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Decreased approach behavior and nucleus accumbens immediate early gene expression in response to Parkinsonian ultrasonic vocalizations in rats

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Abstract

Many individuals with Parkinson disease (PD) have difficulty producing normal speech and voice, resulting in problems with interpersonal communication and reduced quality of life. Translational animal models of communicative dysfunction have been developed to assess disease pathology. However, it is unknown whether acoustic feature changes associated with vocal production deficits in these animal models lead to compromised communication. In rodents, male ultrasonic vocalizations (USVs) have a well-established role in functional inter-sexual communication. To test whether acoustic deficits in USVs observed in a *PINK1* knockout (KO) PD rat model compromise communication, we presented recordings of male *PINK1* KO USVs and normal wild-type (WT) USVs to female rat listeners. We measured approached behavior and immediate early gene expression (c-Fos) in brain regions implicated in auditory processing and sexual motivation. Our results suggest that females show reduced approach in response to *PINK1* KO USVs compared to WT. Moreover, females exposed to *PINK1* KO USVs had lower c-Fos immunolabeling in the nucleus accumbens, a region implicated in sexual motivation. These results are the first to demonstrate that vocalization deficits in a rat PD model result in compromised communication. Thus, the *PINK1* KO PD model may be valuable for assessing treatments aimed at restoring vocal communicative function.

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INTRODUCTION

Successful communication is critical to carrying out functional social interactions. Parkinson disease (PD) is a complex, progressive neurodegenerative disorder that leads to a range of motor and non-motor deficits, including deficits in vocal communication. The disease is associated with significant voice and speech deficits (Goberman & Blomgren, 2008; Harel, Cannizzaro, & Snyder, 2004; Holmes, Oates, Phyland, & Hughes, 2000; Narayana et al., 2009; Stewart et al., 1995) that occur in approximately 90% of affected individuals (Ho, Iansek, Marigliani, Bradshaw, & Gates, 1998). Voice and speech deficits, termed hypokinetic dysarthria, often include a quiet, hoarse, monotonous voice, imprecise articulation and compromised intelligibility (Kim, Kent, & Weismer, 2011; Logemann, Fisher, Boshes, & Blonsky, 1978; Sapir, 2014). These vocal deficits can lead to impaired interpersonal communication that significantly impacts quality of life in PD patients (Duffy, 2006; Fox et al., 2006; Karlsen, Tandberg, Arsland, & Larsen, 2000).

Rodent ultrasonic vocalizations (USVs) have been used to study the relationships between vocal communication deficits and disease mechanisms in multiple models of PD (Johnson, Grant, Schallert, & Ciucci, 2015). Moreover, USVs carry semiotic value and are capable of changing the behavior of the signal recipient, similar to the function of human vocal communication (Arriaga, Zhou, & Jarvis, 2012; Brudzynski & Pniak, 2002; Coffey et al., 2013; Riede, 2011; Wohr & Schwarting, 2007). The overall goal of using a translational model of communicative dysfunction is the discovery and implementation of pharmacologic and surgical treatments as well as behavioral therapies to restore functional communication in PD (Cenci, Whishaw, & Schallert, 2002; Ciucci, Vinney, Wahoske, & Connor, 2010; Johnson et al., 2011). However, it is unknown if the established vocal deficits in these models, as evidenced by changes in acoustic parameters of USVs, interfere with functional communication and subsequent social behavior. Thus, it is imperative that acoustic deficits in vocal output observed in models of PD pathology are evaluated for relevance, reliability, and validity of detrimental impacts in communication in the model species being tested.

Rat USVs have been well-established as sensitive measures of social behavior (Brudzynski, 2009; Knutson, Burgdorf, & Panksepp, 1998; McGinnis & Vakulenko, 2003; Wohr & Schwarting, 2013), particularly 50-kilohertz (kHz) USVs which play a role in establishing or maintaining social contacts (Brudzynski & Pniak, 2002; Knutson et al., 1998; McGinnis & Vakulenko, 2003; Wohr, Houx, Schwarting, & Spruijt, 2008) and facilitating sexual behavior (Barfield, Auerbach, Geyer, & McIntosh, 1979; Bialy, Rydz, & Kaczmarek, 2000; Burgdorf et al., 2008; Sewell, 2009). Recently, we have measured 50-kHz USV production of rodent models in our laboratory to investigate how PD-related pathology influences vocal output (Ciucci, Ma, Kane, Ahrens, & Schallert, 2008; Ciucci et al., 2010; Johnson et al., 2011). Several pharmacological (dopamine receptor antagonists), neurotoxin (6-hydroxydopamine) and genetic rodent models (overexpression of human wild-type alpha synuclein under the

Thy-1 promotor in the mouse and with AAV2/5 in the rat) of PD demonstrate degradation in acoustic features of USVs (Ciucci et al., 2009; Ciucci et al., 2007; Gombash et al., 2013; Grant et al., 2014; Ringel, Basken, Grant, & Ciucci, 2013; Wright, Dobosiewicz, & Clarke, 2013). Specifically, recent evidence from our laboratory indicates that in rats, the complete knockout (KO) of PTEN-induced putative kinase 1 (*PINK1*), a gene associated with autosomal recessive, early onset PD in human populations (Bonifati et al., 2005; Kawajiri, Saiki, Sato, & Hattori, 2011; Tan et al., 2006; Valente et al., 2004), results in early and progressive reductions of USV bandwidth, peak frequency, and intensity as compared to wild-type (WT) controls (Grant, Pultorak, Kelm-Nelson, & Ciucci, 2013). The impact of these *PINK1* KO deficits in USV production on social communication is unknown.

Effective vocal communication requires the production of vocal signals that are capable of being successfully interpreted by a receiver. Playback methodology allows us to analyze vocalization integrity by observing conspecific behavior in response to acoustic stimuli (Burgdorf et al., 2008; Kroodsma, 1989; Musolf, Hoffmann, & Penn, 2010; Seffer, Schwarting, & Wohr, 2014; Snoeren & Agmo, 2013, 2014). Past research has demonstrated that playback of 50-kHz USVs elicit conspecific approach behavior and induce preference over artificially generated tones and noise controls (Burgdorf et al., 2008; Willadsen, Seffer, Schwarting, & Wohr, 2014; Wohr & Schwarting, 2007, 2013), but no studies have shown that rats discriminate between WT 50-kHz USVs and PD disease model USVs. Thus, to assess impact of USV acoustic deficits on communication, we conducted a series of USV playback experiments comparing the behavioral response of female rats to USVs of WT control male and *PINK1* KO male 50-kHz USVs. Specifically, we hypothesized that male *PINK1* KO vocal deficits would negatively impact communication as measured by reduced interest from females, and that females would prefer playback of male USVs over a noise control but prefer WT USVs over *PINK1* KO USVs.

To further assess if USV acoustic deficits affect communication, we also tested whether presentation of *PINK1* KO USVs, as compared to WT USVs, altered c-Fos immediate early gene expression in brain regions related to hearing and social motivation in the female rat listeners. Immediate early genes are transcription factor indicators of neuronal cellular activity and induce downstream gene induction; this method has been used extensively to explore brain activity in response to behavioral experience. Neurons in specific regions of the cortex are activated by novel and familiar auditory stimuli (Wan et al., 2001) as well as differentially activated by USV types conferring different communicative functions (affiliative 50-kHz versus [vs] negative 22-kHz vocalizations) (Sadananda, Wohr, & Schwarting, 2008). However, it is unknown if c-Fos immunoreactivity is affected by rat PD-model vocalizations. Thus, examining increases in this transcription factor expression in association with listening to auditory stimuli can provide indirect evidence of neuronal activity of a subset of cells in a given brain region during a particular behavior. We used c-Fos immunoreactivity to investigate regions that are active in response to the playback of recorded ultrasonic calls. Specifically, we investigated neuronal labeling in auditory processing (primary auditory cortex, (Roger & Arnault, 1989)), interconnected regions regulating auditory processing (periaqueductal gray, (Larson, 1985; Larson & Kistler, 1986)) and attention/decision making (cingulate cortex, (Floresco & Ghods-Sharifi, 2007)). Additionally, we hypothesized that female rats exposed to *PINK1* KO male 50-kHz

vocalizations, as compared to WT male 50-kHz vocalizations, would have decreased c-Fos neuronal labeling in regions associated with and implicated in socio-sexual motivation (medial preoptic area, nucleus accumbens, (Edwards & Einhorn, 1986; Everitt, 1990; Pfaus, Damsma, Wenkstern, & Fibiger, 1995)).

MATERIALS AND METHODS

Animals and Housing

Eighteen female 3-month-old Long Evans rats (Charles River, Raleigh, NC) were used in this study. Prior to arrival at our facility, animals were weaned at 21 days (+/- 1 day) and were housed in female colonies in barrier rooms with positive air pressure in open-top cages. Upon arrival at the University of Wisconsin-Madison, animals were housed in same-sex groups of three in an isolated room, in standard polycarbonate cages with corncob bedding. Animals were not exposed to normal (wild-type) or *PINK1* vocalizations of males until the study. Lights were maintained on a reverse 12:12 hour light/dark cycle with food and water available ad libitum. Behavioral procedures were conducted under partial red illumination within the first four hours of the dark period.

Playback Stimuli

Two 30-second (sec) stimulus tracks were constructed by concatenating strings of 50-kHz USVs previously recorded from 6-month-old Long Evans and 6-month-old *PINK1* KO (Long Evans) male rats. Recordings were made using a CM16 (Avisoft Bioacoustics) ultrasonic microphone placed 15 cm above the male's cage, with a 250-kHz sampling rate and 16-bit resolution (Grant et al., 2013). Male USVs were recorded within 90 sec after the removal of a receptive female following a brief interaction during which the male successfully demonstrated interest (sniffing, chasing, and mounting). Stimulus track wild-type (WT) consisted of USVs from wild-type rats and stimulus track PD model (PD) consisted of USVs from rats with homozygous KO of *PINK1* (Figure 1). USVs of homozygous *PINK1* KO males are significantly diminished in peak frequency, maximum intensity, and bandwidth compared with those of wild-type males (Grant et al., 2013). Since our aim was to determine the impact of acoustic deficits, USV strings used for the PD track were non-randomly selected from *PINK1* KO males that exemplified these acoustic deficits. USVs from three males of each condition were used to reduce possible bias due to individual acoustic characteristics of a particular male (Kroodsma, 1989). Independent-samples t-tests were conducted to confirm significant differences between the stimulus tracks for the three acoustic parameters above (Table 1).

The two playback tracks had a similar proportion of frequency modulated vs flat USVs and a similar total number of USVs (Table 1). The small differences between the tracks in the proportion and number of USVs were due to our sampling of unmodified strings. This sampling method maintained the organization of multiple, rapid vocalizations, which may be important to preserve semantic information in rodents (Chabout, Sarkar, Dunson, & Jarvis, 2015; Holy & Guo, 2005). To reproduce the natural USV intensity, playback intensity of the USV tracks (measured 20 cm from the speaker) was set such that USV intensity was equivalent to recordings. Intensity value comparisons are provided in Table 1 in dBFS

(decibel full scale, where 0 is the maximum possible intensity). Since the USV stimuli tracks contained background noises (ambient room noise and noise from the male rat moving around in its cage), we used a 30-sec recorded segment of background noise in which no USVs were produced as a control stimulus track (henceforth “NOISE”; Figure 1).

Comparison of segments of ambient noise across the three playback tracks confirmed that overall track intensity was presented within 1 decibel (dB).

Apparatus and Equipment

The experimental apparatus (Figure 2) was a 91.5 cm long $\times$ 30.5 cm wide $\times$ 30.5 cm tall acrylic Plexiglas arena, divided into three equal sized compartments (30.5 cm $\times$ 30.5 cm $\times$ 30.5 cm). Compartments were separated by 11.43 cm wide $\times$ 11.43 cm tall openings equipped with a sliding mesh gate mechanism. The distal ends of the apparatus each contained a floor-level 33 cm diameter hole covered with wire mesh, immediately beyond which were the ultrasonic speakers (Vifa Dynamic Ultrasound Speakers; 1–120kHz frequency range, Avisoft Bioacoustics, Glienicke, Germany). Playback was verified prior to the trials using an UltraSoundGate condenser microphone (CM16; Avisoft Bioacoustics). Behavior was monitored by a video camera (Sony HD Handycam Camcorder HDR-CX210, Sony Corporation of America, New York, NY, USA) 90cm from the side of the apparatus.

Procedure

Rats were handled daily beginning two weeks prior to testing. The week prior to testing, rats experienced two 10-minute (min) acclimation sessions on separate days to explore the experimental apparatus. Testing days were separated by a 4–5 day washout period to reduce possible habituation to experimental conditions. Furthermore, we implemented a balanced design such that one subset of rats experienced *Experiment 1* before *Experiment 2*, while the other subset experienced *Experiment 2* before *Experiment 1*. Rats were randomly assigned to experiment order. USV stimulus location (L side vs R side) was balanced for each stimulus presentation. For all trials, rats were placed in the apparatus and stimulus presentation was initiated after 30 sec. The apparatus was deodorized after each use with a 30% ethanol spray solution.

Experiment 1: Determining preference of USVs vs background noise—In *Experiment 1* we used a classical choice paradigm to test the hypothesis that female rats would prefer to visit and spend more time near the USV stimulus (WT or PD) over the simultaneously-playing background noise (NOISE). Each female (n=18) received 9 presentations of 30-sec stimuli sets, each followed by 90 sec of post-stimulus washout silence. The simultaneous stimuli sets WT vs NOISE and PD vs NOISE were each presented twice (alternating between the left and right speakers) and were preceded and followed by a NOISE vs NOISE stimulus set. The female was restricted to the central chamber during the first 15 sec of stimuli playback, and then allowed to explore the entire arena for the latter 15 sec of playback and subsequent 90 sec of post-stimulus silence.

Experiment 2: Determining preference of WT vs PD USVs—In *Experiment 2* we used a classical choice test paradigm to test the hypothesis that female rats would prefer to visit and spend more time near the WT playback stimulus over the PD playback stimulus

during simultaneous presentation of both USV stimuli. In *Experiment 2*, each female (n=18) received a total of 5 stimuli presentations: two simultaneous presentations of the WT vs PD stimuli (counterbalancing L/R speakers) preceded and followed by a presentation of NOISE vs NOISE. As in *Experiment 1*, the female was restricted to the central chamber during the first 15 sec of each presentation and allowed to explore the entire arena for the latter 15 sec of presentation as well as the remaining 90 sec of washout silence.

Experiment 3: c-Fos expression in response to PD and WT USVs—In

Experiment 3 we tested the hypothesis that female rats exposed to PD USVs would show reduced interest in the acoustic stimuli and reduced immediate early gene expression in brain areas related to socio-sexual motivation as compared to females exposed to WT USVs. In order to confirm that animals had not habituated to the USV playback stimuli, we included the NOISE control stimulus as a third condition and assessed behavioral interest across the three playback stimuli conditions (n=6 animals per condition). Unlike *Experiment 1* and *Experiment 2*, females were confined to one distal chamber of the apparatus and USVs were played using a speaker adjacent to that chamber. Females were presented with the identical 30-sec stimulus five times, separated by 90 sec of post-stimulus silence. Ninety min after the initiation of behavioral testing, animals were deeply anesthetized with 5% isoflurane and transcardially perfused with 500mL of cold 4% paraformaldehyde in 1% phosphate buffered saline (PBS). Brains were immediately dissected, post-fixed in paraformaldehyde for 24 hours and cryoprotected in .02% sodium azide solution. Coronal brain sections were sliced at 60 microns on a freezing microtome. Every fifth section was stored at -20°C until processing (described below).

Behavior Scoring

For *Experiment 1* and *Experiment 2*, preference was assessed during two consecutive time periods for each 30-sec stimulus presentation: a “Gate” period and a “Speaker” period. The Gate period consisted of the initial 15 sec of stimuli playback while the female rat was confined to the central chamber between the closed divider gates. The Speaker period consisted of the latter 15 sec of playback after the gate was lifted, allowing the animal to freely explore the entire arena. Interest in each playback stimulus was scored by calculating the duration a rat visited the gate (during the “Gate” period) and the speaker (during the “Speaker” period) corresponding to the side of the apparatus on which the stimulus was presented. A visit was defined as the rat pausing for at least one sec with its nose oriented toward and within three cm of the corresponding gate or speaker. The duration across all visits was calculated to arrive at a total duration measure for each period, similar to previous playback studies in rodents (Burgdorf et al., 2008; Musolf et al., 2010; Snoeren & Agmo, 2014; Willadsen et al., 2014).

For *Experiment 1*, we assessed preference for each USV stimuli (WT and PD) vs the NOISE stimulus by calculating the overall proportion (within-subjects) of the duration of visits to the USV stimuli ($\text{USV duration} / [\text{USV duration} + \text{NOISE duration}]$) for each rat during each measurement period. Similarly, for *Experiment 2*, we assessed preference for the WT stimulus vs the PD stimulus by calculating the overall proportion (within-subjects) of the duration of visits to the WT stimulus ($\text{WT duration} / [\text{WT duration} + \text{PD duration}]$) for each

rat during each measurement period. For *Experiment 3*, we calculated the total duration of visits to the speaker during playback of one stimulus type (between subjects). A speaker visit was defined using the same criteria described for *Experiment 1* and *Experiment 2*.

c-Fos Immunohistochemistry and cell quantification

Brain sections were immunolabeled with c-Fos primary antibody (c-Fos (K-25), sc-253, Santa Cruz Biotechnology, Inc., Dallas, TX, USA Santa Cruz Biotechnology, Inc.). Sections were rinsed in 0.01M phosphate buffered saline (PBS, pH=7.4) with 0.3% Triton X-100 (PBS-T; Tx-100 #21568-2500, Acros®, New Jersey) for 34 min, incubated in 0.5% H₂O₂ for 10 min, rinsed for 34 min, blocked in 20% normal goat serum (NGS, Equitech-Bio Inc, Kerrville, Texas) made in PBS-T with 2% Bovine Serum Albumin (BSA, Fisher Scientific #BP1600-100) solution for 1 hour and then agitated and incubated overnight in 1% NGS (made in PBS-T with 2% BSA) primary solution at 4° C (polyclonal rabbit anti-c-Fos at 1:4000). Sections were then rinsed in PBS-T for 34 min and incubated in conjugated biotinylated secondary solution for 2 hours at room temperature (goat anti-rabbit IgG at 1:500; Millipore LV1602347) in PBS-T and 2% BSA. Sections were then rinsed in PBS-T for 34 min, incubated in avidin-biotin solution (Vectastain Elite ABC, Vector Laboratories) for 1 hour, rinsed in 0.1M PBS for 30 min and the avidin–biotin complex was labeled using filtered 3,3-diaminobenzidine (DAB Easy Tablets, 10mg/tablet; 0.04%, Acros®, New Jersey) with 0.01% hydrogen peroxide solution and incubated for 13 min. Sections were rinsed for 25 min in 0.01M PBS, float mounted onto gelatin-coated slides, dried overnight, dehydrated in a graded series of alcohols and xylenes, and cover slipped (Fisherbrand® Microscope Cover Glass, 12–545-M, 24×60-1) using Cytoseal® 60 (Thermo Scientific Richard-Allan Scientific, 23–244256) mounting medium. All samples were run in a single batch to eliminate batch variability.

Cells demonstrating c-Fos immunoreactive label were quantified using bright-field microscopy with a Nikon microscope with a Spot Camera (Diagnositics Instruments, Inc.) connected to a computer with MetaVue software (Universal Imaging Corp). Quantification methodology was adapted from Ellis and Ritters (2013) and is similar to non-stereological counting methodology and total enumeration methodology (Gombash et al., 2014; Tramontin, Smith, Breuner, & Brenowitz, 1998). This methodology eliminates the possibility of double-counting cells based on our tissue sampling protocol. Quantification took place within standardized rectangles that were selectively placed onto the 20X magnification digital photomicrographs within the boundaries of the following 5 regions according to histological criteria of the rat atlas (Paxinos & Watson, 2005): primary auditory cortex (Au1, Area: 0.88mm × 0.67mm), periaqueductal gray (PAG, Area: 0.95mm × 0.69mm), cingulate cortex (Cg, 1.06mm × 0.71mm), medial preoptic area (MPA, Area: 0.13mm × 0.53mm) and nucleus accumbens (N. Acc, Area: 0.54mm × 0.40mm). The intensity threshold was manually adjusted for each region to differentiate labeled cells from the background. For each distinct region the same intensity threshold was used across all samples. Two measurements (left and right hemisphere) were made from three serial sections of the same region on each hemisphere for a total of six measurements per animal. These measurements were averaged to obtain one measurement for each animal. In cases of tissue damage, adjacent sections were used or the animal was dropped from the analysis. A

researcher masked to experimental conditions manually verified the number of discrete labeled cells that the program automatically labeled above threshold.

Statistical Analysis

All statistical analyses were conducted in R (R-Core-Team, 2014) and GraphPad Prism 5 (La Jolla, CA, USA). In *Experiment 1* and *Experiment 2*, one sample t-tests were used to assess preference as compared to a null hypothesis proportion of 0.5 (i.e. no preference). In the rare case ($n=3$) that an animal did not visit either stimuli during a given measurement period (Gate or Speaker), it was dropped from the corresponding analysis for that period. Although we assessed behavior at two measurement periods (Gate and Speaker), we did not correct for multiple testing in order to reduce inflated risk of Type II Error, which has been documented to be pervasive in animal behavioral studies (Jennions & Moller, 2003; Nakagawa, 2004). Due to small, normality assumption-violating sample sizes in *Experiment 3* ($n=6$ per group), the non-parametric Mann-Whitney U test was used to analyze pair-wise group differences in behavior and c-Fos expression between the NOISE, WT and PD playback stimuli. Pearson correlations were used to assess the relationship between c-Fos expression and behavior across all subjects. All behavior was scored by a single experienced observer masked to playback condition (stimulus type, location and order). Intra-rater and inter-rater reliability measures, assessed by rescoring 10% of the data (6 trials selected at random; total duration at both the gate and speaker), were confirmed to be high (intraclass correlation coefficient (ICC) >0.94 for intra-rater reliability, ICC >0.90 for inter-rater reliability). For all procedures, we used two-tailed tests with an alpha level of 0.05.

Ethics Statement

Protocols used for rat acquisition, housing, and behavioral testing were in adherence to guidelines approved by the *National Institutes of Health Guide for the Care and Use of Laboratory Animals* (DHEW Publication 80–23, Revised 1985, Office of Science and Health Reports, DRR/NIH, Bethesda, MD 20205). The protocol (Permit Number M02221) was approved by the University of Wisconsin-Madison Institutional Animal Care and Use Committee. All perfusion surgeries were performed under isoflurane anesthesia and all efforts were made to minimize suffering.

RESULTS

Experiment 1 - Determining preference of USVs vs background noise

Female rats consistently preferred WT over NOISE, with a mean proportion significantly greater than chance for total duration (Figure 3A) spent at the gate ($.67 \pm .06$, $t(17) = 2.59$, $p = .019$) and speaker ($.86 \pm .05$, $t(15) = 7.29$, $p < 0.0001$). In contrast, female rats showed no preference of PD over NOISE as measured by mean proportion of total duration (Figure 3B) spent at the gate ($.62 \pm .09$, $t(17) = 1.38$, $p = .19$) or speaker ($.52 \pm .10$, $t(16) = 0.21$, $p = .83$).

Experiment 2 - Determining preference of WT vs PD USVs

Females rats preferred WT over PD during the gate period (Figure 4), showing a significantly higher mean proportion of total duration near the WT gate as compared to chance ($.61 \pm .05$, $t(17) = 2.12$, $p = .049$). However, female rats showed no preference of

USV stimulus type (WT vs PD), measured by mean proportion of total duration spent at the WT speaker (Figure 4) as compared to chance ($.35 \pm .09$, $t(16) = -1.60$, $p = .13$).

Experiment 3 - Behavior response and c-Fos expression

Approach Behavior—Animals presented with USVs showed greater interest in the playback stimuli than those presented with NOISE. The mean total duration (Figure 5) spent near the speaker was significantly less in the NOISE condition (18.3 ± 5.0 sec) than in both the PD condition (38.2 ± 8.2 sec; Mann-Whitney $U = 31$, $n_1 = n_2 = 6$, $p = .045$) and the WT condition (38.7 ± 6.4 sec; Mann-Whitney $U = 31$, $n_1 = n_2 = 6$, $p = .045$).

c-Fos Expression and Correlations with Approach Behavior—Females presented with the WT stimulus showed significantly greater c-Fos expression in the N. Acc (Figure 6) than females presented with the PD stimulus (Mann-Whitney $U = 25$, $n_1 = n_2 = 5$, $p = .01$), whereas expression did not differ between NOISE and PD ($U = 12$, $n_1 = 6$, $n_2 = 5$, $p = .65$) or NOISE and WT ($U = 20$, $n_1 = 6$, $n_2 = 5$, $p = .41$) in the N. Acc. No significant differences were found in c-Fos expression (Table 2) across the NOISE, PD, or WT stimuli conditions for the PAG ($p = .21$), MPA ($p = .58$), Au1 ($p = .52$), or Cg ($p = .23$). Total duration spent near the speaker was significantly negatively correlated with c-Fos expression in the PAG, $r(14) = .52$, $p = .04$. No significant correlations were found between total duration spent near the speaker and c-Fos expression in any other brain area: N. Acc, $r(14) = -.02$, $p = .95$; MPA, $r(16) = -.035$, $p = .15$; Au1, $r(14) = -.41$, $p = .12$; and Cg, $r(16) = -.12$, $p = .63$.

DISCUSSION

Our results support our hypothesis that male *PINK1* KO vocal deficits negatively impact communication, demonstrating for the first time that pathology affecting ultrasonic vocalizations in a rodent model of PD can disrupt communication by impacting both the approach behavior and neuronal response of conspecific rat listeners. We replicated previous findings that presentation of male wild-type vocalizations induces approach behavior in females (Willadsen et al., 2014; Wohr & Schwarting, 2007). However, approach behavior toward *PINK1* KO USVs was less robust, showing higher approach compared to NOISE in the between-subjects test, but not in the within-subjects test. When simultaneously presented with WT USVs and *PINK1* KO USVs, female rats showed evidence of preference toward WT. Finally, we demonstrated that female rats presented with *PINK1* KO vocalizations showed decreased neuronal activity in the nucleus accumbens, a mesolimbic region implicated in socio-sexual motivation (Edwards & Einhorn, 1986; Everitt, 1990; Pfaus et al., 1995), as compared to female rats presented with wild-type vocalizations. Thus, our data suggest that vocal deficits observed in *PINK1* KO rodent models of disease have measurable impacts on social communication, producing meaningful changes in behavior and neural activity in conspecific receivers.

Wild-type USVs are preferred over background noise

Consistent with previous playback studies in rodents, presentation of wild-type 50-kHz USVs elicited greater approach responses than presentation of background noise control stimuli (Burgdorf et al., 2008; Sewell, 2009; Willadsen et al., 2014; Wohr & Schwarting,

2007). Our findings were corroborated using two separate experimental paradigms: (1) a within-subjects preference test directly comparing behavior in response to simultaneous presentation of WT USVs vs NOISE (*Experiment 1*) and (2) a between-subjects test comparing behavior of a subset of rats presented with WT USVs to a subset of rats presented with NOISE (*Experiment 3*). In *Experiment 1*, rats demonstrated a preference for wild-type USVs over background noise as indicated by a greater proportion of total visit duration than expected by chance (Figure 3). In *Experiment 3*, rats presented with wild-type USVs spent more time near the speaker than those presented with background noise (Figure 5).

PINK1 KO USVs are not consistently preferred over background noise

When subsets of females were presented with a single stimulus in a between-subjects design (*Experiment 3*), *PINK1* KO USVs yielded more approach behavior than did background noise, similar to wild-type USVs (Figure 5). However, when presented with a choice of simultaneous stimuli (*Experiment 1*), female rats did not show a preference for *PINK1* KO USVs over background noise (Snoeren & Agmo, 2013, 2014) (Figure 3). A lack of preference for USV stimuli over control stimuli using similar assessment techniques has been reported using presentation of 22-kHz USVs (Sales, 1991), which, unlike 50-kHz USVs, have been associated with negative valence and are considered aversive (Burgdorf et al., 2008; Sadananda et al., 2008). One recent study's results indicate approach behavior in response to frequency modulated, but not flat, 50-kHz USVs (Snoeren & Agmo, 2013, 2014). Similarly, presentation of 50-kHz artificial tones, which are distinctively lacking in frequency modulation, fail to induce approach behavior in rats (Wohr & Schwarting, 2007). Thus, it has been proposed that certain acoustic parameters of 50-kHz USVs underlie their appetitive quality, most notably a high degree of frequency modulation (Burgdorf et al., 2008) and high peak frequency (Wohr & Schwarting, 2007). Consistent with these findings, the present study reveals that male *PINK1* KO USVs, which are characterized by a lowered peak frequency and decreased bandwidth (a measure of frequency modulation), are not preferred by female rats over noise controls. This lack of preference is biologically meaningful, suggesting that changes in approach behavior result from the degradation of USV acoustic features seen in the *PINK1* KO model.

Evidence for wild-type USV preference over PINK1 KO USVs in a direct preference test

Female rats preferred wild-type USVs over *PINK1* KO USVs in a direct preference test in which the two stimuli were presented simultaneously (*Experiment 2*). This preference was specifically revealed by analysis of the initial 15 sec (Gate period) of each presentation (Figure 4), in which female rats were confined to a gated area but could choose to orient toward either distal chamber, beyond which were housed the playback speakers. Interestingly, analysis of the latter 15 sec (Speaker period) revealed high variability in proportion of time spent near the two speakers, with no statistical difference from chance in either direction (PD or WT) (Figure 4). Given the robust differences in response toward wild-type and *PINK1* KO USVs in *Experiment 1* and the preference for WT USVs during the Gate period of *Experiment 2*, we were surprised by the lack of preference for WT in the Speaker period of *Experiment 2*. Several explanations may account for this apparent inconsistency.

First, the potentially disorienting nature of simultaneous USV presentation while the animal was exploring the arena may account for behavioral differences. In *Experiment 1*, preference during the Gate period paralleled that of the Speaker period for presentation of USV stimulus vs background noise stimulus (i.e. WT was consistently preferred across the Speaker and Gate periods whereas PD was consistently not preferred across the Speaker and Gate periods). In *Experiment 2*, however, preference during the Gate period did not parallel that of the Speaker period for simultaneous presentation of two stimuli (PD vs WT), suggesting that the high variability in behavior was uniquely a result of simultaneous presentation of two USV stimuli. It is possible that amplitude differences (WT>PD) between the individual USVs in each track may account for some variation in behavior during simultaneous playback (i.e. the louder stimulus obscuring the quieter stimulus, thus affecting behavior), but one would expect WT to be preferred in both the Gate and Speaker periods if this were the case, which was not observed.

Second, it is possible the observed preference during the Gate period, but not the Speaker period, was due to the animals rapidly habituating to the USV presentation; previous studies in rodents have shown that approach behavior declines after the first presentation of repeated exposure to playback of USVs (Hammerschmidt, Radyushkin, Ehrenreich, & Fischer, 2009) or after the first minute of presentation (Wohr & Schwarting, 2012). However, habituation occurring at time scales less than a minute, as would be the case in our experiment, is unlikely. Furthermore, given that we observed significant behavioral differences between presentation of USV stimuli and the noise control stimulus that persisted to the final day of testing (*Experiment 3*), it is unlikely that habituation to USV stimuli was a major concern in the present study.

Third, it is possible that novelty contributed to female rat approach behavior, providing an incentive to switch between stimuli presented (i.e. approach WT in the gate period, then approach PD in the speaker period after sufficiently exploring the WT stimulus). Finally, there may be a bimodal effect in response, with some subjects showing decreased, and others increased, interest in PD compared to WT due to the perhaps unusual nature of the *PINK1* KO vocalizations. This is evidenced by the consistently observed higher variability in response to *PINK1* KO stimulus than wild-type stimulus in *Experiment 1* and *Experiment 3*. Although the total duration of the PD and WT tracks was identical, the total number of individual USVs differed by a small amount (4 USVs). It cannot be ruled out that this slight difference contributed to differences in behavior, but it is unlikely. Future studies should elucidate the precise acoustic features that underlie changes in preference between USVs. Nonetheless, differences in female rat behavior were demonstrated across the USV stimulus types (and background noise control) and uniquely detected in each of our experimental paradigms. As such, we recommend that future studies using USV playbacks incorporate more than one experimental paradigm, given the subtle difficulties of behavior assessment. Ideally, some combination of within-subjects design, between-subjects design, simultaneous stimuli presentation, and non-simultaneous (non-overlapping) stimuli presentation, as done in the present study, appears to be prudent.

Difference in c-Fos expression in response to WT vs PD stimuli

In addition to producing changes in behavior, USV stimulus type affected neural activity in female rat listeners. Female rats that received the wild-type stimulus had a higher number of c-Fos immunolabeled cells in the nucleus accumbens compared to females that received the *PINK1* KO stimulus (Figure 6). Despite the differences in behavior between presentation of the USV stimuli and background noise control (Figure 5), c-Fos measures did not significantly differ between USV stimuli and background noise (Table 2). This is similar to a previous study examining c-Fos measures in response to playback of rat USVs which primarily statistically treated an additional “arena-only” condition (no playback stimulus) as the baseline control condition (Sadananda et al., 2008). It is possible that the relatively short length of stimulus presentation in the present study (totaling 180 sec) might not yield as robust of c-Fos expression differences between playback stimuli as would a longer stimulus presentation regimen; further studies should examine the effect of stimulus duration. Interestingly, when examining all animals across conditions, we found a negative relationship between approach behavior and neural activity in the periaqueductal gray (PAG), but found no differences in neural activity when comparing conditions. Previous work detected greater c-Fos expression in the rostral dorso-medial region of the PAG in response to 22-kHz USVs as compared to 50-kHz USVs, but the same study did not analyze behavior in relation to c-Fos expression (Sadananda et al., 2008). Contrastingly, despite no direct discernable relationship with approach behavior, we found differential c-Fos expression across conditions in the nucleus accumbens, a mesolimbic region implicated in socio-sexual motivation (Becker, Rudick, & Jenkins, 2001; Edwards & Einhorn, 1986; Everitt, 1990; Pfaus et al., 1990; Pfaus et al., 1995) and appetitive behavior (Mogenson, Jones, & Yim, 1980). The nucleus is similarly implicated in female rodent social attachment and pair bonding (Aragona et al., 2006; Gingrich, Liu, Cascio, Wang, & Insel, 2000; Young & Wang, 2004) as it has direct connections to brain circuitry implicated in reward and multiple neuropeptides associated with affiliation and bonding (oxytocin, vasopressin, opioids) (Aragona, Liu, Curtis, Stephan, & Wang, 2003; Young, Lim, Gingrich, & Insel, 2001) as well as goal-directed behaviors (catecholamines) (Goto & Grace, 2005; Mas, Gonzalez-Mora, Louilot, Solé, & Guadalupe, 1990; Robbins & Everitt, 1982). In a study of male rats, nucleus accumbens dopamine release phasically increases during an initial playback of appetitive 50-kHz USVs suggesting that stimulation of dopamine neurotransmission in the nucleus accumbens may activate approach behavior and is critical in the perception of 50-kHz USVs (Willuhn et al., 2014).

In the present study, USVs used for playback were elicited from wild-type and *PINK1* KO Long Evans male rats upon exposure to receptive females. *PINK1* KO rats show a number of neural and gross motor impairments (Dave et al., 2014), and there are many potential mechanisms mediating the observed alterations in characteristics of male vocalizations in addition to neuromuscular deficits, including decreased affect or sexual motivation. In the present study, we were primarily interested in the impact of acoustic feature changes on intraspecific communication as measured by female approach behavior, but future studies should elucidate the causal factors underlying vocalization changes in the *PINK1* KO and other models of disease. Furthermore, unlike a majority of playback studies which have examined male behavior, playback of USVs was presented only to non-estrous females, so it

remains unknown whether males and females (estrous and non-estrous) show disparate patterns of approach and c-Fos expression to male PD USVs. Nonetheless, females preferentially approached and spent more time near the wild-type stimulus and showed increased c-Fos expression in response to wild-type compared to *PINK1* KO USVs. Thus, our data suggest that female rats are less motivated by male *PINK1* KO USVs than normal male USVs, which are widely known to induce a positive affective state and have been argued to play a key role in the facilitation of mating behavior, including female approach (Brudzynski & Pniak, 2002; Dizinno & Whitney, 1977; Pomerantz, Nunez, & Bean, 1983). This, in turn, suggests that the degradation of acoustic features of USVs observed in the *PINK1* KO vocalizations have properties that may result in impediments to successful communication; future studies should address the impact on social interactions and breeding success.

Broader Impacts and Future Directions

Our data are the first to demonstrate that the *PINK1* KO model shows impairments in social communication resultant from acoustic deficits in USVs. Animal models of PD, such as the *PINK1* KO discussed here, are an important tool for assessing disease pathology and elucidating the efficacy of potential treatments (Bezard & Przedborski, 2011; Chesselet & Richter, 2011; Dave et al., 2014). Importantly, assessing the validity of genetic construct models is critical to the application of translational research. Specifically, regarding early vocal deficits, the *PINK1* KO rat model exhibits degradation in acoustic parameters of 50-kHz vocalizations such as decreased bandwidth, intensity and peak frequency compared to wild-type controls. However, until now, the impacts of vocal deficits on intra-specific communication have not been quantified. Identifying and classifying communication deficits in *PINK1* KO will advance our understanding of the mechanisms underlying vocal communication deficits associated with PD. In order to evaluate the mechanisms of dysfunction, rodent models provide a robust research tool to elucidate behavioral dysfunction, pathophysiological mechanisms and potential treatments for deficits. The use of playback studies offers a unique translational tool for characterizing vocal integrity in models of disease by interpreting conspecific behavior and neural correlates in response to acoustic signals. Future studies may incorporate a playback paradigm to allow researchers to evaluate the efficacy of restorative treatments.

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Figure 1. Ultrasonic Vocalization Playback Tracks

Spectrogram showing a representative segment of the (A) wild type, (B) *PINK1* KO [PD], and (C) background noise playback tracks. Bandwidth and duration indicated by arrows. Darker shading indicates greater amplitude (intensity).

Figure 2. Playback Apparatus

Classic three-chambered choice test apparatus with sliding mesh gate mechanism. Playbacks presented first while gates closed ("Gate period"), then while gates lifted ("Speaker period"). Ultrasonic playback speakers are situated immediately beyond 3" diameter meshed holes at the distal ends.

Figure 3. Experiment 1: Preference of USVs vs Background Noise Control

Mean proportion of total duration spent near (A) wild-type [WT] USV stimulus and (B) *PINK1* KO [PD] USV stimulus during simultaneous presentation of WT and background noise or PD and background noise, respectively. Approach behavior measured first toward the gate divider, then to the speaker itself. Boxes indicate interquartile range (IQR), and contain the median (black line) with whiskers extending to 10th and 90th percentile. Observations beyond the 10th and 90th percentile are black circles. *indicates mean proportion significantly greater ($p < 0.05$) than 0.5 (chance).

Figure 4. Experiment 2: Preference of WT vs PD USVs

Mean proportion of total duration spent near wild-type [WT] USV stimulus over the total (WT + PD) during simultaneous presentation of WT vs *PINK1* KO [PD] USV stimulus.

Approach behavior measured first toward the gate divider, then to the speaker. Boxes indicate interquartile range (IQR), and contain the median (black line) with whiskers extending to 10th and 90th percentile. Observations beyond the 10th and 90th percentile are black circles. *indicates mean proportion significantly greater ($p < 0.05$) than 0.5 (chance).

Figure 5. Experiment 3: Approach Behavior

Total duration spent near speaker for each independent stimulus presentation (background noise control [NOISE], *PINK1* KO [PD] ultrasonic vocalizations, and wild-type [WT] ultrasonic vocalizations). Bars show mean $\pm$ SEM. $n=6$ animals per condition. Horizontal lines indicate significant pairwise group differences ($p < 0.05$).

Figure 6. Experiment 3: c-Fos Expression and USV Presentation

Photomicrographs of c-Fos immediate early gene expression (punctate black nuclear staining) in the rat nucleus accumbens after exposure to (A) background noise control [NOISE], (B) *PINK1* KO [PD] USV stimulus or (C) wild-type [WT] USV stimulus. Magnification is 10X, scale bar is 0.10mm; ac= anterior commissure. Mean c-Fos immunoreactivity in the nucleus accumbens (D), Bars show mean $\pm$ SEM. $n=5$ animals per condition. Horizontal line indicates significant pairwise group difference ($p < 0.05$).

Table 1

Acoustic differences of WT and PD playback stimuli. Values are mean $\pm$ SE (ms=milliseconds, kHz=kilohertz, FM=“frequency modulated”, dBFS=decibel full scale, where 0 is the maximum possible intensity).

Stimulus	Total USVs	Duration (ms)	Bandwidth (kHz)	Max intensity (dBFS)	Max peak frequency (kHz)	FM calls	Flat calls
WT	64	40.6 $\pm$ 3.3	30.9 $\pm$ 1.6	-44.86 $\pm$ 0.76	76.4 $\pm$ 1.4	85%	15%
PD	60	39.9 $\pm$ 2.4	13.3 $\pm$ 1.0 *	-51.94 $\pm$ 0.63 *	57.7 $\pm$ 1.0 *	81%	19%

* indicates p<0.001 compared with WT.

Table 2

c-Fos expression cell count (Experiment 3). Values are mean $\pm$ SEM.

	WT	PD	NOISE
PAG	18.5 $\pm$ 6.3 (n=5)	18.3 $\pm$ 2.6 (n=6)	23.6 $\pm$ 5.28 (n=5)
N. Acc *	148.8 $\pm$ 7.9 (n=5)	100.1 $\pm$ 6.2 (n=5)	108.6 $\pm$ 23.7 (n=6)
MPA	104.3 $\pm$ 10.9 (n=6)	92.7 $\pm$ 11.5 (n=6)	101.9 $\pm$ 16.5 (n=6)
Au1	81.4 $\pm$ 17.0 (n=5)	84.9 $\pm$ 18.9 (n=6)	95.1 $\pm$ 18.6 (n=5)
Cg	156.6 $\pm$ 30.9 (n=6)	159.4 $\pm$ 16.0 (n=6)	153.6 $\pm$ 10.1 (n=6)

*indicates PD significantly different from WT (p<0.05).