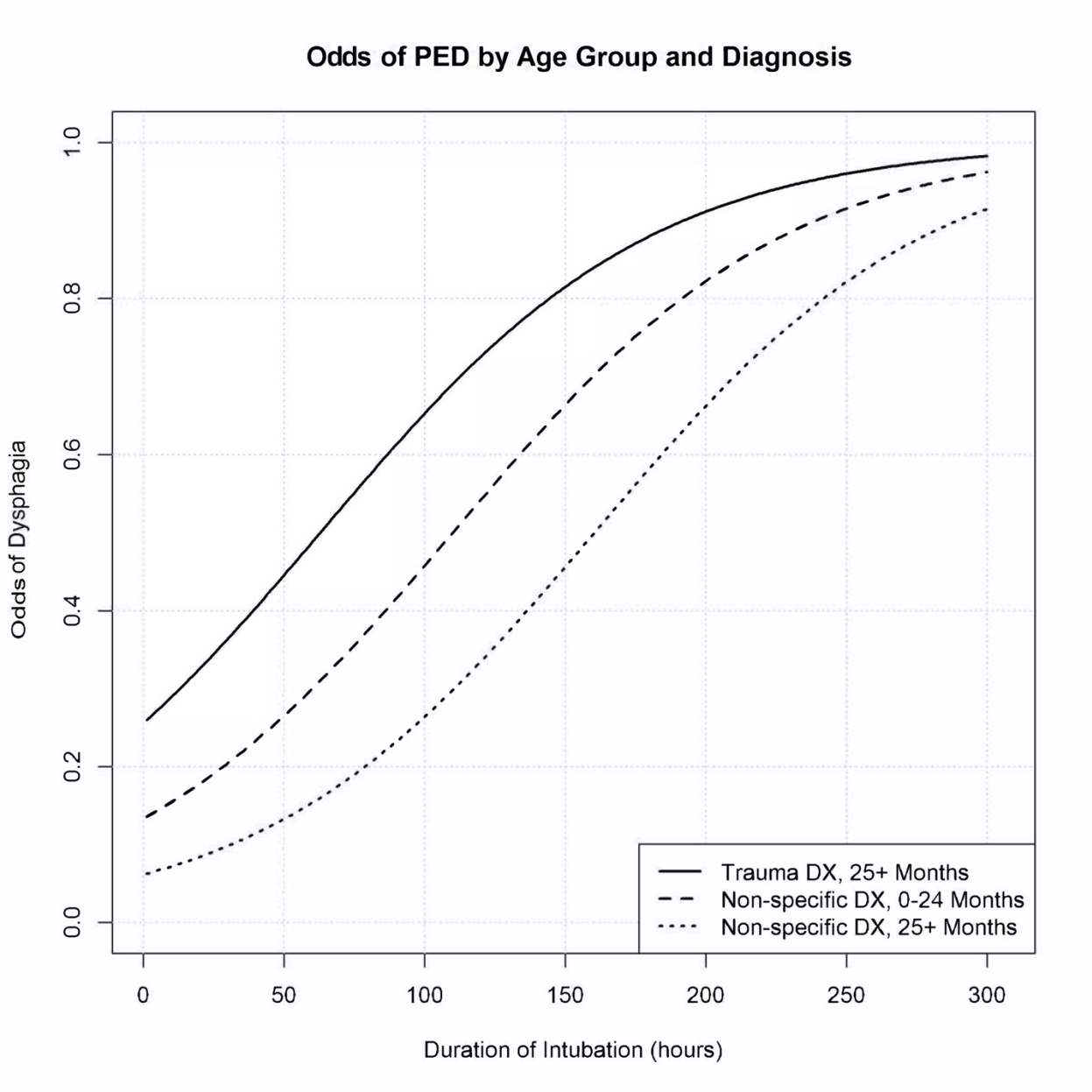
	Increase in length of stay (days)	Time between Extubation and Discharge (days)
Age 0-24 months (compared to 25+ months)	-1.15 days	-1.45 days
p-value (95% CI)	p=.314 (-3.4-1.1)	p=.16 (-3.4 - 0.56)
Dysphagia (Compared to No Dysphagia)	10.95 days	8.65 days
p-value (95% CI)	p<.0001* (8.7-13.2)	p<.0001* (6.6-10.7)
1 or more Complex Chronic Conditions (compared to none)	1.72 days	0.911 days
p-value (95% CI)	p=.177 (-0.8-4.2)	p=.425 (-1.3 - 3.2)
Primary Diagnosis (compared to Systemic or Other Medical Complexity)		
Traumatic Brain Injury	2.57 days	2.78 days
p-value (95% CI)	p=.213 (-1.5-6.7)	p= .13 (-0.9 - 6.4)
Neurologic	1.05 days	1.57 days
p-value (95% CI)	p=.506 (-2.1-4.2)	p= .26 (-1.2 - 4.4)
Pulmonary	1.0 days	-1.04 days
p-value (95% CI)	p=.536 (-2.2-4.2)	p=.47 (-3.9 - 1.8)
Trauma	-.42 days	0.41 days
p-value (95% CI)	p=.857(-5.0-4.2)	p=.84 (-3.7 - 4.5)
Cardiac Diagnosis or Cardiothoracic Surgery	2.09 days	2 days
p-value (95% CI)	p=.22 (-1.3 -5.4)	p=.19 (-1.0 - 5.0)

*=significant (alpha=.05)

Table 4: Distribution of need for alternative means of nutrition by dysphagia status.

	Dysphagia (n=108)	No Dysphagia (n=264)
Alternative Means of Nutrition at Discharge		
Yes:	n=24 (22%)	n=3 (1%)
No:	n=84 (78%)	261(99%)

Figure 1



Odds of Dysphagia increase by 1.7% for every hour of intubation (50% per day). Odds are increased for patients aged 0-24 months versus 25+ months and are further-increased for patients with a primary diagnosis of Trauma (there were no patients aged 0-24 months with a primary diagnosis of Trauma in this study).