Title:

Sex-Specific Vocalization Impairments in A53T α-synuclein mutant Mice

Abstract

Parkinson's Disease is a neurodegenerative disorder that is characterized by both motor and nonmotor symptoms, including vocalization deficits. Among the various causes of Parkinson's Disease, a mutation in the α-synuclein gene is a significant contributor. In this paper, we analyze a dataset containing acoustic vocalization measurements from both A53T mutant and wild-type mice, comparing overall differences between mutant and control groups, as well as differences between males and females. The dataset included parameters such as duration, maximum frequency, minimum frequency, maximum power, and frequency differences between simple and complex vocalizations. Mutant mice exhibited a higher duration of simple vocalization and a lower duration of complex vocalization. Mutant animals exhibited higher frequency and maximum power in simple calls, particularly among females, while complex calls showed little difference, suggesting the A53T mutation primarily disrupts simple vocal patterns. Moreover, both simple and complex vocalizations show statistically significant differences in minimum and maximum frequency between mutant and control groups. These results suggest that the A53T mutation in the a-synuclein gene selectively alters simple vocalization patterns more than complex ones, with a pronounced effect on frequency and power, particularly in females. This suggests that vocal communication deficits in this Parkinson’s disease model may be sex-dependent and more pronounced in specific types of vocalizations.

Key words: PD, vocalization, A-synuclein, sex difference

Introduction

Parkinson's disease (PD) is a neurodegenerative disorder of movement that has features of cognitive impairment, autonomic dysfunction, sleep disorders, hyposmia, and depression (Poewe et al., 2017). PD affected a large portion of the population, approximately 6.1 million people worldwide (Bloem et al., 2021). Although the mechanism has not yet been fully understood, the prevalence of the disease has risen rapidly over the past few decades, and the personal impact of the disease is immense (Bloem et al., 2021). PD includes two types of symptoms: motor and nonmotor. Motor symptoms refer to the movement-related symptoms, including slowness of voluntary movements with progressive reduction in speed, muscular rigidity, and postural instability (Hughes et al., 1992). On the other hand, non-motor symptoms are not related to movements and can appear before motor symptoms do (Hughes et al., 1992). The non-motor symptoms include a lack of emotional involvement, sleep problems, loss of smell and taste, mood disturbances, fatigue, and pain (Pont‐Sunyer et al., 2015). Genetic mutations represent one of the most significant contributing factors among the various causes of Parkinson's disease. (Bloem et al., 2021.) Namely, a-Synclein was the first PD-gene to be discovered (Matsumine et al., 1997). Synucleins are proteins that are abundant in the brain, located in the olfactory bulb, the dorsal motor nucleus of the vagus (DMV), and the Substantia nigra pars compacta in the early stages (Taguchi et al., 2016). Synucleins consist of three members- a-synuclein, b-synuclein, and y-synuclein (Lavedan, 1998).

Among them, a-synuclein is particularly closely linked to PD through mutations such as P.Ala53Thr, a mutation in the Alanine residue. This mutation is known to be associated with

many detrimental symptoms of PD (Choi et al., 2008; Spira et al., 2001). The mouse model of PD with the A53T mutation develops numerous sensorimotor and synaptic impairments, followed by age-associated cognitive and motor deficits (Paumier et al., 2013). Furthermore, A53T α-synuclein in astrocytes contributes to initiating the damaging process that leads to neuron death (Gu et al., 2010). Some papers described the mechanism by mentioning that the AT53 mutation leads to more stable a-synuclein in neurons, which causes it to accumulate to high levels and contributes to the progression of PD (Li et al., 2004). Although dopaminergic therapies remain the standard for managing PD, several unmet needs persist in both motor and non-motor symptoms (Elkouzi et al., 2019). Therefore, the challenges remain in finding ways to change the course of the disease.

On top of the symptoms of common PD patients, it was also discovered that there is an association of vocalization with PD. The vocalization involves the mechanism that supports the accurate coordination of respiration and the adjustment of the vocal fold tension and length (Rektorová et al., 2012). Several studies have found a deep relationship between vocalization and PD (Grant et al., 2014; Rektorová et al., 2012). Vocal fold atrophy can be a potential factor contributing to impaired swallowing safety in PD (Yiu et al., 2020). The study also found that prosodic and acoustic features were the most effective indicators for detecting PD (Bocklet et al., 2011). The rodent model of PD also demonstrates relevance to vocalization. The researchers recorded ultrasonic vocalizations from Thy1-aSyn transgenic mice and wild-type controls at different ages to assess vocalization deficits in the experiment (Grant et al., 2014). Vocalization deficits in Thy1-asyn mice appeared early in life, around two to three months (Grant et al., 2014). Additionally, duration and intensity of calls were comparably reduced, and the call profile was altered in Thy1-asyn mice, especially at two to three months (Grant et al., 2014). Another

study also found that rats with aggregated alpha-synuclein showed earlier and progressive vocal deficits. (Grant et al., 2015). Early and progressive deficits in ultrasonic vocalization and motor functions, including tongue force and biting, were observed (Grant et al., 2015). This pattern suggests that the buildup of alpha-synuclein in the affected region is impacting the areas that control the vocal cords. As vocalization is an essential aspect of PD, the early deficits observed in this model serve as a crucial biomarker for this disease.

It has been suggested that sex is one of the crucial components of the development of PD. Several studies have indicated differences between the sexes (Haaxma et al., 2007). The neurodegenerative disorder is approximately twice as common in men as in women (Baldereschi et al., 2000; Solla et al., 2012). Building on these observations, the study examined gender differences in PD by analyzing symptoms, brain changes, and hormonal effects in 253 patients (Haaxma et al., 2007). It found that women developed PD two years later than men, and this later start of the disease is likely to be related to estrogen-related factors like menopause and childbirth (Haaxma et al., 2007). Women were more likely to experience tremors as their initial symptoms, a pattern associated with a slower progression of the disease and milder motor symptoms. As a result, women tend to show milder degeneration compared to men, suggesting a benign phenotype shown in women (Haaxma et al., 2007). Motor and non-motor symptoms varied based on sex differences. In the beginning, women experienced more nonmotor symptoms, such as fatigue, depression, and pain, whereas motor symptoms such as speech problems were noted in men (Santos-García et al., 2023). However, after two years, men's nonmotor symptoms got worse, and women's abilities to function in daily life declined more (Santos-García et al., 2023). Similarly, Picillo et al. examined how PD progresses differently in men and women over a five-year period (Picillo et al., 2022). Men exhibited a faster decline in

both motor and nonmotor aspects and required higher doses of medications (Picillo et al., 2022). Their symptoms worsened more quickly, especially in daily tasks, even though they were on medications (Picillo et al., 2022). However, another study found contrasting results, suggesting that men do not always experience worse results than women (Abraham et al., 2019). They found that women with PD reported worse disability and quality of life than men, even though doctors did not see the major differences in symptoms (Abraham et al., 2019). Women also experienced more anxiety and had less support compared to men (Abraham et al., 2019). The study suggests that more mental support for women with PD may improve their mood and experiences with PD.

Additionally, by analyzing speech recordings from individuals with PD and matched healthy controls, the researchers found that most acoustic measures—such as pitch variability, speech rate, and vowel articulation—were significantly impacted by PD and sex as separate factors. However, the most notable finding was a sex-specific interaction in articulatory precision, particularly in the production of plosive consonants. Females with PD were more likely to produce multiple bursts during plosive sounds, whereas males with PD often failed to make a burst at all (Houle et al., 2024). This suggests that PD may disrupt articulatory timing and coordination differently in males and females, with females showing more fragmented plosive articulation and males showing reduced articulatory force. (Houle et al., 2024). Another study examined how PD contributes to the ability to express emotion through speech, with an emphasis on differences between males and females (Gnerre et al., 2023). Researchers compared 14 people with PD and 13 people without it, analyzing vocal features such as pitch, intensity, and voice quality during emotional and non-emotional (neutral) speech. The study found that emotional expression was flawed in people with PD, particularly women, while neutral speech showed no differences (Gnerre et al., 2023). Specifically, women with PD had a lower pitch

when expressing pleasure and poorer voice quality in fear and anger (Gnerre et al., 2023). Complementing these findings, an earlier study by Hertrich and Ackermann (1995) also explored sex-specific vocal changes in PD. The study examined how Parkinson's disease (PD) affects the voice of men and women by having them produce long vowel sounds and observing the movement of their vocal cords (Hertrich & Ackermann, 1995). They found that women with PD exhibited more voice irregularities, such as a shaky pitch and unusually low sounds, compared to control groups and men with PD (Hertrich & Ackermann, 1995). These issues are likely to stem from how the vocal cords vibrate differently in PD, suggesting that PD affects the voice of men and women differently, possibly due to differences in the natural size of the vocal cords. These studies suggest that vocal impairments in PD differ by sex, potentially impacting patients' social interactions and quality of life. Studying sex differences in vocalization within PD is significant because the differences can serve as a potential sex-specific biomarker. Identifying these gender-specific vocalization patterns as biomarkers could help track the progression of the disease more precisely, allowing the adjustment of treatment strategies in the early stages.

In this paper, we investigate whether there is a sex difference in vocalization from the A53T PD animal model. Specifically, we aim to explore the distinct differences between males' and females' vocal expression, a nonmotor symptom often observed in PD. This research aims to understand the sex-specific manifestations of non-motor symptoms, which may suggest future approaches to diagnosis, treatment, and personalized care.

Results

This study investigated whether there are sex differences in vocalization phenotypes in an animal model of Parkinson's disease. To determine sex differences in vocalization symptoms, we obtained the vocalization dataset from A53T mutant mice and wild-type mice (the control group). We analyzed the differences in both simple and complex vocalizations between these groups. Simple calls have a constant, non-modulating frequency, while complex calls contain two or more directional changes in frequency, each of which is at least 3 kHz (Krasko et al., 2021). Measuring the differences between simple and complex vocalizations is vital, as these patterns may reflect underlying challenges affected by PD.

First, we investigated whether the duration of vocalization is impaired in mutant animals, which highlights the difference between simple and complex vocalizations. Our analysis also revealed that mutant mice exhibited a significantly longer duration than control mice in the simple vocalization test (Figure 1A; KS test, $p = 0.02$). Mutant mice also exhibited a shorter duration than the control mice in the complex vocalization test (Figure 1B; KS test; $p = 0.04$). Then, to investigate whether there is a sex difference in vocalization duration, we analyzed our data using either female-only or male-only samples. In the simple vocalization test, a comparison between male wild-type and male mutant animals did not exhibit a significant difference. In addition, there is no significant difference between female mutant animals and female wild-type animals. In the complex vocalization test, there is a trend toward lower duration in male mutant animals compared to male wild-type controls (Figure 1D; KS test; $p = 0.08$). Our data show that mutant animals exhibit altered vocalization duration depending on the complexity of the vocalization, with increased duration in simple vocalizations and decreased duration in complex vocalizations, regardless of sex.

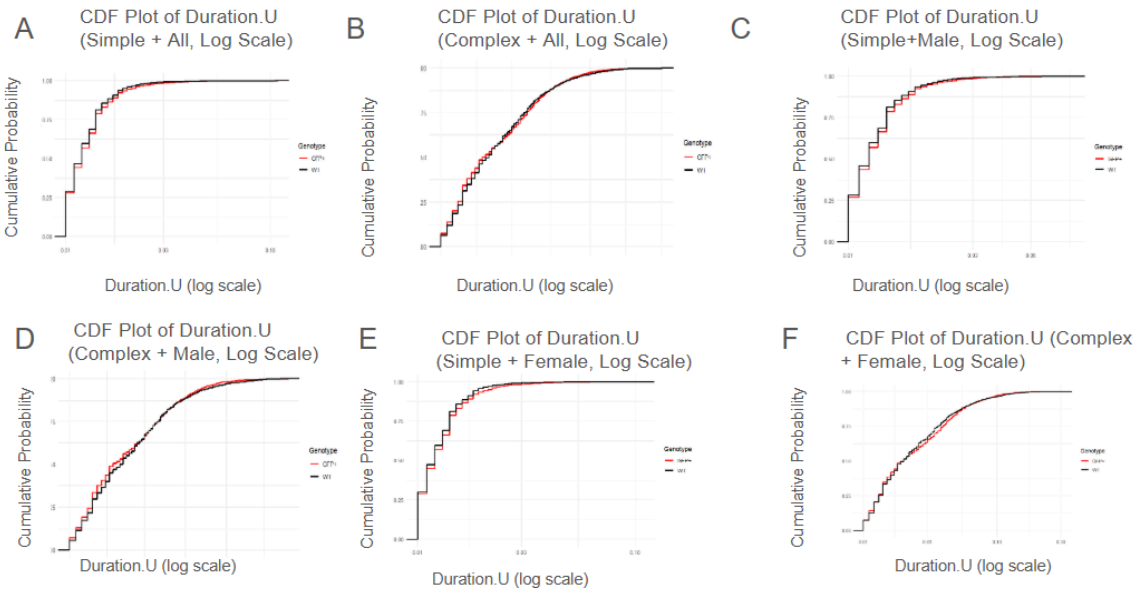

Figure 1. Cumulative distribution plots of vocalization duration (log scale) for wild-type (WT) and mutant mice.
(A) Simple vocalizations (all mice): Mutant mice showed longer durations than WT mice.
(B) Complex vocalizations (all mice): Mutant mice exhibited shorter durations than WT mice.
(C) Simple vocalizations (males only): No significant difference in duration was observed between genotypes.
(D) Complex vocalizations (males only): Mutant males showed slightly shorter durations than WT males.
(E) Simple vocalizations (females only): No significant difference in duration was observed between genotypes.
(F) Complex vocalizations (females only): No significant difference in duration was observed between genotypes.

Secondly, to determine whether the mutation alters the frequency of vocalization, we analyzed the difference between simple and complex vocalization. Mutant mice with simple vocalization showed significantly higher frequency (Figure 2A ; KS test; $p = 0.13$).

To investigate sex differences, we then analyzed the data separately for males and females. Mutant females with simple vocalization also manifested higher frequency (Figure 2E; KS test; $p = 0.03$). In comparison, there was no difference in complex vocalization between the mutant and control groups. Additionally, no differences were found between the simple and

complex in male mice. There was no difference in females with complex vocalization. Our data reveal that the mutation increases vocalization frequency in simple vocalizations, particularly in females, while having no significant effect on complex vocalizations in either sex.

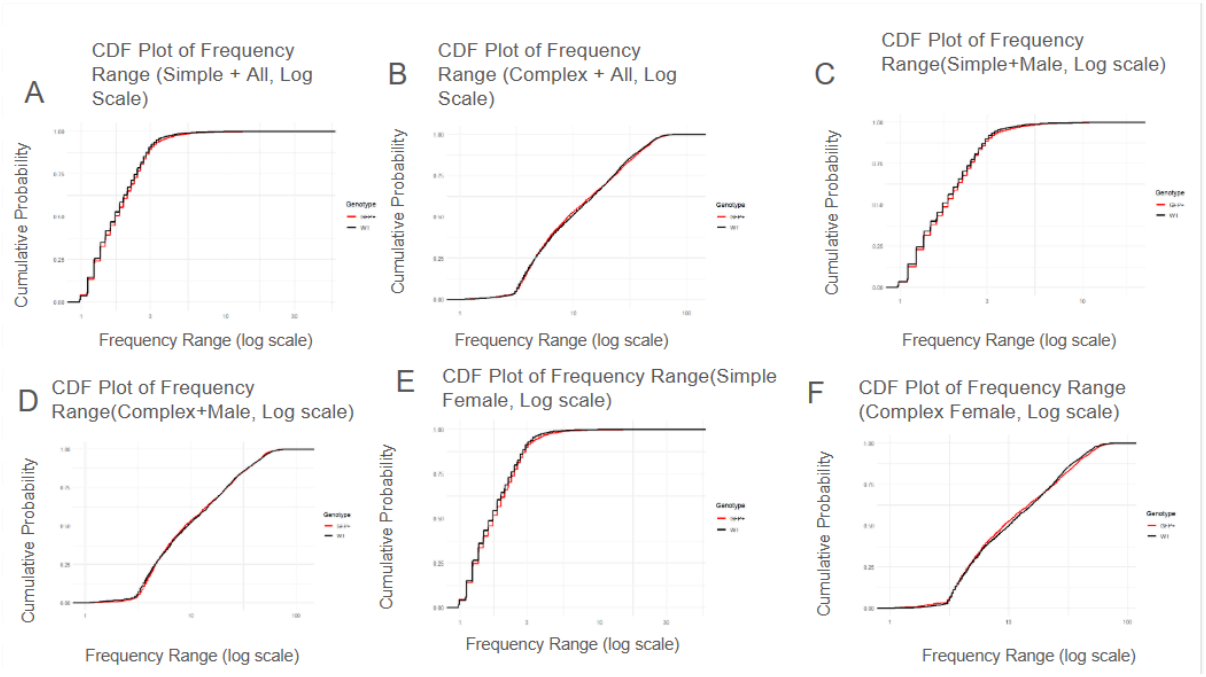

Figure 2. Cumulative distribution plots of vocalization frequency range (log scale) in wild-type (WT) and mutant mice.

(A) Simple vocalizations (all mice): Mutant mice exhibited a wider frequency range than WT mice.
(B) Complex vocalizations (all mice): No strong differences in frequency range were observed between genotypes.
(C) Simple vocalizations (males only): No genotype difference was found in frequency range.
(D) Complex vocalizations (males only): No genotype difference was found.
(E) Simple vocalizations (females only): Mutant females showed a significantly higher frequency range than WT females (p = 0.02, KS test).
(F) Complex vocalizations (females only): No significant difference between genotypes.

Thirdly, we analyzed whether the highest energy level (maximum power) of vocalization reveals a difference between simple and complex vocalizations. Mutant mice with simple vocalization showed significantly higher max acoustic power than the control in mice with simple vocalization (Figure 3A; KS test; p = 0.03). To identify a sex difference, we analyzed the

data separately for males and females. Female mutants with simple vocalization exhibited higher max acoustic power than the female control with simple vocalizations (Figure 3E; KS test; p = 0.02). There are no significant differences between mutant and wild-type animals in complex vocalization for either males or females. Our data indicate that mutation influences vocal intensity primarily in simple vocalizations, with a greater effect on females, while complex vocalizations remain unaffected.

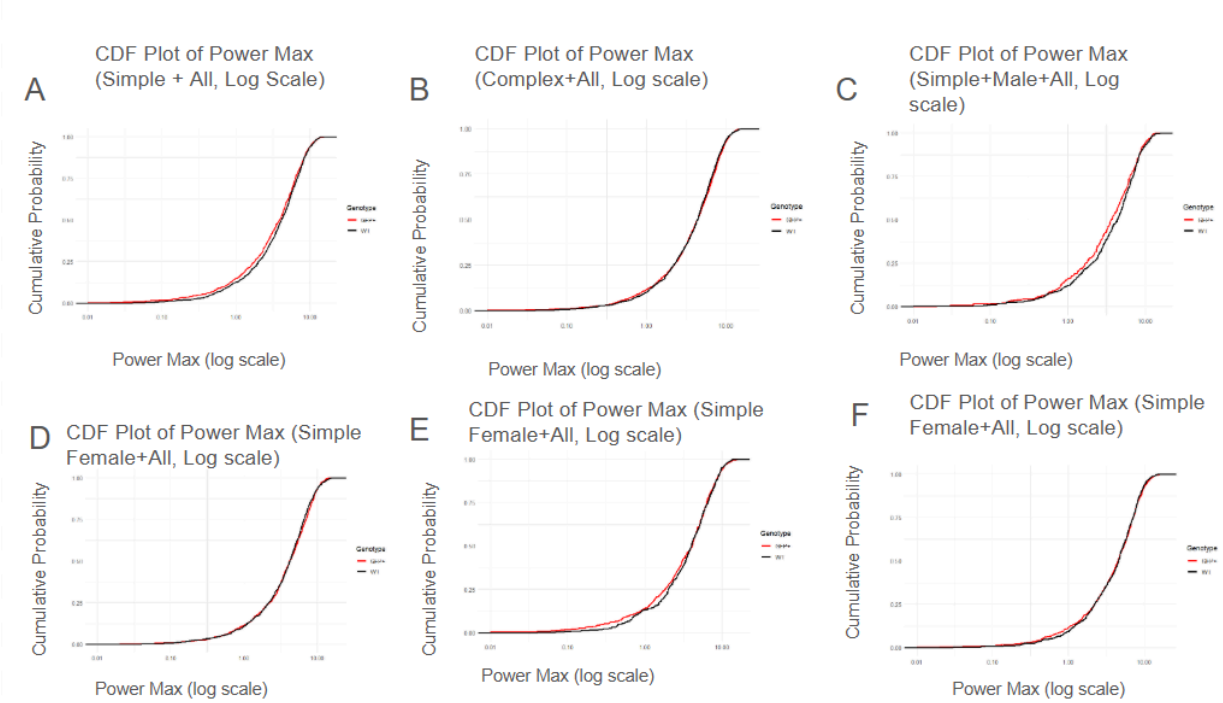

Figure 3. Cumulative distribution plots of maximum acoustic power (log scale) in wild-type (WT) and mutant mice.
(A) Simple vocalizations (all mice): Mutant mice showed significantly higher maximum acoustic power than WT mice.
(B) Complex vocalizations (all mice): No significant difference was observed between genotypes.
(C) Simple vocalizations (males only): No significant genotype-related difference in maximum acoustic power.
(D) Complex vocalizations (males only): No difference was found between genotypes.
(E) Simple vocalizations (females only): Mutant females exhibited significantly higher maximum acoustic power than WT females.
(F) Complex vocalizations (females only): No significant difference between genotypes.

Fourthly, we investigated whether the minimum frequency of vocalization shows some difference between simple and complex vocalization. Mutant mice with simple vocalization showed a significantly higher minimum frequency than the control mice with simple vocalization (Figure 4A; KS test; $p < 0.01$). Mutant mice with complex vocalizations showed a considerably higher minimum frequency than the control mice (Figure 4B; KS test; $p < 0.01$). We have also utilized the data for males and females separately to identify the differences between them. Male mutants with simple vocalization showed a higher frequency minimum than the male control with simple vocalizations (Figure 4C; KS test; $p < 0.01$). Male mutants with complex vocalizations exhibited a lower frequency minimum than the male control with complex vocalizations (Figure 4D; KS test, $p = 0.05$). Female mutants with simple vocalizations exhibited a higher minimum frequency than the female control with simple vocalizations (Figure 4E; KS test; $p < 0.01$). Female mutants with complex vocalizations exhibited a higher minimum frequency than the female control with complex vocalizations (Figure 4F; KS test; $p < 0.01$). Our data indicate that the mutation affects the frequency minimum of vocalizations, with effects varying depending on the complexity of the call and sex.

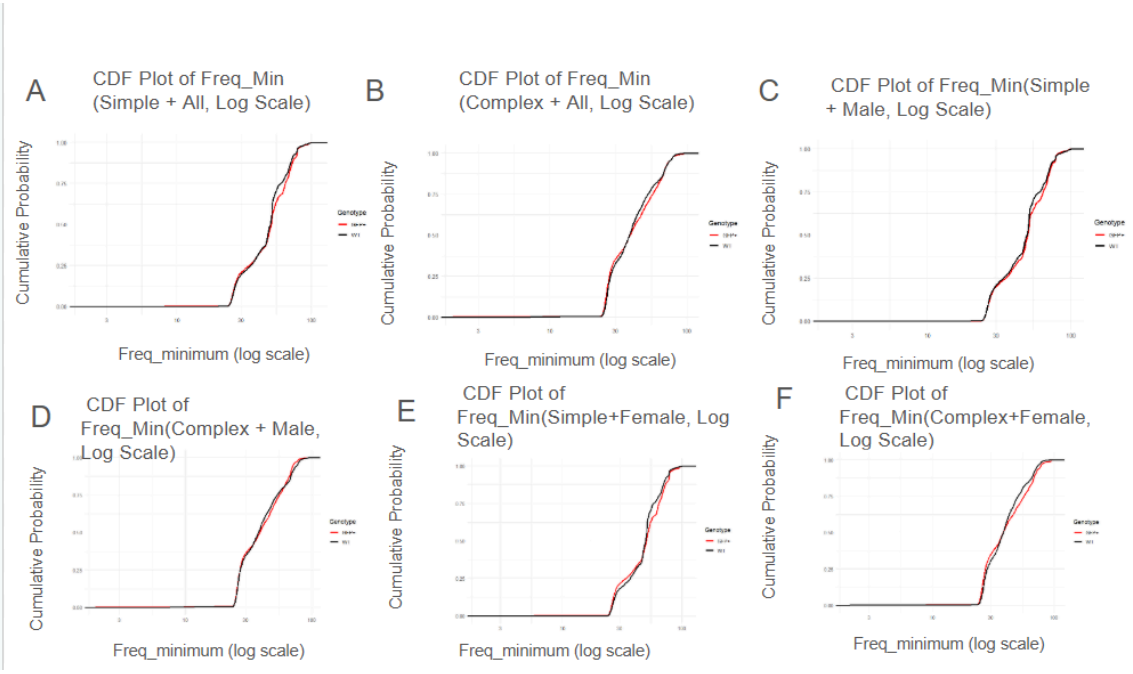

Figure 4. Cumulative distribution plots of minimum vocalization frequency (log scale) in wild-type (WT) and mutant mice.
(A) Mutant mice with simple vocalizations showed significantly higher minimum frequency than wild-type mice.
(B) In complex vocalizations, mutant mice also exhibited a higher minimum frequency than controls.
(C) Among males with simple vocalizations, mutant mice showed a higher minimum frequency than wild-type males.
(D) In contrast, for complex vocalizations, male mutants showed a lower minimum frequency than male wild-type mice.
(E) Female mutants with simple vocalizations exhibited a higher minimum frequency than female wild-type mice.
(F) Female mutants with complex vocalizations also showed a higher minimum frequency than their wild-type counterparts.

Then, we investigated whether the frequency maximum of vocalization shows some difference between simple and complex vocalization. Mutant mice with simple vocalizations exhibited a significantly higher maximum frequency than the control mice (Figure 5A; KS test; $p < 0.01$). Mutant mice with complex vocalizations showed a considerably higher maximum frequency than the control mice (Figure 5B; KS test; $p < 0.01$). We also analyzed the data separately for males and females to identify any sex-specific differences. Male mutants with

simple vocalization showed a higher frequency maximum than the male control with simple vocalizations (Figure 5C; KS test; $p < 0.01$). Male mutants with complex vocalization displayed a lower frequency minimum than the male control with complex vocalizations (Figure 5D; KS test; $p < 0.01$). Female mutants with simple vocalization showed a higher frequency minimum than the female control with simple vocalizations (Figure 5E; KS test; $p < 0.01$). Female mutants with complex vocalization showed a lower frequency minimum than the female control with complex vocalizations (Figure 5F; KS test; $p < 0.01$).

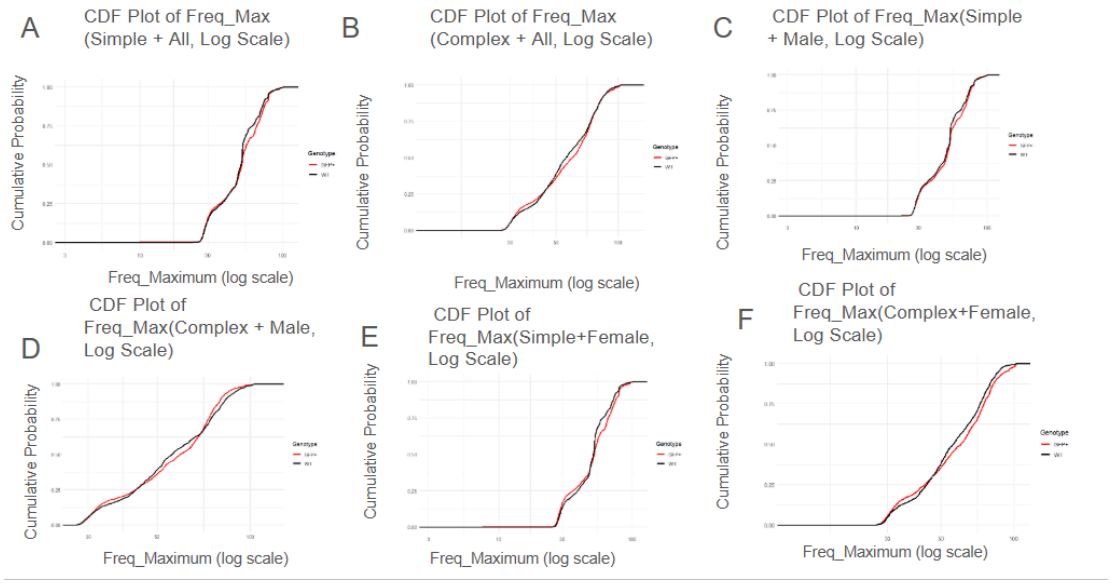

Figure 5. Cumulative distribution plots of maximum vocalization frequency (log scale) in wild-type (WT) and mutant mice.

(A) Mutant mice with simple vocalizations exhibited a significantly higher maximum frequency than wild-type mice.
(B) In complex vocalizations, mutant mice also showed a higher maximum frequency than controls.
(C) Among males with simple vocalizations, mutant mice showed higher maximum frequency than wild-type males.
(D) In contrast, for complex vocalizations, male mutants exhibited lower maximum frequency than male wild-type mice.
(E) Female mutants with simple vocalizations showed higher maximum frequency than female wild-type mice.

(F) In complex vocalizations, female mutants showed lower maximum frequency than their wild-type counterparts.

Finally, we compared the frequencies at which we detected vocalization events, including simple and complex vocalizations, which differed between the sexes. Mutant mice with simple vocalizations had a call rate that averaged significantly lower than that of wild-type mice (Figure 6A; KS test, $p < 0.01$). But complex vocalizations were not found to differ between control and mutant groups. Furthermore, females and males both lacked differences between mutant and wild-type groups, further suggesting that the observed reduction in simple call rate is not sex-specific but a universal effect of the mutation.

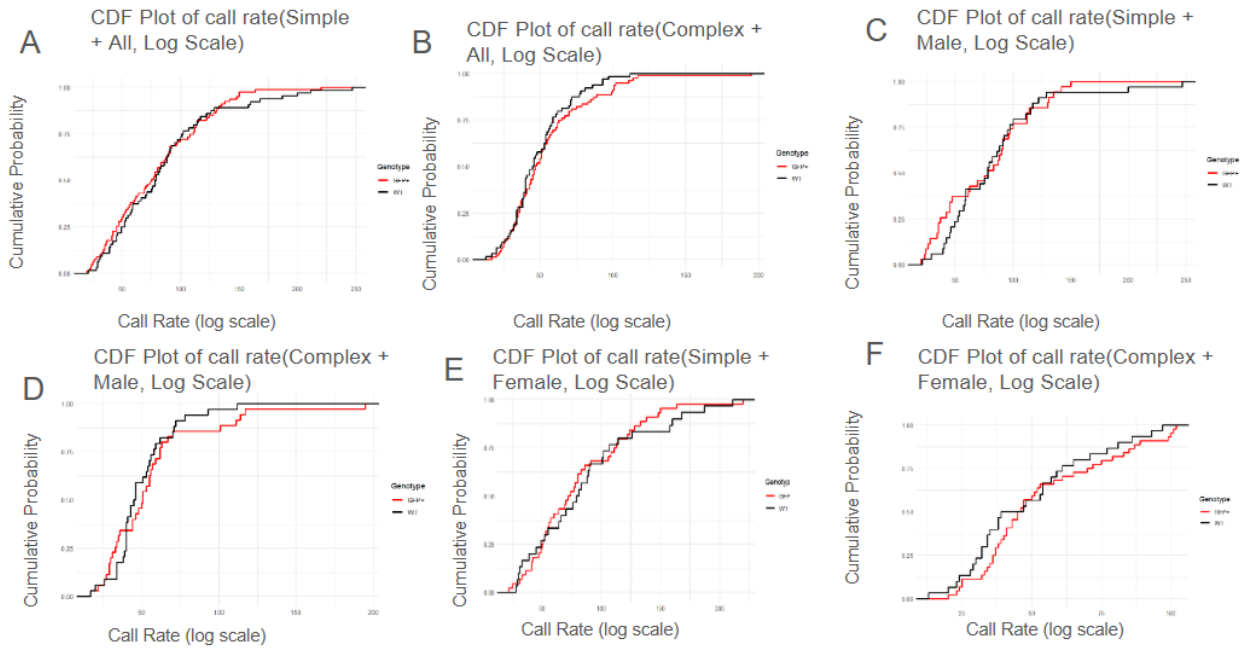

Figure 6. Cumulative distribution plot of call rate (log scale) in wild-type (WT) and mutant mice.
(A) Simple vocalizations (all mice): Mutant mice showed a significantly lower call rate compared to WT mice.
(B) Complex vocalizations (all mice): No significant difference in call rate was observed between genotypes.
(C) Simple vocalizations (males only): No genotype-related difference was found.
(D) Complex vocalizations (males only): No difference between WT and mutant groups.

(E) Simple vocalizations (females only): No significant difference was observed.
(F) Complex vocalizations (females only): No difference between genotypes.

Method:

The Purdue University Research Repository is the source of this information (Abraham et al., 2019; Schaser & Rodgers, 2024). A publicly accessible vocalization dataset from A53T mutant mice and wild-type mice (which served as the control group) was used for our analysis. We focused on several key acoustic parameters: Duration, Frequency Maximum, Frequency Minimum, Power Maximum, and frequency range, to compare vocalization characteristics between simple and complex call types. To statistically evaluate the variations in the distributions of these variables between the two vocalization types, we used R to perform the Kolmogorov-Smirnov (KS) test. We were able to determine whether the observed differences were statistically significant by using the KS test. Results with p-values below 0.05 were considered statistically significant.

Discussion

In this study, we have analyzed whether there are sex differences in vocalization phenotypes in an animal model of Parkinson's disease by comparing simple and complex vocalization between mutant and control groups. By comparing the mutant and non-mutant vocalizations between simple and complex vocalizations, we examined multiple acoustic

properties, including vocalization duration, frequency, maximum power, maximum frequency, and minimum frequency. Our findings revealed that the duration of simple vocalization increased, while the duration of complex vocalization decreased, regardless of sex. However, an insidious sex-specific trend emerged in males, characterized by complex vocalizations, which shows a tendency toward shorter durations. Furthermore, we found that mutant animals exhibited a higher frequency and maximum power in simple calls, particularly among females, whereas complex calls did not show a significant difference, suggesting that the A53T mutation may disrupt simple vocal patterns. Moreover, both simple and complex vocalizations exhibit statistically significant differences in minimum and maximum frequency between mutant and control groups; however, these differences are more pronounced in simple vocalizations, particularly among females. These results suggest that the A53T mutation selectively disrupts simple vocalization patterns by increasing their frequency and power, with more pronounced effects observed in females. In contrast, complex vocalizations are less affected overall but still exhibit some frequency alterations.

Alpha-synuclein has been identified as playing a significant role in PD (Matsumine et al., 1997). Alpha-synuclein is a protein that is typically broken down by the cell's waste systems, mainly including proteasomes and lysosomes (Bennett et al., 1999; Junn et al., 2009). The lysosomes also clear alpha-synuclein in its soluble and aggregated forms through chaperone-mediated autophagy(CMA) (Cuervo et al., 2004; Lee et al., 2004). However, alpha-synuclein could also disrupt CMA itself, disturbing the clearance of other proteins (Vogiatzi et al., 2008). In PD, this balance is disrupted, leading to the misfolding of alpha-synuclein and its accumulation, which creates harmful aggregates that damage cells (Junn et al., 2009). Mutations in the GBA1 gene, which is linked to Gaucher's disease, degrade

lysosomal function, in turn impairing alpha-synuclein clearance and amplifying the risk of the development of PD (Manning-Boğ et al., 2009; Sidransky et al., 2009). These mutations not only impair alpha-synuclein clearance but also increase membrane glucocerebroside levels, which in turn promote the aggregation of alpha-synuclein at lipid-rich membrane sites (Fortin et al., 2004; Mazzulli et al., 2011). Overall, reduced clearance and abnormal interactions with cell membranes lead to the accumulation of alpha-synuclein, which forms toxic aggregates that cause damage to brain cells, a hallmark of PD (Breydo et al., 2012; Goedert et al., 2013). These mechanisms highlight Alpha-Synuclein's central role in PD progression.

A previous study utilized a genetically modified mouse, known as the Thy1-aSyn mouse, which overproduces alpha-synuclein (Grant et al., 2014). The study examined early symptoms, including reduced duration and frequency of ultrasonic vocalizations, as well as later-stage motor symptoms associated with dopamine loss, which occurred 14 months later (Grant et al., 2014). Thy-aSyn mice showed a significantly reduced call of duration compared to the wild-type mice (Grant et al., 2014). The vocalization of mutant mice was quieter (less intense) than that of wild-type mice (Grant et al., 2014). The types and calls of mutant mice were altered, resulting in abnormal vocalization patterns compared to the wild-type (Grant et al., 2014). The frequency of the mutant mice also decreased with age compared to the wild-type (Grant et al., 2014). These findings suggest that alpha-synuclein overexpression causes early and progressive vocal impairments in the mutant mice.

Similar to Grant et al., 2014, our study examined the vocalization phenotypes in an animal model of Parkinson's disease between mutant and control groups(Grant et al.). While Grant et al., 2014 reported that mutant mice exhibited a significantly shorter duration than the control group, our study found a more detailed pattern: simple vocalizations had increased

duration, whereas complex vocalizations showed decreased duration in the mutant mice. Additionally, Grant et al., 2014 showed that the frequency decreased in the mutant mice, but our study revealed different patterns. The mutant mice with simple vocalizations showed an increased frequency, while those with complex vocalizations did not exhibit significant differences compared to the control group. These differences may be attributable to variations in methodologies, including animal age, vocalization types, and the techniques employed to analyze the acoustics, underscoring the importance of utilizing standardized classification and analysis methods when studying vocalization phenotypes to ensure consistent comparability across studies.

Complementing these findings, Gombash et al., 2013 have explored vocalizations in rat models of alpha-synuclein pathology (Gombash et al., 2013). They found that mutant mice displayed deficits in call intensity and call rate than the control group (Gombash et al., 2013). Other genres, such as durations and frequency, were found to be not significantly affected (Gombash et al., 2013). The study concluded that they found selective vocal deficits.

Compared to our study, this study also shows some discrepancies in duration, frequency, and call rate. While our findings revealed significant differences between mutant and wild-type rats in both simple and complex vocalizations, the other study reported fewer changes across acoustic parameters. However, both studies were consistent in terms of call rate: each demonstrated a significantly lower call rate in the α-synuclein overexpression group compared to controls.

Likewise, Paumier et al., 2015 found that injection of a-synuclein PFFs in rats led to significant disruptions in vocalization (Paumier et al., 2015). Specifically, the study revealed that

PFF-injected rats exhibited decreased maximum frequency, shorter maximum call durations, and a reduced call rate compared to the control group (Paumier et al., 2015).

In contrast, our study revealed that rats with aggregated alpha-synuclein exhibited higher maximum frequencies in both types of vocalization: simple and complex. Although Paumier et al., 2015 also showed a shorter duration in mutant mice, our study provided a more nuanced understanding. Duration of mutant mice with simple vocalization showed a higher duration than the wild-type, while duration of mutant mice with complex vocalization expressed a lower duration. Our study revealed a reduced call rate in modified mice exhibiting simple vocalizations, which aligns with Paumier et al. (2015). However, the modified mice with complex vocalizations did not show a significant difference.

These different results suggest that the impact of alpha-synuclein aggregation on vocalization is complex and may depend largely on the type of vocalization produced. The difference in how long the call lasts between complex and simple vocalization implies that different parts of the brain regulate these two types of sounds, and each part of the brain might be affected differently by the mutation.

Further research is needed to explore the neural mechanisms underlying the altered vocalization phenotypes observed in mutant mice. Specifically, it would be valuable to examine the distinct brain regions involved in producing vocalizations to understand how mutations affect these areas and contribute to differences in vocal behavior. Simple vocalizations primarily rely on the basal ganglia, brainstem, and primary motor cortex, whereas complex vocalizations engage a broader network, including the prefrontal cortex, supplementary motor area (SMA), cerebellum, auditory cortex, and basal ganglia. The observation that complex vocalizations are

less affected by the mutation suggests that this extended network may provide compensatory mechanisms to mitigate the impact of neurodegeneration.

Additionally, conducting longitudinal studies that track vocal symptoms over time would enable researchers to monitor disease progression related to age and severity. Such studies could reveal how vocal impairments evolve and correlate with neurodegenerative changes. Longitudinal designs enable us to observe how vocal impairment fluctuates, develops, or worsens, which cross-sectional studies cannot capture properly. By consistently monitoring vocal symptoms, longitudinal studies can help identify early markers of disease onset, track the progression of symptoms, and potentially identify different stages of neurodegeneration. Moreover, these studies facilitate the evaluation of how interventions or environmental factors influence vocal function over time, ultimately supporting the development of targeted therapeutic strategies and improving prognostic assessments.

LSVT Method and Its Effectiveness in Addressing Vocal Deficits in Parkinson's Disease

Building on these insights, animal models have contributed to understanding the neural mechanisms underlying vocal deficits in PD. However, human populations have demonstrated that behavioral and speech therapy interventions can alleviate these vocal deficits. Silveira & Brasolotto (2005 have used Lee Silverman Voice Treatment(LSVT) method to assess the voice before and after the therapy (Silveira & Brasolotto, 2005). The LSVT is an intensive, evidence-based speech therapy designed to enhance vocal loudness and communication in individuals with PD through high-effort and repetitive vocal exercises (Ramig et al., 2001). The

study found that patients who employed the method improved clarity and articulation, increased intensity, enhanced vocal control, and satisfaction (Silveira & Brasolotto, 2005).

In a related study, Searl et al., 2011 also explored the feasibility of LSVT in a group format as a potential treatment option for individuals with PD. The second goal of the study was to measure and report changes in the participants' vocal abilities before and after the program (Searl et al., 2011). The study found that LSVT works well in a group setting, although it is typically used one-on-one. It also saw a significant increase in vocal intensity, maximum fundamental frequency, and frequency, indicating improvement (Searl et al., 2011). These findings support that LSVT is an effective treatment in both individual and group settings.

Similarly, Cannito et al., 2012 investigated the effectiveness of LSVT methods, and the researchers measured the sound pressure level (SPL) of vowel productions before and after the treatment (Cannito et al., 2012). Before the study, the mean vocal intensity was 82.83 dB SPL, and after, it was 91.51 dB SPL, indicating a significant statistical difference and confirming the treatment effect on vocal intensity (Cannito et al., 2012). The finding confirms that LSVT successfully targets and addresses vocal deficits in PD, specifically reduced loudness. These results align with previous studies, which indicate that LSTV is effective and serves as a remedy.

Lastly, (Sapir et al., 2007) examined whether LSVT helps improve vowel articulations in individuals with PD (Sapir et al., 2007). The results showed the improvements in vocal intensity, indicating stronger and more hearable speech (Sapir et al., 2007). This improvement is vital for individuals with vocal deficits in PD. Additionally, the second formant (F2) of the vowel was enhanced, which reflects better control of tongue articulation and positioning. (Sapir et al.,

2007). Finally, the perceptual vowel rating improved, suggesting that the speech of treated individuals sounded clearer and of better quality than before (Sapir et al., 2007).

Together, these studies underscore the effectiveness of the LSVT method in addressing vocal deficits. Its effect is evident in various speech dimensions, including vocal intensity, articulation, clarity, and listeners' perceptions.

2. Pharmacological and Sensory-Based Approaches

Another possible treatment available is pharmacological. The study aimed to investigate the effects of levodopa medication, vocal exercise, and environmental factors on vocal communication in PD (Kelm-Nelson et al., 2016). Levodopa is a medication that increases dopamine levels in the brain, and it is the primary treatment for PD (Hauser, 2009). The study found that levodopa alone had a limited effect on improving vocal communication in the rat model of PD (Kelm-Nelson et al., 2016). Nevertheless, when combined with vocal exercise therapy, it showed significant improvement in vocal quality (Kelm-Nelson et al., 2016). This suggests that while levodopa can be helpful, it does not fully address the vocal deficits on its own. Therefore, integrating pharmacological treatment with therapies like LSVT may be a powerful approach..

Likewise, De Letter et al. sought to determine whether the medication levodopa affects the rate at which patients with PD speak and the extent of the change (De Letter et al., 2006). The results showed that levodopa itself did not affect the rate at which they said, and no significant change in overall speech rate was found (De Letter et al., 2006). However, the medication led to variability in speech rate, meaning they sometimes spoke more slowly and at

other times faster (De Letter et al., 2006). This inconsistency might be attributable to the side effects of levodopa affecting breathing or an increase in speech disruptions.

To further explore the impact of levodopa on speech-related functions, Ho et al. (2008 conducted a study to test the effect of levodopa on speech in PD patients. (Ho et al., 2008). The study showed that it increased speech loudness and speed (Ho et al., 2008). However, vocal intensity declined over time, even though pitch and articulation remained unchanged (Ho et al., 2008). This implies that although levodopa can enhance some aspects of vocal speech, it may not provide sustained improvements in vocal control.

Given the mixed results of levodopa on vocal outcomes, evaluating responses to pharmacological treatments in mutant mice could clarify the relationship between vocal symptoms and therapeutic efficacy (Mak et al., 2015). Ideally, future research would test multiple treatment options to identify those most effective in restoring vocal function to levels comparable to control animals, thereby advancing potential therapeutic strategies.

One of the major limitations of the study is that it employed a cross-sectional design, which collects data from diverse groups of individuals at a single point in time. As we were unable to track the vocal changes over time, we could not determine how the disease progressed or the stage of each mouse during the study. The other limitation is that the hormonal cycle of females was not controlled. The hormonal cycle may play a role in vocalization, influencing factors such as frequency or intensity (Geyer & Barfield, 1978). Some differences we thought were caused by mutants might be caused by hormone levels.

Our findings suggest that A53T mutations in Parkinson's disease lead to distinct changes in vocalization, which vary in complexity and extent by sex. These results suggest that vocal deficits can serve as a non-invasive biomarker for the early symptoms of Parkinson's disease. These changes were particularly conspicuous in the duration, frequency, power, and frequency ranges of simple vocalizations, which were affected more than the complex vocalizations. This suggests that non-modulating frequencies are more vulnerable to the early neurodegenerative changes.

Furthermore, subtle sex-specific changes were observed in frequency and power, which could be due to underlying hormonal or neurological mechanisms that affect neurodegenerative disorders. Understanding these mechanisms could enhance our diagnostic tools, trends, and therapeutic approaches.

References:

Abraham, D. S., Gruber-Baldini, A. L., Magder, L. S., McArdle, P. F., Tom, S. E., Barr, E., Schrader, K., & Shulman, L. M. (2019). Sex differences in Parkinson's disease presentation and progression. Parkinsonism & Related Disorders, 69, 48–54.

Baldereschi, M., Di Carlo, A., Rocca, W. A., Vanni, P., Maggi, S., Perissinotto, E., Grigoletto, F., Amaducci, L., & Inzitari, D. (2000). Parkinson's disease and parkinsonism in a longitudinal study: Two-fold higher incidence in men. Neurology, 55(9), 1358–1363.

Bennett, M. C., Bishop, J. F., Leng, Y., Chock, P. B., Chase, T. N., & Mouradian, M. M. (1999). Degradation of α-synuclein by proteasome. Journal of Biological Chemistry, 274(48), 33855–33858.

Bloem, B. R., Okun, M. S., & Klein, C. (2021). Parkinson's disease. The Lancet, 397(10291), 2284–2303.

Bocklet, T., Nöth, E., Stemmer, G., Ruzickova, H., & Rusz, J. (2011). Detection of persons with Parkinson's disease by acoustic, vocal, and prosodic analysis. 478–483.

Breydo, L., Wu, J. W., & Uversky, V. N. (2012). α-Synuclein misfolding and Parkinson's disease. Biochimica et Biophysica Acta (BBA)-Molecular Basis of Disease, 1822(2), 261–285.

Cannito, M. P., Suiter, D. M., Beverly, D., Chorna, L., Wolf, T., & Pfeiffer, R. M. (2012). Sentence intelligibility before and after voice treatment in speakers with idiopathic Parkinson's disease. Journal of Voice, 26(2), 214–219.

Choi, J. M., Woo, M. S., Ma, H.-I., Kang, S. Y., Sung, Y.-H., Yong, S. W., Chung, S. J., Kim, J.-S., Shin, H., & Lyoo, C. H. (2008). Analysis of PARK genes in a Korean cohort of early-onset Parkinson disease. Neurogenetics, 9, 263–269.

Cuervo, A. M., Stefanis, L., Fredenburg, R., Lansbury, P. T., & Sulzer, D. (2004). Impaired degradation of mutant α-synuclein by chaperone-mediated autophagy. Science, 305(5688), 1292–1295.

De Letter, M., Santens, P., De Bodt, M., Boon, P., & Van Borsel, J. (2006). Levodopa-induced alterations in speech rate in advanced Parkinson's disease. Acta Neurologica Belgica, 106(1), 19–22.

Elkouzi, A., Vedam-Mai, V., Eisinger, R. S., & Okun, M. S. (2019). Emerging therapies in Parkinson disease—Repurposed drugs and new approaches. Nature Reviews Neurology, 15(4), 204–223.

Fortin, D. L., Troyer, M. D., Nakamura, K., Kubo, S., Anthony, M. D., & Edwards, R. H. (2004). Lipid rafts mediate the synaptic localization of α-synuclein. Journal of Neuroscience, 24(30), 6715–6723.

Geyer, L. A., & Barfield, R. J. (1978). Influence of gonadal hormones and sexual behavior on ultrasonic vocalization in rats: I. Treatment of females. Journal of Comparative and Physiological Psychology, 92(3), 438.

Gnerre, M., Malaspina, E., Di Tella, S., Anzuino, I., Baglio, F., Silveri, M. C., & Biassoni, F. (2023). Vocal emotional expression in Parkinson's disease: Roles of sex and emotions. Societies, 13(7), 157.

Goedert, M., Spillantini, M. G., Del Tredici, K., & Braak, H. (2013). 100 years of Lewy pathology. Nature Reviews Neurology, 9(1), 13–24.

Gombash, S. E., Manfredsson, F. P., Kemp, C. J., Kuhn, N. C., Fleming, S. M., Egan, A. E., Grant, L. M., Ciucci, M. R., MacKeigan, J. P., & Sortwell, C. E. (2013). Morphological and behavioral impact of AAV2/5-mediated overexpression of human wildtype

alpha-synuclein in the rat nigrostriatal system. PloS One, 8(11), e81426.

Grant, L. M., Kelm‐Nelson, C. A., Hilby, B. L., Blue, K. V., Paul Rajamanickam, E. S., Pultorak, J. D., Fleming, S. M., & Ciucci, M. R. (2015). Evidence for early and progressive ultrasonic vocalization and oromotor deficits in a PINK1 gene knockout rat model of Parkinson's disease. Journal of Neuroscience Research, 93(11), 1713–1727.

Grant, L. M., Richter, F., Miller, J. E., White, S. A., Fox, C. M., Zhu, C., Chesselet, M.-F., & Ciucci, M. R. (2014). Vocalization deficits in mice over-expressing alpha-synuclein, a model of pre-manifest Parkinson's disease. Behavioral Neuroscience, 128(2), 110.

Gu, X.-L., Long, C.-X., Sun, L., Xie, C., Lin, X., & Cai, H. (2010). Astrocytic expression of Parkinson's disease-related A53T α-synuclein causes neurodegeneration in mice. Molecular Brain, 3, 1–16.

Haaxma, C. A., Bloem, B. R., Borm, G. F., Oyen, W. J., Leenders, K. L., Eshuis, S., Booij, J., Dluzen, D. E., & Horstink, M. W. (2007). Gender differences in Parkinson's disease. Journal of Neurology, Neurosurgery & Psychiatry, 78(8), 819–824.

Hauser, R. A. (2009). Levodopa: Past, present, and future. European Neurology, 62(1), 1–8.

Hertrich, I., & Ackermann, H. (1995). Gender-specific vocal dysfunctions in Parkinson's disease: Electroglottographic and acoustic analyses. Annals of Otology, Rhinology & Laryngology, 104(3), 197–202.

Ho, A. K., Bradshaw, J. L., & Iansek, R. (2008). For better or worse: The effect of levodopa on speech in Parkinson's disease. Movement Disorders: Official Journal of the Movement Disorder Society, 23(4), 574–580.

Houle, N., Feaster, T., Mira, A., Meeks, K., & Stepp, C. E. (2024). Sex Differences in the Speech of Persons with and without Parkinson's Disease. American Journal of Speech-Language

Pathology, 33(1), 96–116.

Hughes, A. J., Daniel, S. E., Kilford, L., & Lees, A. J. (1992). Accuracy of clinical diagnosis of idiopathic Parkinson's disease: A clinico-pathological study of 100 cases. Journal of Neurology, Neurosurgery & Psychiatry, 55(3), 181–184.

Junn, E., Lee, K.-W., Jeong, B. S., Chan, T. W., Im, J.-Y., & Mouradian, M. M. (2009). Repression of α-synuclein expression and toxicity by microRNA-7. Proceedings of the National Academy of Sciences, 106(31), 13052–13057.

Kelm-Nelson, C. A., Brauer, A. F., & Ciucci, M. R. (2016). Vocal training, levodopa, and environment effects on ultrasonic vocalizations in a rat neurotoxin model of Parkinson disease. Behavioural Brain Research, 307, 54–64.

Krasko, M. N., Hoffmeister, J. D., Schaen-Heacock, N. E., Welsch, J. M., Kelm-Nelson, C. A., & Ciucci, M. R. (2021). Rat models of vocal deficits in Parkinson's disease. Brain Sciences, 11(7), 925.

Lavedan, C. (1998). The synuclein family. Genome Research, 8(9), 871–880.

Lee, H.-J., Khoshaghideh, F., Patel, S., & Lee, S.-J. (2004). Clearance of α-synuclein oligomeric intermediates via the lysosomal degradation pathway. Journal of Neuroscience, 24(8), 1888–1896.

Li, W., Lesuisse, C., Xu, Y., Troncoso, J. C., Price, D. L., & Lee, M. K. (2004). Stabilization of α-synuclein protein with aging and familial Parkinson's disease-linked A53T mutation. Journal of Neuroscience, 24(33), 7400–7409.

Manning-Boğ, A. B., Schüle, B., & Langston, J. W. (2009). Alpha-synuclein-glucocerebrosidase interactions in pharmacological Gaucher models: A biological link between Gaucher disease and parkinsonism. Neurotoxicology, 30(6), 1127–1132.

Matsumine, H., Saito, M., Shimoda-Matsubayashi, S., Tanaka, H., Ishikawa, A., Nakagawa-Hattori, Y., Yokochi, M., Kobayashi, T., Igarashi, S., & Takano, H. (1997). Localization of a gene for an autosomal recessive form of juvenile Parkinsonism to chromosome 6q25. 2-27. American Journal of Human Genetics, 60(3), 588.

Mazzulli, J. R., Xu, Y.-H., Sun, Y., Knight, A. L., McLean, P. J., Caldwell, G. A., Sidransky, E., Grabowski, G. A., & Krainc, D. (2011). Gaucher disease glucocerebrosidase and α-synuclein form a bidirectional pathogenic loop in synucleinopathies. Cell, 146(1), 37–52.

Paumier, K. L., Luk, K. C., Manfredsson, F. P., Kanaan, N. M., Lipton, J. W., Collier, T. J., Steece-Collier, K., Kemp, C. J., Celano, S., & Schulz, E. (2015). Intrastriatal injection of pre-formed mouse α-synuclein fibrils into rats triggers α-synuclein pathology and bilateral nigrostriatal degeneration. Neurobiology of Disease, 82, 185–199.

Paumier, K. L., Sukoff Rizzo, S. J., Berger, Z., Chen, Y., Gonzales, C., Kaftan, E., Li, L., Lotarski, S., Monaghan, M., & Shen, W. (2013). Behavioral characterization of A53T mice reveals early and late stage deficits related to Parkinson's disease. PloS One, 8(8), e70274.

Picillo, M., LaFontant, D.-E., Bressman, S., Caspell-Garcia, C., Coffey, C., Cho, H. R., Burghardt, E. L., Dahodwala, N., Saunders-Pullman, R., & Tanner, C. M. (2022). Sex-related longitudinal change of motor, non-motor, and biological features in early Parkinson's disease. Journal of Parkinson's Disease, 12(1), 421–436.

Poewe, W., Seppi, K., Tanner, C. M., Halliday, G. M., Brundin, P., Volkmann, J., Schrag, A.-E., & Lang, A. E. (2017). Parkinson disease. Nature Reviews Disease Primers, 3(1), 1–21.

Pont‐Sunyer, C., Hotter, A., Gaig, C., Seppi, K., Compta, Y., Katzenschlager, R., Mas, N.,

Hofeneder, D., Brücke, T., & Bayés, A. (2015). The O nset of N onmotor S ymptoms in P arkinson's disease (T he ONSET PD S tudy). Movement Disorders, 30(2), 229–237.

Ramig, L. O., Sapir, S., Countryman, S., Pawlas, A. A., O'Brien, C., Hoehn, M., & Thompson, L. L. (2001). Intensive voice treatment (LSVT®) for patients with Parkinson's disease: A 2 year follow up. Journal of Neurology, Neurosurgery & Psychiatry, 71(4), 493–498.

Rektorová, I., Mikl, M., Barrett, J., Marecek, R., Rektor, I., & Paus, T. (2012). Functional neuroanatomy of vocalization in patients with Parkinson's disease. Journal of the Neurological Sciences, 313(1–2), 7–12.

Santos-García, D., Laguna, A., Hernández-Vara, J., de Deus Fonticoba, T., Cores Bartolomé, C., Feal Painceiras, M. J., Íñiguez-Alvarado, M. C., García Díaz, I., Jesús, S., & Boungiorno, M. T. (2023). Sex differences in motor and non-motor symptoms among Spanish patients with Parkinson's disease. Journal of Clinical Medicine, 12(4), 1329.

Sapir, S., Spielman, J. L., Ramig, L. O., Story, B. H., & Fox, C. (2007). Effects of intensive voice treatment (the Lee Silverman Voice Treatment [LSVT]) on vowel articulation in dysarthric individuals with idiopathic Parkinson disease: Acoustic and perceptual findings.

Searl, J., Wilson, K., Haring, K., Dietsch, A., Lyons, K., & Pahwa, R. (2011). Feasibility of group voice therapy for individuals with Parkinson's disease. Journal of Communication Disorders, 44(6), 719–732.

Sidransky, E., Nalls, M. A., Aasly, J. O., Aharon-Peretz, J., Annesi, G., Barbosa, E. R., Bar-Shira, A., Berg, D., Bras, J., & Brice, A. (2009). Multicenter analysis of glucocerebrosidase mutations in Parkinson's disease. New England Journal of Medicine, 361(17), 1651–1661.

Silveira, D. N., & Brasolotto, A. G. (2005). Vocal rehabilitation in patients with Parkinson disease: Interfering factors. Pró-Fono Revista de Atualização Científica, 17, 241–250.

Solla, P., Cannas, A., Ibba, F. C., Loi, F., Corona, M., Orofino, G., Marrosu, M. G., & Marrosu, F. (2012). Gender differences in motor and non-motor symptoms among Sardinian patients with Parkinson's disease. Journal of the Neurological Sciences, 323(1–2), 33–39.

Spira, P. J., Sharpe, D. M., Halliday, G., Cavanagh, J., & Nicholson, G. A. (2001). Clinical and pathological features of a Parkinsonian syndrome in a family with an Ala53Thr α‐synuclein mutation. Annals of Neurology, 49(3), 313–319.

Taguchi, K., Watanabe, Y., Tsujimura, A., & Tanaka, M. (2016). Brain region‐dependent differential expression of alpha‐synuclein. Journal of Comparative Neurology, 524(6), 1236–1258.

Vogiatzi, T., Xilouri, M., Vekrellis, K., & Stefanis, L. (2008). Wild type α-synuclein is degraded by chaperone-mediated autophagy and macroautophagy in neuronal cells. Journal of Biological Chemistry, 283(35), 23542–23556.

Yiu, Y., Curtis, J. A., Perry, S. E., & Troche, M. S. (2020). Relationship of vocal fold atrophy to swallowing safety and cough function in Parkinson's disease. The Laryngoscope, 130(2), 303–308.

Review for “Sex-Specific Vocalization Impairments in A53T α-synuclein Mutant Mice”

Final Recommendation: Revise and Resubmit (Major Revisions Required)

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1. Originality & Significance

The paper explores vocalization changes in a well-known mouse model of Parkinson’s disease (PD), with attention to sex differences and call type. This is a potentially novel and worthwhile line of inquiry, especially for a high school journal, and the idea of reanalysing open-source data is commendable.

However, the paper does not sufficiently highlight what is novel or why these analyses are significant. The rationale for focusing on vocalization metrics and the choice of subgroups (e.g., separating simple vs. complex calls and male vs. female animals) is never clearly articulated. The study lacks a defined hypothesis or overarching goal, which reduces the perceived novelty and leaves the reader uncertain about the contribution to the field.

The manuscript includes a brief aim in the introduction, stating an intent to examine sex differences in vocalisation in a PD mouse model. While this provides a useful starting point, it is not framed as a clear, testable hypothesis. Strengthening this aim and ensuring it is consistently reflected in the analyses and discussion would improve the overall coherence and perceived significance of the study.

Suggestions:

- Clearly articulate a research question or hypothesis in the introduction.
- Explain the potential scientific relevance of the work (e.g., how vocalizations may reflect underlying motor or cognitive impairment in PD).
- Clarify how your analysis adds something new compared to the original dataset publication.

2. Clarity & Structure

The paper’s structure needs significant improvement. The introduction is overly long, repetitive, and unfocused. It presents a large number of loosely connected studies without clearly building a rationale for the present study. Findings from these studies are summarised, sometimes with unnecessary detail, such as the number of participants, without linking them clearly to the research question. As a result, the reader is left unsure of the central question or how the cited work leads to the current analysis.

A clearer structure for the introduction could help orient the reader. One possible sequence might be:

- Overview of Parkinson's disease, including motor and non-motor symptoms.
- Introduction to vocalization deficits as a non-motor symptom of PD, with references to relevant human and animal literature.
- Discussion of sex differences in PD, including both motor and vocal effects.
- Rationale for using the A53T mouse model and why vocalisation in mice can be informative.
- Statement of the research question or hypothesis, explaining what will be analysed and why.

The results section is also difficult to follow. Dozens of comparisons and figures are presented in rapid succession, with minimal guidance. The writing is often descriptive but not interpretive and readers are not told what matters or why. Without summaries or integration of findings, the reader must interpret the significance of each result on their own.

Suggestions:

- Revise the introduction following a clear thematic sequence and remove redundant or loosely connected citations.
- Explicitly state the research question or hypothesis at the end of the introduction.
- Use section subheadings in the Results and Discussion to organise the findings by call type, sex, or age group.
- Reduce the number of figures or combine them into summary plots. Focus on the most meaningful comparisons.
- Conclude the Results section with a paragraph synthesising the key findings and their relevance.- Edit the introduction to remove repetition and improve focus. Group ideas logically (e.g., start with PD, then vocalization, then the mouse model).
    - Use section subheadings in the Results and Discussion to organise the findings.
    - Limit the number of figures or group them into summary plots. Highlight only the most informative comparisons.
    - End the Results section with a short paragraph that summarises the key takeaways.

3. Use of Evidence & Research Methods

The statistical analysis is one of the weakest aspects of the paper. The widespread use of Kolmogorov–Smirnov (KS) tests across numerous variables, age groups, sexes, and call types is not justified or explained. There is no rationale provided for choosing this nonparametric test, and it is unclear whether the data meet the assumptions required for its use. In many cases, KS tests are used where more informative and interpretable methods (e.g., t-tests, ANOVA, or mixed-effects models) would be more appropriate.

There is no correction for multiple comparisons despite what appears to be dozens of statistical tests, severely undermining the reliability of the reported p-values. This exploratory approach, without correction or clear prioritisation, increases the likelihood of false positives and makes it difficult for the reader to discern which effects are robust or meaningful.

Critically, the unit of analysis is never clearly defined. The reader is left to guess whether the tests compare distributions of individual vocalisations, animal-level means, or something else. Repeated measures and nested data structures (e.g., multiple calls per animal) are not acknowledged or accounted for statistically. This omission casts doubt on the validity of all statistical conclusions.

Figures are used excessively, with many plots presenting non-significant results. This adds to the reader's confusion and dilutes the impact of any meaningful patterns. Not every comparison needs to be visualised in the main paper. Non-significant or exploratory results could be moved to supplementary material.

Suggestions:

- Add a dedicated "Statistical Analysis" subsection in the Methods, clearly defining the unit of analysis (e.g., individual vocalisations vs. per-animal averages), sample sizes, and any steps taken to preprocess the data (e.g., removal of outliers).
- Justify the use of the KS test. If the goal is to compare group averages, simpler and more interpretable tests such as the independent t-test or Mann–Whitney U test (for nonparametric comparisons) may be more appropriate.
- Report both the number of animals and the number of calls per condition. If calls were pooled across animals, this should be explicitly stated, and the implications of that decision should be discussed.
- To avoid inflating the false positive rate, reduce the number of comparisons by focusing on a small number of clearly motivated contrasts. Apply corrections for multiple comparisons (e.g., Holm–Bonferroni) if many tests are retained.
- Limit visualisations to key comparisons. Results that are exploratory or non-significant can be moved to an appendix or supplemental material.
- Ensure that each analysis is introduced with a rationale and followed by a clear interpretation of the result. Help the reader understand not just what was found, but why it was analysed in the first place.
- Place figures after the first paragraph that reports the associated results
- Consider computing **animal-level summary statistics** (e.g., average pitch or call duration per mouse) and comparing these across groups. This avoids pseudoreplication and is much easier to interpret.
- Axis labels are too small, resolution is poor, and significance markers are inconsistently applied. I recommend exporting all figures as "svg" or "eps" files from R, with high DPI (330 or above). If needed, you can edit them using Illustrator or the free software Inkscape. Ensure to export as high DPI PNG images.

- Rather than displaying every metric and subgroup, prioritise a smaller selection of meaningful plots and ensure they are fully interpretable. Include clear titles, legends, and indications of sample size. A summary table of group-level vocalisation metrics (means, SDs, p-values) may be useful.

4. Engagement with Literature

The paper cites a number of appropriate sources and introduces key terms from the Parkinson's and neurodegeneration literature. However, many of these references are clustered at the start, and their integration into the argument is uneven. For example, the function of α-synuclein is mentioned, but the implications for behaviour or vocalization are not explained. While the introduction includes many relevant studies on vocal deficits and sex differences in human PD patients, the relevance of these citations to the current mouse vocalisation paradigm remains unclear. The author should clarify how ultrasonic vocalisation parameters such as duration or frequency relate to human speech features (e.g., prosody, articulatory precision, emotional expression) affected in PD. A more concise synthesis explaining how sex-specific vocal differences in humans motivate analogous exploration in mice would strengthen the rationale and improve the paper's coherence.

There is also no discussion of the original study from which the dataset was taken. Without referencing the prior publication, it's unclear what findings are being replicated, extended, or reinterpreted.

The Discussion section attempts to revisit key studies from the introduction, but this is often done in a descriptive rather than interpretive way. Prior studies are listed with limited critical evaluation, and little effort is made to explain how the current results confirm, diverge from, or extend existing findings. Key studies using similar mouse models (e.g., Grant et al., Gombash et al.) are cited, but differences in methodology (e.g., strain, call type, acoustic features, age range) are not discussed. This weakens any claims about novelty or divergence. Additionally, a lengthy paragraph on LSVT and levodopa treatments in human PD appears late in the Discussion, without being clearly linked to the preceding results or vocalisation metrics in mice. While this content is interesting, its placement and relevance are unclear and should be integrated more tightly, or removed if not directly informative. Since the primary focus of this paper is on sex differences in mouse vocalisation phenotypes, not on treatment, it would be more appropriate to summarise these human intervention findings more concisely, perhaps in a single paragraph on translational implications.

Overall, the paper would benefit from a clearer conceptual bridge between the cited literature, the behavioural findings in mice, and their translational relevance to human speech impairment in Parkinson's. Maintaining focus on vocalisation-specific mechanisms will help avoid tangential material and improve scientific cohesion.

Suggestions:

- Improve integration of sources into the argument. For example, don't just define α-synuclein, explain why its overexpression might affect vocalization.
- Include a brief summary and citation of the original study that generated the dataset in the introduction.
- In the Discussion, contrast your findings with those of the original study to demonstrate your unique contribution.
- In the Discussion, go beyond restating prior work: explain how your results compare to existing studies using similar methods or models. Highlight methodological differences where relevant.
- Clarify how mouse vocalisation measures (e.g., call duration, frequency modulation) relate to the human PD symptoms cited earlier (e.g., impaired prosody, reduced articulation).
- Either better integrate the paragraph on LSVT and levodopa into a discussion of translational relevance or remove it to maintain focus on your core findings.

5. Grammar & Language

The writing needs substantial editing to improve clarity, sentence structure, and grammatical correctness. There are repeated sentence openings, incomplete thoughts, inconsistent terminology (e.g., "non-motor" vs. "nonmotor"), and misplaced punctuation (e.g., "Various causes of Parkinson's disease. (Bloem et al., 2021.)").

That said, the student clearly understands the biological concepts and terminology, and their ambition is evident. With careful revision, the writing could be greatly improved.

Suggestions:

- Review the paper for sentence clarity and grammar. Use tools like Grammarly or ask a mentor or peer to help.
- Standardise scientific terminology throughout (e.g., "α-synuclein," "non-motor symptoms").
- Reduce redundancy and vary sentence structure to improve flow.
- Pay close attention to figure labels, axis titles, and legends—these must be legible and consistently formatted.
- Revise citation usage to follow a consistent academic style. For example:
    - If the author's name is included in the sentence (e.g., "Gombash et al. found…"), use the year in parentheses: Gombash et al. (2013). Also, do not place the entire citation in parentheses again at the end of the sentence.
    - When citing the same study across multiple consecutive sentences, it is not necessary to repeat the full citation each time.

- Refer to the APA citation manual and resources linked on the Convergence website to ensure all references are formatted accordingly.

Concluding Remarks

This paper shows initiative and an eagerness to engage with real scientific data, an impressive feat for an early-career researcher. The author has tackled a complex biological question and should be proud of taking on such a challenging task. That said, substantial revisions are needed to clarify the purpose, improve statistical transparency, and streamline the results. While the project draws on advanced topics in neuroscience and neurodegeneration, the revised version should aim to explain technical concepts clearly and frame findings in a way that engages both experts and curious non-specialists.

These revisions present an excellent opportunity to learn how to think like a scientist: forming hypotheses, selecting appropriate analyses, and communicating findings effectively. I encourage the author to embrace this process and continue building their skills, this paper has potential once its structure and analysis are improved.

Manuscript Title: Sex-Specific Vocalization Impairments in A53T α-synuclein mutant Mice

Recommendation: Accept with minor revisions

Overall: Author has written a very comprehensive coverage of vocalizations impact due to PD. In addition, the author has discussed in detail the contribution of alpha-synuclein in PD progression, and has demonstrated an excellent familiarity with literature of the field. The research theme is interesting and the results support the author's claim that the A53T mutation alters vocalization in the mouse model used.

Some points for revision:

1. Introduction: *"Some papers described the mechanism by mentioning that the AT53 mutation leads to more stable a-synuclein in neurons, which causes it to accumulate to high levels and contributes to the progression of PD (Li et al., 2004)."* Please elaborate, how does accumulation of alpha-synuclein result in PD?

2. For Methods section, the author has cited "*Schaser & Rodgers, 2024*". However, this is not in the list of references.

3. It is unclear where source of data comes from. The author has written "*A publicly accessible vocalization dataset from A53T mutant mice and wild-type mice (which served as the control group) was used for our analysis.*" Please provide dataset link. If they are from previously published papers, the papers also need to be cited.

4. In this paper, the author has analyzed a study of control and mutant male and female mice. However, it is not stated how many mice were used per condition. Please clarify this point.

5. Kolmogorov-Smirnov (KS) test used is appropriate for statistical analysis. However, this statistics test is sensitive to sample sizes, therefore, it is crucial to state the number of mice used (see above point as well).

6. The figures/graphs need a better resolution and to be bigger in proportion to the text. Axis/numbers need to be bigger.

7. The author has stated in figure legends which comparisons are statistically significant. That is a great descriptive help. Please also add the p-value of those that are statistically significant, even if they have already been mentioned in the text.

8. Consider simplifying graphs titles/axes for a cleaner look. E.g. *Fig 1A. "Duration.U"*: what is U? this is not described in the legend. The title can be revised to: *CDF Plot of Duration (Simple, All)*, or just *Duration of Vocalization*. The words "*Log Scale*" can be removed, as it is already on the x-axis. The type of plot, CDF, can be described in the legend. Please also write "Cumulative Distribution Function (CDF)" the first time you use it in the text, before using the acronym CDF in the rest of the paper.

9. This paragraph in Discussion: "*Alpha-synuclein has been identified as playing a significant role in PD (Matsumine et al., 1997). ... Overall, reduced clearance and abnormal interactions with cell membranes lead to the accumulation of alpha-synuclein, which forms toxic aggregates that cause*

damage to brain cells, a hallmark of PD (Breydo et al., 2012; Goedert et al., 2013). These mechanisms highlight Alpha-Synuclein's central role in PD progression." This paragraph is more suited in the introduction, and can actually be used as a bridge in explaining the role of alpha-synuclein in PD (see also point 1).

10. Author has done an excellent job plotting the results in graphs. However, this reviewer would like to see more discussion of the results from this project. Consider adding an "Analysis" section before the "Discussion" section to address this.

11. The Discussion section is detailed and references many other papers, demonstrating the author's knowledge of other studies. The author did a very thorough comparison of his results with other studies. However, there needs to be more discussion of author's own results, which can be remedied by adding an analysis section (see point 9). My suggestion is to have an Analysis section where the focus is on this paper's results, and a Discussion section where comparisons with other studies will be elaborated upon.

12. While detailed, the Discussion section is also longer than expected. Consider better structuring, or using subsections with titles, e.g. "*Comparisons with other studies*", "*Limitations of this study*", "*Future research directions*", "*Clinical treatment strategies*" (For LSVT and pharmacological approaches".

13. Author has mentioned rats several times as part of this study, even though it was stated that the experiments were done in mice: "*In contrast, our study revealed that rats with aggregated alpha-synuclein exhibited higher maximum frequencies in both types of vocalization: simple and complex.*", and "*While our findings revealed significant differences between mutant and wild-type rats in both simple and complex vocalizations, the other study reported fewer changes across acoustic parameters.*". Please clarify and be consistent. This is also where citing the source data is crucial so readers can look at the data themselves.

Title:

Sex-Specific Vocalization Impairments in A53T α-synuclein mutant Mice

Abstract

Parkinson's Disease is a neurodegenerative disorder that is characterized by both motor and non-motor symptoms, including vocalization deficits. Among the various causes of Parkinson's Disease, a mutation in the α-synuclein gene is a significant contributor. In this paper, we analyze a dataset containing acoustic vocalization measurements from both A53T mutant and wild-type mice, comparing WT vs. A53T within males and within females for simple vocalizations. The dataset included parameters such as duration, maximum power, and frequency ranges of simple vocalizations. Mutant mice did not exhibit significant differences in the duration of simple vocalizations compared to the control group. However, they showed a significantly broader frequency range in simple calls in females, with no measurable difference in males. In addition, female mutant mice exhibited higher maximum acoustic power than female controls, while no differences were observed in males. These findings indicate that the A53T mutation in the α-synuclein gene selectively alters frequency-related aspects of simple vocalizations, with additional effects on vocal intensity that are more pronounced in females. This suggests that vocal communication deficits in this Parkinson's Disease model may be sex-dependent, with frequency range providing the most sensitive marker of genotype-related differences.

Keywords: PD, vocalization, α-synuclein, sex differences

Introduction

Parkinson's disease (PD) is a neurodegenerative disorder of movement that has features of cognitive impairment, autonomic dysfunction, sleep disorders, hyposmia, and depression (Poewe et al., 2017). PD affected a large portion of the population, approximately 6.1 million people worldwide (Bloem et al., 2021). Although the mechanism has not yet been fully understood, the prevalence of the disease has risen rapidly over the past few decades, and the personal impact of the disease is immense. PD includes two types of symptoms: motor and non-motor. Motor symptoms refer to the movement-related symptoms, including slowness of voluntary movements with progressive reduction in speed, muscular rigidity, and postural instability (Hughes et al., 1992).

On the other hand, non-motor symptoms are not related to movements and can appear before motor symptoms do. The non-motor symptoms include a lack of emotional involvement, sleep problems, loss of smell and taste, mood disturbances, fatigue, and pain (Pont‐Sunyer et al., 2015). Genetic mutations are among the most significant contributing factors to the various causes of PD. (Bloem et al., 2021.)

Among them, α-synuclein is particularly closely linked to PD through mutations such as A53T, a mutation in the Alanine residue. This mutation is known to be associated with many detrimental symptoms of PD (Choi et al., 2008; Spira et al., 2001). The mouse model of PD with the A53T mutation develops numerous sensorimotor and synaptic impairments, followed by age-associated cognitive and motor deficits (Paumier et al., 2013). Furthermore, A53T α-synuclein in astrocytes contributes to initiating the damaging process that leads to neuron death (Gu et al., 2010).

Because α-synuclein aggregates disrupt dopaminergic and brainstem circuits, they can impair fine motor behaviors such as respiration–phonation coupling and vocal fold control (Rektorová et al., 2012; Ciucci et al., 2007). In rodents, this is manifested as altered call duration and frequency, whereas in humans, it parallels prosodic flattening and reduced articulatory precision in PD speech (Bocklet et al., 2011; Gnerre et al., 2023). These results suggest that overexpression and aggregation of α-synuclein disrupt vocal control circuits, highlighting vocalization as a sensitive biomarker of PD.

Vocalization deficits are increasingly recognized as a non-motor symptom of PD. Patients often show altered prosody, reduced acoustic variability, and vocal fold atrophy, which may also contribute to impaired swallowing (Rektorová et al., 2012; Bocklet et al., 2011; Yiu et al., 2020). Animal models also replicate these findings: α-synuclein transgenic rodents exhibit early-onset and progressive impairments in ultrasonic vocalization, including reduced call duration, intensity, and altered profiles, which often appear by two to three months of age and precede motor decline (Grant et al., 2014; Grant et al., 2015). These results suggest that α-synuclein accumulation disrupts vocal control circuits, highlighting vocalization as a sensitive biomarker of PD.

Measures, such as call duration, frequency modulation, and vocal intensity in mice, are considered counterparts of human PD vocal deficit symptoms. In mice, changes in call duration match prosodic timing deficits in PD speech (Gnerre et al., 2023; Rektorová et al., 2012), reduced frequency modulation represents the monotone voice and pitch variation of patients (Bocklet et al., 2011; Houle et al., 2024), and lower call intensity mirrors hypophonia or reduced loudness, which are symptoms of PD (Grant et al., 2014, 2015; Ramig et al., 2001).

Sex Differences in Parkinson's Disease

Epidemiological studies suggest that sex is a crucial factor in PD development. The disease is approximately twice as common in men as in women (Baldereschi et al., 2000; Solla et al., 2012). Women tend to develop PD about two years later than men, likely due to estrogen-related protective factors such as menopause and childbirth (Haaxma et al., 2007). Women are more likely to present with tremors as an initial symptom, associated with a slower progression and milder motor course. By contrast, men tend to exhibit more severe motor symptoms, including speech problems, while women experience more non-motor symptoms such as fatigue, depression, and pain (Santos-García et al., 2023). Longitudinal studies confirm these patterns: men show faster decline in both motor and non-motor aspects, requiring higher medication doses and experiencing greater impairment in daily tasks (Picillo et al., 2022). However, other reports suggest that women with PD sometimes report worse disability, quality of life, and greater anxiety despite physicians observing no major symptom differences (Abraham et al., 2019). These findings highlight the complex and sometimes contradictory nature of sex differences in PD progression and symptom burden.

Sex Differences in Vocal Effects

Sex-related differences also extend to vocal impairments in PD. Analysis of speech recordings revealed that while most acoustic measures—such as pitch variability, speech rate, and vowel articulation—are affected by both PD and sex independently, there are sex-specific interactions. For example, females with PD more often produced multiple bursts during plosive consonants, whereas males frequently failed to make a burst, indicating sex-dependent disruptions in articulatory timing and force (Houle et al., 2024). Similarly, Gnerre et al. (2023) showed that emotional expression in speech was impaired in PD patients, particularly women, who demonstrated lower pitch when expressing pleasure and poorer voice quality when expressing

fear and anger. Neutral speech, however, showed no sex-related differences. Complementing these findings, Hertrich and Ackermann (1995) reported that women with PD exhibited more irregularities in sustained vowel production, such as shaky pitch and abnormally low sounds, compared to both controls and men with PD. These results suggest that PD affects male and female voices differently, potentially due to inherent differences in vocal cord structure and function.

In this paper, we investigate whether there are sex differences in vocalization phenotypes in the A53T mouse model of Parkinson's Disease (PD). Specifically, we aim to explore distinct differences between male and female vocal expression, a non-motor symptom often observed in PD. To address this, we analyzed an existing vocalization dataset from A53T mutant mice and wild-type controls, focusing on simple vocalizations. Simple calls are defined as those with a constant, non-modulating frequency (Krasko et al., 2021). Because simple calls rely more heavily on basic motor pathways, they may be particularly vulnerable to early disruptions in PD (Holy & Guo, 2005). Thus, simple calls are especially sensitive to alterations in timing and intensity, paralleling hypophonia and prosodic timing deficits observed in PD.

We hypothesize that A53T mutant mice will exhibit sex-specific alterations in simple vocalizations. Specifically, we predict that females will show greater variability and disruption in acoustic parameters than males, consistent with evidence that sex hormones influence dopaminergic circuits involved in vocal motor control (Gillies & McArthur, 2010; Van Den Eeden et al., 2003; Ciucci et al., 2007).

By testing this hypothesis, we aim to clarify how non-motor symptoms manifest across sex, shedding light on potential mechanisms underlying sex-specific vulnerabilities in PD. While the original dataset provides raw acoustic measures of A53T mutant and wild-type mice, it did not

explore how these alterations vary by sex. Our analysis builds on this by uncovering sex-specific patterns in simple vocalizations that suggest distinct neural mechanisms and highlight the potential for more precise biomarkers of Parkinson's disease.

3. Results

3.1 Vocalization Duration

This study investigated whether there are sex differences in vocalization phenotypes in an animal model of Parkinson's disease. To explore this question, we obtained the vocalization dataset from A53T mutant mice and wild-type mice (the control group). We focused our analysis on simple vocalizations, which are defined as calls with a constant, non-modulating frequency (Krasko et al., 2021). Examining simple vocalizations by sex is important for understanding whether male and female mice exhibit distinct alterations in vocalization duration.

First, we investigated whether there is a sex difference in vocalization duration by analyzing samples from males and females in the simple vocalization test. The cumulative distribution plots showed no significant difference between male wild-type and male mutant animals (Figure 1, left panel; KS test, $p = 0.182$). Similarly, female wild-type and female mutant animals also did not differ significantly in their vocalization duration (Figure 1, right panel; KS test, $p = 0.176$). These results indicate that, when separated by sex, mutant mice do not show altered duration of simple vocalizations compared to wild-type controls.

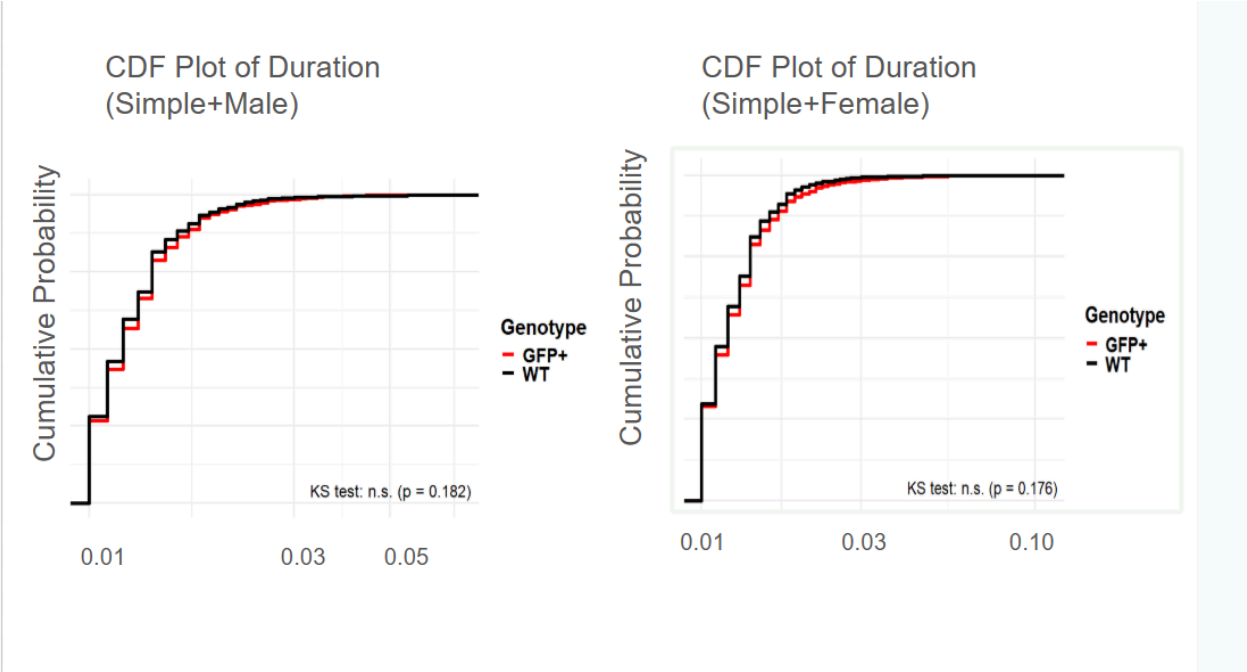

Figure 1. Cumulative distribution plots of vocalization duration (log scale) for wild-type (WT) and mutant mice. The left panel shows simple male vocalizations, and the right panel shows simple female vocalizations.

3.2 Vocalization Frequency Range

Secondly, to determine whether the mutation alters the frequency range of simple vocalizations, we examined male and female mice separately. Male mutant mice did not differ significantly from their wild-type controls (KS test, $p = 0.253$). In contrast, female mutant mice exhibited a significantly broader frequency range compared to female wild-type controls (KS test, $p = 0.0287$) (Figure 2).

Taken together, these findings indicate that the A53T mutation increases the frequency range of simple vocalizations in females, while males show no measurable differences.

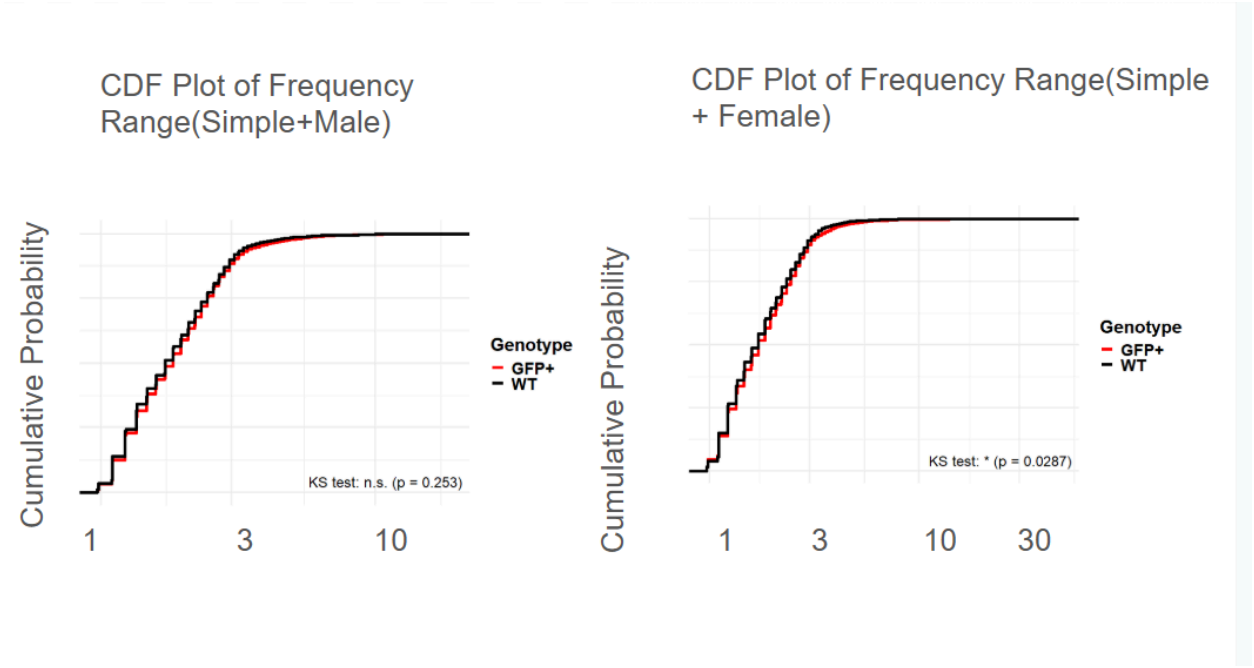

Figure 2. Cumulative distribution plots of vocalization frequency range (log scale) for wild-type (WT) and mutant mice. The left panel shows simple male vocalizations, and the right panel shows simple female vocalizations.

3.3 Maximum Acoustic Power

Thirdly, we analyzed whether the maximum energy level (maximum acoustic power) of vocalizations differs between wild-type and mutant mice. When examining simple vocalizations, male mutant mice did not differ significantly from male controls (Figure 3, left panel; KS test, $p = 0.379$). In contrast, female mutant mice exhibited significantly higher maximum acoustic power compared to female controls (Figure 3, right panel; KS test, $p = 0.017$). These results suggest that the A53T mutation influences vocal intensity primarily in simple vocalizations, with a more pronounced effect in females, while males remain unaffected.

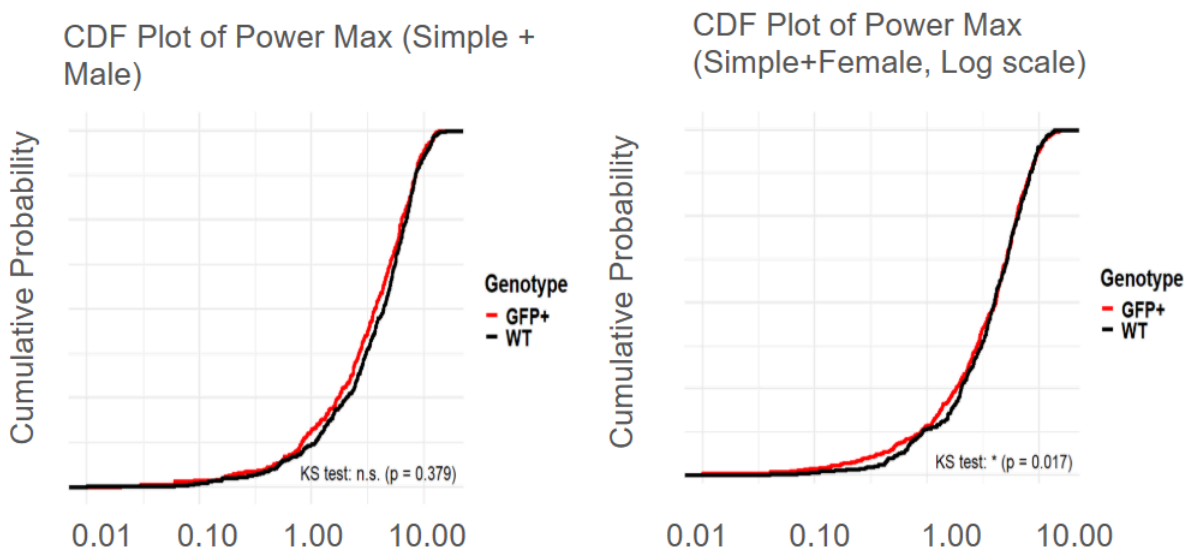

Figure 3. Cumulative distribution plots of maximum acoustic power (log scale) for wild-type (WT) and mutant mice. The left panel shows simple male vocalizations, and the right panel shows simple female vocalizations.

Summary of Results

Overall, the analyses revealed that simple vocalizations were differentially affected by the A53T mutation in male and female mice. For vocalization duration, no significant differences were observed between mutant and wild-type animals in either sex. In contrast, frequency range showed a selective genotype effect: female mutant mice exhibited significantly broader frequency ranges compared to their controls, while males showed no measurable difference. For maximum acoustic power, differences were also sex-specific, with female mutants showing considerably higher values than female controls, while no difference was found in males. Together, these findings suggest that frequency-related measures are the most sensitive markers

of genotype differences in females, while vocal intensity changes are likewise more pronounced in females.

Method:

The Purdue University Research Repository is the source of this information. This dataset is publicly accessible at: https://purr.purdue.edu/publications/4583/supportingdocs/1. A publicly accessible vocalization dataset from A53T mutant mice and wild-type mice (which served as the control group) was used for our analysis. Our analyses included 7 WT males, 7 GFP+ males, 6 WT females, and 9 GFP+ females. We focused on several key acoustic parameters: Duration, Power Maximum, and frequency range, comparing WT vs. A53T within each sex for simple calls. To evaluate distributional differences between genotypes (WT vs. A53T) within sex for simple calls, we used R to perform two-sample Kolmogorov–Smirnov (KS) tests. The KS test was chosen because it is a non-parametric method that does not assume normality or equal variances and is appropriate for comparing two distributions. Unlike tests that compare only group averages (e.g., t-test or Mann–Whitney U), the KS test evaluates differences across the entire distribution, making it suitable for detecting shifts in both central tendency and variability of acoustic parameters. We were able to determine whether the observed differences were statistically significant by using the KS test. Results with p-values below 0.05 were considered statistically significant.

4. Discussion

Summary of Key Findings

4.1 Vocalization Duration

Simple vocalizations were examined to assess whether the A53T mutation altered call timing across sexes. Mutant mice did not differ significantly from wild-type controls in the duration of simple calls for either males or females (Figure 1). This indicates that call length is not measurably influenced by the mutation, suggesting that duration is a relatively stable parameter across genotypes and sexes.

4.2 Frequency Range

Analysis of simple calls revealed a female-specific effect of the A53T mutation. Mutant females exhibited significantly broader frequency ranges compared to WT females, whereas no measurable differences were observed in males. This indicates that sex may modulate the acoustic breadth of simple calls, with frequency range serving as a sensitive marker of genotype-related differences in females.

4.3 Acoustic Power

Maximum acoustic power showed a sex-specific effect of the A53T mutation in simple vocalizations. Female mutants exhibited significantly higher values than WT females, whereas no measurable differences were observed in males. These findings suggest that simple calls are sensitive to mutation-driven changes in vocal intensity, with effects that are more pronounced in females.

4.4 Comparisons with Other Studies

A previous study using Thy1-aSyn rats reported a reduction in the duration, frequency, and intensity of ultrasonic vocalizations, with these abnormalities worsening over time (Grant et al., 2014). In contrast, our analysis of A53T mice revealed a significantly broader frequency range in females, as well as higher maximum power in females, while duration showed no significant differences. These discrepancies likely reflect methodological variations such as species, age, classification criteria, or acoustic analysis approaches. Importantly, unlike the original study, which emphasized overall impairments, our analysis uncovered female-specific alterations, highlighting that α-synuclein pathology may differentially affect vocal motor circuits across sexes.

Similarly, Gombash et al. (2013) found reduced vocal intensity in rats, but no consistent changes in frequency or duration. In contrast, our analysis of A53T mice revealed a broader frequency range and higher maximum power in females, while duration showed no significant differences. These findings suggest that, although reduced intensity is a common outcome of α-synuclein pathology, additional alterations in frequency may emerge in a female-specific manner.

Paumier et al. (2015) reported that PFF-injected rats exhibited shorter call durations and reduced vocal intensity. In contrast, our analysis of A53T mice revealed no significant differences in duration but did find a broader frequency range and higher maximum power in females. These differences may be explained by model-specific factors, since PFF injections produce progressive pathology, whereas A53T transgenics exhibit constitutive α-synuclein overexpression.

Together, these findings suggest that although α-synuclein pathology consistently affects vocal intensity, its impact on duration and frequency range may depend on the disease model and sex.

4.5 Implications for Neural Mechanisms

The alterations we observed in simple vocalizations suggest that α-synuclein pathology disrupts neural circuits critical for basic vocal motor control. Simple ultrasonic calls are thought to be mediated by brainstem and basal ganglia pathways, with dopaminergic modulation playing a key role in shaping frequency and intensity (Ciucci et al., 2007; Tschida et al., 2019). The broader frequency ranges in females and increased vocal intensity in females may reflect dysfunction in basal ganglia–brainstem circuits, alongside sex-dependent modulation of dopaminergic pathways. These findings highlight the basal ganglia as a central locus of vulnerability in PD-related vocal deficits and suggest that sex-specific factors, such as hormonal influences, may amplify circuit dysfunction.

These findings underscore the scientific relevance of vocalization analysis in PD models, as changes in call structure may serve as behavioral readouts of underlying neural dysfunction. Because vocal production depends on the precise motor control of respiration and laryngeal muscles, as well as cognitive sequencing of call patterns, disruptions in vocalizations can reflect both motor and cognitive impairments characteristic of PD (Rektorová et al., 2012; Grant et al., 2014; Ciucci et al., 2007). This highlights the potential of vocalization metrics as non-invasive biomarkers for early detection and monitoring of disease progression (Bocklet et al., 2011; Grant et al., 2015).

4.6 Translational Relevance of Behavioral and Pharmacological Approaches

Human studies have demonstrated the effectiveness of the Lee Silverman Voice Treatment (LSVT) in improving vocal deficits in Parkinson’s disease. LSVT enhances vocal loudness, articulation, and clarity through high-effort vocal exercises, with improvements reported in vocal

intensity, articulation, and perceptual speech quality (Ramig et al., 2001; Silveira & Brasolotto, 2005; Searl et al., 2011; Cannito et al., 2012; Sapir et al., 2007). Pharmacological treatments such as levodopa, in contrast, have shown mixed effects on vocal outcomes. Some studies have reported limited improvements in vocal quality (De Letter et al., 2006; Kelm-Nelson et al., 2016), while others have observed increased loudness and speech speed, but without sustained benefits (Ho et al., 2008). These findings suggest that combining pharmacological treatments with behavioral therapies, such as LSVT, may offer stronger outcomes than medication alone. Testing such strategies in animal models could therefore clarify underlying mechanisms and therapeutic potential. By connecting the variability observed in human interventions with the patterns identified in our animal vocalization data, we provide a translational framework that highlights how preclinical studies can inform combined treatment approaches for Parkinson's vocal deficits.

4.7 Limitations of This Study

Two limitations should be noted. First, the cross-sectional design prevents conclusions about the progression of vocal impairments over time. Second, the hormonal cycle in females was not controlled, which may influence vocalization parameters such as frequency or intensity (Geyer & Barfield, 1978). Some observed differences could therefore be partially attributable to hormone-related variability.

4.8 Future Research Directions

Future work should employ longitudinal designs to track vocal symptoms over time and correlate them with neurodegenerative changes. Such studies could identify early markers of disease progression and clarify how vocal deficits develop over time with age.

Additionally, targeted neurobiological investigations are needed to map the specific brain regions controlling simple vocalizations and to determine how A53T alters these circuits. Integrating vocal-behavior studies with therapeutic interventions, including both behavioral and pharmacological approaches, could accelerate the development of non-invasive biomarkers and treatment strategies for Parkinson's disease.

References:

Abraham, D. S., Gruber-Baldini, A. L., Magder, L. S., McArdle, P. F., Tom, S. E., Barr, E., Schrader, K., & Shulman, L. M. (2019). Sex differences in Parkinson's disease presentation and progression. *Parkinsonism & Related Disorders*, *69*, 48–54.

Baldereschi, M., Di Carlo, A., Rocca, W. A., Vanni, P., Maggi, S., Perissinotto, E., Grigoletto, F., Amaducci, L., & Inzitari, D. (2000). Parkinson's disease and parkinsonism in a longitudinal study: Two-fold higher incidence in men. *Neurology*, *55*(9), 1358–1363.

Bloem, B. R., Okun, M. S., & Klein, C. (2021). Parkinson's disease. *The Lancet*, *397*(10291), 2284–2303.

Bocklet, T., Nöth, E., Stemmer, G., Ruzickova, H., & Rusz, J. (2011). *Detection of persons with Parkinson's disease by acoustic, vocal, and prosodic analysis*. 478–483.

Choi, J. M., Woo, M. S., Ma, H.-I., Kang, S. Y., Sung, Y.-H., Yong, S. W., Chung, S. J., Kim, J.-S., Shin, H., & Lyoo, C. H. (2008). Analysis of PARK genes in a Korean cohort of early-onset Parkinson disease. *Neurogenetics*, *9*, 263–269.

Ciucci, M. R., Ma, S. T., Fox, C., Kane, J. R., Ramig, L. O., & Schallert, T. (2007). Qualitative changes in ultrasonic vocalization in rats after unilateral dopamine depletion or haloperidol: A preliminary study. *Behavioural Brain Research*, *182*(2), 284–289.

Elkouzi, A., Vedam-Mai, V., Eisinger, R. S., & Okun, M. S. (2019). Emerging therapies in Parkinson disease—Repurposed drugs and new approaches. *Nature Reviews Neurology*, *15*(4), 204–223.

Gillies, G. E., & McArthur, S. (2010). Estrogen actions in the brain and the basis for differential action in men and women: A case for sex-specific medicines. *Pharmacological Reviews*, *62*(2), 155–198.

Gnerre, M., Malaspina, E., Di Tella, S., Anzuino, I., Baglio, F., Silveri, M. C., & Biassoni, F. (2023). Vocal emotional expression in Parkinson's disease: Roles of sex and emotions. *Societies*, *13*(7), 157.

Grant, L. M., Kelm‐Nelson, C. A., Hilby, B. L., Blue, K. V., Paul Rajamanickam, E. S., Pultorak, J. D., Fleming, S. M., & Ciucci, M. R. (2015). Evidence for early and progressive ultrasonic vocalization and oromotor deficits in a PINK1 gene knockout rat model of Parkinson's disease. *Journal of Neuroscience Research*, *93*(11), 1713–1727.

Grant, L. M., Richter, F., Miller, J. E., White, S. A., Fox, C. M., Zhu, C., Chesselet, M.-F., & Ciucci, M. R. (2014). Vocalization deficits in mice over-expressing alpha-synuclein, a model of pre-manifest Parkinson's disease. *Behavioral Neuroscience*, *128*(2), 110.

Gu, X.-L., Long, C.-X., Sun, L., Xie, C., Lin, X., & Cai, H. (2010). Astrocytic expression of Parkinson's disease-related A53T α-synuclein causes neurodegeneration in mice. *Molecular Brain*, *3*, 1–16.

Haaxma, C. A., Bloem, B. R., Borm, G. F., Oyen, W. J., Leenders, K. L., Eshuis, S., Booij, J., Dluzen, D. E., & Horstink, M. W. (2007). Gender differences in Parkinson's disease. *Journal of Neurology, Neurosurgery & Psychiatry*, *78*(8), 819–824.

Hertrich, I., & Ackermann, H. (1995). Gender-specific vocal dysfunctions in Parkinson's disease:

Electroglottographic and acoustic analyses. *Annals of Otology, Rhinology & Laryngology*, *104*(3), 197–202.

Holy, T. E., & Guo, Z. (2005). Ultrasonic songs of male mice. *PLoS Biology*, *3*(12), e386.

Houle, N., Feaster, T., Mira, A., Meeks, K., & Stepp, C. E. (2024). Sex Differences in the Speech of Persons with and without Parkinson's Disease. *American Journal of Speech-Language Pathology*, *33*(1), 96–116.

Hughes, A. J., Daniel, S. E., Kilford, L., & Lees, A. J. (1992). Accuracy of clinical diagnosis of idiopathic Parkinson's disease: A clinico-pathological study of 100 cases. *Journal of Neurology, Neurosurgery & Psychiatry*, *55*(3), 181–184.

Krasko, M. N., Hoffmeister, J. D., Schaen-Heacock, N. E., Welsch, J. M., Kelm-Nelson, C. A., & Ciucci, M. R. (2021). Rat models of vocal deficits in Parkinson's disease. *Brain Sciences*, *11*(7), 925.

Lavedan, C. (1998). The synuclein family. *Genome Research*, *8*(9), 871–880.

Li, W., Lesuisse, C., Xu, Y., Troncoso, J. C., Price, D. L., & Lee, M. K. (2004). Stabilization of α-synuclein protein with aging and familial Parkinson's disease-linked A53T mutation. *Journal of Neuroscience*, *24*(33), 7400–7409.

Matsumine, H., Saito, M., Shimoda-Matsubayashi, S., Tanaka, H., Ishikawa, A., Nakagawa-Hattori, Y., Yokochi, M., Kobayashi, T., Igarashi, S., & Takano, H. (1997). Localization of a gene for an autosomal recessive form of juvenile Parkinsonism to chromosome 6q25. 2-27. *American Journal of Human Genetics*, *60*(3), 588.

Middleton, F. A., & Strick, P. L. (2000). Basal ganglia and cerebellar loops: Motor and cognitive circuits. *Brain Research Reviews*, *31*(2–3), 236–250.

Nestor, K., Rasero, J., Betzel, R., Gianaros, P. J., & Verstynen, T. (2024). Cortical network reconfiguration aligns with shifts of basal ganglia and cerebellar influence. *ArXiv*, arXiv-2408.

Paumier, K. L., Sukoff Rizzo, S. J., Berger, Z., Chen, Y., Gonzales, C., Kaftan, E., Li, L.,

Lotarski, S., Monaghan, M., & Shen, W. (2013). Behavioral characterization of A53T mice reveals early and late stage deficits related to Parkinson's disease. *PloS One*, *8*(8), e70274.

Picillo, M., LaFontant, D.-E., Bressman, S., Caspell-Garcia, C., Coffey, C., Cho, H. R., Burghardt, E. L., Dahodwala, N., Saunders-Pullman, R., & Tanner, C. M. (2022). Sex-related longitudinal change of motor, non-motor, and biological features in early Parkinson's disease. *Journal of Parkinson's Disease*, *12*(1), 421–436.

Poewe, W., Seppi, K., Tanner, C. M., Halliday, G. M., Brundin, P., Volkmann, J., Schrag, A.-E., & Lang, A. E. (2017). Parkinson disease. *Nature Reviews Disease Primers*, *3*(1), 1–21.

Pont‐Sunyer, C., Hotter, A., Gaig, C., Seppi, K., Compta, Y., Katzenschlager, R., Mas, N., Hofeneder, D., Brücke, T., & Bayés, A. (2015). The O nset of N onmotor S ymptoms in P arkinson's disease (T he ONSET PD S tudy). *Movement Disorders*, *30*(2), 229–237.

Ramig, L. O., Sapir, S., Countryman, S., Pawlas, A. A., O'Brien, C., Hoehn, M., & Thompson, L. L. (2001). Intensive voice treatment (LSVT®) for patients with Parkinson's disease: A 2 year follow up. *Journal of Neurology, Neurosurgery & Psychiatry*, *71*(4), 493–498.

Rektorová, I., Mikl, M., Barrett, J., Marecek, R., Rektor, I., & Paus, T. (2012). Functional neuroanatomy of vocalization in patients with Parkinson's disease. *Journal of the Neurological Sciences*, *313*(1–2), 7–12.

Santos-García, D., Laguna, A., Hernández-Vara, J., de Deus Fonticoba, T., Cores Bartolomé, C., Feal Painceiras, M. J., Íñiguez-Alvarado, M. C., García Díaz, I., Jesús, S., & Boungiorno, M. T. (2023). Sex differences in motor and non-motor symptoms among Spanish patients with Parkinson's disease. *Journal of Clinical Medicine*, *12*(4), 1329.

Solla, P., Cannas, A., Ibba, F. C., Loi, F., Corona, M., Orofino, G., Marrosu, M. G., & Marrosu, F. (2012). Gender differences in motor and non-motor symptoms among Sardinian patients with Parkinson's disease. *Journal of the Neurological Sciences*, *323*(1–2), 33–39.

Spira, P. J., Sharpe, D. M., Halliday, G., Cavanagh, J., & Nicholson, G. A. (2001). Clinical and

pathological features of a Parkinsonian syndrome in a family with an Ala53Thr α-synuclein mutation. *Annals of Neurology*, *49*(3), 313–319.

Taguchi, K., Watanabe, Y., Tsujimura, A., & Tanaka, M. (2016). Brain region-dependent differential expression of alpha-synuclein. *Journal of Comparative Neurology*, *524*(6), 1236–1258.

Van Den Eeden, S. K., Tanner, C. M., Bernstein, A. L., Fross, R. D., Leimpeter, A., Bloch, D. A., & Nelson, L. M. (2003). Incidence of Parkinson's disease: Variation by age, gender, and race/ethnicity. *American Journal of Epidemiology*, *157*(11), 1015–1022.

Yiu, Y., Curtis, J. A., Perry, S. E., & Troche, M. S. (2020). Relationship of vocal fold atrophy to swallowing safety and cough function in Parkinson's disease. *The Laryngoscope*, *130*(2), 303–308.

Zhang, W., Wang, T., Pei, Z., Miller, D. S., Wu, X., Block, M. L., Wilson, B., Zhang, W., Zhou, Y., & Hong, J.-S. (2005). Aggregated α-synuclein activates microglia: A process leading to disease progression in Parkinson's disease. *The FASEB Journal*, *19*(6), 533–542.

Supplementary Table 1. Duration comparisons (p-values)

Condition	WT vs mutant	Male WT vs mutant	Female WT vs mutant
Duration / Simple	0.02	0.18	0.18
Duration / Complex	0.04	0.09	0.13

Supplementary Table 1. Comparison of vocalization duration (p-values) between wild-type (WT) and mutant mice. Results are shown separately for simple and complex calls, and further divided by sex.

Supplementary Table 2. Frequency comparisons (p-values)

Condition	WT vs mutant	Male WT vs mutant	Female WT vs mutant
Frequency / Simple	0.01	0.25	0.03
Frequency / Complex	0.35	0.58	0.40

Supplementary Table 2. Frequency comparisons (p-values) between wild-type (WT) and mutant mice. Results are shown separately for simple and complex vocalizations, and further divided by sex.

Supplementary Table 3. Power_max comparisons (p-values)

Condition	WT vs mutant	Male WT vs mutant	Female WT vs mutant
Power_max / Simple	0.03	0.38	0.02
Power_max / Complex	0.21	0.23	0.21

Supplementary Table 3. Power comparisons (p-values) between wild-type (WT) and mutant mice. Results are shown separately for simple and complex vocalizations, and further divided by sex.

Dear Editor and Reviewers,

Thank you for all of your insightful and valuable feedback, as well as the opportunity to revise my manuscript. I appreciate the time and effort that you and the reviewers have invested in providing comments to improve my work. After carefully considering all comments, I have revised the manuscript accordingly. I approached the revision process with care and diligence, and I hope my responses adequately address all the concerns raised.

Reviewer 1:

1. Originality & Significance

The paper explores vocalization changes in a well-known mouse model of Parkinson's disease (PD), with attention to sex differences and call type. This is a potentially novel and worthwhile line of inquiry, especially for a high school journal, and the idea of reanalysing open-source data is commendable.

However, the paper does not sufficiently highlight what is novel or why these analyses are significant. The rationale for focusing on vocalization metrics and the choice of subgroups (e.g., separating simple vs. complex calls and male vs. female animals) is never clearly articulated. The study lacks a defined hypothesis or overarching goal, which reduces the perceived novelty and leaves the reader uncertain about the contribution to the field.

The manuscript includes a brief aim in the introduction, stating an intent to examine sex differences in vocalisation in a PD mouse model. While this provides a useful starting point, it is not framed as a clear, testable hypothesis. Strengthening this aim and ensuring it is consistently reflected in the analyses and discussion would improve the overall coherence and perceived significance of the study.

Suggestions:

· Clearly articulate a research question or hypothesis in the introduction.

->Thank you for your comments. I have now added a clear hypothesis paragraph in the introduction.

Page 5 : "We hypothesize that A53T mutant mice will exhibit sex-specific alterations in simple vocalizations. Specifically, we predict that females will show greater variability and disruption in acoustic parameters than males, consistent with evidence that sex hormones influence dopaminergic circuits involved in vocal motor control (Gillies & McArthur, 2010; Van Den Eeden et al., 2003; Ciucci et al., 2007)."

· Explain the potential scientific relevance of the work (e.g., how vocalizations may reflect underlying motor or cognitive impairment in PD).

->Thank you for this helpful suggestion. We have added a paragraph in the Implications for Neural Mechanisms section (page 15) that "Because vocal production depends on the precise motor control of respiration and laryngeal muscles, as well as cognitive sequencing of call patterns, disruptions in vocalizations can reflect both motor and cognitive impairments characteristic of PD (Rektorová et al., 2012; Grant et al., 2014; Ciucci et al., 2007). This highlights the potential of vocalization metrics as non-invasive biomarkers for early detection and monitoring of disease progression (Bocklet et al., 2011; Grant et al., 2015)."

Clarify how your analysis adds something new compared to the original dataset publication.

->Thank you for your comments. I have added clear difference between the original dataset and analysis. Page 6: "While the original dataset provides raw acoustic measures of A53T mutant and wild-type mice, it did not explore how these alterations vary by sex. Our analysis builds on this by uncovering sex-specific patterns in simple vocalizations that suggest distinct neural mechanisms and highlight the potential for more precise biomarkers of Parkinson's disease."

Revise the introduction following a clear thematic sequence and remove redundant or loosely connected citations.

Thank you for your feedback, we revised the structure so the thematic sequence now flows as: PD → Vocal deficits in PD → General sex differences → Sex differences in vocal effects → Hypothesis (with rationale)

· Explicitly state the research question or hypothesis at the end of the introduction.

->Thank you for your suggestion. I've now explicitly stated the research question/hypothesis at the end of the introduction. Page 5:"We hypothesize that A53T mutant mice will exhibit sex-specific alterations in simple vocalizations. Specifically, we predict that females will show greater variability and disruption in acoustic parameters than males, consistent with evidence that sex hormones influence dopaminergic circuits involved in vocal motor control (Gillies & McArthur, 2010; Van Den Eeden et al., 2003; Ciucci et al., 2007)."

· Use section subheadings in the Results and Discussion to organise the findings by call type, sex, or age group.

-> ***I completely agree with your point—it was harder to follow before. I've now reorganized it to make everything much clearer and easier to read. Thank you for your helpful comments.***

· Reduce the number of figures or combine them into summary plots. Focus on the most meaningful comparisons.

->**We appreciate this suggestion. In the revision, we have reduced the number of figures by combining related results into summary plots. Specifically, we focused on acoustic parameters of simple male and simple female vocalizations, which are central to our study, and consolidated them into clearer, more concise figures. In addition, we decided to remove Figures 4, 5, and 6 to focus on the main findings presented in Figures 1, 2, and 3.This approach highlights the key findings while avoiding redundancy.**

· Conclude the Results section with a paragraph synthesising the key findings and their relevance.- Edit the introduction to remove repetition and improve focus. Group ideas logically (e.g., start with PD, then vocalization, then the mouse model).

o Use section subheadings in the Results and Discussion to organise the findings.

->Thank you for this suggestion. We have revised the Results section and reorganized the subsections as follows: 3.1 Duration, 3.2 Vocalization Frequency Range, 3.3 Maximum Acoustic Power, and a final subsection summarizing the results. We have revised the Discussion section and reorganized the subsections as follows: 4.1 Vocalization Duration, 4.2 Frequency Range, 4.3 Acoustic Power, 4.4 Implications for Neural Mechanisms, 4.5 Translational Relevance of Behavioral and Pharmacological Approaches, 4.6 Limitations of This Study, and 4.7 Future Research Directions.

o Limit the number of figures or group them into summary plots. Highlight only the most informative comparisons.

-> Thank you for your suggestion. I understand that the original figures were confusing. I have now included only the figures for simple males and simple females, along with the supplementary table of p-value comparisons. In addition, we decided to remove Figures 4, 5, and 6 to focus on the main findings presented in Figures 1, 2, and 3. This should clarify where significant differences were observed and where they were not.

o End the Results section with a short paragraph that summarises the key takeaways.

-> ***Thank you for the suggestion. On page 9, I have added the section called "Summary of Results"***

Add a dedicated "Statistical Analysis" subsection in the Methods, clearly defining the unit of analysis (e.g., individual vocalisations vs. per-animal averages), sample sizes, and any steps taken to preprocess the data (e.g., removal of outliers).

-> We thank the reviewer for raising this important point. Rather than adding a separate Statistical Analysis subsection, we have revised the Methods to clearly specify the statistical approach. We now explicitly state that the unit of analysis was individual vocalization events, not per-animal averages. Each data point represents a unique call emitted by one of the 7 WT males, 7 GFP+ males, 6 WT females, and 9 GFP+ females (29 animals in total). The number of calls per group is reported in the Supplementary Materials. Data normality was assessed using the Shapiro–Wilk test, which indicated non-normality ($p < 0.05$). No additional preprocessing or outlier removal was performed.

· Justify the use of the KS test. If the goal is to compare group averages, simpler and more interpretable tests such as the independent t-test or Mann–Whitney U test (for nonparametric comparisons) may be more appropriate.

->Thank you for your comments. Page 10:"The KS test was chosen because it is a non-parametric method that does not assume normality or equal variances and is appropriate for comparing two distributions. Unlike tests that compare only group averages (e.g., t-test or Mann–Whitney U), the KS test evaluates differences across the entire distribution, making it suitable for detecting shifts in both central tendency and variability of acoustic parameters."

· Report both the number of animals and the number of calls per condition. If calls were pooled across animals, this should be explicitly stated, and the implications of that decision should be discussed.

-> We have revised the text to explicitly report both the number of animals (7 WT males, 7 GFP+ males, 6 WT females, 9 GFP+ females) and the number of vocalizations per condition, which are now included in Supplementary Table S1. We also state that calls were pooled across animals, and we have added a brief discussion of the implications of this choice. Specifically, pooling provides a comprehensive view of the distribution of vocalization features but does not account for repeated measures at the animal level. To avoid inflating the false positive rate, reduce the number of comparisons by focusing on a small number of clearly motivated contrasts. Apply corrections for multiple comparisons (e.g., Holm–Bonferroni) if many tests are retained.

->We have revised the manuscript to prioritize the most conceptually important comparisons (simple male and simple female groups). Exploratory subgroup results have been moved to the Supplementary Materials to reduce the number of contrasts presented in the main text. While we did not apply a Holm–Bonferroni correction, we

minimized the number of statistical tests to lower the risk of inflated false positives. A full table of all tests and raw p-values is provided in the Supplementary Materials.

· Limit visualisations to key comparisons. Results that are exploratory or non-significant can be moved to an appendix or supplemental material.

-> We have limited the main figures to only the key comparisons (wild-type vs. mutant for simple male and simple female vocalizations). Non-significant or exploratory results have been moved to the Supplementary Materials. This ensures that the main text highlights only the most interpretable and conceptually important findings.

· Ensure that each analysis is introduced with a rationale and followed by a clear interpretation of the result. Help the reader understand not just what was found, but why it was analysed in the first place.

->We have revised the Results section so that each analysis is preceded by a clear rationale and followed by a concise interpretation. For example, we now explicitly state that the KS test was used to evaluate differences in the shape of call distributions, not just means, and we explain how significant results inform our understanding of vocalization differences between genotypes.

· Place figures after the first paragraph that reports the associated results

->We have repositioned all figures so that they appear immediately after the first paragraph reporting the corresponding results.

· Consider computing **animal-level summary statistics** (e.g., average pitch or call duration per mouse) and comparing these across groups. This avoids pseudoreplication and is much easier to interpret.

-> Thank you for the suggestion. In our analysis, we did not compute animal-level summary statistics, because our goal was to examine the entire distribution of acoustic features rather than reduce the data to a single summary value per animal. Aggregating the data at the animal level would obscure meaningful variation within each group and potentially miss distributional differences. Therefore, instead of using a rank-based test such as the Mann–Whitney U test, which focuses on central tendency, we employed the KS test to compare the full distributions across groups. This approach allows us to detect not only differences in central tendency but also differences in variability and distributional shape, which are critical for our research question.

· Axis labels are too small, resolution is poor, and significance markers are inconsistently applied. I recommend exporting all figures as “svg” or “eps” files from R, with high DPI (330 or above). If needed, you can edit them using Illustrator or the free software Inkscape. Ensure to export as high DPI PNG images.

->All figures have been regenerated at high resolution (≥330 DPI) and exported as SVG/PNG files to ensure clarity. We have standardized axis label sizes, improved readability, and applied consistent significance markers across all figures.

· Rather than displaying every metric and subgroup, prioritise a smaller selection of meaningful plots and ensure they are fully interpretable. Include clear titles, legends, and indications of sample size. A summary table of group-level vocalisation metrics (means, SDs, p-values) may be useful.

-> We have streamlined the figures to highlight only the most relevant metrics and comparisons. Each figure now includes a clear title, full legend, and sample size annotations. Additionally, we provide a summary table of p-values in the Supplementary Materials for transparency and ease of interpretation.

Improve integration of sources into the argument. For example, don’t just define α-synuclein, explain why its overexpression might affect vocalization.

-> Thank you for this suggestion. I revised the Introduction to go beyond simply defining α-synuclein. Page 3: “Because α-synuclein aggregates disrupt dopaminergic and brainstem circuits, they can impair fine motor behaviors such as respiration–phonation coupling and vocal fold control (Rektorová et al., 2012; Ciucci et al., 2007). In rodents, this is manifested as altered call duration and frequency, whereas in humans, it parallels prosodic flattening and reduced articulatory precision in PD speech (Bocklet et al., 2011; Gnerre et al., 2023). These results suggest that overexpression and aggregation of α-synuclein disrupt vocal control circuits, highlighting vocalization as a sensitive biomarker of PD.”

· Include a brief summary and citation of the original study that generated the dataset in the introduction.

-> While the dataset used in this study is publicly available, it has not yet been published in a peer-reviewed format. We updated the source of this dataset in the method section.

· In the Discussion, contrast your findings with those of the original study to demonstrate your unique contribution.

->Since it hasn’t been published in a peer-reviewed format using this dataset, direct comparison with our results is limited. However, we compared our results with previous literature regarding the vocalization phenotypes associated with Parkinson’s disease.

->We agree and made the section "Comparision with other studies" on discussion section.

· In the Discussion, go beyond restating prior work: explain how your results compare to existing studies using similar methods or models. Highlight methodological differences where relevant.

->***Since it hasn't been published in a peer-reviewed format using this dataset, direct comparison with our results is limited.***

Clarify how mouse vocalisation measures (e.g., call duration, frequency modulation) relate to the human PD symptoms cited earlier (e.g., impaired prosody, reduced articulation).

-> ***Thank you for this helpful suggestion. We added text in the Introduction to explain why these vocalisation parameters are relevant to PD symptoms. Specifically, we now note that:***

Page 3 : " Animal models also mirror these findings: α-synuclein transgenic rodents exhibit early-onset and progressive impairments in ultrasonic vocalization, including reduced call duration, intensity, and altered profiles, often appearing by two to three months of age and preceding motor decline (Grant et al., 2014; Grant et al., 2015). These results suggest that α-synuclein accumulation disrupts vocal control circuits, highlighting vocalization as a sensitive biomarker of PD. "

· Either better integrate the paragraph on LSVT and levodopa into a discussion of translational relevance or remove it to maintain focus on your core findings.

Thank you for the helpful suggestion. We revised this section to explicitly frame it as Translational Relevance and to connect the LSVT and levodopa literature to our animal vocalization findings. We retained all original content and citations and added two linking sentences that explain how our results provide a preclinical platform to evaluate combined behavioral–pharmacological strategies.

Review the paper for sentence clarity and grammar. Use tools like Grammarly or ask a mentor or peer to help.

->Thank you for the comments. I have asked a mentor for help.

· Standardise scientific terminology throughout (e.g., "α-synuclein," "non-motor symptoms").

->Thank you for the suggestion. I agree on the importance of standardizing terminology, and I have revised the manuscript accordingly.

· Reduce redundancy and vary sentence structure to improve flow.

-> Thank you for your comments. I have substantially shortened the introduction to eliminate overlapping and redundant content. In addition, I revised the phrasing in the Results section to improve clarity and reduce repetition

· Pay close attention to figure labels, axis titles, and legends—these must be legible and consistently formatted.

->We thank the reviewer for this helpful suggestion. All figure labels, axis titles, and legends have been revised to ensure they are legible, consistently formatted, and stylistically uniform across all figures.

· Revise citation usage to follow a consistent academic style. For example:

o If the author's name is included in the sentence (e.g., "Gombash et al. found…"), use the year in parentheses: Gombash et al. (2013). Also, do not place the entire citation in parentheses again at the end of the sentence.

->Thank you for your comments. It has been fixed.

o When citing the same study across multiple consecutive sentences, it is not necessary to repeat the full citation each time.

->Thank you for your comments. It has been fixed.

o Refer to the APA citation manual and resources linked on the Convergence website to ensure all references are formatted accordingly.

-> Thank your comments. It is APA.

Reviewer 2:

1. Introduction: *"Some papers described the mechanism by mentioning that the AT53 mutation leads to more stable a-synuclein in neurons, which causes it to accumulate to high levels and contributes to the progression of PD (Li et al., 2004)."* Please elaborate, how does accumulation of alpha-synuclein result in PD?

->Thank you for your comments. Following the reviewer's suggestion, I have moved point 9 earlier in the introduction, so the mechanism of alpha-synuclein accumulation is now elaborated there."

Page 3: "Because α-synuclein aggregates disrupt dopaminergic and brainstem circuits, they can impair fine motor behaviors such as respiration–phonation

coupling and vocal fold control (Rektorová et al., 2012; Ciucci et al., 2007). In rodents this appears as altered call duration and frequency, while in humans it parallels prosodic flattening and reduced articulatory precision in PD speech (Bocklet et al., 2011; Gnerre et al., 2023). These results suggest that overexpression and aggregation of α-synuclein disrupt vocal control circuits, highlighting vocalization as a sensitive biomarker of PD."

2. For Methods section, the author has cited "*Schaser & Rodgers, 2024*". However, this is not in the list of references.

-> We thank the reviewer for noting this inconsistency. The brief mention of "Schaser & Rodgers, 2024" has been removed from the Methods, as the dataset is directly cited through the Purdue University Research Repository.

3. It is unclear where source of data comes from. The author has written "*A publicly accessible vocalization dataset from A53T mutant mice and wild-type mice (which served as the control group) was used for our analysis.*" Please provide dataset link. If they are from previously published papers, the papers also need to be cited.

-> We thank the reviewer for highlighting the need to clarify the data source. We have revised the Methods section to clearly state that the dataset was obtained from the Purdue University Research Repository (PURR) and have added the direct dataset link for transparency. Page 10:"The Purdue University Research Repository is the source of this information. This dataset is publicly accessible at: https://purr.purdue.edu/publications/4583/supportingdocs/1. "

4. In this paper, the author has analyzed a study of control and mutant male and female mice. However, it is not stated how many mice were used per condition. Please clarify this point.

-> We thank the reviewer for pointing this out. We have now clarified the number of animals used per condition. Specifically, our analyses included 7 WT males, 7 GFP+ males, 6 WT females, and 9 GFP+ females. Page 10: "Our analyses included 7 WT males, 7 GFP+ males, 6 WT females, and 9 GFP+ females."

5. Kolmogorov-Smirnov (KS) test used is appropriate for statistical analysis. However, this statistics test is sensitive to sample sizes, therefore, it is crucial to state the number of mice used (see above point as well).

-> Thank you for the comment. The data used in this analysis is from 29 mice in total (7 WT males, 7 GFP+ males, 6 WT females, and 9 GFP+ females). To assess the overall distribution of the dataset, we combined all individual data points from these 29 mice. Since our aim was to evaluate the distribution of the entire dataset rather than compare between groups, we performed the Kolmogorov-Smirnov (KS) test on the pooled data.

Page 10:"The KS test was chosen because it is a non-parametric method that does not assume normality or equal variances and is appropriate for comparing two distributions. Unlike tests that compare only group averages (e.g., t-test or Mann–Whitney U), the KS test evaluates differences across the entire distribution, making it suitable for detecting shifts in both central tendency and variability of acoustic parameters. "

6. The figures/graphs need a better resolution and to be bigger in proportion to the text. Axis/numbers need to be bigger.

->Thank you for your suggestion — I have updated the figures to improve resolution and increased the axis labels and numbers for better readability.

7. The author has stated in figure legends which comparisons are statistically significant. That is a great descriptive help. Please also add the p-value of those that are statistically significant, even if they have already been mentioned in the text.

-> Thank you for your comments. We have revised the figure legends to include the exact p-values for all statistically significant comparisons, even when already mentioned in the text. In addition, we have attached the supplementary tables for clarity.

8. Consider simplifying graphs titles/axes for a cleaner look. E.g. *Fig 1A. "Duration.U"*: what is U? this is not described in the legend. The title can be revised to: *CDF Plot of Duration (Simple, All)*, or just *Duration of Vocalization*. The words "*Log Scale*" can be removed, as it is already on the x-axis. The type of plot, CDF, can be described in the legend. Please also write "Cumulative Distribution Function (CDF)" the first time you use it in the text, before using the acronym CDF in the rest of the paper.

-> ***Thank you for your comments. We have simplified the letters in the figures and removed unnecessary parts.***

9. This paragraph in Discussion: "*Alpha-synuclein has been identified as playing a significant role in PD (Matsumine et al., 1997). … Overall, reduced clearance and abnormal interactions with cell membranes lead to the accumulation of alpha-synuclein, which forms toxic aggregates that cause damage to brain cells, a hallmark of PD (Breydo et al., 2012; Goedert et al., 2013). These mechanisms highlight Alpha-Synuclein's central role in PD progression.*" This paragraph is more suited in the

introduction, and can actually be used as a bridge in explaining the role of alpha-synuclein in PD (see also point 1).

-> Thank you for your comments. As suggested, I have moved this paragraph to the introduction, where it now serves as a bridge to explain the role of alpha-synuclein in PD (see also point 1).

10. Author has done an excellent job plotting the results in graphs. However, this reviewer would like to see more discussion of the results from this project. Consider adding an "Analysis" section before the "Discussion" section to address this.

->We appreciate the reviewer's helpful suggestion. To strengthen the manuscript, we expanded the "Summary of Results" subsection to provide more detailed interpretation of the findings, effectively serving as an Analysis section that bridges the Results and Discussion. This addition highlights the key statistical outcomes and their immediate implications prior to the broader discussion.

11. The Discussion section is detailed and references many other papers, demonstrating the author's knowledge of other studies. The author did a very thorough comparison of his results with other studies. However, there needs to be more discussion of author's own results, which can be remedied by adding an analysis section (see point 9). My suggestion is to have an Analysis section where the focus is on this paper's results, and a Discussion section where comparisons with other studies will be elaborated upon.

-> We thank the reviewer for this helpful suggestion. In the revised manuscript, we expanded the "Summary of Results" subsection at the end of the Results to serve as a dedicated Analysis section. This part now emphasizes interpretation of our own findings—such as the stronger and more consistent genotype effects on frequency range and the sex-specific differences in acoustic power—before moving to the broader Discussion, which focuses on comparisons with other studies. We believe this restructuring clarifies the distinction between analyzing our results and situating them in the wider literature.
Page 9: "Overall, the analyses revealed that simple vocalizations were differentially affected by the A53T mutation in male and female mice. For vocalization duration, no significant differences were observed between mutant and wild-type animals in either sex. In contrast, frequency range showed the strongest and most consistent genotype effects: both male and female mutant mice exhibited significantly broader frequency ranges compared to their respective controls……."

12. While detailed, the Discussion section is also longer than expected. Consider better structuring, or using subsections with titles, e.g. "*Comparisons with other studies*",

"*Limitations of this study*", "*Future research directions*", "*Clinical treatment strategies*" (For LSVT and pharmacological approaches".

->Thank you for this helpful suggestion. I have restructured the Discussion section by adding subsections with titles, including "Comparisons with Other Studies," "Limitations of This Study," "Future Research Directions," and "Clinical Treatment Strategies." This improves clarity and readability while retaining the detailed content.

13. Author has mentioned rats several times as part of this study, even though it was stated that the experiments were done in mice: "*In contrast, our study revealed that rats with aggregated alpha-synuclein exhibited higher maximum frequencies in both types of vocalization: simple and complex.*", and "*While our findings revealed significant differences between mutant and wild-type rats in both simple and complex vocalizations, the other study reported fewer changes across acoustic parameters.*". Please clarify and be consistent. This is also where citing the source data is crucial so readers can look at the data themselves.

->We thank the reviewer for this helpful comment and for pointing out the inconsistency. To clarify, all experiments conducted in our study were performed in A53T α-synuclein mutant mice. Mentions of rats in the manuscript refer to previously published studies that we cited for comparison. We have revised the relevant sentences to explicitly distinguish between our mouse findings and rat data from the literature, and we have added citations so readers can directly consult the source data.

Review for Revised Manuscript on Sex Differences in Vocalization in A53T Mice
Final Recommendation: Revise and Resubmit (Minor to Moderate Revisions)

The revised manuscript demonstrates strong improvements in structure and clarity, and the overall flow of the paper is now much more cohesive. However, several formatting, structural, and conceptual issues remain that must be addressed before the paper can be considered for publication.

1. Figures and Visualisation

- Please ensure that all x-axes in your figures include both an axis title and unit. For example, in Figure 2, the x-axis could easily be misinterpreted as representing 1–30 Hz, while I assume you mean 30 kHz, given the known frequency range of mouse ultrasonic vocalisations. Clear axis labeling is essential, especially since your figures appear to plot different metrics (e.g., duration, frequency range).

2. Manuscript Structure

- The Methods section should typically follow the Introduction and precede the Results, unless the journal format explicitly requires it to be placed after the Discussion. Please move the Methods section so that it appears directly after the Introduction.

3. Clarity and Redundancy

- On page 3, the following two sentences appear at the end of consecutive paragraphs and are nearly identical:
  "*These results suggest that overexpression and aggregation of α-synuclein disrupt vocal control circuits, highlighting vocalization as a sensitive biomarker of PD.*"
  "*These results suggest that α-synuclein accumulation disrupts vocal control circuits, highlighting vocalization as a sensitive biomarker of PD.*"
  Considering the similarity in conclusion the two paragraphs can easily be combined into a single, concise paragraph that avoids repetition while still conveying the core point.
- In the Discussion, be cautious not to repeat the exact phrasing from the Results section. The Discussion should summarise and interpret the findings, rather than reiterate them. Consider reviewing phrasing in peer-reviewed literature for examples of how to summarise results effectively without duplication.
- Throughout the manuscript, there is frequent use of phrases like "These findings…" at the beginning of sentences. While this is common, using it

repeatedly can make the text feel monotonous. Look for opportunities to vary sentence structure and improve flow.

4. Discussion: Interpretation of Results

- The Discussion section includes a subheading titled **“**Translational Relevance of Behavioral and Pharmacological Approaches”, but it is not clear what the actual translational implications of your findings are. You mention LSVT and pharmacological interventions, but do not relate them directly to your current results. You also state that you provided a “translational framework”, which I unable to find. Please define what that framework is and explain how your data supports it or remove the claim. As it stands, this section feels disconnected from the rest of the manuscript and should either be revised substantially or removed.
- Most importantly, the sex differences, which are a central part of your results, are not discussed in sufficient depth. You show that effects on frequency range and acoustic power were only observed in female mice, yet this is not explored in the Discussion. Why might this be? Does it align with findings in the human literature on Parkinson’s disease? You reference studies that report similar effects in rodents but stop short of interpreting the biological or clinical meaning of this result. I strongly encourage you to expand this into the focus of your discussion and it would enhance the novelty and significance of your paper.

I have read the revised manuscript, and my recommendation is **acceptance with minor edits.**

My comments: The author has addressed my previous comments. Even though the revised manuscript is now shorter in length, it is more concise and the discussion is done in a structured and focused format.

Suggested edits:

1. For all 3 figures, the x-axis needs a title. The y-axis needs numerical labels.
2. In "4.4 Comparison with other studies", Please write out the full term of PFF in "PFF-injected rats", before using the abbreviation.
3. There are 3 supplementary tables with analysis of p-values, but this is not referred to at all in the text. Consider adding a reference in the text to the supplementary tables.
4. In Supplementary Table 1, for Duration/Simple, the p-value for WT vs mutant is 0.02, yet when separated into male and female groups for analysis, the p-values are 0.18 for both. This suggests that the p-value for WT vs mutant is likely to be larger than 0.02. Please check the p-values again.

Title:

Sex-Specific Vocalization Impairments in A53T α-synuclein mutant Mice

Abstract

Parkinson's Disease is a neurodegenerative disorder that is characterized by both motor and non-motor symptoms, including vocalization deficits. Among the various causes of Parkinson's Disease, a mutation in the α-synuclein gene is a significant contributor. In this paper, we analyze a dataset containing acoustic vocalization measurements from both A53T mutant and wild-type mice, comparing WT vs. A53T within males and within females for simple vocalizations. The dataset included parameters such as duration, maximum power, and frequency ranges of simple vocalizations. Mutant mice did not exhibit significant differences in the duration of simple vocalizations compared to the control group. However, they showed a significantly broader frequency range in simple calls in females, with no measurable difference in males. In addition, female mutant mice exhibited higher maximum acoustic power than female controls, while no differences were observed in males. These findings indicate that the A53T mutation in the α-synuclein gene selectively alters frequency-related aspects of simple vocalizations, with additional effects on vocal intensity that are more pronounced in females. This suggests that vocal communication deficits in this Parkinson's Disease model may be sex-dependent, with frequency range providing the most sensitive marker of genotype-related differences.

Keywords: PD, vocalization, α-synuclein, sex differences

Introduction

Parkinson's disease (PD) is a neurodegenerative disorder of movement that has features of cognitive impairment, autonomic dysfunction, sleep disorders, hyposmia, and depression (Poewe et al., 2017). PD affected a large portion of the population, approximately 6.1 million people worldwide (Bloem et al., 2021). Although the mechanism has not yet been fully understood, the prevalence of the disease has risen rapidly over the past few decades, and the personal impact of the disease is immense. PD includes two types of symptoms: motor and non-motor. Motor symptoms refer to the movement-related symptoms, including slowness of voluntary movements with progressive reduction in speed, muscular rigidity, and postural instability (Hughes et al., 1992).

On the other hand, non-motor symptoms are not related to movements and can appear before motor symptoms do. The non-motor symptoms include a lack of emotional involvement, sleep problems, loss of smell and taste, mood disturbances, fatigue, and pain (Pont-Sunyer et al., 2015). Genetic mutations are among the most significant contributing factors to the various causes of PD. (Bloem et al., 2021.)

Among them, α-synuclein is particularly closely linked to PD through mutations such as A53T, a mutation in the Alanine residue. This mutation is known to be associated with many detrimental symptoms of PD (Choi et al., 2008; Spira et al., 2001). The mouse model of PD with the A53T mutation develops numerous sensorimotor and synaptic impairments, followed by age-associated cognitive and motor deficits (Paumier et al., 2013). Furthermore, A53T α-synuclein in astrocytes contributes to initiating the damaging process that leads to neuron death (Gu et al., 2010).

Because α-synuclein aggregates disrupt dopaminergic and brainstem circuits, they can impair fine motor behaviors such as respiration–phonation coupling and vocal fold control (Rektorová et al., 2012; Ciucci et al., 2007). In humans, this manifests as prosodic flattening, reduced articulatory precision, and vocal fold atrophy, which may also contribute to impaired swallowing (Bocklet et al., 2011; Rektorová et al., 2012; Yiu et al., 2020). Similarly, rodent models show parallel deficits: α-synuclein transgenic mice exhibit early-onset and progressive impairments in ultrasonic vocalization, including reduced call duration, intensity, and altered spectral profiles that often appear by two to three months of age and precede motor decline (Grant et al., 2014; Grant et al., 2015). Collectively, these findings indicate that α-synuclein overexpression and aggregation disrupt vocal control circuits across species, highlighting vocalization as a sensitive biomarker of Parkinson's disease (PD) (Gnerre et al., 2023).

Measures such as call duration, frequency modulation, and vocal intensity in mice are considered counterparts of human PD vocal deficit symptoms. In mice, changes in call duration match prosodic timing deficits in PD speech (Gnerre et al., 2023; Rektorová et al., 2012), reduced frequency modulation represents the monotone voice and pitch variation of patients (Bocklet et al., 2011; Houle et al., 2024), and lower call intensity mirrors hypophonia or reduced loudness, which are symptoms of PD (Grant et al., 2014, 2015; Ramig et al., 2001).

Sex Differences in Parkinson's Disease

Epidemiological studies suggest that sex is a crucial factor in PD development. The disease is approximately twice as common in men as in women (Baldereschi et al., 2000; Solla et al., 2012). Women tend to develop PD about two years later than men, likely due to estrogen-related protective factors such as menopause and childbirth (Haaxma et al., 2007). Women are more

likely to present with tremors as an initial symptom, associated with a slower progression and milder motor course. By contrast, men tend to exhibit more severe motor symptoms, including speech problems, while women experience more non-motor symptoms such as fatigue, depression, and pain (Santos-García et al., 2023). Longitudinal studies confirm these patterns: men show faster decline in both motor and non-motor aspects, requiring higher medication doses and experiencing greater impairment in daily tasks (Picillo et al., 2022). However, other reports suggest that women with PD sometimes report worse disability, quality of life, and greater anxiety despite physicians observing no major symptom differences (Abraham et al., 2019). These findings highlight the complex and sometimes contradictory nature of sex differences in PD progression and symptom burden.

Sex Differences in Vocal Effects

Sex-related differences also extend to vocal impairments in PD. Analysis of speech recordings revealed that while most acoustic measures—such as pitch variability, speech rate, and vowel articulation—are affected by both PD and sex independently, there are sex-specific interactions. For example, females with PD more often produced multiple bursts during plosive consonants, whereas males frequently failed to make a burst, indicating sex-dependent disruptions in articulatory timing and force (Houle et al., 2024). Similarly, Gnerre et al. (2023) showed that emotional expression in speech was impaired in PD patients, particularly women, who demonstrated lower pitch when expressing pleasure and poorer voice quality when expressing fear and anger. Neutral speech, however, showed no sex-related differences. Complementing these findings, Hertrich and Ackermann (1995) reported that women with PD exhibited more irregularities in sustained vowel production, such as shaky pitch and abnormally low sounds, compared to both controls and men with PD. These results suggest that PD affects male and

female voices differently, potentially due to inherent differences in vocal cord structure and function.

In this paper, we investigate whether there are sex differences in vocalization phenotypes in the A53T mouse model of Parkinson's Disease (PD). Specifically, we aim to explore distinct differences between male and female vocal expression, a non-motor symptom often observed in PD. To address this, we analyzed an existing vocalization dataset from A53T mutant mice and wild-type controls, focusing on simple vocalizations. Simple calls are defined as those with a constant, non-modulating frequency (Krasko et al., 2021). Because simple calls rely more heavily on basic motor pathways, they may be particularly vulnerable to early disruptions in PD (Holy & Guo, 2005). Thus, simple calls are especially sensitive to alterations in timing and intensity, paralleling hypophonia and prosodic timing deficits observed in PD.

We hypothesize that A53T mutant mice will exhibit sex-specific alterations in simple vocalizations. Specifically, we predict that females will show greater variability and disruption in acoustic parameters than males, consistent with evidence that sex hormones influence dopaminergic circuits involved in vocal motor control (Gillies & McArthur, 2010; Van Den Eeden et al., 2003; Ciucci et al., 2007).

By testing this hypothesis, we aim to clarify how non-motor symptoms manifest across sex, shedding light on potential mechanisms underlying sex-specific vulnerabilities in PD. While the original dataset provides raw acoustic measures of A53T mutant and wild-type mice, it did not explore how these alterations vary by sex. Our analysis builds on this by uncovering sex-specific patterns in simple vocalizations that suggest distinct neural mechanisms and highlight the potential for more precise biomarkers of Parkinson's disease.

2. Method

The Purdue University Research Repository is the source of this information. This dataset is publicly accessible at: https://purr.purdue.edu/publications/4583/supportingdocs/1. A publicly accessible vocalization dataset from A53T mutant mice and wild-type mice (which served as the control group) was used for our analysis. Our analyses included 7 WT males, 7 GFP+ males, 6 WT females, and 9 GFP+ females. We focused on several key acoustic parameters: Duration, Power Maximum, and frequency range, comparing WT vs. A53T within each sex for simple calls. To evaluate distributional differences between genotypes (WT vs. A53T) within sex for simple calls, we used R to perform two-sample Kolmogorov–Smirnov (KS) tests. The KS test was chosen because it is a non-parametric method that does not assume normality or equal variances and is appropriate for comparing two distributions. Unlike tests that compare only group averages (e.g., t-test or Mann–Whitney U), the KS test evaluates differences across the entire distribution, making it suitable for detecting shifts in both central tendency and variability of acoustic parameters. We were able to determine whether the observed differences were statistically significant by using the KS test. Results with p-values below 0.05 were considered statistically significant.

3. Results

3.1 Vocalization Duration

This study investigated whether there are sex differences in vocalization phenotypes in an animal model of Parkinson's disease. To explore this question, we obtained the vocalization dataset from A53T mutant mice and wild-type mice (the control group). We focused our analysis on simple vocalizations, which are defined as calls with a constant, non-modulating frequency (Krasko et al., 2021). Examining simple vocalizations by sex is important for understanding whether male and female mice exhibit distinct alterations in vocalization duration.

First, we investigated whether there is a sex difference in vocalization duration by analyzing samples from males and females in the simple vocalization test. The cumulative distribution plots showed no significant difference between male wild-type and male mutant animals (Figure 1, left panel; KS test, $p = 0.182$). Similarly, female wild-type and female mutant animals also did not differ significantly in their vocalization duration (Figure 1, right panel; KS test, $p = 0.176$). These results indicate that, when separated by sex, mutant mice do not show altered duration of simple vocalizations compared to wild-type controls.

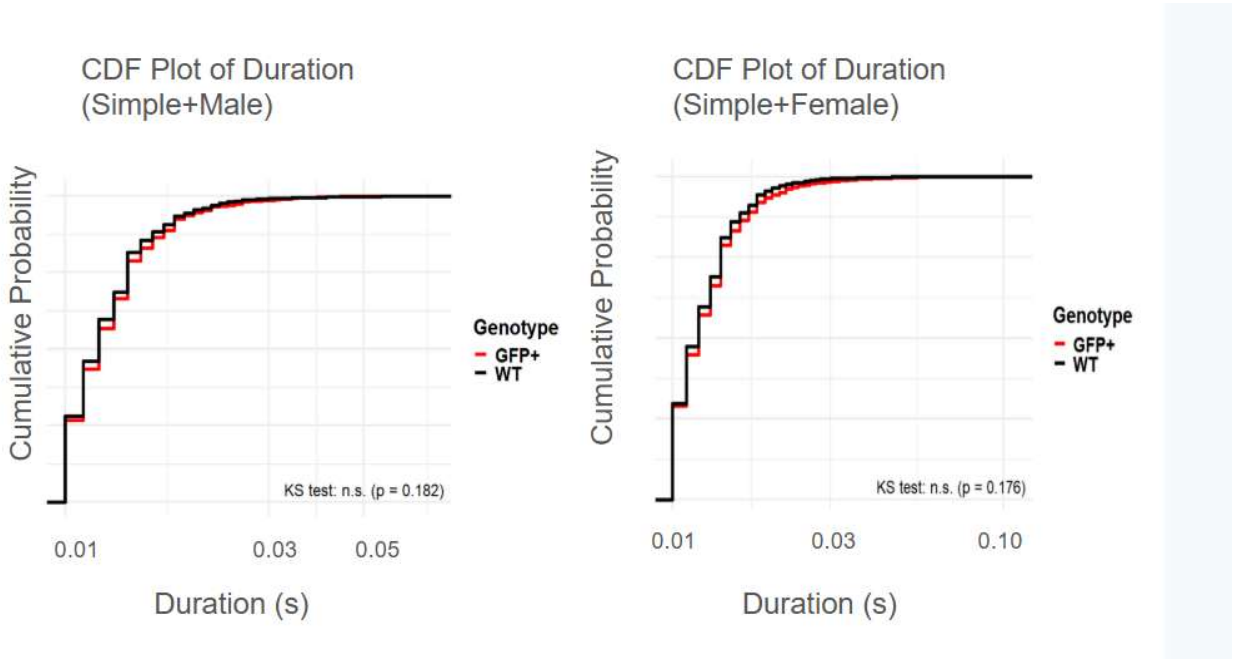

Figure 1. Cumulative distribution plots of vocalization duration (log scale) for wild-type (WT) and mutant mice. The left panel shows simple male vocalizations, and the right panel shows simple female vocalizations.

3.2 Vocalization Frequency Range

Secondly, to determine whether the mutation alters the frequency range of simple vocalizations, we examined male and female mice separately. Male mutant mice did not differ significantly from their wild-type controls (KS test, $p = 0.253$). In contrast, female mutant mice exhibited a significantly broader frequency range compared to female wild-type controls (KS test, $p = 0.0287$) (Figure 2).

Taken together, these findings indicate that the A53T mutation increases the frequency range of simple vocalizations in females, while males show no measurable differences.

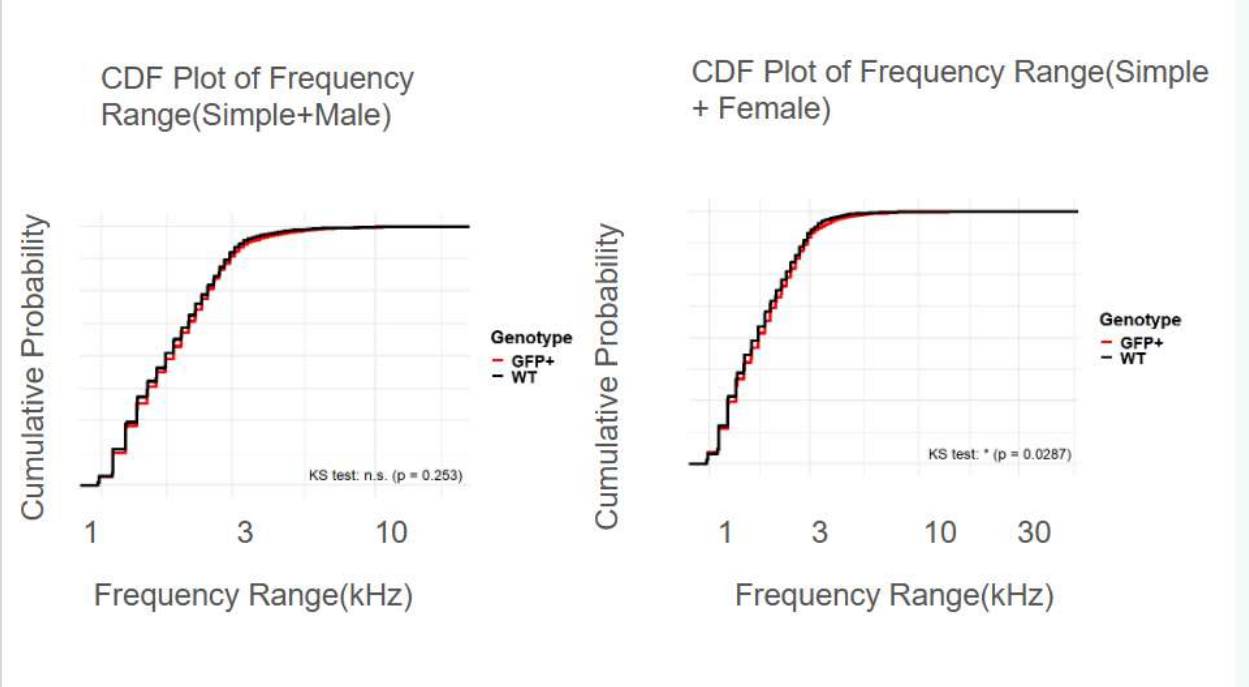

Figure 2. Cumulative distribution plots of vocalization frequency range (log scale) for wild-type (WT) and mutant mice. The left panel shows simple male vocalizations, and the right panel shows simple female vocalizations.

3.3 Maximum Acoustic Power

Thirdly, we analyzed whether the maximum energy level (maximum acoustic power) of vocalizations differs between wild-type and mutant mice. When examining simple vocalizations, male mutant mice did not differ significantly from male controls (Figure 3, left panel; KS test, $p = 0.379$). In contrast, female mutant mice exhibited significantly higher maximum acoustic power compared to female controls (Figure 3, right panel; KS test, $p = 0.017$). These results suggest that the A53T mutation influences vocal intensity primarily in simple vocalizations, with a more pronounced effect in females, while males remain unaffected.

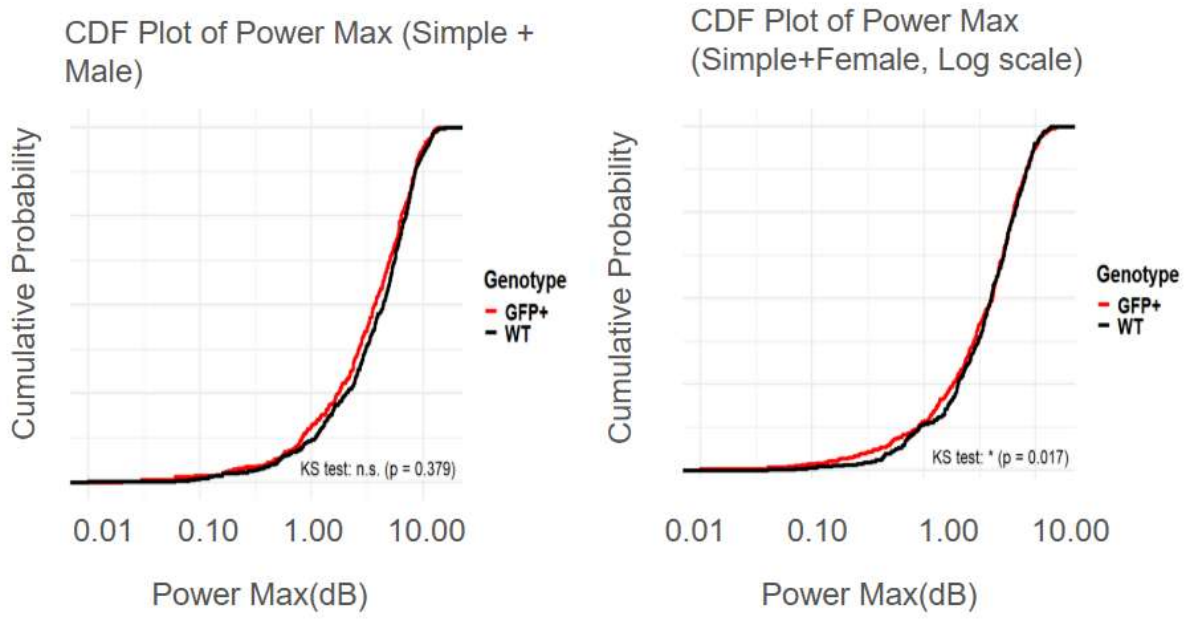

Figure 3. Cumulative distribution plots of maximum acoustic power (log scale) for wild-type (WT) and mutant mice. The left panel shows simple male vocalizations, and the right panel shows simple female vocalizations.

Summary of Results

Overall, the analyses revealed that simple vocalizations were differentially affected by the A53T mutation in male and female mice. For vocalization duration, no significant differences were observed between mutant and wild-type animals in either sex. In contrast, frequency range showed a selective genotype effect: female mutant mice exhibited significantly broader frequency ranges compared to their controls, while males showed no measurable difference. For maximum acoustic power, differences were also sex-specific, with female mutants showing considerably higher values than female controls, while no difference was found in males. Together, these findings suggest that frequency-related measures are the most sensitive markers of genotype differences in females, while vocal intensity changes are likewise more pronounced in females.

4. Discussion

Summary of Key Findings

4.1 Vocalization Duration

Simple vocalizations were examined to assess whether the A53T mutation altered call timing across sexes. Mutant mice did not differ significantly from wild-type controls in the duration of simple calls for either males or females (Figure 1). This indicates that call length is not

measurably influenced by the mutation, suggesting that duration is a relatively stable parameter across genotypes and sexes.

4.2 Frequency Range

Analysis of simple calls revealed a female-specific effect of the A53T mutation. Mutant females exhibited significantly broader frequency ranges compared to WT females, whereas no measurable differences were observed in males. This indicates that sex may modulate the acoustic breadth of simple calls, with frequency range serving as a sensitive marker of genotype-related differences in females.

4.3 Acoustic Power

Maximum acoustic power showed a sex-specific effect of the A53T mutation in simple vocalizations. Female mutants exhibited significantly higher values than WT females, whereas no measurable differences were observed in males. These findings suggest that simple calls are sensitive to mutation-driven changes in vocal intensity, with effects that are more pronounced in females.

4.4 Comparisons with Other Studies

A previous study using Thy1-aSyn rats reported a reduction in the duration, frequency, and intensity of ultrasonic vocalizations, with these abnormalities worsening over time (Grant et al., 2014). In contrast, our analysis of A53T mice revealed a significantly broader frequency range in females, as well as higher maximum power in females, while duration showed no significant differences. These discrepancies likely reflect methodological variations such as species, age, classification criteria, or acoustic analysis approaches. Importantly, unlike the original study,

which emphasized overall impairments, our analysis uncovered female-specific alterations, highlighting that α-synuclein pathology may differentially affect vocal motor circuits across sexes.

Similarly, Gombash et al. (2013) found reduced vocal intensity in rats, but no consistent changes in frequency or duration. In contrast, our analysis of A53T mice revealed a broader frequency range and higher maximum power in females, while duration showed no significant differences. These findings suggest that, although reduced intensity is a common outcome of α-synuclein pathology, additional alterations in frequency may emerge in a female-specific manner.

Paumier et al. (2015) reported that α-synuclein pre-formed fibril (PFF)-injected rats exhibited shorter call durations and reduced vocal intensity. In contrast, our analysis of A53T mice revealed no significant differences in duration but did find a broader frequency range and higher maximum power in females. These differences may be explained by model-specific factors, since PFF injections produce progressive pathology, whereas A53T transgenics exhibit constitutive α-synuclein overexpression.

Together, these findings suggest that although α-synuclein pathology consistently affects vocal intensity, its impact on duration and frequency range may depend on the disease model and sex.

4.5 Implications for Neural Mechanisms

The alterations we observed in simple vocalizations suggest that α-synuclein pathology disrupts neural circuits critical for basic vocal motor control. Simple ultrasonic calls are thought to be mediated by brainstem and basal ganglia pathways, with dopaminergic modulation playing a key role in shaping frequency and intensity (Ciucci et al., 2007; Tschida et al., 2019). The broader frequency ranges in females and increased vocal intensity in females may reflect dysfunction in

basal ganglia–brainstem circuits, alongside sex-dependent modulation of dopaminergic pathways. These findings highlight the basal ganglia as a central locus of vulnerability in PD-related vocal deficits and suggest that sex-specific factors, such as hormonal influences, may amplify circuit dysfunction.

Sex differences in dopaminergic function and hormonal modulation may underlie the female-specific effects observed. Estrogen and related factors influence dopamine signaling within basal ganglia circuits, and disruption of these interactions by α-synuclein overexpression could exaggerate alterations in vocal frequency and intensity in females. Human studies report similar patterns: although women generally show a lower incidence and slower motor progression of Parkinson's disease, they often present with distinct non-motor symptoms, including differences in speech prosody and vocal control (Baldereschi et al., 2000; Haaxma et al., 2007; Solla et al., 2012; Abraham et al., 2019; Picillo et al., 2022; Santos-García et al., 2023). These parallels suggest that sex hormones and dopaminergic tone jointly modulate the vulnerability of vocal circuits, offering a biological explanation for the female-specific alterations identified in A53T mice.

These findings underscore the scientific relevance of vocalization analysis in PD models, as changes in call structure may serve as behavioral readouts of underlying neural dysfunction. Because vocal production depends on the precise motor control of respiration and laryngeal muscles, as well as cognitive sequencing of call patterns, disruptions in vocalizations can reflect both motor and cognitive impairments characteristic of PD (Rektorová et al., 2012; Grant et al., 2014; Ciucci et al., 2007). This highlights the potential of vocalization metrics as non-invasive biomarkers for early detection and monitoring of disease progression (Bocklet et al., 2011; Grant et al., 2015).

4.6 Translational Relevance of Behavioral and Pharmacological Approaches

Our findings reveal female-specific alterations in frequency range and acoustic power in A53T mice, suggesting that α-synuclein pathology interacts with sex-dependent dopaminergic modulation of vocal control circuits. These results form the basis of a translational framework that links preclinical vocal biomarkers to clinical interventions targeting the same neural pathways. The increased vocal power observed in female A53T mice may reflect compensatory activation within dopaminergic circuits of the basal ganglia and brainstem—pathways that are similarly targeted by behavioral therapies in humans. This correspondence between preclinical and clinical patterns supports the use of vocalization metrics as a bridge for evaluating circuit-level effects of treatment interventi

Human studies have demonstrated the effectiveness of the Lee Silverman Voice Treatment (LSVT) in improving vocal deficits in Parkinson's disease. LSVT enhances vocal loudness, articulation, and clarity through high-effort vocal exercises, with improvements reported in vocal intensity, articulation, and perceptual speech quality (Ramig et al., 2001; Silveira & Brasolotto, 2005; Searl et al., 2011; Cannito et al., 2012; Sapir et al., 2007). Pharmacological treatments such as levodopa, in contrast, have shown mixed effects on vocal outcomes. Some studies have reported limited improvements in vocal quality (De Letter et al., 2006; Kelm-Nelson et al., 2016), while others have observed increased loudness and speech speed, but without sustained benefits (Ho et al., 2008). These findings suggest that combining pharmacological treatments with behavioral therapies, such as LSVT, may offer stronger outcomes than medication alone.

Testing such strategies in animal models could therefore clarify underlying mechanisms and therapeutic potential. By connecting the variability observed in human interventions with the patterns identified in our animal vocalization data, we provide a translational framework that highlights how preclinical studies can inform combined treatment approaches for Parkinson's vocal deficits.

4.7 Limitations of This Study

Two limitations should be noted. First, the cross-sectional design prevents conclusions about the progression of vocal impairments over time. Second, the hormonal cycle in females was not controlled, which may influence vocalization parameters such as frequency or intensity (Geyer & Barfield, 1978). Some observed differences could therefore be partially attributable to hormone-related variability.

4.8 Future Research Directions

Future work should employ longitudinal designs to track vocal symptoms over time and correlate them with neurodegenerative changes. Such studies could identify early markers of disease progression and clarify how vocal deficits develop over time with age.

Additionally, targeted neurobiological investigations are needed to map the specific brain regions controlling simple vocalizations and to determine how A53T alters these circuits. Integrating vocal-behavior studies with therapeutic interventions, including both behavioral and pharmacological approaches, could accelerate the development of non-invasive biomarkers and treatment strategies for Parkinson’s disease.

References:

Abraham, D. S., Gruber-Baldini, A. L., Magder, L. S., McArdle, P. F., Tom, S. E., Barr, E., Schrader, K., & Shulman, L. M. (2019). Sex differences in Parkinson's disease presentation and progression. *Parkinsonism & Related Disorders*, *69*, 48–54.

Baldereschi, M., Di Carlo, A., Rocca, W. A., Vanni, P., Maggi, S., Perissinotto, E., Grigoletto, F., Amaducci, L., & Inzitari, D. (2000). Parkinson's disease and parkinsonism in a longitudinal study: Two-fold higher incidence in men. *Neurology*, *55*(9), 1358–1363.

Bloem, B. R., Okun, M. S., & Klein, C. (2021). Parkinson's disease. *The Lancet*, *397*(10291), 2284–2303.

Bocklet, T., Nöth, E., Stemmer, G., Ruzickova, H., & Rusz, J. (2011). *Detection of persons with Parkinson's disease by acoustic, vocal, and prosodic analysis*. 478–483.

Choi, J. M., Woo, M. S., Ma, H.-I., Kang, S. Y., Sung, Y.-H., Yong, S. W., Chung, S. J., Kim, J.-S., Shin, H., & Lyoo, C. H. (2008). Analysis of PARK genes in a Korean cohort of early-onset Parkinson disease. *Neurogenetics*, *9*, 263–269.

Ciucci, M. R., Ma, S. T., Fox, C., Kane, J. R., Ramig, L. O., & Schallert, T. (2007). Qualitative changes in ultrasonic vocalization in rats after unilateral dopamine depletion or haloperidol: A preliminary study. *Behavioural Brain Research*, *182*(2), 284–289.

Elkouzi, A., Vedam-Mai, V., Eisinger, R. S., & Okun, M. S. (2019). Emerging therapies in Parkinson disease—Repurposed drugs and new approaches. *Nature Reviews Neurology*, *15*(4), 204–223.

Gillies, G. E., & McArthur, S. (2010). Estrogen actions in the brain and the basis for differential action in men and women: A case for sex-specific medicines. *Pharmacological Reviews*,

62(2), 155–198.

Gnerre, M., Malaspina, E., Di Tella, S., Anzuino, I., Baglio, F., Silveri, M. C., & Biassoni, F. (2023). Vocal emotional expression in Parkinson's disease: Roles of sex and emotions. *Societies*, *13*(7), 157.

Grant, L. M., Kelm-Nelson, C. A., Hilby, B. L., Blue, K. V., Paul Rajamanickam, E. S., Pultorak, J. D., Fleming, S. M., & Ciucci, M. R. (2015). Evidence for early and progressive ultrasonic vocalization and oromotor deficits in a PINK1 gene knockout rat model of Parkinson's disease. *Journal of Neuroscience Research*, *93*(11), 1713–1727.

Grant, L. M., Richter, F., Miller, J. E., White, S. A., Fox, C. M., Zhu, C., Chesselet, M.-F., & Ciucci, M. R. (2014). Vocalization deficits in mice over-expressing alpha-synuclein, a model of pre-manifest Parkinson's disease. *Behavioral Neuroscience*, *128*(2), 110.

Gu, X.-L., Long, C.-X., Sun, L., Xie, C., Lin, X., & Cai, H. (2010). Astrocytic expression of Parkinson's disease-related A53T α-synuclein causes neurodegeneration in mice. *Molecular Brain*, *3*, 1–16.

Haaxma, C. A., Bloem, B. R., Borm, G. F., Oyen, W. J., Leenders, K. L., Eshuis, S., Booij, J., Dluzen, D. E., & Horstink, M. W. (2007). Gender differences in Parkinson's disease. *Journal of Neurology, Neurosurgery & Psychiatry*, *78*(8), 819–824.

Hertrich, I., & Ackermann, H. (1995). Gender-specific vocal dysfunctions in Parkinson's disease: Electroglottographic and acoustic analyses. *Annals of Otology, Rhinology & Laryngology*, *104*(3), 197–202.

Holy, T. E., & Guo, Z. (2005). Ultrasonic songs of male mice. *PLoS Biology*, *3*(12), e386.

Houle, N., Feaster, T., Mira, A., Meeks, K., & Stepp, C. E. (2024). Sex Differences in the Speech of Persons with and without Parkinson's Disease. *American Journal of Speech-Language Pathology*, *33*(1), 96–116.

Hughes, A. J., Daniel, S. E., Kilford, L., & Lees, A. J. (1992). Accuracy of clinical diagnosis of idiopathic Parkinson's disease: A clinico-pathological study of 100 cases. *Journal of*

Neurology, Neurosurgery & Psychiatry, *55*(3), 181–184.

Krasko, M. N., Hoffmeister, J. D., Schaen-Heacock, N. E., Welsch, J. M., Kelm-Nelson, C. A., & Ciucci, M. R. (2021). Rat models of vocal deficits in Parkinson's disease. *Brain Sciences*, *11*(7), 925.

Lavedan, C. (1998). The synuclein family. *Genome Research*, *8*(9), 871–880.

Li, W., Lesuisse, C., Xu, Y., Troncoso, J. C., Price, D. L., & Lee, M. K. (2004). Stabilization of α-synuclein protein with aging and familial Parkinson's disease-linked A53T mutation. *Journal of Neuroscience*, *24*(33), 7400–7409.

Matsumine, H., Saito, M., Shimoda-Matsubayashi, S., Tanaka, H., Ishikawa, A., Nakagawa-Hattori, Y., Yokochi, M., Kobayashi, T., Igarashi, S., & Takano, H. (1997). Localization of a gene for an autosomal recessive form of juvenile Parkinsonism to chromosome 6q25. 2-27. *American Journal of Human Genetics*, *60*(3), 588.

Middleton, F. A., & Strick, P. L. (2000). Basal ganglia and cerebellar loops: Motor and cognitive circuits. *Brain Research Reviews*, *31*(2–3), 236–250.

Nestor, K., Rasero, J., Betzel, R., Gianaros, P. J., & Verstynen, T. (2024). Cortical network reconfiguration aligns with shifts of basal ganglia and cerebellar influence. *ArXiv*, arXiv-2408.

Paumier, K. L., Sukoff Rizzo, S. J., Berger, Z., Chen, Y., Gonzales, C., Kaftan, E., Li, L., Lotarski, S., Monaghan, M., & Shen, W. (2013). Behavioral characterization of A53T mice reveals early and late stage deficits related to Parkinson's disease. *PloS One*, *8*(8), e70274.

Picillo, M., LaFontant, D.-E., Bressman, S., Caspell-Garcia, C., Coffey, C., Cho, H. R., Burghardt, E. L., Dahodwala, N., Saunders-Pullman, R., & Tanner, C. M. (2022). Sex-related longitudinal change of motor, non-motor, and biological features in early Parkinson's disease. *Journal of Parkinson's Disease*, *12*(1), 421–436.

Poewe, W., Seppi, K., Tanner, C. M., Halliday, G. M., Brundin, P., Volkmann, J., Schrag, A.-E., &

Lang, A. E. (2017). Parkinson disease. *Nature Reviews Disease Primers*, *3*(1), 1–21.

Pont-Sunyer, C., Hotter, A., Gaig, C., Seppi, K., Compta, Y., Katzenschlager, R., Mas, N., Hofeneder, D., Brücke, T., & Bayés, A. (2015). The O nset of N onmotor S ymptoms in P arkinson's disease (T he ONSET PD S tudy). *Movement Disorders*, *30*(2), 229–237.

Ramig, L. O., Sapir, S., Countryman, S., Pawlas, A. A., O'Brien, C., Hoehn, M., & Thompson, L. L. (2001). Intensive voice treatment (LSVT®) for patients with Parkinson's disease: A 2 year follow up. *Journal of Neurology, Neurosurgery & Psychiatry*, *71*(4), 493–498.

Rektorová, I., Mikl, M., Barrett, J., Marecek, R., Rektor, I., & Paus, T. (2012). Functional neuroanatomy of vocalization in patients with Parkinson's disease. *Journal of the Neurological Sciences*, *313*(1–2), 7–12.

Santos-García, D., Laguna, A., Hernández-Vara, J., de Deus Fonticoba, T., Cores Bartolomé, C., Feal Painceiras, M. J., Íñiguez-Alvarado, M. C., García Díaz, I., Jesús, S., & Boungiorno, M. T. (2023). Sex differences in motor and non-motor symptoms among Spanish patients with Parkinson's disease. *Journal of Clinical Medicine*, *12*(4), 1329.

Solla, P., Cannas, A., Ibba, F. C., Loi, F., Corona, M., Orofino, G., Marrosu, M. G., & Marrosu, F. (2012). Gender differences in motor and non-motor symptoms among Sardinian patients with Parkinson's disease. *Journal of the Neurological Sciences*, *323*(1–2), 33–39.

Spira, P. J., Sharpe, D. M., Halliday, G., Cavanagh, J., & Nicholson, G. A. (2001). Clinical and pathological features of a Parkinsonian syndrome in a family with an Ala53Thr α-synuclein mutation. *Annals of Neurology*, *49*(3), 313–319.

Taguchi, K., Watanabe, Y., Tsujimura, A., & Tanaka, M. (2016). Brain region-dependent differential expression of alpha-synuclein. *Journal of Comparative Neurology*, *524*(6), 1236–1258.

Van Den Eeden, S. K., Tanner, C. M., Bernstein, A. L., Fross, R. D., Leimpeter, A., Bloch, D. A., & Nelson, L. M. (2003). Incidence of Parkinson's disease: Variation by age, gender, and race/ethnicity. *American Journal of Epidemiology*, *157*(11), 1015–1022.

Yiu, Y., Curtis, J. A., Perry, S. E., & Troche, M. S. (2020). Relationship of vocal fold atrophy to swallowing safety and cough function in Parkinson's disease. *The Laryngoscope*, *130*(2), 303–308.

Zhang, W., Wang, T., Pei, Z., Miller, D. S., Wu, X., Block, M. L., Wilson, B., Zhang, W., Zhou, Y., & Hong, J.-S. (2005). Aggregated α-synuclein activates microglia: A process leading to disease progression in Parkinson's disease. *The FASEB Journal*, *19*(6), 533–542.

Supplementary Table 1. Duration comparisons (p-values)

Condition	WT vs mutant	Male WT vs mutant	Female WT vs mutant
Duration / Simple	0.02	0.18	0.18
Duration / Complex	0.04	0.09	0.13

Supplementary Table 1. Comparison of vocalization duration (p-values) between wild-type (WT) and mutant mice. Results are shown separately for simple and complex calls, and further divided by sex.

Supplementary Table 2. Frequency comparisons (p-values)

Condition	WT vs mutant	Male WT vs mutant	Female WT vs mutant
Frequency / Simple	0.01	0.25	0.03
Frequency / Complex	0.35	0.58	0.40

Supplementary Table 2. Frequency comparisons (p-values) between wild-type (WT) and mutant mice. Results are shown separately for simple and complex vocalizations, and further divided by sex.

Supplementary Table 3. Power_max comparisons (p-values)

Condition	WT vs mutant	Male WT vs mutant	Female WT vs mutant
Power_max / Simple	0.03	0.38	0.02
Power_max / Complex	0.21	0.23	0.21

Supplementary Table 3. Power comparisons (p-values) between wild-type (WT) and mutant mice. Results are shown separately for simple and complex vocalizations, and further divided by sex.