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Exercise Effects on Early Vocal Ultrasonic Communication Dysfunction in a *PINK1* Knockout Model of Parkinson's Disease

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Abstract

Background—Parkinson's disease (PD) is a complex neurodegenerative disease with vocal communication deficits that manifest early, progress, and are largely resistant to medical interventions; however, they do respond to exercise-based speech and voice therapies.

Objective and Methods—To study how exercise-based vocal treatment can affect the progression of communication deficits related to PD, we studied ultrasonic vocalizations (USVs) in rats with homozygous knockout ($-/-$) of *PINK1*, a gene mutation known to cause PD, under the manipulation of a behavioral vocal exercise paradigm that allows us to precisely control dose and timing of exercise in the prodromal (prior to diagnosis) stages.

Results—We show that intensive vocal-training rescues frequency range and intensity deficits as well as leads to an increase in call complexity and duration of calls compared to sham-training; however, over time this training regime loses significant effect as the disease progresses. We also show effects of frequent handling and conspecific (male-female) interaction in the sham-training group as they demonstrated significantly higher call rate, intensity, frequency range, and call complexity compared to rats without any form of training and consequently less handling/interaction. Further, we confirm that this model exhibits progressive gross motor deficits that indicate neurodegeneration.

Discussion—This study suggests that the evolving nature of vocal communication deficits requires an adjustment of therapy targets and more intensive training over the course of this progressive disease and demonstrates the importance of frequent social experiences.

Keywords

Parkinson's disease; *PINK1*; ultrasonic vocalization; cranial sensorimotor; exercise; rat; communication

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INTRODUCTION

Communication deficits in Parkinson's disease (PD) are common, and as the disease progresses, up to 90% of individuals have significant voice and speech deficits [1] that have a negative impact on social interactions and quality of life [2–6]. These evolving vocal communication deficits often manifest early, prior to hallmark basal ganglia dopamine depletion, and can be difficult to treat because they do not benefit from standard medical therapies such as levodopa or deep brain stimulation [3, 7–9]. However, communication deficits respond to some degree to exercise-based speech and voice intervention [10–14]. Although interventions show promise, they are not optimized due to our incomplete knowledge of the full mechanisms of disease pathology and the heterogeneous nature of PD in human treatment interventions such as dose, timing, and intensity.

Animal models of disease are useful to study the onset, disease progression over time, and effects of behavioral treatment in a controlled experimental setting. Moreover, rodent ultrasonic vocalization models are useful for studying the relationships between vocal communication deficits and disease mechanisms. Rodents produce ultrasonic vocalizations (USVs) that are communicative in nature [15–17], vulnerable to dopamine-depletion [18], and exhibit genetic Parkinsonian phenotypes [19, 20]. Specifically, in the rat, unilateral degeneration of nigrostriatal dopamine induced by the neurotoxin 6-hydroxydopamine (6-OHDA) leads to degradation of the bandwidth and peak amplitude of 50-kHz frequency modulated calls [18]. Additionally, targeted-sensorimotor training (vocal exercise-training) in this rat model increases the number of calls and acoustic integrity during training sessions [21]. However, the 6-OHDA lesion model only parallels significant dopamine loss in the mid-late stages of PD.

To address early and progressive vocalization dysfunction, prior to significant nigrostriatal dopamine loss, we used a recently characterized genetic rat model of PD, *PINK1* knockout (*PINK1* $-/-$) [22, 23]. Genetic rodent models of PD are ideal to identify behavioral deficits and pathology as well as evaluate courses of treatment. Rats in this specific genetic model express a form of PD that is due to the complete knockout of the *PINK1* gene, loss of function mutation, which is related to the PARK6 phenotype of human familial PD. In humans, *PINK1* genetic variants contribute to early onset of PD with progressive deficits over time, extensive nigrostriatal dopamine depletion in the late stages of the disease, as well as other motor and non-motor deficits [24–26]. *PINK1* $-/-$ rats have comparable symptom profiles to sporadic PD patients; specifically, this model exhibits significant vocalization deficits beginning at 2 months (mo) of age (decreased vocal loudness) that persist and progress (decreased peak frequency and bandwidth) at 4, 6 and 8 mo of age [20]. Recent data from our laboratory demonstrate that female rat conspecifics respond differently to the *PINK1* $-/-$ calls as compared to non-affected wildtype control calls, indicative of a communication impairment [27]. Moreover, recent characterization of this model demonstrates motor impairment and moderate nigrostriatal dopamine loss at 8 mo of age that mimics the progression of the disease in humans [20, 22]. Thus, this progressive genetic model is useful to examine the effects of vocal exercise training in the early stages of the disease (prior to 8 months of age).

Social experience has distinct effects on a variety of behavioral outcomes. In PD, patients benefit from an enriched environment. For example, music-based movement therapy benefits gait and balance deficits [28, 29]. A larger social network and a socially active lifestyle are positively associated with higher cognitive function in patients with neurodegenerative diseases [30, 31]. Additionally, group occupational therapy has been shown to improve physical symptoms and quality of life compared to patients with PD without group therapy [32]. Furthermore, voice deficits in PD patients are significantly improved in social conditions such as motivational interviewing [33].

In rats, the effects of enriched social experiences have been well documented. Enriched environments impact a variety of biological processes including behavioral, cellular, and molecular genetic processes (reviewed in [34]). For example, daily handling can have a significant impact not only on behavior, but also on brain morphology [35, 36] and has been shown to be neuroprotective [37]; however, these effects have not been studied in a genetic rat model of PD.

To evaluate changes in USVs with vocal exercise, we performed a behavioral study on the *PINK1* $-/-$ rat model and compared acoustic parameters of vocal exercise-trained rats and sham-exercise-trained as well as a control group (received no manipulation). Changes in 50-kHz frequency modulated vocalizations' acoustic parameters (duration, bandwidth, peak amplitude (intensity), and peak frequency) as well as call rate and percent complex calls were assessed before and after training in both exercise groups. Because social enrichment can affect a variety of behavioral and brain functions, we also compared vocal exercise- and sham-exercise-trained groups to a separate group of 8 mo old *PINK1* $-/-$ rats that did not receive any kind of behavioral training. Additionally, we assayed the exercise training groups for other gross motor behaviors to verify the progression of motor deficits in this PD rat model. We hypothesized that vocal exercise training would improve deficits in acoustic parameters over the course of the longitudinal study compared to sham-trained control animals. Due to the influence of conspecific social interaction, we also predicted that the social aspect of the training paradigm (vocalization and sham) would lead to improved communication compared to control animals that did not receive any training. Additionally, we hypothesized that there would be significant declines in gross motor function over time, as gross motor function was not a target of therapy.

MATERIAL AND METHODS

Animals and habituation

Twelve male Long-Evans rats with a homozygous knockout of *PINK1* (SAGETM Research Labs, Boyertown, PA, USA[38]), aged 5 weeks at the time of pre-study habituation, and female wildtype stimulus rats ($n = 6$) were housed in same-sex groups of two in standard polycarbonate cages with sawdust bedding on a reversed 12:12 hour light: dark cycle. Male *PINK1* $-/-$ rats were randomly divided into two groups, vocal exercise-trained and sham-trained. An additional 10 *PINK1* $-/-$ rats were housed in groups of two and used as training controls for USV analysis at 8 mo of age. All testing occurred during the dark period of the cycle using partial red illumination. Animals were handled each day for 7 days prior to the commencement of experiments. Food was available ad libitum. Water was restricted as

described below for the training animals (vocal exercise, sham exercise only), and all animals were sexually experienced per protocol [39]. All procedures were approved by the University of Wisconsin-Madison Animal Care and Use Committee (IACUC) and were conducted in accordance with the United States Public Health Service Guide for the Care and Use of Laboratory Animals.

Behavioral assays and testing

USV and sham exercise—After sexual experiencing, male rats in the vocal exercise- and sham-training groups were placed on a water restriction paradigm (water was restricted except for 3 hours per day after testing) [40]. This water restriction protocol allows access to water for hydration and comfort and maximizes training compliance with water as a reward. Animal weight was monitored and recorded twice weekly. During training, each male rat was placed alone in his home cage underneath the microphone (see below for specifications). A sexually receptive female stimulus rat was placed in the chamber with the male; the female rat was removed from the cage when the male showed signs of interest (mounting, chasing). For vocal exercise-trained animals, when the male rat produced a 50-kHz frequency modulated ultrasonic call, a pen-click sound followed by 2 sec of a water reward was delivered. Using the classic method of reinforcing successive approximation, the click followed by water was used to reinforce calls of increasing complexity and loudness on a variable ratio 5 schedule of reinforcement [18]. Sham-exercised animals were reinforced with a water reward in a similar manner described above for going to a specific site in their home cage after brief encounter with the female as described above. Animals were vocal exercised or sham-exercised five times a week (approximately 20 reinforcements per day for each group) for 6 mo total (age 2 to 8 mo).

USV testing—USVs were recorded in a sound-isolated room and analyzed from male rats in the vocal and sham exercise groups at 2 (baseline), 4, 6, and 8 mo of age (corresponding training timepoints: baseline, 2 mo, 4 mo, 6 mo). At 2.5 mo of age, or after 2 weeks of training, only 8 animals were assayed as pilot data for studying immediate effects of intervention. Additionally, social control animals were tested at 8 mo. An ultrasonic microphone with high directional properties for recording 50-kHz frequency modulated USVs (CM16, Avisoft, Germany; flat frequency response of up to 150-kHz; working frequency response range of 10–180-kHz) was used to record vocalizations. The subject male rat was placed in his home cage with the microphone, and calls were elicited by pairing with a receptive female as described above. Vocalizations were recorded for 90 sec with the male rat alone in the home cage to ensure analyses of only male USVs.

Offline acoustic analysis was performed by building spectrograms from each waveform with Fast Fourier Transform (FFT) of 512 points, frame size of 100%, flat top window, and temporal resolution of 75% overlap. A high pass filter was used to reduce noise below 25-kHz. Calls were slowed down by a factor of 10 in order to listen and categorize the call type. Two raters, masked to condition, categorized each call (simple, frequency-modulated, harmonic) [18, 21, 41]. Specifically, call types were used to determine call rate (calls/sec) and call profile (% complex, the ratio of simple to complex calls). Acoustic analyses were performed on 50-kHz vocalizations using SASLab Pro (Avisoft, Germany) including

bandwidth (Hertz-Hz), peak frequency (kHz), intensity (decibel-dB), and duration (milliseconds-ms). In order to gauge the effects on overall performance during the testing session, the average of all calls (overall performance) as well as the average of the top 10 performances (subset of trials) and maximum (best) performance were calculated for duration, intensity, bandwidth, and peak frequency variables. This approach was determined because PD often affects average performance greater than maximal performance [42]. Therefore, the average values represent the overall performance during a testing session and maximum values represent the “best” performance [39].

Catalepsy: general limb motor impairment—The behavioral cataleptic test measures the time the rat takes to disengage its forelimbs from a horizontal bar. All trained rats were placed alone in a standard cage with a 1 cm diameter, fixed stable bar that ran parallel to the testing surface. The cataleptic (time to disengage) descent time, duration between initial forelimb contact and return to the testing surface (sec) for three trials was analyzed at 6 and 8 mo of age [43–45].

Spontaneous movement: forelimb and hindlimb use—All trained rats were tested for forelimb and hindlimb use at 6 and 8 mo of age. Rats were placed in an upright translucent acrylic cylinder, measuring 30 cm high and 20 cm in diameter, to encourage rearing and vertical exploration with the forepaws (similar to [46]). Two masked raters analyzed video recordings of the task, and the number of forelimb- and hindlimb-contacts was totaled over a 2 min period.

Challenging beam: motor limb function—At 6 and 8 mo of age, trained rats were assayed for gross and fine motor limb function as each rat traversed a 165 cm long ledged, tapered beam (Lafayette Instrument Company, Lafayette, IN) [46]. The last 1/3 of the challenge beam has a tapered, reduced diameter that increases task complexity. After, acclimation to the testing procedures, each rat was placed on a loading platform and allowed to traverse the beam toward his homepage for a total of 5 trials. Masked raters then reviewed video footage and assessed the time to traverse the beam (sec), time to traverse the final third of the beam (sec), and the total number of foot faults.

Statistical analysis

All statistical analyses were conducted with SigmaPlot® 12.5 (Systat Software, Inc., San Jose, CA). Due to small sample sizes and pilot nature of the work, the critical level for significance was set at 0.10 for all testing. Explicitly, comparisons between exercise treatment groups (exercise and sham) were analyzed for the average, average of the top 10, and average maximum for: bandwidth, peak frequency, intensity, and duration as well as call rate and % complex calls across timepoints. Additionally, we compared for gross motor deficits, both as distinct training groups and with their data collapsed into one *PINK1* group. Due to the exploratory nature of the experiments and the associated statistical type II error, no corrections were made for multiple comparisons. Additionally, as to be transparent, we have provided all of the data.

In order to study the effects of vocal exercise training over time, an Analysis of Variance (ANOVA) examined interactions and main effects. Specifically, a mixed-model repeated measures 2×5 ANOVA for the two categorical independent group variables (exercise-trained, sham-trained) was performed for: (A) acoustic and call variables (bandwidth, peak frequency, intensity, duration, call rate, and % complex calls), and (B) each of the gross motor dependent variables (catalepsy, cylinder contacts, challenging beam traversal) over time (5 levels within groups: baseline, 2 weeks, 2 mo, 4 mo, 8 mo) and *post-hoc* analysis was performed with a conservative Fisher's Least Significant Differences (LSD) test. Additionally, we performed a priori planned comparisons (independent student's *t*-tests) between treatment groups and USV measures at 2 weeks because this was an exploratory timepoint with a limited sample size. Furthermore, to assess gross motor function in the *PINK1* genotype across time, independent student's *t*-tests were used to assess changes when treatment groups were collapsed (catalepsy, hindlimb and forelimb movements) as well as ANOVA for challenging beam variables (time to traverse, foot faults, and time to traverse the last 1/3). At the 8 mo timepoint a one-way ANOVA was used to compare acoustic and call variables (bandwidth, peak frequency, intensity, duration, call rate, and % complex calls) between exercise-trained, sham-trained and control *PINK1* $-/-$ groups. If necessary, data variables were transformed (rank transformed) if data failed to conform to assumptions, normality (using the Shapiro-Wilk test) and equal variance. *Post-hoc* analysis was performed with Fisher's LSD.

Inter and intra-rater reliability was calculated for 10% of the randomly sampled acoustic and motor data using the intra-class correlation coefficient (ICC) on both training groups; a correlation coefficient of 0.90 or greater was considered reliable.

RESULTS

Ultrasonic vocalizations

Average of 50-kHz frequency modulated calls—Average USV data are displayed in Table 1; data comparisons between vocal exercise and sham-exercise groups.

Duration: There was no significant interaction between group and timepoint ($F(4, 34) = 0.90, p = 0.475$). There was significant main effect of timepoint ($F(4, 34) = 4.30, p = 0.006$). Specially, post-hoc comparisons between training groups demonstrated differences between baseline and 2 weeks of training ($p < 0.001$) as well as baseline to 2 mo of training ($p = 0.001$) (data not displayed). At the 2-week and 2 mo timepoint, duration was increased compared to baseline. There was no main effect for group ($F(1, 34) = 0.0023, p = 0.96$).

Bandwidth: There was no significant interaction between group and timepoint ($F(4, 34) = 0.86, p = 0.50$). There was a significant main effect of timepoint ($F(1, 34) = 5.206, p = 0.002$); there were differences between baseline and 2 weeks of training ($p < 0.001$) as well as baseline and 2 mo ($p = 0.002$) (data not displayed). At the 2-week and 2 mo timepoint, call bandwidth was increased compared to baseline. There was no main effect for group ($F(1, 34) = 2.47, p = 0.14$).

Intensity: There was no significant interaction for average intensity ($F(1, 34) = 1.02, p = 0.41$), main effects between treatment groups ($F(1, 34) = 0.92, p = 0.36$), or over time ($F(4, 34) = 1.35, p = 0.27$).

Peak Frequency: There was no significant interaction for average peak frequency ($F(4, 34) = 0.29, p = 0.88$), main effects between treatment groups ($F(1, 34) = 0.08, p = 0.78$), or over time ($F(4, 34) = 0.702, p = 0.60$).

Average of the top 10 50-kHz frequency modulated calls—Average of the top 10 calls USV data are displayed in Table 2; data comparisons between vocal exercise and sham-exercise groups.

Duration: There were no significant group $\times$ timepoint interactions ($F(4, 35) = 0.49, p = 0.75$). There was a significant main effect of timepoint ($F(1, 35) = 3.91, p = 0.01$). Specifically, there were differences between baseline and 2 weeks ($p = 0.012$), 2 mo ($p = 0.003$), 4 mo ($p = 0.005$), and 6 mo ($p = 0.022$), respectively (Fig. 1A). All testing timepoints had increased duration of calls compared to baseline. There was no main effect for group ($F(1, 35) = 0.24, p = 0.63$).

Bandwidth: There were no significant group $\times$ timepoint interactions ($F(4, 35) = 1.12, p = 0.36$). There was a significant main effect of timepoint ($F(1, 35) = 6.79, p < 0.001$). Specifically, there were significant differences between baseline and 2 weeks ($p < 0.001$), 2 mo ($p = 0.002$), 4 mo ($p < 0.001$), and 6 mo ($p = 0.009$) for trained animals. All testing timepoints had increased bandwidth of calls compared to baseline. There was no significant main effect of group ($F(1, 35) = 2.60, p = 0.13$). However, when comparing training groups after 2 weeks of training, data show vocal exercised-trained rats had significantly increased average of the top 10 calls for bandwidth ($t(6) = 2.96, p = 0.012$; Fig. 1B) compared to sham-trained rats.

Intensity: There were no significant group $\times$ timepoint interactions ($F(4, 35) = 0.27, p = 0.90$) (Fig. 2). There was an effect of timepoint ($F(1, 35) = 4.57, p = 0.005$). Specifically, there was a significant increase in intensity between baseline and 4 mo ($p = 0.001$) and 4 mo and 6 mo ($p = 0.007$). There was no main effect of group ($F(1, 35) = 0.89, p = 0.37$). However, after 2 weeks of training, vocal exercised-trained rats had significantly increased intensity ($t(6) = 1.54, p = 0.087$; Fig. 1C) compared to sham-exercise rats.

Peak Frequency: There was no significant interaction for average peak frequency ($F(4, 35) = 0.07, p = 0.99$), main effects between treatment groups ($F(1, 35) = 0.022, p = 0.89$), or over time ($F(4, 35) = 0.47, p = 0.76$).

Maximum value of 50-kHz frequency modulated calls—Maximum USV data are displayed in Table 3; data comparisons between vocal exercise and sham-exercise groups.

Duration: There were no significant group $\times$ timepoint interactions ($F(4, 46) = 0.66, p = 0.62$). There were significant effects of timepoint ($F(1, 46) = 4.501, p = 0.004$). Specifically, there were differences between baseline and 2 weeks ($p = 0.005$), 2 mo ($p = 0.002$), 4 mo (p

= <0.001), and 6 mo ($p = 0.012$) (data not displayed). All testing timepoints had increased duration of calls compared to baseline. There was no significant main effect of group ($F(1, 46) = 0.11$, $p = 0.74$).

Bandwidth: There were no significant group $\times$ timepoint interactions ($F(4, 35) = 0.42$, $p = 0.79$). There were significant main effects of timepoint ($F(1, 35) = 5.45$, $p = 0.002$); there were significant increases between baseline and 2 weeks ($p < 0.001$) and 4 mo ($p = 0.005$). Additionally, there was a significant main effect of group ($F(1, 35) = 3.08$, $p = 1.0$) (data not displayed), after 2 mo of training, vocal exercise-trained rats had increased maximum bandwidth.

Intensity: There were no significant group $\times$ timepoint interactions ($F(4, 35) = 0.25$, $p = 0.91$). There were significant effects of timepoint ($F(1, 35) = 5.23$, $p = 0.002$). There were significant increases between baseline and 2 mo ($p = 0.004$) and 4 mo ($p < 0.001$) as well as differences between 4 mo and 6 mo ($p = 0.003$) (data not displayed). There was no main effect of group ($F(1, 35) = 0.43$, $p = 0.52$).

Peak Frequency: There was no significant interaction for average peak frequency ($F(4, 35) = 0.39$, $p = 0.81$), main effects between treatment groups ($F(1, 35) = 0.25$, $p = 0.63$), or over time ($F(4, 35) = 1.74$, $p = 0.16$).

Call rate—USV data for call rate and percent complex calls are displayed in Table 4; data comparisons between vocal exercise and sham-exercise groups.

There were no significant group $\times$ timepoint interactions ($F(4, 38) = 1.40$, $p < 0.001$). There were significant main effects of group ($F(1, 38) = 3.46$, $p = 0.087$). Specifically, *post-hoc* comparisons demonstrate significant increases between baseline and, 2 weeks ($p < 0.001$), 2 mo ($p < 0.001$), 4 mo ($p = 0.052$), and 6 mo ($p < 0.001$). Additionally, there was a significant main effect for timepoint ($F(1, 38) = 12.97$, $p < 0.001$). Within timepoint comparisons revealed after 2 mo of training, exercise-trained rats had significantly higher call rate ($F(1, 38) = 3.46$, $p = 0.087$; Fig. 3A).

Percent complex calls—Data comparisons between vocal exercise and sham-exercise groups. There were no significant group $\times$ timepoint interactions ($F(4, 35) = 0.28$, $p = 0.89$). There was a significant main effect of timepoint ($F(1, 35) = 7.74$, $p < 0.001$); specifically, there were significant decreases between the 2 week timepoint and 2 ($p < 0.001$), 4 ($p < 0.001$), and 6 ($p = 0.007$) mo as well as a significant decrease in percent complex calls between baseline and 6 mo ($p = 0.003$) (Fig. 3B). There was no main effect for group ($F(1, 35) = 0.15$, $p = 0.70$).

USV comparison to non-exercise/non-socialized *PINK1* $-/-$ animals: Average USV data for social control animals are displayed in Table 5; data comparisons between vocal exercise, sham-exercise, and social control *PINK1* $-/-$ groups.

At 8 mo of age (testing timepoint 6 mo of training), there were significant main effects between vocal exercise-trained animals and control *PINK1* $-/-$ group for call rate ($F(2, 21)$

= 12.870, $p < 0.001$; Fig. 4A) and average intensity ($F(2, 20) = 2.837$, $p = 0.085$; Fig. 4B). Specifically, *post-hoc* testing demonstrate significant increases in call rate of the vocal exercise-trained animals compared to the control ($p < 0.001$) and average intensity of the frequency modulated calls ($p = 0.029$, Fig. 4C), but there were no significant differences between vocal exercise-trained and sham-trained animals. Additionally, there were no significant differences in any other USV measures ($p > 0.10$ for all variables; Table 6).

Motor assays

Gross motor data are displayed in Table 7. Data comparisons for motor assays were performed between vocal exercise and sham-exercise groups.

Catalepsy—There were no significant group $\times$ timepoint interactions ($F(1, 20) = 1.85$, $p = 0.19$, Fig. 5A). Additionally, there were no significant main effects of treatment group ($F(1, 20) = 0.80$, $p = 0.38$) or main effects of timepoint ($F(1, 20) = 0.78$, $p = 0.038$).

Spontaneous movement

Hindlimb contacts: There was no interaction between group $\times$ timepoint ($F(1, 19) = 0.56$, $p = 0.406$). Additionally, there were no main effects for treatment group ($F(1, 19) = 0.72$, $p = 0.406$) or timepoint ($F(1, 19) = 1.56$, $p = 0.23$). There was a significant difference between timepoints when the data from both treatment groups were collapsed ($t(10) = 1.66$, $p = 0.06$) (Fig. 5B). In general, animals at 8 mo of age made fewer hindlimb contacts compared to 6 mo of age.

Forelimb contacts: There was no interaction between treatment group $\times$ timepoint ($F(1, 9) = 0.009$, $p = 0.93$). There were no significant main effects for treatment group ($F(1, 9) = 0.05$, $p = 0.82$) or timepoint ($F(1, 9) = 1.77$, $p = 0.22$). The data for each timepoint was collapsed and paired-tests were used to evaluate changes over time; there was a significant difference between timepoints ($t(10) = 1.42$, $p = 0.09$) (Fig. 5C). Animals at 8 mo of age made fewer forelimb contacts compared to 6 mo of age.

Challenging beam—There was no significant treatment group $\times$ timepoint interaction for the average time to traverse the beam ($F(1, 27) = 0.94$, $p = 0.403$; Fig. 5D), nor were there any main effects of group ($F(1, 27) = 0.84$, $p = 0.37$) or timepoint ($F(2, 27) = 0.89$, $p = 0.42$).

There was no group $\times$ timepoint interaction for the average time to traverse the last 1/3 of the beam ($F(2, 27) = 0.94$, $p = 0.04$); there were no main effect of group ($F(1, 27) = 0.098$, $p = 0.76$). There was a main effect of time ($F(2, 27) = 3.92$, $p = 0.032$). There was a statistically significant difference for the time to traverse the last 1/3 of the beam between 8 mo and 4 mo ($p = 0.04$) and 6 mo and 4 mo ($p = 0.09$) (Fig. 5F). With age, animals took a significantly longer time to traverse the last 1/3 (reduced diameter) of the beam.

There was no group $\times$ timepoint interaction for the average number of foot faults ($F(2, 28) = 0.64$, $p = 0.53$) or main effect of group ($F(1, 28) = 0.91$, $p = 0.35$). There was a significant effect of time ($F(2, 28) = 3.63$, $p = 0.39$). Specifically, there were differences between 8 mo

and 6 mo ($p = 0.06$) and 8 mo and 4 mo ($p = 0.06$) (Fig. 5E). Animals made significantly more foot faults at 8 mo of age compared to 4 or 6 mo.

Rater reliability

Inter-rater reliability ICC index for duration, bandwidth, intensity, and peak frequency were 0.99, 0.99, 0.97 and 0.93, respectively. Intra-rater reliability ICC index for duration, bandwidth, intensity and peak frequency were 0.93, 0.91, 0.99, and 1.0, respectively. Spontaneous activity inter-rater reliability ICC index was 0.97; intra-rater reliability was 0.95. Challenging beam inter-rater reliability ICC index was 1.0 for total time to traverse, 1.0 for time to traverse the last 1/3, and 1.0 for total foot-faults; intra-rater reliability was 1.0 for time to traverse the last 1/3 and 1.0 for total foot-faults.

DISCUSSION

The primary aim of this investigation was to assess the efficacy of an intensive (5 days per week) vocal exercise therapy on USVs in the *PINK1* $-/-$ genetic rat model of PD. Our results partially support the hypothesis that vocal exercise improves acoustic parameters of USVs, as vocal training had significant effects on intensity and bandwidth. However, while there was an effect at early time points following vocal exercise (2.5, 2 mo), there were no significant differences between vocal and sham exercise groups at later time points (4, 6, 8 mo), though both groups maintained improvements in acoustic parameters at these time points compared to baseline. Evidence from this work also supports the hypothesis that those animals that receive either sham or vocal exercise training show significant differences in both acoustic and call profile properties (call rate, percent complex) compared to controls. Overall, these findings suggest that targeted vocal exercise can ameliorate some aspects of USV deficits in the short-term and that general training exercises and socialization can improve long-term measurements of vocalization quality in a genetic rat model of PD.

Previous work from our lab has found that this vocal exercise paradigm effectively improves the USV deficits following unilateral dopamine depletion with 6-OHDA. 6-OHDA infusion to the medial forebrain bundle produces a relatively static neuropathology confined to changes associated with striatal dopamine loss that is responsive to a 4 week intensive vocal exercise training [21], compared to sham training. However, the *PINK1* $-/-$ model shows progressive behavioral deficits that result from progressive underlying neuropathology [20]. Compared to our past data, we observe similar deficits in the *PINK1* $-/-$ groups compared to age-matched WT controls. Specifically, we observe sustained levels of intensity deficit throughout training. Additionally, intensity measures are reduced in the social control *PINK1* $-/-$ compared to the WT (WT mean = 45, SEM = 0.50). All *PINK1* $-/-$ animals also demonstrate differences in other acoustic parameters compared to past work; bandwidth is reduced at 4 mo (WT mean = 24679, SEM = 11191) and 6 mo (WT mean = 23470, SEM = 999), and peak frequency is significantly affected at 6 mo (WT mean = 56658, SEM = 949) and 8 mo (WT mean = 55494, SEM = 911) of age [20]. Grant et al. (2015) also observe increases in vocal duration in WT and *PINK1* $-/-$ which is consistent with the present study. Of note, WT animals for comparison did not undergo daily handling and social contact, which may influence USV results. As such, the neuropathology in this PD model is more

dynamic. Because training did not vary or become more challenging as the animals aged and progressed in their disease course, vocal exercise training was not effective in protecting vocalizations over time, as compared to the calls of wild type animals [20]. In other words, as disease progresses, training targets should also progress to counteract the exacerbation of deficits. However, it is noted that continued training (exercise or sham) resulted in maintenance of acoustic properties compared to no-exercise *PINK1* $-/-$ controls. This notion has translational implications for voice therapy in individuals with PD. Current behavioral interventions, including LSVT-LOUD[®], consist of intensive training sessions for one month. Though this is effective in enhancing speech quality, in terms of both behavioral and physiological output, for up to two years from initial training [13, 47, 48], our results suggest that continuing treatment that advances as the disease progresses would achieve optimal therapeutic outcomes for the long-term.

Interestingly, both sham and vocal exercise training produced significant changes in call rate and average intensity of USVs as compared to untrained *PINK1* $-/-$ control at 8 mo. As compared to the WT, the sham and vocal exercise animals demonstrated similar results (see above); specifically, all trained rats produce more complex calls and maintain intensity over time [20]. However, in this study control *PINK1* $-/-$ animals had reduced intensity measures compared to WT animals. Animals that received training maintained the integrity of USVs over time while those that did not receive training demonstrated degradation of these call parameters over time. These results suggest that general therapies that involve increased socialization and goal-directed behavior can produce targeted improvements in evolving vocal deficits. The benefits of social enrichment in rats are well documented and include enhanced BDNF expression as well as enhanced resiliency to stress [49–54]. Moreover, recent evidence also suggests that environmental and social enrichment leads to increased 50-kHz vocalizations rates and social approach behavior [55]. Thus, treatments that serve to provide positive social interaction and reinforcement for patients with PD could be considered neuroprotective against some aspects of the disease. There were no significant differences in peak frequency (which has been particularly associated with affective communication in rodents) between the vocal exercise, sham exercise, or control groups at any time point. Moreover, all three groups experienced declines in peak frequency at proceeding time points. Willuhn et al., demonstrated that peak frequency, the frequency at which the most intense part of the call is emitted, is particularly correlated to eliciting phasic dopamine release from the nucleus accumbens, which demonstrates the affective nature of 50-kHz USVs in rats [56]. White noise played at the same intensity, bandwidth, and duration as a 50-kHz USV was ineffective in eliciting these dopamine transients from the accumbens, and thus, peak frequency is of particular importance when translating rodent vocalizations to human communication [56]. Training, vocal or sham, was not effective in improving this critical parameter, and thus, focused training that challenges the animal to produce calls with a higher peak frequency may be necessary in constructing vocal therapy to better counteract PD pathology, at least in this animal model. Naturally, therapy targets for humans would correspond to increasing intensity and prosodic elements of speech production, such as phonation range. There were no differences in gross motor behavior between sham and exercised animals at any time point, but there was a significant decline in gross motor ability over time in both groups. This is consistent with our past data that demonstrate gross motor

deficits progressively worsen in this animal model over time [20], which provides validity to the *PINK1* $-/-$ rat model as a means to assess treatments and therapeutic outcomes of PD.

Our work demonstrates the benefits of vocal therapy as well as social activity as mediators of vocal communication in the *PINK1* $-/-$ rat model of PD. These results possess important implications for optimizing voice therapy strategies in humans and shed light on possible novel behavioral interventions that could aid in improving quality of life and standard of care for PD patients.

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Fig. 1.

Vocal exercise training of 50kHz frequency modulated ultrasonic vocalizations in *PINK1*^{-/-} rats. Average from the top 10 calls for duration, bandwidth and intensity (A-C). Sham-trained (black bars) and vocal exercised-trained (white bars) + SEM. Significant within timepoint comparisons (* $p < 0.05$; # $p < 0.10$); brackets indicate significant differences between treatment timepoints ($p < 0.05$).

Fig. 2.

Intensity of *PINK1*^{-/-} 50kHz frequency modulated ultrasonic vocalizations with training over time. Representative sample ultrasonic vocalization spectrograms from one (A) vocal exercise-trained and one (B) sham-trained animal at baseline and after 2 weeks, 2, 4 and 6 months of behavioral training. Relative intensity is encoded by darkness of the signal; louder is darker.

Fig. 3. *PINK1* $-/-$ 50kHz frequency modulated ultrasonic vocalization call data. A) call rate B) percent complex calls in vocal exercise-trained (white bar) and sham-trained (black bar) + SEM. Significant within timepoint comparisons ($\# p < 0.10$); brackets indicate significant differences between treatment timepoints ($p < 0.05$).

Fig. 4.

Endpoint differences in treatment groups compared to *PINK1* $-/-$ social control. A) Call rate + SEM. B) Average Frequency Modulated Intensity + SEM. Brackets indicate significant group differences ($p < 0.05$). C) Representative sample ultrasonic vocalization spectrogram from one control, one sham-trained animal, and one vocal exercise-trained animal at 8 months of age.

Fig. 5.

Gross motor neurodegeneration over time of *PINK1*^{-/-} rats. A. catalepsy; B. cylinder-hindlimb; C. cylinder-forelimb; D. time to traverse beam; E. number of foot faults; F. time to traverse the last 1/3. X-axis is timepoint and Y-axis is behavior + SEM. Brackets indicate significant differences between testing timepoints ($p < 0.05$).

Table 1

50kHz frequency modulated ultrasonic vocalization data; Average. Means (SEM)

	Group	Baseline	2 weeks	2 mo	4 mo	6 mo
Duration	Exercise	0.051 (0.0071)	0.062 (0.010)	0.0734 (0.0051)	0.062 (0.0057)	0.062 (0.0091)
	Sham	0.045 (0.0034)	0.080 (0.014)	0.066 (0.0055)	0.061 (0.0055)	0.065 (0.0069)
Bandwidth	Exercise	20064.89 (695.18)	27105.82 (629.93)	23469.12 (734.63)	22810.10 (1197.25)	22524.423 (1377.92)
	Sham	18439.77 (1186.21)	21840.91 (2070.46)	22362.16 (1916.83)	21069.611 (2437.22)	20226.20 (1603.44)
Intensity	Exercise	-44.47 (0.885)	-42.98 (0.60)	-43.04 (0.72)	-44.57 (0.42)	-44.04 (1.12)
	Sham	-45.26 (1.03)	-43.29 (1.35)	-44.66 (0.58)	-44.41 (1.281)	-46.450 (1.084)
Peak Frequency	Exercise	50406.86 (1438.58)	48632.545 (2524.41)	50153.44 (1249.64)	49677.61 (1384.41)	49253.60 (1286.30)
	Sham	52623.42 (2820.53)	47033.67 (3488.16)	51062.84 (1963.01)	50116.95 (1046.54)	51357.623 (1920.75)

Group averages for ultrasonic vocalization call data displayed in figures. Means (SEM) for ultrasonic vocalization duration (seconds), bandwidth (Hz), intensity (dB) and peak frequency (Hz) for vocal exercise- and sham-trained animals at baseline, 2 weeks, 2 mo, 4 mo and 6 mo testing timepoints.

Table 2

50kHz frequency modulated ultrasonic vocalization data; Average of the top 10. Means (SEM)

	Group	Baseline	2 weeks	2 mo	4 mo	6 mo
Duration	Exercise	0.109 (0.023)	0.15 (0.04)	0.19 (0.023)	0.18 (0.025)	0.15 (0.029)
	Sham	0.085 (0.014)	0.19 (0.028)	0.15 (0.022)	0.17 (0.021)	0.16 (0.028)
Bandwidth	Exercise	34390 (1526.80)	51822.5 (1816.28)	42255 (2363.36)	44301.67 (2384.35)	40928.33 (3078.78)
	Sham	30026.67 (2761.79)	4012 (4188.81)	38191.67 (5073.07)	40401.67 (5536.86)	36598.333 (5209.69)
Intensity	Exercise	-33.25 (1.35)	-29.34425 (0.73)	-30.19 (1.29)	-29.18 (0.29)	-31.82 (1.21)
	Sham	-35.47 (2.12)	-31.82 (1.71)	-31.97 (1.53)	-30.934 (2.47)	-35.54 (2.92)
Peak Frequency	Exercise	63056.67 (2690.39)	60945 (3684.36)	61121.67 (1152.95)	60596.67 (1393.33)	59981.67 (1362.57)
	Sham	63620 (2667.58)	57635 (4660.88)	61301.67 (1858.53)	61453.33 (1545.54)	60275 (2411.20)

Average of the top 10 ultrasonic vocalization call data displayed in figures. Means (SEM) for ultrasonic vocalization duration (seconds), bandwidth (Hz), intensity (dB) and peak frequency (Hz) for vocal exercise- and sham-trained animals at baseline, 2 weeks, 2 mo, 4 mo, and 6 mo testing timepoints.

Table 3

50kHz frequency modulated ultrasonic vocalization data; Average maximum. Means (SEM)

	Group	Baseline	2 weeks	2 mo	4 mo	6 mo
Duration	Exercise	0.17 (0.037)	0.27 (0.093)	0.33 (0.062)	0.32 (0.05)	0.274 (0.067)
	Sham	0.11 (0.02)	0.36 (0.103)	0.24 (0.038)	0.31 (0.05)	0.26 (0.05)
Bandwidth	Exercise	44616.67 (1982.76)	62850 (2224.67)	53366.67 (3828.81)	52500 (3539.49)	52633.33 (3463.01)
	Sham	36883.33 (3908.06)	50050 (5992.56)	44283.33 (4774.89)	48016.67 (6293.722)	44266.67 (5914.09)
Intensity	Exercise	-28.74 (1.21)	-26.26 (0.87)	-25.56 (1.28)	-23.97 (0.78)	-28.70 (1.31)
	Sham	-31.13 (1.90)	-28.23 (1.62)	-25.38 (2.0)	-25.49 (2.23)	-30.15 (2.27)
Peak Frequency	Exercise	68983.33 (3879.03)	67575 (2441.44)	64316.67 (1583.32)	64566.67 (1742.35)	63666.67 (1304.52)
	Sham	69916.67 (2186.54)	63800 (5803.02)	68233.33 (2787.31)	66500 (1556.71)	64483.33 (2511.76)

Average maximum ultrasonic vocalization call data displayed in figures. Means (SEM) for ultrasonic vocalization duration (seconds), bandwidth (Hz), intensity (dB) and peak frequency (Hz) for vocal exercise- and sham-trained animals at baseline, 2 weeks, 2 mo, 4 mo, and 6 mo testing timepoints.

Table 4

50kHz frequency modulated ultrasonic vocalization data; Call Information. Means (SEM)

	Group	Baseline	2 weeks	2 mo	4 mo	6 mo
Call Rate	Exercise	1.88 (0.34)	2.52 (0.12)	3.069 (0.062)	3.28 (0.206)	2.77 (0.371)
	Sham	1.65 (0.27)	2.34 (0.27)	2.44 (0.15)	2.96 (0.19)	2.38 (0.17)
% Complex	Exercise	72.34 (6.45)	84.56 (3.49)	66.38 (5.11)	59.43 (5.74)	52.26 (5.99)
	Sham	63.91 (4.79)	80.07 (5.16)	60.95 (7.81)	58.75 (4.781)	45.87 (6.80)

Average call data displayed in figures. Means (SEM) for ultrasonic vocalization call rate and percent complex calls for vocal exercise- and sham-trained animals at baseline, 2 weeks, 2 mo, 4 mo, and 6 mo testing timepoints.

Table 5

Control Non-socialized animals, 50kHz frequency modulated ultrasonic vocalization data. Means (SEM)

Call Parameter	Average	Average of the Top 10	Average Maximum
Duration	0.069 (0.0079)	0.16 (0.022)	0.28 (0.035)
Bandwidth	21361.83 (975.26)	39186 (2316.534)	48780 (3529.58)
Intensity	-47.26 (0.64)	-35.92 (1.99)	-29.78 (1.018)
Peak Frequency	50869.30 (810.87)	60551 (1044.64)	64610 (1667.10)
Call Rate	1.99 (0.18)	n/a	n/a
% Complex	0.58 (0.043)	n/a	n/a

Average data for social control animals. Means (SEM) for ultrasonic vocalization acoustic parameters (duration (seconds), bandwidth (Hz), intensity (dB) and peak frequency (Hz)) and call data (call rate and percent complex calls) for social control animals at 8 mo of age.

Table 6

One-Way ANOVA Data

Variable	F (df)	p value
FM Duration	0.92 (2, 18)	0.42
FM Bandwidth	0.803 (2, 18)	0.46
FM Peak Frequency	0.62 (2, 18)	0.54
Top 10 Duration	0.05 (2, 19)	0.96
Top 10 Bandwidth	0.34 (2, 19)	0.71
Top 10 Intensity	H= 1.970 (2)	0.37
Top 10 Peak Frequency	0.04 (2, 19)	0.97
Max Duration	H = 0.245 (2)	0.89
Max Bandwidth	0.79 (2, 19)	0.47
Max Intensity	0.22 (2, 19)	0.80
Max Peak Frequency	0.07 (2, 19)	0.93
% Complex	1.28 (2, 19)	0.301

When normality assumptions failed a Kruskal-Wallis ANOVA on Ranks was used and H values are reported. ANOVA data for treatment and control groups. F test statistics (degrees of freedom) and p values for USV acoustic parameters (duration (seconds), bandwidth (Hz), intensity (dB) and peak frequency (Hz)) and call data (call rate and percent complex calls).

Table 7

Motor behavioral data. Means (SEM)

	Group	4 mo age	6 mo age	8 mo age
Catalepsy	Exercise	Not tested	2.47 (0.36)	2.25 (0.28)
	Sham	Not tested	2.25 (0.52)	3.31 (0.52)
Spontaneous Activity: Forelimb	Exercise	Not tested	18.8 (3.09)	15.33 (1.65)
	Sham	Not tested	18.67 (1.41)	15.67 (2.45)
Spontaneous Activity: Hindlimb	Exercise	Not tested	8.0 (1.41)	7.17 (1.56)
	Sham	Not tested	10.67 (2.26)	7.33 (1.09)
Beam: Total time to traverse	Exercise	2.47 (0.19)	2.93 (0.47)	5.0 (0.75)
	Sham	3.07 (0.64)	4.4 (0.92)	4.87 (0.82)
Beam: Time to traverse last 1/3	Exercise	1.27 (0.14)	1.73 (0.28)	3.37 (0.71)
	Sham	1.47 (0.35)	2.83 (0.70)	2.93 (0.42)
Beam: Foot faults	Exercise	0.93 (0.46)	0.367 (0.12)	0.87 (0.14)
	Sham	0.17 (0.10)	0.53 (0.18)	1.35 (0.26)

Average motor behavioral data displayed in figures. Means (SEM) for catalepsy, spontaneous activity forelimb and hindlimb (total number of steps), and challenging beam traversal (sec), time to traverse last third (seconds), foot faults (average over 5 trials) for vocalexercise-trained and sham-trained animals at 4, 6 and 8 mo of age.