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Vocal training, levodopa, and environment effects on ultrasonic vocalizations in a rat neurotoxin model of Parkinson disease

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Abstract

Levodopa does not improve dysarthria in patients with Parkinson Disease (PD), although vocal exercise therapy, such as “LSVT/LOUD[®]”, does improve vocal communication. Most patients receive vocal exercise therapy while concurrently being treated with levodopa, although the interaction between levodopa and vocal exercise therapy on communication in PD is relatively unknown. Further, carryover of vocal exercise therapy to novel situations is critical for successful outcomes, but the influence of novel situations on rehabilitated vocal communication is not well understood. To address the influence of exercise, medications, and environment on vocal communication with precise experimental control, we employed the widely used 6-OHDA rat neurotoxin model of PD (infusion to the medial forebrain bundle), and assessed ultrasonic vocalizations after: vocal exercise, vocal exercise with levodopa, levodopa alone, and control conditions. We tested USVs in the familiar training environment of the home cage and a novel cage. We hypothesized that parkinsonian rats that undergo vocal exercise would demonstrate significant improvement of ultrasonic vocalization (USV) acoustic parameters as compared to the control exercise and levodopa-only treatment groups. We further hypothesized that vocal exercise in combination with levodopa administration, similar to what is common in humans, would lead to improvement in USV outcomes, particularly when tested in a familiar versus a novel environment. We found that the combination of exercise and levodopa lead to some improvement in USV acoustic parameters and these effects were stronger in a familiar vs. a novel environment. Our results suggest that although treatment can improve aspects of communication, environment can influence the benefits of these effects.

Keywords

Parkinson disease; Rat; Ultrasonic vocalization; Levodopa; Vocal exercise; 6-OHDA

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1. Introduction

Parkinson Disease (PD) is a complex neurodegenerative condition primarily attributed to dopamine depletion and the degeneration of the nigrostriatal pathways [1–4], although the pathology of PD is vastly complex [1,5–7]. Sensorimotor deficits have devastating effects on quality of life in people with PD [8]. Voice and speech deficits are common early-onset complications of PD and in humans, clinical speech and voice therapy (exercise), such as LSVT/LOUD[®], has been shown to improve vocalizations [9–15]. However, patients must transition and carry over therapy techniques from the clinic to everyday activities in order to have successful speech treatment outcomes and drive long-lasting changes [16]. For example, weak responders to LSVT/LOUD[®] may require additional or longer training as well as increases in communication during daily activities [17]. Thus, there is a significant gap in our understanding of how the effects of exercise are influenced by the communication environment. Further, vocal exercise therapy is typically conducted while patients are also being treated with pharmacological methods of dopamine augmentation such as levodopa [15,18]. The effects of therapy, levodopa, and the environment have not been studied with precise experimental control due to the inherent variability associated with studying human patients. Thus, we studied the effects of medication, exercise, and environment in a widely accepted rat model of Parkinsonism: unilateral infusion of the neurotoxin 6-OHDA to the medial forebrain bundle [19–27].

Rodent models are a powerful research tool to study sensorimotor deficits associated with PD [28,29]. Previous studies in rats with unilateral infusion 6-OHDA suggest that targeted exercise may reverse and/or slow disease progression by sparing striatal dopamine and decreasing signs of Parkinsonism [30–32]. For example, forced use of a limb results in behavioral sparing when exercise training is initiated before or early after the administration of a dopamine-depleting neurotoxin [31,33–35]. Chronic levodopa therapy improves some gross motor deficits such as rotational responses [36], but not skilled forelimb movements including reaching and grasping for food [37]. In rats, early exercise intervention (therapy) has been shown to protect viable dopamine neurons in the substantia nigra as well as improve behavioral sensorimotor outcomes [20,26,35,38,39]. However, exercise paradigms are typically carried-out in the drug-free state, and as with humans, and the potential interactions between levodopa and exercise are currently unknown.

Rats are known to communicate through ultrasonic vocalizations (USVs) in a variety of social situations including mating [40–46], and have been used to investigate how PD-related pathology influences vocalizations [20,23,25,47,48]. Rats produce several types of USVs that are classified by frequency and complexity of the waveform. Rats produce social USVs during a mating paradigm that fall within the 50-kilohertz (kHz) range [46] and are composed of short constant frequency calls (simple) or frequency modulated (FM, complex) subtypes [42,44]. These calls are a useful model to study phonatory deficits associated with PD as they parallel human vocal communication in many ways [40,42,44,49]. Recent work has shown that unilateral 6-OHDA infusion to the medial forebrain bundle is sufficient to degrade acoustic parameters of 50-kHz calls (reduced bandwidth, intensity and peak frequency), and that altering dopaminergic synaptic transmission with haloperidol is sufficient to degrade the acoustic signal in rat 50-kHz USVs [25]. Furthermore, rat 50-kHz

USVs are influenced by dopaminergic modulation. For example, pharmacological antagonism of dopamine D1 and D2 receptor subtypes results in reductions in call rate and complexity [23,25,50,51]. Together, these studies suggest that rat 50-kHz USVs are susceptible to neurodegenerative processes and modulated to some degree by dopaminergic systems.

The purpose of the present study was to determine the extent of improvement and/or recovery of vocal function in rats with unilateral 6-OHDA infusions after administration of targeted vocal exercise and/or levodopa treatment. We analyzed the acoustic properties of USVs in rats with unilateral infusion of 6-OHDA after 4 weeks of: sham-controls, exercise, levodopa, exercise and levodopa. These effects were measured in the animal's home cage (where they were housed and trained-referred to as a familiar environment) and in a novel cage, to measure effects of environment on 50-kHz USV production. To confirm lesions, striatal dopamine depletion was quantified using a combination of behaviors (cylinder forelimb placing and apomorphine rotations) and histologic measures of tyrosine hydroxylase (TH) immunohistochemistry in the striatum. We hypothesized that intervention using targeted-vocal exercise would improve acoustic parameters in animals with 6-OHDA infusion. We also hypothesized that exercise in combination with levodopa treatment would significantly improve USV production compared to levodopa and exercise alone. Additionally, we hypothesized that USV production would be significantly different (improved) in the familiar home cage versus a novel test cage.

2. Materials and methods

2.1. Animals and habituation

Twenty-four male Long-Evans rats (Charles River Laboratories, Raleigh, NC, USA), aged 3 months at the time of study onset, were housed in single-sex groups of two in standard polycarbonate cages with corn cob bedding on a reversed 12:12 h (hr) light: dark cycle. All testing occurred during the dark period of the cycle under red light. Food and water was available ad libitum except during vocalization and control exercise, where water was periodically restricted (see below). Animals were handled daily starting at 2 months of age. Female rats ($n = 15$) were used to elicit vocalizations from male rats. The female rats were brought into estrous through subcutaneous injections of 10 μ g of β -estradiol (Sigma Aldrich, St. Louis, MO, USA) and 500 μ g of progesterone (Sigma Aldrich, St. Louis, MO, USA) in sterile vehicle (sesame seed oil; Fisher Scientific, Pittsburgh, PA, USA) at 48 h and 4 h before behavioral testing, respectively. Female rats were not included in the data analysis and were only used for eliciting vocalizations for vocal exercise, control exercise, and testing in familiar and novel cages (see below). All protocols and 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 (National Institutes of Health, Bethesda, MA, USA).

2.2. Overview of experimental testing

Refer to Fig. 1. Rats were handled daily by the experimenter, habituated to the recording apparatus and procedures, and were provided with sexual experience with a female

conspecific starting at 2 months of age. At 3 months of age, the cylinder test for spontaneous activity was used to assess general motor behavior at baseline. Following baseline testing and habituation, animals were randomized into one of 4 treatment groups and received a unilateral 6-OHDA infusion to the medial forebrain bundle. Treatment groups were: 1) sham exercise and vehicle (control group, $n = 7$), 2) vocal exercise and vehicle (exercise group, $n = 5$), 3) levodopa and sham exercise (levodopa group, $n = 6$), or 4) levodopa and vocal exercise (exercise + levodopa, $n = 6$). Animals received therapy five days per week for four weeks. After treatment, animals underwent behavioral testing for USV and limb sensorimotor function. These tests include: USV recording the home cage, USV testing in a novel cage, and cylinder test. After behavioral testing, animals were euthanized and brains were extracted and processed for tyrosine hydroxylase (TH) immunohistochemistry. All researchers were masked to the treatment conditions throughout testing and data analyses.

2.3. 6-OHDA infusion

All males received a unilateral infusion of 6-OHDA to the medial forebrain bundle. This method causes partial, unilateral degeneration of dopaminergic and noradrenergic neurons. Briefly, rats were anesthetized with an intraperitoneal injection of 90 mg/kg ketamine plus 10 mg/kg xylazine. When deeply anesthetized, rats were infused with 7 μ g (free base weight) of 6-OHDA hydrobromide dissolved in 3 μ l of artificial cerebrospinal fluid containing 0.05% (w/v) ascorbic acid to the medial forebrain bundle (-3.3 AP; ± 1.7 ML; -8.0 DV) at a rate of 0.3 μ l/min (min) for 10 min. 6-OHDA was infused into the hemisphere contralateral to the rat's preferred limb, as determined from baseline behavioral data on baseline forelimb-use (cylinder test). After surgery, rats were given an intraperitoneal injection of 0.05 mg/kg buprenorphine for analgesia, allowed to recover in a humidified incubator, and upon waking were returned to their home cages. After 72 h, animals underwent treatments in their respective groups.

2.4. Pharmacotherapy

Rats self-administered one daily therapeutic oral dose (2 mL per day) of 5/20 mg carbidopa (Lodosyn)/levodopa (Henry Schein Inc., Melville, NY, USA) mixed with 14 g of a grape jelly vehicle (J.M. Smucker's, Orrville, OH, USA), while they were housed singly for 30–60 min (depending on how readily they ingested the mixture). Carbidopa inhibits peripheral metabolism of levodopa which indirectly allows for penetration of levodopa across the blood brain barrier for a central nervous system effect [52,53]. The percent ingested was recorded to ensure that at least 90% of the mixture was ingested by each rat.

2.5. Behavioral testing

2.5.1. USV exercise—Male rats were placed on a water removal paradigm (water was restricted except for 3 h per day after testing) which has shown to be safely tolerated without causing distress to the animal [54]. This paradigm was used initially until we successfully transitioned from an unconditioned reward (water) to a conditioned reward (click sound). This typically took about 2 weeks for each animal. Thus, the water restriction paradigm was employed for the first two weeks of the 4 week training period. Animal weight was monitored weekly. For vocal exercise training, each male rat was placed in the home cage

underneath the ultrasonic microphone. To elicit vocalizations, a sexually receptive female rat was placed in the chamber with the male and the female was removed as soon as the male showed signs of interest (mounting, chasing). A learning paradigm was used where 50-kHz ultrasonic vocalizations elicited from the male were reinforced with a water reward on a variable ratio 5 schedule and paired with an audible click. Over time, the water reward is associated with the click as a conditioned reward. Thus, the click became the reward and we gradually transitioned from a water to a click reward. On average, using the method of reinforcing successive approximation, the rat received a water/click reward randomly for every 1–5 calls produced. The experimenter assessed the parameters of the calls (call type, complexity, bandwidth, and relative intensity) in real-time from the Avisoft-generated spectrogram output to provide immediate reward to the rat. Animals were exercised 5 days a week for 4 weeks post-surgery. Each session was approximately 10 min in duration. The sham-exercise animals (control group) were reinforced with a water/click reward in a similar manner described above for going to a specific site in their home cage after brief encounter with the female; the water/click reward was linked to appropriate positioning in the cage, rather than eliciting complex vocalizations. The levodopa plus exercise and sham exercise groups were administered levodopa 30–60 min prior to training.

2.5.2. USV testing—After the completion of all treatments, USV testing occurred twice for each animal, once in the home cage (290 mm × 533 mm × 210 mm) and once in the novel cage (245 mm × 453 mm × 210 mm), with a randomized order on the same day. An ultrasonic microphone with high directional properties and flat frequency response of up to 150-kHz, and a working frequency response range of 10–180-kHz was used for recording 50-kHz USVs (CM16, Avisoft, Germany). Recording parameters were a 16-bit depth with a sampling rate of 250-kHz. The microphone was attached to a panel in the top center of a 10 cm × 10 cm × 12 cm sound-isolated Plexiglas chamber. The rat was placed in the home cage or the novel cage within this chamber. Male-only vocalizations were recorded for 90 seconds (sec). Offline acoustic analysis was performed with a customized automated program using SASLab Pro (Avisoft, Germany). Spectrograms were built from each Avisoft-generated waveform with a Fast Fourier Transform (FFT) of 512 points, frame size of 100%, flat top window, and temporal resolution set to display 75% overlap frame set-up. A high pass filter was used to eliminate noise below 25-kHz including any 22-kHz ultrasonic vocalizations (although none of these calls were observed in the raw data). Calls were slowed down in order to listen and categorize the call type as reported by [25,44,46]. Two experienced raters, masked to experimental conditions, categorized each call into simple or frequency-modulated [19,25,47]. For each call category (simple, frequency modulated), acoustic analyses were performed using SASLab Pro (Avisoft, Germany) and include: bandwidth (Hertz-Hz), peak frequency (kHz), intensity (decibel-dB), and duration (milliseconds-ms). In addition, we analyzed the call rate (calls per sec) and the percent of complex calls produced. In order to analyze performance during the testing session each acoustic variable was calculated for 1) average of all calls, 2) the average of the top 10 performances, and 3) maximum performance. These three measures were analyzed because PD often affects the average motor performance more than the maximal performance [55]. The average of the top 10 performances is indicative of sustaining maximal performance.

2.6. Validation of dopamine depletion

2.6.1. Cylinder: forelimb use asymmetry—Rats were tested for forelimb use asymmetry as a baseline for determining side of 6-OHDA infusion, and 2 days post-exercise completion (4 weeks after surgery) to validate striatal dopamine depletion. 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 [56,57]). Forelimb-use asymmetry was calculated as ipsilateral limb wall contacts, plus 1/2 the number of “both” contacts, divided by the total number of contacts (limited to 20 per session) [21,58,59].

2.6.2. Apomorphine rotations—To validate successful striatal dopamine depletion, at 6 days post-surgery, animals were treated subcutaneously with the dopamine receptor agonist apomorphine (0.1 mg/kg, in 0.9% sterile saline). The net number of ipsiversive and contraversive quarter turns made during a 5 min period (20–25 min post-drug injection) was recorded to estimate the level of degeneration [24,26,60].

2.6.3. Striatal tyrosine hydroxylase immunohistochemistry—After completion of the end-point behavioral testing, rats were deeply anesthetized with 5% isoflurane and transcardially perfused with 500 mL of cold 4% paraformaldehyde in 1% phosphate buffered saline (PBS). Fixed brains were excised, post-fixed for 24 h in 4% paraformaldehyde, submerged in 0.02% sodium azide in 0.1 M PBS solution, sectioned at 60 µm on a freezing microtome, and stored in a cryoprotectant solution (ethylene glycol, glycerol and PBS) at –20 °C until they were stained for immunoreactivity. Coronal, free-floating sections were stained for tyrosine hydroxylase (TH) immunoreactivity on every 5th section. Sections containing the basal ganglia (approximately Bregma 2.52 mm to –2.40 mm) were incubated in A) a primary antibody solution: rabbit anti-TH at 1:2000 (AB152, Millipore, Billerica, MA, USA), B) a biotinylated goat-anti rabbit secondary antibody solution (1:500 dilution, Millipore, Billerica, MA, USA), C) amplified using VECTASTAIN Elite ABC avidin-biotin system (Vector Laboratories, Burlingame, CA, USA), and D) visualized with a 3,3'-diaminobenzidine as described previously [26]. All sections were float mounted onto gelatin-coated slides, dehydrated in a series of alcohols and Histo-Clear™ (HS-200, National Diagnostics®, Atlanta, Georgia, USA) and coverslipped.

Brain sections were imaged on an Epson Perfection V500 Photo Scanner and analyzed as in previous publications Ref. [26,24] using ImageJ software (National Institutes of Health, Bethesda, MD). Briefly, a customized software program developed in Image J detected the optical density of threshold values for tissue positive for TH immunoreactivity. The striatum (caudate and putamen) in each hemisphere was identified and selected manually, run through the software script, and values were expressed as a percent of threshold values compared to the non-infused hemisphere. The lesioned hemisphere is characterized as a percent loss of dopaminergic immunoreactivity. Moderate-to-severe 6-OHDA infusions were characterized by a percent loss greater than 70% [24,26].

2.7. Statistical analysis

All statistical analyses were conducted in SigmaPlot® 12.5 System (Systat Software, Inc., San Jose, CA). Data variables were transformed (rank transformed) if data failed to conform to assumptions for Analysis of Variance (ANOVA) statistical models, normality (using the Shapiro-Wilk test), and equal variance (Levene's test for homogeneity of variance). For data analysis, a 2×4 ANOVA for the categorical independent group variables (4 treatment groups and 2 cage conditions) was performed for each of the dependent variables, acoustic and call variables (bandwidth, peak frequency, intensity, duration, call rate, and % complex calls) for the average, average of the top 10 and average maximum data points, detailed above. General linear model main effects and interactions were examined, and post-hoc analysis was performed with the Holm-Sidak test. The critical level for statistical significance was set a priori at 0.05.

In order to quantify lesion severity, three different measures were used to validate successful infusion surgeries: cylinder contacts (increase from baseline), apomorphine rotations, and TH scoring (Fig. 1A). These measures are indicative of a severe unilateral deficit, thus confirming 6-OHDA infusion with behavior [59,61].

Inter and intra-rater reliability was calculated for 10% of the randomly sampled acoustic data using the intra-class correlation coefficient (ICC); a correlation coefficient of 0.9 or greater was considered reliable.

3. Results

3.1. Rater reliability

Intra-rater reliability ICC index for acoustic parameters duration, intensity, bandwidth and peak frequency was 0.90, 0.97, 0.95, 0.94, respectively. Inter-rater reliability for duration, intensity, bandwidth, and peak frequency was 0.92, 0.93, 0.99, and 0.97, respectively.

3.2. Validation of dopamine depletion

There were no significant differences at baseline testing ($F(3, 20) = 0.33$, $p = 0.80$; mean = 7.15 contacts) demonstrating that all groups were similar (Fig. 2A). After surgery, all animals had increased contacts in the cylinder compared to baseline, where higher cylinder scores indicate greater behavioral deficits [58]. There were no significant differences between groups ($F(3, 20) = 2.63$, $p = 0.079$). Six days after 6-OHDA infusion, all animals exhibited apomorphine rotations (Fig. 2B). There were no significant differences between groups ($F(3, 20) = 2.27$, $p = 0.11$). The percent loss of striatal TH immunoreactivity was greater than 86% (Fig. 2C, D) in all animals. There were no significant differences between groups ($F(3, 19) = 1.22$, $p = 0.33$). This indicates all animals in treatment groups had approximately the same degree of nigrostriatal dopamine depletion.

3.3. Post treatment ultrasonic vocalization analysis

3.3.1. Call rate—There was no significant interaction between treatment group (Sham Control, Exercise, Levodopa, Exercise +Levodopa) and cage condition (Home cage vs. Novel cage) ($F(3, 50) = 1.219$, $p = 0.312$; Table 1). There was a significant main effect of

cage condition ($F(1, 50) = 52.37, p < 0.001$; Fig. 3). Specially, post hoc comparisons demonstrated an increase in call rate in the home cage compared to the novel cage ($t = 7.237, p < 0.001$). There were no main effects for treatment group ($F(3, 50) = 0.78, p = 0.51$).

3.3.2. Percent complex calls—There was no significant interaction between treatment group and cage condition ($F(3, 47) = 0.47, p = 0.702$; Table 2). There were no main effects for treatment group ($F(3, 47) = 1.67, p = 0.19$) or cage condition ($F(1, 47) = 0.29, p = 0.60$).

3.3.3. Call duration—There was no significant interaction between treatment group and cage condition ($F(3, 47) = 0.47, p = 0.706$; Table 3) for the average duration. However, there was a significant main effect of cage condition on the average duration of the calls ($F(1, 47) = 19.252, p < 0.001$) (Fig. 4A); there was a statistically significant increase in duration in the home cage compared to the novel cage ($t = 4.39, p < 0.001$). There was no significant main effect of treatment group for the average duration of the FM calls ($F(3, 54) = 2.695, p = 0.057$).

There was no significant interaction between treatment and cage condition for the average of the top 10 ($F(3, 47) = 1.85, p = 0.15$) and the maximum average ($F(3, 47) = 1.58, p = 0.21$) (Table 3). There were significant main effects of cage condition in both the average of the top 10 ($F(1, 54) = 43.181, p < 0.001$) and the maximum average duration ($F(1, 54) = 36.95, p < 0.001$) (Fig. 4B and C, respectively). Specifically, the duration was increased in the home cage compared to the novel cage for the average of the top 10 calls ($t = 6.57, p < 0.001$) and the maximum average ($t = 6.08, p < 0.001$). There were no main effects of treatment group for the duration average of the top 10 ($F(3, 54) = 2.088, p = 0.12$), or the maximum average ($F(3, 54) = 2.374, p = 0.082$).

3.3.4. Bandwidth—There was no significant interaction between treatment group and cage condition for the average bandwidth ($F(3, 47) = 1.17, p = 0.33$), average of the top 10 ($F(3, 47) = 1.57, p = 0.21$) and maximum average ($F(3, 47) = 1.32, p = 0.28$) (Table 4). There was no main effect for the average bandwidth ($F(1, 47) = 2.70, p = 0.11$; Fig. 5A); however, there were significant main effects of cage condition for the average of the top 10 ($F(1, 47) = 17.58, p < 0.001$; Fig. 5B) and maximum average ($F(1, 47) = 6.53, p = 0.014$; Fig. 5C). Post hoc comparisons revealed significant increases in bandwidth for FM calls produced in the home cage compared to the novel cage for the average of the top 10 calls ($t = 4.19, p < 0.001$) and the maximum average bandwidth ($t = 2.56, p = 0.014$). There was no significant main effect of treatment group for average bandwidth ($F(3, 54) = 1.176, p = 0.33$), average of the top 10 ($F(3, 54) = 0.487, p = 0.693$), or maximum average ($F(3, 54) = 0.585, p = 0.628$).

3.3.5. Intensity—There were no significant interactions between treatment group and cage condition for intensity average ($F(3, 47) = 0.36, p = 0.78$), average of the top 10 ($F(3, 47) = 0.77, p = 0.52$), and maximal average ($F(3, 47) = 0.85, p = 0.47$) (Table 5). There were no significant main effects of cage condition for the average intensity ($F(3, 47) = 1.84, p = 0.15$), average of the top 10 ($F(3, 47) = 1.06, p = 0.38$), and maximum average ($F(1, 47) = 1.46, p = 0.23$). There were no main effects of treatment group for the average intensity ($F(3,$

47) = 1.84, $p = 1.54$), average of the top 10 ($F(1, 47) = 3.07$, $p = 0.09$) and maximum average ($F(3, 47) = 0.62$, $p = 0.61$).

3.3.6. Peak frequency—There was no significant interaction between treatment and cage condition for the average peak frequency ($F(3, 47) = 1.887$, $p = 0.15$). However, there was a main effect of as well as cage condition ($F(1, 47) = 5.87$, $p = 0.02$) as well as treatment group ($F(3, 47) = 14.5$, $p < 0.001$) (Fig. 6). Specifically, post hoc analysis demonstrated significant increases in peak frequency for calls produced in the home cage compared to the novel cage ($t = 2.42$, $p = 0.019$). There were significant increases in the average peak frequency in the exercise + levodopa treatment group compared to sham control ($t = 5.83$, $p < 0.001$), exercise ($t = 5.50$, $p < 0.001$), and levodopa groups ($t = 4.89$, $p < 0.001$) (Fig. 7A).

There was no significant interaction for treatment and cage condition for peak frequency average of the top 10 ($F(3, 47) = 2.67$, $p = 0.06$). There was no main effect of cage condition for the average of the top 10 ($F(1, 47) = 3.0$, $p = 0.09$). However, there was a significant treatment effect ($F(1, 47) = 12.9$, $p < 0.001$; (Fig. 7B). Specifically, there were significant increases in peak frequency in the exercise + levodopa group compared to sham control ($t = 5.41$, $p < 0.001$), exercise ($t = 5.16$, $p < 0.001$), and levodopa groups ($t = 4.80$, $p < 0.001$).

For peak frequency maximum average, there was a significant interaction between treatment groups and cage condition ($F(3, 47) = 5.26$, $p = 0.003$). Specifically, pairwise multiple comparison procedures revealed significant increases in maximum peak frequency in the home cage compared to the novel cage for the exercise treatment group ($t = 2.97$, $p = 0.005$) as well as the sham control treatment group ($p = 0.022$), but not the levodopa or exercise + levodopa treatment groups ($p > 0.05$). Analysis during testing within the home cage demonstrated significant increases in the maximum peak frequency produced in the exercise + levodopa treatment group compared to the sham control ($t = 5.50$, $p < 0.001$) and the levodopa group ($t = 3.08$, $p = 0.017$). The exercise treatment group also had increased peak frequency maximums compared to the sham control ($p = 0.025$). Within the novel cage condition, exercise + levodopa differed significantly from the exercise only group ($t = 4.39$, $p < 0.001$) and levodopa only ($t = 3.50$, $p = 0.006$).

4. Discussion

The primary purpose of this experiment was to address the therapeutic effects of vocal exercise, levodopa administration, combined effects of exercise plus levodopa, and influences of familiar vs. novel post-treatment testing on vocalization acoustics in a unilateral 6-OHDA Parkinsonian rat model. All rats in this study showed parkinsonian deficits similar to previous research [19,21,24,25]. Consistent with our hypothesis, results demonstrate improvement in USV peak frequency when animals received a combination of vocal exercise and concurrent levodopa administration. This was the only acoustic parameter, out of 6 tested, that was significantly affected by combined treatment; however, this finding was likely influenced by the effects of testing environment. Our results show that animals produced fewer calls and had reductions in USV duration and bandwidth when tested in a novel cage environment compared to the animal's home cage regardless of treatments. These findings suggest that testing environment can affect USV production.

Importantly, we demonstrate a significant interaction between treatment (exercise + levodopa condition) and environment. Specifically, there was an increase in the maximum peak frequency in the novel cage. Overall, our data suggest that the combination of behavioral vocalization treatment and pharmacological levodopa administration improve the social parameters of USV production, peak frequency, in familiar and novel conditions. Although not all USV parameters were improved with drugs or exercise equally, we interpret the improvement in peak frequency in both the home cage and novel cage as being compelling evidence that aspects of vocal communication can be modulated with treatment.

The production of frequency-modulated 50-kHz USVs in rats occurs in appetitive contexts including social approach [44,62–64], and are modulated, in part, by the neurotransmitter dopamine [51,65]. The 6-OHDA infusion produces a relatively static pathology in the medial forebrain bundle, associated with striatal dopamine loss; even a unilateral infusion drastically changes the ultrasonic acoustic parameters [19,20,24,25]. For example, [19] reports reduction reductions in bandwidth, duration and intensity as well as irregular frequency modulation [19]. Studies have also shown that 4 weeks post 6-OHDA infusion results in significant degradation of USVs including decreases in bandwidth, intensity, and complexity of vocalizations [19,25] and that maximum duration at 4 weeks post-toxin infusion is specifically correlated to reductions in striatal dopamine as well as reductions in call complexity [24]. Thus, we sought to reverse these deficits with levodopa, exercise, or a combination of levodopa plus exercise.

One of the main goals of the study was to examine improvement of acoustic deficits in USV production, with drugs and exercise. Thus, we employed the widely used, albeit imperfect, model of unilateral disruption to the medial forebrain bundle, which depletes striatal dopamine and results in robust cranial and limb sensorimotor deficits [19,21,22,24,25,27,33,35,66,67]. Our main finding was that combined levodopa and exercise improved peak frequency. However, we recognize that modulating dopamine neurotransmission can also impair goal-directed behavior, including the production of social vocalizations, which could be a limitation to this study [23,51]. In rats, peak frequency is highly correlated with pro-social signals [68], suggesting that this acoustic measure is translatable to human vocal communication. Our data are the first to demonstrate that a combination of exercise and levodopa is most effective in increasing peak frequency in this model of PD. Specifically, we observe a significant increase in average, average of the top 10, and maximum peak frequency values in rats that received exercise and levodopa treatments compared to rats receiving levodopa or exercise alone and compared to the control group. We also observe significant increases in maximum peak frequency in the exercise-treated groups compared to the control group. Exercise training, therefore, modulates behavioral and neurochemical outcomes. For example, specific exercise-training results in the rescue of tongue function deficits in the 6-OHDA model [26], and general systemic exercise improves gross motor function [34,39,69]. Our results show that targeted vocal exercise modulates behavioral recovery of peak frequency dysfunction in this model.

Multiple studies have shown that levodopa has no effect on vocalization deficits in humans [15]. Exercise-based speech and voice therapies such as LSVT/LOUD® have been shown to improve vocal performance, but these therapies are almost exclusively conducted in

medicated patients. Thus, the interaction between therapy and pharmacology is unexplored in humans and likely influences outcomes. Our data show that vocal exercise in combination with levodopa improves the peak frequency measure. Levodopa has profound behavioral effects including increasing fine motor control, and doses may induce motor changes that contribute to improved peak frequency in this model.

Levodopa administration has effects on striatal dopamine denervation and chronic levodopa treatment may alter striatal function [70]. Likewise, levodopa has effects other neuromodulators including striatal choline acetyltransferase, dynorphin, and substance P suggesting that chronic levodopa treatment may alter striatal function through multiple pathways [70]. In our study, rats were tested after receiving a daily, single dose of levodopa which is not reflective of the dosing pattern that PD patients receive. In the early stages of PD, administration of levodopa is controversial [71]; it remains unclear whether levodopa is beneficial or harmful to disease-related plastic changes, especially in conjunction with exercise. In general, our data suggest that social vocal production may be improved by levodopa's effect on fine motor control, where other acoustic parameters including duration, bandwidth and intensity are not improved.

We show that USV parameters significantly decline in a novel cage as compared to the animal's home cage regardless of treatment conditions, suggesting that this variable has profound importance on animal behavior. The environment has significant effects on behavioral outcomes. For example, patients with PD benefit from enriched social conditions [72–74]. Further, the carryover of specific therapy techniques is critical for successful speech treatment and maintenance [16]. Our data show that USV acoustic parameters are further reduced when tested in a novel cage compared to a home cage environment. This effect of cage condition on call rate, duration, and bandwidth demonstrates the need for additional training in novel environments. However, we observe an increase or sustained value for maximum peak frequency, a measure of upper limit of the amplitude of the vocal call, within the novel cage environment for animals treated with a combination of levodopa and exercise. In the novel cage environment, this treatment combination improved maximum performance compared to all other treatments. We suggest that the important social aspects of this parameter are facilitated by exercise as well as additional motor improvements as a result of levodopa treatments. We observed a significant increase in peak frequency in the control group from home cage to novel cage; we hypothesize that improvements in this peak frequency parameter may also be linked to other compensatory neurotransmitter systems not affected including, for example, opioid, noradrenergic, or serotonergic systems.

Unilateral infusions of the neurotoxin will result in both dopamine and norepinephrine loss [75–77]. Previous work has demonstrated a significant contribution of the noradrenergic system to acoustic variables; for example, call rate is increased with noradrenergic system treatments in adult rats [78]. However, the effect on acoustic parameters including intensity, bandwidth and peak frequency are currently unknown. In humans, both dopamine and norepinephrine systems are compromised in PD (reviewed in [79]), and future work should address the contributions of both systems to vocalization deficits in this model. Depletion of the catecholamines in this model, leads to reductions in the sensorimotor striatum (caudate and putamen) and also the nucleus accumbens (core and shell) and olfactory tubercle (Fig.

2). Our unpublished data demonstrate that rats with 6-OHDA lesions do not show impairments in olfaction (novel odor discrimination) or reductions in motivation (latency to mount; also demonstrated in [25]). We do not rule out the possibility that some of the changes we see in the 6-OHDA model are related to sexual motivation. However, in all conditions, rats continued to sniff chase, mount, as well as vocalize, suggesting minimal influence of motivation and arousal state.

Past studies have shown that 6-OHDA as well as low doses of the dopamine antagonist haloperidol result in USV changes [25]. For example, in a bilateral 6-OHDA model, rats exhibited a reduction in vocalizations where injections of haloperidol, a dopamine antagonist, into the nucleus accumbens, results in an increase in the number of 50-kHz USVs [51,80]. We acknowledge that incentive salience/anhedonia may be affected in this model; however, the data do not appear to reflect a decrease in the salience of the female stimulus used to elicit the vocalizations. Likewise, a significant limitation of using a unilateral infusion model is the contribution from the intact hemisphere and the effect of compensatory mechanisms; behavioral consequences are also affected by the extent of the dopamine depletion, and effects of nigrostriatal subregions including the caudate-putamen complex (reviewed in [27]). The 6-OHDA model lacks the progressive, age-dependent effects of PD as well as additional PD related pathologies including aggregation of alpha-synuclein which have been implicated in rodent USV deficits [81,82].

Despite significant clinical implications, voice deficits in PD are undertreated as they do not respond to standard pharmaceutical and surgical interventions. In humans, speech/voice therapy has been shown to improve communication with exercise therapy, and in patients it is typically conducted with dopamine-augmenting drugs such as levodopa. Early exercise has been shown to protect viable dopamine neurons and improve behavioral sensorimotor outcomes in animal models. However, in animal models, exercise paradigms are carried-out in the drug-free state. Based on the results of this study, we provide evidence from an animal model regarding the patterns of behavior associated with voice/speech therapy in patients with PD either on or off levodopa. Our results suggest that the combination of pharmaceutical and therapeutic approaches improves social communication as well as the cross-over from training environment to a novel environment. Accordingly, this study is an important first step in translating basic science research to clinical studies and understanding the mechanisms underlying cranial sensorimotor dysfunction in PD.

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HIGHLIGHTS

- Ultrasonic vocalizations are modulated by levodopa and vocal exercise in the unilateral 6-OHDA model.
- Ultrasonic vocalizations and treatments are influenced by testing environments in the unilateral 6-OHDA model.
- Acoustic parameters of ultrasonic vocalizations in the unilateral 6-OHDA model are diminished when tested in a novel versus a familiar environment.

Fig. 1.
Experimental timeline in weeks. Overview of the experimental timeline, conditions and testing.

Fig. 2.

6-OHDA infusion validation.

A. Average number of cylinder contacts $\pm$ standard error of the means (SEM) in the forelimb asymmetry test (cylinder test) 2 days after the completion of testing. Dashed line represents baseline (before surgery) average (mean = 7.15 contacts). There were no differences at baseline between groups; all groups were increased after 6-OHDA infusion surgeries. B. Average number of rotations $\pm$ SEM in the apomorphine rotation test 6 days after lesion surgery; there were no significant differences between groups. C. Average TH (tyrosine hydroxylase) score $\pm$ SEM represented as a percent loss of striatal dopamine as measures by TH immunoreactivity (out of 100%). D. Coronal view of a rat brain section (40 μ m) immunolabeled for anti-tyrosine hydroxylase with 3,3-Diaminobenzidine. The right side received a 6-OHDA infusion and displays a dopamine loss of 94.59%.

Fig. 3.

Post-treatment call rate and duration of calls.

A. Means $\pm$ standard error of the means (SEM) of the average call rate (calls per second) in the home cage (light bar) and novel cage (dark bar) environments post experimental treatments. B. Average duration (in seconds) mean and standard errors of the mean (SEM) in the home cage and novel cage environments after treatment. C. Average of the top 10 calls. Means (SEM) of the top 10 longest call durations in the home cage and novel cage post-treatment. D. Average of the maximum durations. Means (SEM) of the maximum durations in the home cage and novel cage environments post-treatment. Sample size is $n = 24$. Asterisks (*) indicate significant differences ($p < 0.001$).

Fig. 4.

Post-treatment bandwidth of calls.

A. Average bandwidth. Means and standard errors of the mean (SEM) of the average bandwidths in the home cage (light bar) and novel cage (dark bar) environments after treatments. B. Average of the top 10 bandwidth. Means (SEM) of the average top 10 bandwidths in the home and novel cage environments post-treatment. C. Average maximum bandwidth. Means (SEM) of the average maximum bandwidths in the home and novel cage environments post-treatment. Sample size is $n = 24$. Asterisks (*) indicate significant differences ($p < 0.001$); # indicates significant difference ($p < 0.05$).

Fig. 5.

Post-treatment peak frequency of calls.

A. Average peak frequency. Means and standard errors of the mean (SEM) of the average peak frequency the home cage (light bar) and novel cage (dark bar) environments after treatments. Sample size is $n = 24$. Asterisks (*) indicate significant differences ($p < 0.001$); # indicates significant difference ($p < 0.05$).

Fig. 6.

Peak frequency treatment effects.

A. Average peak frequency. Means and standard errors of the mean (SEM) of the average peak frequency in the 4 treatment groups: control (white bar), exercise (diagonal bars), levodopa (gray bar), exercise + levodopa (gray with diagonal bars). B. Average of the top 10 peak frequencies. Means (SEM) of the top 10 peak frequencies of the 4 treatment groups. C. Maximum peak frequencies. Means (SEM) of the maximum peak frequencies of the 4 treatment groups. Black bars with asterisks (*) indicate significant differences ($p < 0.001$).

Fig. 7.

Post-treatment average maximum peak frequency.

Average maximum peak frequency means and standard errors of the mean (SEM) in the home cage (left) and novel cage (right) environments. Treatment groups on the x-axis: control (white bar), exercise (diagonal solid bars), levodopa (gray bar), exercise + levodopa (gray with diagonal solid bars). Within cage condition significant differences are represented by black bars; between cage condition significant differences are represented by bracketed black bars with asterisks (*) indicate significant differences ($p < 0.001$); \$ indicates significant difference ($p < 0.01$); # indicates significant difference ($p < 0.05$).

Table 1

Summary statistics: % complex calls.

Treatment Group	Cage Condition	Mean (SEM)
Control	Home	8.30 (5.02)
Exercise	Home	53.30 (7.77)
Levodopa	Home	40.19 (7.10)
Exercise + Levodopa	Home	47.29 (7.09)
Control	Novel	59.53 (5.79)
Exercise	Novel	52.30 (7.77)
Levodopa	Novel	41.36 (7.09)
Exercise + Levodopa	Novel	46.30 (7.09)

Table 2

Summary statistics: call rate.

Treatment Group	Cage Condition	Mean (SEM)
Control	Home	2.12 (0.22)
Exercise	Home	2.66 (0.33)
Levodopa	Home	1.59 (0.31)
Exercise + Levodopa	Home	2.27 (0.31)
Control	Novel	0.67 (0.22)
Exercise	Novel	0.54 (0.33)
Levodopa	Novel	0.69 (0.31)
Exercise + Levodopa	Novel	0.72 (0.31)

Table 3

Summary statistics: duration.

Treatment Group	Cage Condition	Average Duration Mean (SEM)	Top 10 Duration Mean (SEM)	Maximum Duration Mean (SEM)
Control	Home	0.053 (0.003)	0.094 (0.007)	0.150 (0.015)
Exercise	Home	0.055 (0.004)	0.120 (0.011)	0.193 (0.024)
Levodopa	Home	0.047 (0.004)	0.079 (0.010)	0.124 (0.022)
Exercise + Levodopa	Home	0.043 (0.004)	0.079 (0.010)	0.123 (0.022)
Control	Novel	0.043 (0.003)	0.063 (0.008)	0.108 (0.018)
Exercise	Novel	0.037 (0.004)	0.042 (0.011)	0.058 (0.024)
Levodopa	Novel	0.037 (0.004)	0.044 (0.010)	0.059 (0.022)
Exercise + Levodopa	Novel	0.036 (0.004)	0.046 (0.010)	0.064 (0.022)

Table 4

Summary statistics: bandwidth.

Treatment Group	Cage Condition	Average Bandwidth Mean (SEM)	Top 10 Bandwidth Mean (SEM)	Maximum Bandwidth Mean (SEM)
Control	Home	17149.04 (1532.29)	31163.33 (2651.54)	42616.67 (3722.65)
Exercise	Home	20178.66 (2373.81)	37494.00 (4107.75)	46300.00 (5767.10)
Levodopa	Home	22073.99 (2166.98)	33248.33 (3749.85)	42333.33 (5264.62)
Exercise + Levodopa	Home	22117.10 (2166.33)	35905.00 (3749.85)	46050.00 (5264.62)
Control	Novel	18447.93 (1769.334)	27250.00 (3061.74)	41655.56 (4298.54)
Exercise	Novel	16133.67 (2373.81)	18639.84 (4107.75)	25680.00 (5767.10)
Levodopa	Novel	16773.28 (2166.98)	21392.98 (3749.85)	35850.00 (5264.62)
Exercise + Levodopa	Novel	20373.12 (2166.98)	27276.67 (3749.85)	37100.00 (5264.62)

Table 5

Summary statistics: intensity.

Treatment Group	Cage Condition	Average Intensity Mean (SEM)	Top 10 Intensity Mean (SEM)	Maximum Intensity Mean (SEM)
Control	Home	-49.80 (1.98)	-40.92 (2.49)	-35.89 (2.74)
Exercise	Home	-46.84 (3.07)	-35.03 (3.85)	-29.95 (4.24)
Levodopa	Home	-42.84 (2.80)	-34.47 (3.52)	-28.79 (3.87)
Exercise + Levodopa	Home	-43.08 (2.80)	-33.29 (3.52)	-27.91 (3.87)
Control	Novel	-45.82 (2.29)	-40.13 (2.87)	-32.88 (3.16)
Exercise	Novel	-45.99 (3.07)	-43.85 (3.85)	-36.81 (4.24)
Levodopa	Novel	-43.16 (2.80)	-38.77 (3.00)	-32.45 (3.87)
Exercise + Levodopa	Novel	-43.25 (2.80)	-37.91 (3.52)	-33.27 (3.87)