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Relationships among rat ultrasonic vocalizations, behavioral measures of striatal dopamine loss, and striatal tyrosine hydroxylase immunoreactivity at acute and chronic time points following unilateral 6-hydroxydopamine-induced dopamine depletion

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Abstract

Voice deficits in Parkinson disease (PD) emerge early in the disease process, but do not improve with standard treatments targeting dopamine. Experimental work in the rat shows that severe and chronic unilateral nigrostriatal dopamine depletion with 6-OHDA results in decreased intensity, bandwidth, and complexity of ultrasonic vocalizations. However, it is unclear if mild/acute dopamine depletion, paralleling earlier stages of PD, results in vocalization deficits, or to what degree vocalization parameters are correlated with other dopamine-dependent indicators of lesion severity or percent of tyrosine hydroxylase (%TH) loss. Here, we assayed ultrasonic vocalizations, forelimb asymmetry, and apomorphine rotations in rats with a range of unilateral dopamine loss resulting from 6-OHDA or vehicle control infusions to the medial forebrain bundle at acute (72 hours) and chronic (4 weeks) time points post-infusion. The %TH loss was evaluated at 4 weeks. At 72 hours, forelimb asymmetry and %TH loss were significantly correlated, while at 4 weeks, all measures of lesion severity were significantly correlated with each other. Call complexity was significantly correlated with all measures of lesion severity at 72 hours but only with %TH loss at 4 weeks. Bandwidth was correlated with forelimb asymmetry at both time points. Duration was significantly correlated with all dopamine depletion measures at 4 weeks. Notably, not all

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parameters were affected universally or equally across time. These results suggest that vocalization deficits may be a sensitive index of acute and **catecholamine loss** and further underscores the need to characterize the neural mechanisms underlying vocal deficits in PD.

Keywords

ultrasonic vocalization; Parkinson disease; dopamine; striatum; rat; 6-OHDA

Introduction

Parkinson disease (PD) leads to deficits in vocal sensorimotor control resulting in hypophonia (reduced vocal loudness and pitch) [1-6] that have a profound impact on communication and quality of life.[6-8] Unfortunately, standard pharmacological and surgical interventions that target nigrostriatal dopamine pathways do not consistently improve vocal deficits.[6, 9-18] Given this, the relationship between the primary disease pathology of nigrostriatal dopamine depletion and vocal deficits is unclear.

Previous work in rats has shown that unilateral 6-hydroxydopamine (6-OHDA) infusion to the medial forebrain bundle, causing severe striatal dopamine loss (greater than 85%), leads to decreased bandwidth (frequency range), intensity (loudness), and complexity of ultrasonic vocalizations.[19, 20] Thus, significant depletions in **catecholamines including** dopamine are sufficient to disrupt vocal function in a **6-OHDA rat model of PD**. However, voice deficits are often present in the early stages of PD,[21-23] prior to the emergence of cardinal motor deficits in the limbs (bradykinesia, tremor, muscle rigidity) associated with significant and bilateral nigrostriatal degeneration. It is unknown whether mild (<15%) or moderate (15-85%) striatal dopamine loss also degrades the acoustic signal in ultrasonic vocalization. We hypothesized that some aspects of ultrasonic vocalizations are affected even at low levels of striatal dopamine depletion, paralleling early dopamine loss and voice deficits in PD, but would not be necessarily correlated with limb deficits.[24]

Severe striatal dopamine loss **following 6-OHDA infusions** results in more predictable and reliable deficits to the limb sensorimotor system compared to aspects of cranial (oromotor) function, such as licking and chewing,[25-27] suggesting that these two systems may have a different sensitivity to dopamine depletion. However, the relationships among behavioral measures of lesion severity in the limb, such as forelimb use and apomorphine rotations, and ultrasonic vocalizations are unknown, particularly with mild dopamine depletion. Previous studies have shown that the degree of rotational behavior and end-point or qualitative measures of dopamine loss are not strongly related, especially with mild dopamine depletion.[28, 29] The post-lesion neuromodulation responsible for these behavioral deficits, such as upregulation of post-synaptic dopamine receptors in the striatum,[30, 31] is likely dependent on deficit severity and time after infusion. Thus, it is also likely that the severity of acoustic deficits in ultrasonic vocalizations uniquely respond to time course and severity of dopamine depletion. Understanding the relationships between limb deficits and vocal deficits in the context of acute versus chronic and mild versus severe dopamine depletion

may be helpful in designing medical and behavioral treatments for voice disorders in PD, because they are often refractory to those that target striatal dopamine.[6, 9-18]

To address these questions, we employed a 6-OHDA rat model of unilateral nigrostriatal dopamine depletion. Rats received either a unilateral infusion of 6-OHDA or vehicle to the medial forebrain bundle, resulting in a range of dopamine depletion (0-100% loss). We conducted behavioral assays of striatal dopamine loss (forelimb use asymmetry test and apomorphine rotations) and recorded ultrasonic vocalizations *in-vivo* 72 hours and 4 weeks post neurotoxin infusion. We also performed post-mortem immunohistochemical analysis of the striatum for tyrosine hydroxylase (TH) positive cells in all animals to verify the amount of striatal dopamine depletion at 4 weeks. We hypothesized that at 72 hours, deficits in acoustic measures of ultrasonic vocalizations would be related to striatal dopamine depletion (%TH loss), as voice deficits are thought to emerge in the early stages of PD. In contrast, we hypothesized that apomorphine rotations and forelimb asymmetry would not be related to each other, ultrasonic vocalization measures, or %TH loss, as post-synaptic receptor upregulation that is related to rotational behavior are present at approximately 14 days post-neurotoxin infusion and forelimb deficits are related to severe depletion, which may not have completely manifested at the 72 hour timepoint.[30] At 4 weeks post-lesion, we expected the behavioral measures of dopamine depletion (forelimb asymmetry, apomorphine rotations) to be strongly correlated with each other and with %TH loss. We also expected vocalizations to be correlated to some degree with forelimb asymmetry, apomorphine rotations, and %TH loss at 4 weeks.

Methods

Animals

Forty-nine 3 month-old male Long-Evans rats were used in this study at the 72 hour testing period (Charles River, Raleigh, NC). A randomized subset of these rats (n=24) were retested after 4 weeks to examine the effects of striatal dopamine depletion over a longer time course. (The remaining 25 rats were randomized to undergo a vocalization training paradigm and were not included in the 4 week analysis). Twelve female Long-Evans rats (ages 4-14 months) were used to sexually experience the males in order to elicit vocalizations on acclimation and recording days and were not used in the acoustic, behavioral or tissue analyses. [19, 20, 32] The male rats were housed in pairs in standard polycarbonate cages with corncob bedding and were on a reverse 12:12 hour light:dark cycle. All testing occurred during their dark period with partial red light illumination. Food and water were provided *ad libitum*. All animals were handled, light cycle reversed, and sexually experienced for a 2 week introductory period prior to neurotoxin infusion. We tested at three time points: baseline, and 72 hours and 4 weeks post-infusion. Testing involved administration of the forelimb asymmetry test, apomorphine rotations (72 hours and 4 weeks only), and ultrasonic vocalization recording. Results are reported as a change from baseline. Following behavioral and vocalization testing at 4 weeks, all animals were euthanized, and the amount of striatal TH was quantified as an indirect measure of dopamine levels using immunohistochemical methods. For all analyses, raters were masked to experimental condition. 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 (National Institutes of Health, Bethesda, MA, USA).

Ultrasonic Vocalization Recording and Analysis

Immediately prior to vocalization recording, a sexually receptive female rat in estrous was placed in the test cage until the male rat showed signs of interest (chasing, mounting). Estrous status was confirmed behaviorally, by the presence of darting, ear wiggling, and lordosis. The male rat was allowed to mount the female rat twice, the female was removed, and vocalizations were recorded for 120 seconds. Vocalizations were recorded with an ultrasonic microphone with a flat frequency response up to 150-kHz and a working frequency response range of 10-180 kHz (CM16, Avisoft, Germany) with 16-bit depth resolution and sampled at a rate of 250-kHz. The microphone was mounted approximately 15 cm above a standard polycarbonate rat cage (25 cm × 46 cm). Offline acoustic analysis was performed with a customized automated program using SASLab Pro (Avisoft, Germany). Spectrograms were built from each waveform with the frequency resolution set to an FFT of 512 points with a frame size of 100 % and flat top window and the temporal resolution set to display 75 % overlap. Extraneous low frequency noise was removed from the spectrogram using a high pass filter set to exclude sounds below 25-kHz. A rater masked to condition manually classified calls by their acoustic properties into simple, frequency modulated, or harmonic (Figure 1c).[19, 20] In addition, each call was inspected to ensure that extraneous noise did not interfere with the automatic parameter measurements; removal of noise and adjustments of duration and bandwidth were made as necessary to ensure that the software was capturing the whole call, and only the call. The call intensity, peak frequency, bandwidth and duration were collected automatically by the software and compiled in a spreadsheet. Acoustic parameters of were compared within the simple and frequency modulated categories. We did not include the harmonic category, as this type of call is infrequent and not universally produced. However, when determining the proportion of complex to simple calls, frequency modulated and harmonic calls were considered complex.

The following acoustic variables were measured: average and maximum bandwidth in hertz (Hz), average and maximum intensity in decibels (dB) and average and maximum duration in seconds (s) for calls within the simple and frequency modulated categories. We also measured the proportion of time spent producing complex calls, by dividing the summed duration of complex calls by the summed duration of all calls and multiplying that number by 100, for percent of complex calls.

6-OHDA and Sham Surgery

Rats were anesthetized with 2-4% isoflurane and placed in a stereotaxic frame. All rats received left-sided unilateral infusions of 7 µg 6-OHDA hydrobromide (free base weight) dissolved in 3 µl artificial cerebrospinal fluid (composition: NaCl, KCl, CaCl₂, MgCl₂*6H₂O) containing 0.05% (weight/volume) ascorbic acid or a 3 µl vehicle infusion of artificial cerebrospinal fluid only. Infusion coordinates were measured from bregma (−3.3 mm AP; −1.7 mm ML; −8.0 mm DV from dural surface), and infusions were delivered at a

rate of 0.3 μl /min for 10 min. Post-operation analgesia (0.1 ml bupivacaine, sub-cutaneous) was administered after suturing. Following surgery, animals were placed in a humidified incubator to prevent hypothermia, and on waking were returned to their home cages.

Forelimb Asymmetry Test

The forelimb asymmetry test, or “Cylinder Test”, and the association between forelimb use asymmetry and striatal dopamine depletion have been described elsewhere.[33, 34] Briefly, rats were placed in an upright acrylic cylinder (20 cm diameter) to encourage rearing and exploration with the forelimbs. The number of contacts made by either forelimb or by both forelimbs simultaneously was recorded. The ratio of contacts made by the non-impaired forelimb (ipsilateral to lesion) relative to the total number of contacts was calculated using the formula: ipsilateral limb contacts plus both [simultaneous or rapidly alternating limb contacts] divided by the total number of contacts. Scores significantly above 50% indicate a greater reliance on the ipsilateral limb for voluntary movement. [33, 34]

Apomorphine Rotation

Rats were injected with 0.5 mg/kg subcutaneous apomorphine hydrochloride, and the net number of contraversive turns during a 2 minute trial was recorded 25 minutes post-injection and reported as revolutions per minute (modified from Herrera-Marschitz, Casas, & Ungerstedt, 1988).[35]

Immunohistochemistry

All immunohistochemical methods and analysis have been described previously.[25] After completion of post-lesion measures at the 4 week time point, rats were deeply anesthetized with 2.5 – 4.0% isoflurane and intra-aortically perfused with 250 mL cold physiological saline 1 minute after an intracardial injection of 100 units of heparin. Immediately following, 500 mL of ice cold 4% paraformaldehyde in 0.1 M phosphate buffered saline (PBS) was perfused to fix brain tissue. Whole brains were removed and postfixed in ice cold fixative for 1-4 hours. Brains were then cryoprotected for 48-96 hours in a 20% sucrose/5% glycerol solution in 0.1M PBS at 4°C. Brains were mounted on a freezing microtome and one in every five 60 μm coronal slices throughout the basal ganglia were harvested and stored in PBS with 0.02% NaN_3 at 4°C until processing.

Floating slices were immunolabeled for TH using a rabbit anti-tyrosine hydroxylase primary antibody (1:2000 dilution, Millipore, Billerica, MA, USA) and a biotinylated goat anti-rabbit secondary antibody (1:500 dilution, Millipore, Billerica, MA, USA). The signal was amplified using the VECTASTAIN Elite ABC avidin-biotin system (Vector Laboratories, Burlingame, CA, USA). Slices were incubated in primary antibody for 16 hours, in secondary for 3 hours, and in avidin-biotin solution for 1 hour at room temperature. Labeling was visualized with 3,3'-diaminobenzidine (DAB) chromogen developed with a 0.3% peroxidase reaction for 90 seconds. Slices were quenched, counterstained with haematoxylin, and mounted on gelatin-coated slides.

Brain slices were imaged on an Epson Perfection V500 Photo Scanner and uploaded to a computer (Dell Optiplex 960) and analyzed using ImageJ software (National Institutes of

Health, Bethesda, MD). A custom-designed software program was developed in Image J to detect the optical density of thresholded values for neurons that were positive for TH immunoreactivity. The region of interest (striatum in each hemisphere) was identified manually, run through the analysis script, and values were expressed as a percent of thresholded values as compared with the intact hemisphere. Thus, the lesioned hemisphere is expressed as percent loss of dopaminergic neurons in the striatum.

Statistical Analysis

Difference scores from baseline were obtained for forelimb asymmetry and acoustic measures of ultrasonic vocalizations at 72 hours ($n=47$) and 4 weeks ($n=24$). Apomorphine rotations values and percent TH loss obtained from post-mortem analysis at 4 weeks for all animals were not a difference scores. Variables were plotted and tested for normal distribution. A regression was run for both a linear and a quadratic term for each of the acoustic variables and found that there was not a strong association between measures of striatal dopamine and any of the dependent variables for the quadratic term. As such, we used correlation analysis to make comparisons among variables. Since most data were not normally distributed, relationships among variables were tested with a Spearman's Rank Order Correlation (SAS Institute, Inc, Cary, NC). In order to test the relative contribution of severe lesions on vocalization and limb behavior deficits, we repeated the correlations with a subgroup of severe animals (85-100% depletion, as measured by %TH loss) ($n=17$) at the 4 week time point. A critical level for significance was set *a priori* at $p<0.05$. Given the exploratory nature of this work and the associated risk of beta error, no corrections were made for multiple comparisons. We acknowledge the risk of Type I error and have provided the data to be transparent in our analyses. This was decided *a priori* after weighing the strengths and weaknesses of correcting for multiple comparisons.

Results

Lesion severity (based on %TH loss) had a bimodal distribution, with 18 rats in the severe category (85-100% loss) and 24 in the none-to-mild category (0-15% loss), while only 7 rats were in the moderate range of 15-85% loss (Figure 1a-b). Correlation matrices for all comparisons are presented in Figure 2.

At 72 hours ($N=49$), the only significant relationships among measures of striatal dopamine loss were %TH loss and forelimb asymmetry test scores ($r=0.72$, $p<0.001$) (Figure 3a). Apomorphine rotation scores were not related to forelimb asymmetry test scores ($r=0.011$, $p=0.55$) or %TH loss ($r=0.18$, $p=0.34$) (Figure 3b, c). The ratio of time spent producing complex calls (call complexity) was significantly negatively correlated with all measures of striatal dopamine loss (Figure 4), including the forelimb asymmetry test ($r=-0.29$, $p=0.04$), apomorphine rotations ($r=-0.38$, $p=0.03$), and %TH loss ($r=-0.51$, $p<0.001$) (Figure 4a-c).

For frequency modulated calls at 72 hours ($N=49$), average bandwidth was correlated with the forelimb asymmetry test ($r=0.36$, $p=0.01$) and apomorphine rotations ($r=0.38$, $p=0.03$), but not %TH loss ($r=0.29$, $p=0.05$) (Figure 5a-c). This positive relationship indicates that bandwidth was larger with more severe indices of lesion at 72 hours. Maximum bandwidth was not related to forelimb asymmetry ($r=0.18$, $p=0.22$), apomorphine rotations ($r=-0.24$,

$p=0.19$) or %TH loss ($r=0.06$, $p=0.67$) (data not shown). Average intensity of frequency modulated calls was only related to apomorphine rotations ($r=0.37$, $p=0.04$) (Figure 5d), a not forelimb asymmetry ($r=0.08$, $p=0.58$) or %TH loss ($r=0.01$, $p=0.93$) (data not shown). Maximum intensity of frequency modulated calls was unrelated to forelimb asymmetry ($r=-0.14$, $p=0.35$), apomorphine rotations ($r=-0.16$, $p=0.38$) or %TH loss ($r=-0.08$, $p=0.61$) (data not shown). Neither average nor maximum duration of frequency modulated calls was correlated with forelimb asymmetry ($r=0.21$, $p=0.15$; $r=0.02$, $p=0.87$), apomorphine rotations ($r=-0.02$, $p=0.93$; $r=-0.26$, $p=0.14$), or %TH loss ($r=0.12$, $p=0.43$; $r=0.003$, $p=0.98$) (data not shown).

For simple calls at 72 hours (**N=49**), no significant relationships were identified among average measures of acoustic parameters of vocalizations (duration, bandwidth, and intensity) and forelimb asymmetry ($r=-0.04$, $p=0.75$; $r=0.04$, $p=0.78$; $r=-0.06$, $p=0.71$), apomorphine ($r=-0.21$, $p=0.25$; $r=0.16$, $p=0.40$; $r=-0.25$, $p=0.18$), or %TH loss ($r=-0.05$, $p=0.76$; $r=-0.19$, $p=0.19$; $r=-0.22$, $p=0.13$) (data not shown). Likewise, no significant relationships were identified among maximum duration, bandwidth, and intensity and forelimb asymmetry ($r=-0.02$, $p=0.87$; $r=0.12$, $p=0.43$; $r=-0.05$, $p=0.76$), apomorphine ($r=-0.26$, $p=0.15$; $r=0.11$, $p=0.54$; $r=0.2$, $p=0.28$), or %TH loss ($r=0.11$, $p=0.46$; $r=0.03$, $p=0.82$; $r=0.02$, $p=0.88$) (data not shown).

At 4 weeks (**N=24**), the forelimb asymmetry ratio was significantly correlated with %TH loss ($r=0.77$, $p<0.001$) and apomorphine rotation ($r=0.81$, $p<0.001$) and %TH loss was significantly correlated with apomorphine rotations ($r=0.71$, $p<0.001$) (Figure 6a-c). Call complexity was related to %TH loss ($r=-0.45$, $p=0.03$) (Figure 7a) but not forelimb asymmetry ($r=-0.34$, $p=0.10$) or apomorphine ($r=-0.31$, $p=0.14$) (data not shown). Maximum bandwidth of frequency modulated calls had a negative relationship with forelimb asymmetry ($r=-0.46$, $p=0.02$) and %TH loss ($r=-0.53$, $p<0.007$) (Figure 7 b, c), but not apomorphine rotations ($r=-0.38$, $p=0.06$) (data not shown). This indicates bandwidth was smaller with indices of more severe lesions at 4 weeks. Average bandwidth of frequency modulated calls, however, was not related to forelimb asymmetry ($r=0.11$, $p=0.61$), apomorphine rotations ($r=0.16$, $p=0.46$) or %TH loss ($r=0.16$, $p=0.45$) (data not shown). Neither average nor maximum intensity of frequency modulated calls was significantly related to forelimb asymmetry ($r=0.10$, $p=0.64$; $r=-0.32$, $p=0.13$), apomorphine rotations ($r=0.11$, $p=0.6$; $r=-0.21$, $p=0.32$), or %TH loss ($r=0.16$, $p=0.46$; $r=-0.21$, $p=0.32$) (data not shown). Maximum duration of frequency modulated calls was related to forelimb asymmetry ($r=-0.43$, $p=0.04$), apomorphine rotations ($r=-0.46$, $p=0.03$) or %TH loss ($r=-0.51$, $p=0.01$) (Figure 8 a-c), while average duration of frequency modulated calls was not related to any of the three measures of striatal dopamine loss (forelimb asymmetry [$r=-0.12$, $p=0.57$], apomorphine rotations [$r=-0.13$, $p=0.56$] or %TH loss [$r=-0.08$, $p=0.71$]) (data not shown).

For simple calls at 4 weeks (**N=24**), average intensity was related to %TH loss ($r=0.49$, $p=0.01$), but not forelimb asymmetry ($r=0.26$, $p=0.21$) or apomorphine rotations ($r=0.23$, $p=0.28$) (data not shown). Maximum intensity of simple calls was not related to forelimb asymmetry ($r=0.05$, $p=0.82$), apomorphine rotations ($r=0.03$, $p=0.90$) or %TH loss ($r=0.11$, $p=0.60$) (data not shown). Neither average nor maximum bandwidth of simple calls was

related to forelimb asymmetry ($r=0.18, p=0.40$; $r=-0.07, p=0.75$), apomorphine rotations ($r=-0.06, p=0.78$; $r=0.0007, p=0.99$), or %TH loss ($r=-0.002, p=0.99$; $r=-0.04, p=0.85$) (data not shown). Likewise, there were no significant relationships between average or maximum duration of simple calls and forelimb asymmetry ($r=-0.09, p=0.68$; $r=-0.08, p=0.72$), apomorphine rotations ($r=-0.23, p=0.27$; $r=-0.21, p=0.33$), or %TH loss ($r=-0.16, p=0.45$) (data not shown).

For the severely lesioned animals at 4 weeks ($N=17$), forelimb asymmetry was not related to apomorphine rotations ($r=0.21, p=0.55$) or %TH loss ($r=0.08, p=0.84$), nor were apomorphine rotations related to %TH loss ($r=0.17, p=0.63$). Call complexity was significantly negatively correlated with %TH loss ($r=-0.66, p=0.04$), but not forelimb asymmetry ($r=0.006, p=0.99$) or apomorphine rotations ($r=-0.24, p=0.51$). Average bandwidth of frequency modulated calls was significantly negatively correlated with apomorphine rotations ($r=-0.64, p=0.04$), but not forelimb asymmetry ($r=-0.31, p=0.38$) or %TH loss ($r=-0.25, p=0.48$). Maximum bandwidth was not related to forelimb asymmetry ($r=-0.34, p=0.34$), apomorphine rotations ($r=-0.24, p=0.50$), or %TH loss ($r=-0.35, p=0.32$) (data not shown). For frequency modulated calls, neither average nor maximum duration were related to forelimb asymmetry ($r=-0.14, p=0.70$; $r=0.15, p=0.69$), apomorphine rotations ($r=-0.38, p=0.29$; $r=-0.32, p=0.36$) or %TH loss ($r=-0.03, p=0.93$; $r=-0.31, p=0.39$) (data not shown). Likewise, average and maximum intensity of frequency modulated calls were not related to forelimb asymmetry ($r=-0.19, p=0.60$; $r=-0.45, p=0.19$), apomorphine rotations ($r=-0.30, p=0.40$; $r=0.05, p=0.89$) or %TH loss ($r=-0.22, p=0.54$; $r=-0.34, p=0.34$) (data not shown).

For severely lesioned rats at 4 weeks ($N=17$), the average and maximum bandwidths of simple calls were significantly correlated with apomorphine rotations ($r=0.67, p=0.03$; $r=0.65, p=0.04$), but not forelimb asymmetry ($r=-0.09, p=0.80$; $r=-0.13, p=0.73$) or %TH loss ($r=0.52, p=0.11$; $r=0.23, p=0.53$). For simple calls average and maximum duration were not related to forelimb asymmetry ($r=0.13, p=0.72$; $r=0.21, p=0.57$), apomorphine rotations ($r=0.38, p=0.28$; $r=0.33, p=0.35$) or %TH loss ($r=0.44, p=0.19$; $r=0.55, p=0.10$) (data not shown). Similarly, average and maximum intensity of simple calls were not correlated with forelimb asymmetry ($r=0.09, p=0.80$; $r=0.33, p=0.35$), apomorphine rotations ($r=0.45, p=0.19$; $r=0.36, p=0.30$) or %TH loss ($r=0.47, p=0.17$; $r=0.40, p=0.26$) (data not shown).

Discussion

This study examined the relationship among measures of striatal dopamine loss and ultrasonic vocalizations at 72 hours and 4 weeks following unilateral infusion of the neurotoxin 6-OHDA to the medial forebrain bundle as a model of nigrostriatal dopamine depletion in rats. Findings at 72 hours indicate that the forelimb asymmetry test may be sensitive to early cell death as it was significantly related to end-point immunohistochemical measures of striatal dopamine depletion (%TH loss). We were not surprised that apomorphine rotations were not related to forelimb asymmetry or %TH measures at 72 hours, as the mechanisms that lead to upregulation of post-synaptic dopamine receptors that cause rotational behavior with a dopamine agonist have a longer time course.[30, 31]

Consistent with our hypothesis, at 4 weeks all behavioral and immunohistochemical measures of striatal dopamine were strongly and significantly associated with each other.

At 72 hours, only a few acoustic components of frequency modulated calls were related to measures of striatal dopamine loss. The ratio of time spent producing complex calls was negatively associated with forelimb asymmetry test scores, apomorphine rotational behavior, and %TH loss, indicating acute dopamine loss is related to fewer complex calls. The average bandwidth of frequency modulated calls was positively correlated to forelimb asymmetry test scores and apomorphine rotational behavior at 72 hours. This indicates that when behavioral measures of dopamine loss are more severe, bandwidth increased instead of decreased. Previous data have shown that at 5 weeks post-neurotoxin infusion, bandwidth was significantly reduced.[36] However, similar changes in bandwidth have been found in the acute post-dopamine depletion time point with MPTP in bats.[37] One possible explanation is that in the acute phase, a transient increase in synaptic striatal dopamine causes over-excitability and thus modulation of fine sensorimotor control of the larynx can lead to ‘spread’ of the energy of the call with a wider bandwidth. This trend was not present at the 4 week time point, where average bandwidth was not correlated with any measures of striatal dopamine loss. However, maximum bandwidth was negatively correlated with forelimb asymmetry and %TH loss, indicating that when dopamine loss was more severe, bandwidth was smaller; the finding at this time point is consistent with other studies.[19, 38, 39] Overall, data suggest that even acute and mild disruption of dopamine signaling is associated with degrading the complexity of vocalizations, but does not necessarily affect all acoustic parameters of ultrasonic vocalizations equally.

Although apomorphine rotations were not related to other measures of striatal dopamine loss at 72 hours (forelimb asymmetry and %TH loss), there was a significant relationship between apomorphine rotation scores and intensity of frequency modulated calls. Upon examining the scatterplots, however, it appears that the more severe lesions are driving these correlations (see Figure 3c). This is consistent with the findings that a more severe depletion is required in order to cause rotational behavior. In the acute phase, this would require a very severe depletion, and this appears to be true for intensity of calls as well.

Interestingly, simple calls and frequency modulated calls were not uniformly affected and related to measures of striatal dopamine depletion. This was particularly evident with simple calls, in which acoustic measures were not related to measures of dopamine loss at 72 hours and intensity was only related to %TH loss at 4 weeks. Further, it appears that the severe lesions were driving this correlation. Bandwidth of frequency modulated calls was related to forelimb asymmetry and apomorphine rotations at 72 hours, while at 4 weeks, bandwidth was related to forelimb asymmetry and %TH loss. Duration was related to all three measures of dopamine loss at 4 weeks, but not at 72 hours. Taken together, these data suggest that not all call types are equally vulnerable to **6-OHDA induced catecholamine** depletion, and underscores the importance of classifying calls for detailed analyses. This is particularly important when investigating subtleties associated with fine sensorimotor control (such as vocal control) and is consistent with other studies examining modulation of ultrasonic vocalizations in rats demonstrating the importance of call classification.[38, 40, 41] With respect to ultrasonic vocalizations and fine sensorimotor control in the current study,

frequency modulated calls are clearly vulnerable to mild and moderate **catecholamine** depletion, while other calls, such as simple, seem to be less sensitive to changes in neurotransmitter levels. It is worth noting that innervation of the head and neck is bilateral while limb sensorimotor control is unilateral, with only the contralateral hemisphere managing function. These different patterns of innervation could account for the differential manifestation of cranial versus limb sensorimotor deficits in PD and variability sensitivity of specific acoustic properties.

Thus, with **6-OHDA induced catecholamine** depletion, rats produce fewer frequency modulated calls overall, and the acoustic parameters associated with those complex calls are most vulnerable to striatal dopamine depletion (compared to simple calls). It is not clear what this pattern of sensitivity to **catecholamine** depletion indicates. Perhaps the combination or additive effect of trying to compensate for degradation of multiple acoustic components (intensity, bandwidth) secondary to dopamine depletion may overtax the system for calls with rapid modulations, and it becomes relatively “easier” to produce a simple call type. This is consistent with laryngeal EMG studies in the rat demonstrating that fine sensorimotor control of laryngeal muscles is critical for producing ultrasonic vocalizations. [42] Intrinsic laryngeal muscle activity patterns during production of flat (simple) 50-kHz calls are characterized by tonic EMG patterns, while frequency modulated calls are associated with rapid alternating bursts, reflecting the differential complexity of producing each type of call.[42] Thus, mild to moderate dopamine depletion may challenge this dynamic system and result in less complex calling, while with severe compromise of dopamine, all call types are vulnerable and manifest acoustic deficits.

Differential behavioral profiles at acute versus chronic time points likely reflect the effects of neurotoxin-induced cell death and post-synaptic modulation over time. This has important implications for understanding the manifestation of sensorimotor deficits in relation to **catecholamine loss** in diseases such as PD, particularly for deficits such as dysphonia which are thought to manifest early and are refractory to dopamine-modulating treatments. This also has implications for early diagnosis in general, as a system challenged in the early stages may not have a predictable onset and progression of deficits.[43]

In contrast to previous work suggesting that duration of frequency modulated calls is not related to dopamine loss,[19, 39] maximum duration at 4 weeks was correlated with all measures of striatal dopamine loss. One explanation for this discrepancy is that the previous study only looked at average values of acoustic parameters. While average values are useful, the process of averaging may inherently mask values at the extremes. As individuals with PD may be able to perform a task maximally, they frequently have difficulty grading and sustaining fine motor movements over time,[44] and performance degrades. Thus, the inclusion of maximum and average acoustic values of ultrasonic vocalizations in the current study may be a more comprehensive approach to study vocal deficits in a system challenged following dopamine depletion. Interestingly, mice overexpressing human wild type alpha-synuclein, as a model of pre-manifest PD, demonstrate reductions in duration that are associated with alpha-synuclein aggregation in the periaqueductal gray, though bandwidth and nigrostriatal dopamine are not affected.[45] This suggests, as is the case with other behaviors, that multiple pathologic processes may independently be sufficient to disrupt

function in complex sensorimotor behaviors such as vocal control. Likely, voice deficits seen in humans are a result of multiple pathologies that occur prior to and in addition to dopamine depletion. [46, 47]

Prior to the current study, we assumed that more severe lesions would drive the correlations, as the behavioral tests, particularly apomorphine rotations, are more sensitive to severe lesions.[28, 29] However, our results indicate that when analyzing only severely lesioned animals at 4 weeks, these correlations did not endure. While the percent of complex calls produced was correlated with %TH loss, it was not correlated with other measures of striatal dopamine depletion, similar to when all animals were included in the analysis. In addition, bandwidth of frequency modulated and simple calls were correlated with apomorphine rotations. These data suggest that even low levels of striatal dopamine depletion are related to ultrasonic vocalization deficits and perhaps these deficits are a more sensitive behavioral index of striatal dopamine depletion than limb deficits. Recent work in speech acoustics in individuals with PD supports this hypothesis,[21-23, 48-50] and suggests that early vocal deficits may be a promising early biomarker for diagnosis.

As described in the results, the range of lesion severity (based on %TH loss) had a bimodal distribution, with 22 rats in the mild category (0-15% loss), 17 rats in the severe category (85-100% loss), and only 10 rats in the moderate range (15-85%). And, although statistically correlated, the Spearman's ρ values for most of the findings are considered weak to moderate, especially at 72 hours. The strongest correlations were for the severely depleted animals, but even these are considered moderately strong. Thus, although the degeneration of nigrostriatal dopaminergic neurons is implicated in acoustic deficits, it is certainly not the whole picture, as not all acoustic variables were related to striatal dopamine depletion. Acoustic parameters that were the most vulnerable to striatal dopamine depletion were call complexity and bandwidth at 72 hours, and bandwidth and duration at 4 weeks, with call complexity being relatively less affected at 4 weeks.

The overarching aim of this work is to better understand the mechanisms underlying cranial sensorimotor dysfunction in Parkinson disease. To that end, this study related ultrasonic vocalization deficits in a neurotoxin model of Parkinson disease with measures of limb sensorimotor dysfunction and dopamine depletion in the striatum, as cranial sensorimotor dysfunction may not manifest in the same manner as limb sensorimotor deficits. Importantly, 6-OHDA is toxic to not only dopamine, but also other catecholaminergic neurotransmitters, such as norepinephrine. Consequently, unilateral 6-OHDA infusions to the medial forebrain bundle will result in dopamine and norepinephrine loss.[51] Given this, it is possible that some aspects of the observed vocalization deficits are related to norepinephrine loss in addition to dopamine loss. It has been demonstrated that selectively agonizing or antagonizing noradrenergic receptors results in differential alterations to call rate in profile,[52] though the contribution of norepinephrine to other acoustic parameters such as intensity, bandwidth and peak frequency are unknown. In addition, preliminary work in our lab indicates that systemic injections of the noradrenergic neurotoxin DSP-4 results in acute, transient alterations to acoustic parameters of ultrasonic vocalizations such as intensity and bandwidth (unpublished). However it has been previously demonstrated that acoustic parameters such as bandwidth and intensity are vulnerable to dopamine

antagonism,[20, 38] establishing a clear relationship between acoustic deficits and compromise of dopamine. In addition, the present study demonstrates significant correlations between acoustic parameters and tyrosine hydroxylase immunoreactivity in the striatum, underscoring the relationship between vocal sensorimotor control and dopamine. Given the evidence that both dopaminergic and noradrenergic mechanisms are involved in the production of ultrasonic vocalizations coupled with the fact that neurodegeneration in Parkinson disease includes compromise of both of these neurotransmitter systems, it is likely that both neurotransmitters are important to appropriate cranial sensorimotor control and future work will be important to teasing out the contribution of each neurotransmitter to specific vocalization components.

Overall, several vocalization measures at 72 hours (bandwidth and intensity of frequency modulated calls) and 4 weeks (intensity of simple calls, bandwidth and duration of frequency modulated calls) were significantly correlated with measures of striatal dopamine loss. This is in contrast to what we hypothesized based on work done by Plowman *et al.* (2013) demonstrating that behavioral measures of oromotor function, such as lick rate, lick force, and bite force, do not correlate well with striatal dopamine loss following unilateral 6-OHDA induced dopamine loss.[24] It is unclear why different measures of cranial sensorimotor function appear to demonstrate a differential sensitivity to dopamine loss. One possible reason is that the neural substrates that modulate control of these behaviors are different; with vocal control being relatively more sensitive to **catecholamine loss**. An alternative, but not mutually exclusive, reason is that the range of lesion severity in the Plowman *et al.* (2013) study was not as broad, with a small sample of mild-moderate lesions and primarily severe lesions. Given this, it is possible that subtle alterations in oromotor function with mild dopamine loss were not captured (as was arguably the case with vocalizations in the present study). Further, in the Plowman *et al.* (2013) study, limb deficits and oromotor deficits were measures in a block design, and oromotor deficits were studied 6 weeks after the limb, where some spontaneous recovery of licking and chewing may have occurred. Finally, while both studies employ a unilateral 6-OHDA paradigm, Plowman *et al.* (2013) lesion the striatum directly, while the medial forebrain bundle was the target in the present study. As the medial forebrain bundle includes fibers from other neurotransmitter tracts vulnerable to 6-OHDA, such as norepinephrine, the deficits here may be the result of additive effects of more than one compromised neurotransmitter system.

Conclusions

Despite their early emergence, prevalence and negative impact on quality of life, voice deficits in PD are relatively undertreated as they do not respond to standard pharmaceutical and surgical interventions that replace or modulate dopamine. Results from this study indicate that multiple aspects of vocalization deficits resulting from 6-OHDA induced **catecholamine** depletion are sensitive to mild dopamine depletion at both 72 hours and 4 weeks. However, not all aspects of vocalizations were affected universally (simple calls are relatively unaffected) and equally (complexity was more strongly related at 72 hours, while duration only correlated with measures at 4 weeks) suggesting that deficits in the acoustic signal are not necessarily related to the degree of **neurotransmitter** loss. This has important

implications for better characterizing and treating voice deficits in PD, as they are refractory to dopaminergic medications and are therefore undertreated.

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Highlights

- ▶ ▶ Ultrasonic vocalizations are affected by acute, mild dopamine loss.
- ▶ Some vocalization deficits correlated with behavioral measures of dopamine loss.
- ▶ The nature of vocalization deficits was not consistently related to dopamine loss.
- ▶ Vocalization deficits were not consistently related to time post neurotoxin infusion.
- ▶ Ultrasonic vocalization deficits may not be related to the degree of dopamine loss.

Figure 1.

A) Sham (top) vs. lesioned tissue along the anterioposterior axis (remaining images). B) Histogram illustrating the percent of tyrosine hydroxylase loss in the lesioned striatum relative to intact hemisphere. C) Representative simple (left), frequency modulated (middle) and harmonic calls (right) at baseline (top row) and following dopamine depletion (bottom row).

a	FAS	Apo	% Complex	Simple Duration	Simple Bandwidth	Simple Intensity	FM Duration	FM Bandwidth	FM Intensity
FAS	-	-	-0.29 0.04	-0.05 0.75	0.04 0.78	-0.06 0.71	0.21 0.15	0.36 0.01	0.08 0.58
				-0.02 0.87	0.12 0.43	-0.05 0.76	0.02 0.87	0.18 0.22	-0.14 0.35
Apo	0.011 0.55	-	-0.38 0.03	-0.21 0.25	0.16 0.40	-0.25 0.18	-0.02 0.93	0.38 0.03	0.37 0.04
				-0.26 0.15	0.11 0.54	0.2 0.28	-0.26 0.14	-0.24 0.19	-0.16 0.38
TH	0.72 <u><0.001</u>	0.18 0.34	-0.51 <u><0.001</u>	-0.05 0.76	-0.19 0.2	-0.22 0.13	0.12 0.43	0.29 0.05	0.01 0.93
				0.11 0.46	0.03 0.82	0.02 0.88	0.003 0.98	0.06 0.67	-0.08 0.61

b	FAS	Apo	% Complex	Simple Duration	Simple Bandwidth	Simple Intensity	FM Duration	FM Bandwidth	FM Intensity
FAS	-	-	-0.34 0.10	-0.09 0.68	0.18 0.40	0.26 0.21	-0.12 0.57	0.11 0.61	0.1 0.64
				-0.08 0.72	-0.07 0.75	0.05 0.82	-0.43 0.04	-0.46 0.02	-0.32 0.13
Apo	0.81 <u><0.001</u>	-	-0.31 0.14	-0.23 0.27	-0.06 0.78	0.23 0.28	-0.13 0.56	0.16 0.46	0.11 0.6
				-0.21 0.33	0.0007 0.99	0.03 0.9	-0.46 0.03	-0.38 0.06	-0.21 0.32
TH	0.77 <u><0.001</u>	.71 <u><0.001</u>	-0.45 0.03	-0.16 0.45	-0.002 0.99	0.49 0.01	-0.08 0.71	0.16 0.45	0.16 0.46
				-0.19 0.38	-0.04 0.85	0.11 0.60	-0.51 0.01	-0.53 0.07	-0.21 0.32

c	FAS	Apo	% Complex	Simple Duration	Simple Bandwidth	Simple Intensity	FM Duration	FM Bandwidth	FM Intensity
FAS	-	-	0.006 0.99	0.13 0.72	-0.09 0.80	0.09 0.80	-0.14 0.70	-0.31 0.38	-0.19 0.60
				0.21 0.57	-0.13 0.73	0.33 0.35	0.15 0.69	-0.34 0.34	-0.45 0.19
Apo	0.21 0.55	-	-0.24 0.51	0.38 0.28	0.67 0.03	0.45 0.19	-0.38 0.29	-0.64 0.04	-0.30 0.40
				0.33 0.35	0.65 0.04	0.36 0.30	-0.32 0.36	-0.24 0.50	0.05 0.89
TH	0.08 0.84	0.17 0.64	-0.66 0.04	0.44 0.19	0.52 0.11	0.47 0.17	-0.03 0.93	-0.25 0.48	-0.22 0.54
				0.55 0.10	0.23 0.53	0.4 0.26	-0.31 0.39	-0.35 0.32	-0.34 0.34

Figure 2.

Relationships among striatal dopamine measures and USV measures at 72 hours (N=49) (a), 4 weeks (N=24) (b) and 4 weeks for the animals with severe lesions (N=17; 85-100 %TH loss) (c) (TH is from 4 weeks). Correlation coefficients (top number, bold) and p-value (bottom) for dependent variables. Average (top cell) and maximum (shaded, bottom cell) correlation coefficients and p-values for duration, bandwidth and intensity of simple and frequency modulated calls; significant values underlined. Abbreviations: Apo=Apomorphine, TH=tyrosine hydroxylase, FM=frequency modulated call, FAS=forelimb asymmetry score.

Figure 3.

Plots of correlations between measures of striatal dopamine depletion at 72 hours post 6-OHDA infusion. There was a significant relationship between %TH loss and forelimb asymmetry ratio (a), $r=72$, $p<0.001$, but not for apomorphine rotations (revolutions per minute) and forelimb asymmetry ratio (b) $r=0.011$, $p=0.55$, or %TH loss and apomorphine rotations (revolutions per minute) (c), $r=0.18$, $p=0.34$. FAS= Forelimb asymmetry score. Dashed line indicates “typical” unimpaired forelimb use.

Figure 4.

Plots of correlations between call complexity and measures of striatal dopamine depletion at 72 hours post 6-OHDA infusion (N=49; % TH at 4 week). Call complexity was significantly negatively correlated with forelimb asymmetry (a), $r=-0.29$, $p=0.04$, apomorphine rotations (revolutions per minute) (b), $r=-0.38$ $p=0.02$, and % tyrosine hydroxylase loss (c), $r=-0.51$ $p=0.002$. Dashed line indicates “typical” unimpaired forelimb use.

Figure 5.

Plots of correlations between average bandwidth (a-c) and average intensity (d) of frequency modulated calls and measures of striatal dopamine depletion at 72 hours (N=49; % tyrosine hydroxylase at 4 weeks). For frequency modulated calls, bandwidth was correlated with the forelimb asymmetry ratio (a) $r=0.36$ $p=0.01$, apomorphine rotations (revolutions per minute) (b) $r=0.38$ $p=.03$, and % tyrosine hydroxylase loss (c) $r=0.29$, $p=.04$. Intensity of frequency modulated calls was significantly correlated to apomorphine rotations (revolutions per minute) (d) $r=0.37$, $p=0.04$. Dashed line indicates “typical” unimpaired forelimb use.

Figure 6.

Plots of correlations between measures of striatal dopamine depletion at 4 weeks (N=24).

Forelimb asymmetry was significantly correlated with % tyrosine hydroxylase loss (a)

$r=0.77$, $p<0.001$ and apomorphine rotation (revolutions per minute) (b) $r=0.81$, $p<0.001$ and

% tyrosine hydroxylase loss was significantly correlated with apomorphine rotations

(revolutions per minute) (c) $r=0.76$, $p<0.001$. Dashed line indicates "typical" unimpaired forelimb use.

Figure 7.

Plots of correlations between percent complex (a) and maximum bandwidth (b-c) at 4 weeks (N=24). Percent complex was significantly correlated with % tyrosine hydroxylase loss (a) $r=-0.45$, $p=0.03$ and maximum bandwidth correlated with forelimb asymmetry (b) ($r=-0.46$, $p=0.02$) and % tyrosine hydroxylase loss (c) ($r=0.53$, $p<0.007$). Dashed line indicates “typical” unimpaired forelimb use.

Figure 8.

Plots of correlations between maximum duration and measures of striatal dopamine loss at 4 weeks (N=24). Maximum duration was significantly correlated with forelimb asymmetry (a) $r=-0.43$, $p=0.04$, apomorphine rotations (revolutions per minute) (b) $r=-0.46$, $p=0.03$ and % tyrosine hydroxylase loss (c) $r=-0.51$, $p=0.01$. Dashed line indicates “typical” unimpaired forelimb use.