HHS Public Access

Author manuscript

Behav Brain Res. Author manuscript; available in PMC 2020 January 13.

Published in final edited form as:

Behav Brain Res. 2020 January 13; 377: 112175. doi:10.1016/j.bbr.2019.112175.

Early-onset Parkinsonian behaviors in female Pink1−/− rats

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Abstract

Parkinson disease (PD) is a progressive, neurological disease that affects millions of individuals worldwide. Although instability, rigidity, tremor, and bradykinesia are considered hallmark motor signs of the disease, these are not apparent until mid-to-late stage. In addition to limb motor impairment, individuals with PD also exhibit early-onset speech dysfunction and reduced vocal intelligibility as well as anhedonia and anxiety. Many of these clinical signs vary according to sex in humans with PD. In this study, a translational genetic rat model of early-onset PD (Pink1−/−) was used to address significant gaps in knowledge concerning sex-specific characteristics of limb sensorimotor deficits, vocal motor dysfunction, and changes in affective state. Traditional behavioral tests of limb function, ultrasonic vocalization, anxiety, and anhedonia in the Pink1−/− female rat and wildtype controls were used to test the hypothesis that behavioral performance would significantly differ between genotypes, and that these differences would increase with disease progression (age of the rat). Results demonstrate that Pink1−/− female rats do not exhibit limb sensorimotor deficits but do have significantly reduced intensity (loudness) of vocalizations, and present with anhedonia and anxiety by 8 months of age. Consistent with an early-disease model, Pink1−/− female rats do not exhibit significant decreases in nigrostriatal catecholamines/metabolites, as measured by HPLC. These results are significant in expanding knowledge of early-onset deficits in the female Pink1−/− genetic rat model of PD.

Keywords

Parkinson disease; Rat; Pink1; Ultrasonic vocalization; Catecholamine; Estrous

1. Introduction

Parkinson disease (PD) is a complex, progressive neurodegenerative disease that affects nearly 10 million people worldwide [1]. The etiology of PD in the early (*i.e.* preclinical) phases is not well understood. Whereas the hallmark signs of PD include limb motor

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Declaration of Competing Interest

None

impairments, in most cases, individuals with PD are affected by a variety of less recognized signs/symptoms that appear early in the disease including vocalization deficits (*e.g.* reduced pitch, monotone voice) and changes in anxiety levels as well as affective state (depression) [2–5]. In addition, there are well documented differences in how these signs/symptoms manifest in males compared to females, yet few basic research studies focus on female-specific impairments in the early stages of the disease.

Research demonstrates a significant effect of sex in the incidence and prevalence of PD, clinical manifestation, and progression of PD signs and symptoms, including pre-limb-motor issues [6–17]. Classical limb motor deficits in males occur at an earlier average age of onset and have a greater severity of motor symptoms [16]. Individuals with PD can also experience numerous other signs of the disease, including speech impairment (hypokinetic dysarthria) [3]. Notable sex differences have been found in multiple parameters of dysarthria, including intonation and prosody [6,10,18]. Impairments in functional communication can lead to social isolation and the aggravation of comorbidities including cognitive impairment and mood disorders such as depression and anxiety (affective state) [19–21]. Recent research indicates that changes to affective state are common in PD cases; for example, anxiety affects up to 60% of individuals with PD, most commonly affecting females and those diagnosed at a young age [3]. The frequent appearance of anxiety before the manifestation of motor signs suggests it may be mediated by neural mechanisms outside of the classical nigrostriatal pathway dysfunction, which is implicated in pathological motor execution and suggests a complex early-disease pathology. In addition to anxiety, major depression in PD occurs in 30–40% of patients and often involves apathy and anhedonia, or the inability to experience pleasure [22]. Similarly, multiple studies have reported higher rates and severity of depression in females [23]. Sex differences in both age of onset and limb motor signs have been postulated to result from the neuroprotective aspects of the hormone estrogen [24]. Given the complexity of PD and the known sex differences in its progression, it is necessary to understand the interplay of behavior and early-onset pathology in order to improve assessment and treatment.

To that end, several genetic rodent models have been developed that allow researchers to study various aspects of PD in the early (*i.e.* preclinical) stages of disease progression. The *PINK1* gene is one of the more common genes known to play a role in the development of PD in humans [25–27]. The *Pink1−/−* rat model replicates the progression of PD through both preclinical and mid-symptomatic phases, displaying characteristic features such as early-onset, slow progression of sensorimotor deficits, and brainstem neuropathology [28]. Previous research has extensively assayed this model for limb motor deficits as well as ultrasonic vocalization and swallowing deficits [29–35]. An additional consideration in the *Pink1−/−* rat model is the acknowledgement of sex-differences in research designs and the resulting potential sex-biased results, as primary research using the *Pink1−/−* rat model has been done exclusively in males. The female hormonal estrous cycle is known to alter behavioral states (outcomes) and is thought to interfere with interpretations of behavioral assays [36–38]. Previous research designs focusing on sex-differences in a rodent nigrostriatal lesion model of PD targeted the hormonal impact on cognition [39] and the neuroprotective aspects of estrogen [40,41]; however, this is the first study to address sex differences in a genetic and progressive PD model.

The purpose of this study was to examine progressive behavioral differences in female *Pink1−/−* rats versus age-matched wildtype controls using behavioral assays of limb sensorimotor function (tapered balance beam, cylinder limb test, nociception), ultrasonic vocalization, anhedonia (sucrose preference test), and anxiety (elevated plus maze, light/dark box) while simultaneously evaluating estrous cycle and body weight. We hypothesized that there would be significant differences in behavioral performance between females in estrus compared to females not in estrus on these tasks. Despite variability due to estrous state, we further hypothesized that the absence of the *Pink1* gene would significantly impair behavioral performance with disease progression (over time). More specifically, with disease progression, we hypothesized that ultrasonic vocalization acoustic and non-acoustic parameters would be significantly reduced, and *Pink1−/−* rats would show increases in anxiety and anhedonia compared to controls. Here, we tested these hypotheses.

2. Experimental methods

2.1. Animals and housing

A total of ten female Long-Evans rats with a homozygous *Pink1−/−* knockout and ten female Long-Evans wildtype rats (SAGE Laboratories (Horizon Discovery Group®)) were used in this study. A separate cohort of wildtype Long-Evans stimulus males (n = 6) were used in the experimental paradigm of collecting of ultrasonic vocalizations; these rats were not included in any analysis. All behavioral testing was completed at 2, 4, 6, and 8 months (mo) of age with the exception of the Light/Dark box, which was recorded at 3, 5, and 7 mo of age. Age of the rat was used as the quantifiable marker of disease progression. A figure of the experimental design is outlined in Appendix Figure A.1.

Rats arrived at 5 weeks old and were housed in groups of two (within genotypes) in standard polycarbonate cages (17 cm × 28 cm × 12 cm) with corncob bedding. Rats were handled every day for 7 days prior to the commencement of experimentation by both male and female research technicians [42]. Food and water were provided *ad libitum*, except during the Sucrose Preference Test, which involved overnight water restriction. All protocols and procedures were approved by the University of Wisconsin-Madison Animal Care and Use Committee (IACUC) and were conducted in accordance with the United States Public Health Service Guide for the Care and Use of Laboratory Animals (National Institutes of Health, Bethesda, MA, USA) under the protocol number M005177-R01.

2.2. Light cycle reversal

Because rats are nocturnal, a standard light cycle reversal program to establish a 12:12 h light: dark cycle was used for all rats throughout the study to ensure they were in an alert state during data collection. All experimental procedures (except for light/dark box and nociception) occurred under partial red illumination during the dark cycle to maintain light reversal effects.

2.3. Body weights

All rats were weighed (g) weekly per protocol to monitor health and weighed at each testing timepoint using a digital scale.

2.4. Estrous cycle

Because estrous phase can influence rat behavior [43], on each test day the female's current stage of estrous cycle (4 stages: proestrus, metestrus, estrus, diestrus; Appendix Fig. A1) was determined through vaginal lavage. A pipette was inserted approximately 1 cm into the vaginal canal and 0.20–0.25 ml of saline was used to flush the canal and then recollected by the pipette. Samples were then mounted on a slide and stained to confirm the estrous stage cytologically using the Wright staining technique for cytodiagnosis (Wright's Stain, Rapid Formula (Ricca, #9350)) [44]. To distinguish between the 4 stages, two raters noted the presence, quantity, and type of leukocytes and epithelial cells contained in the sample by visualizing under a microscope (Olympus FV1000 Laser Scanning Confocal Microscope, Madison, Wisconsin). Interrater reliability was over 0.95 for determination of stage.

Estrous stage was ultimately defined as 'Estrus' or 'Non-Estrus' in the statistical analysis. Non-estrus included pro-, meta- and diestrus phases. Characteristic estrus behaviors, such as darting, lordosis, and ear wiggling were also recorded during ultrasonic vocalization testing to cross-reference with lavage results.

2.5. Limb sensorimotor assays

2.5.1. Tapered balance beam—All rats were assessed for limb motor function as they traversed a 165 cm long tapered beam [45,46]. The final 1/3 of the beam had a tapered, reduced diameter to increase the complexity of the motor task. During the acclimation period (3 days at each testing timepoint), rats were re-introduced to experimental conditions prior to testing to reduce any novelty effects. On testing days, each rat was placed on a platform for loading and was allowed 5 trials to traverse the beam toward her home cage. To analyze motor performance, masked experimental raters reviewed video footage of the beam traversal; the variables analyzed were total time to traverse the beam (s), time to traverse the final 1/3 tapered part of the beam (s), and total number of foot-faults, or instances where the rat steps off the central portion of the beam. The average of the five trials was calculated per rat at each testing timepoint.

2.5.2. Cylinder test—Overall limb motor activity was measured in a transparent cylinder (20 cm × 30 cm) [47]. The cylinder was placed on a piece of glass, and a camera (Sony HDR-CX210) was positioned below to allow a clear view of movements along the ground and the walls of the cylinder. Recordings were viewed in slow motion and rated by a rater masked to genotype. The numbers of hindlimb and forelimb movements and number of rears and lands over a 1 min period were measured for each rat at each testing timepoint.

2.5.3. Nociception—Acute nociception was measured by the latency of hindlimb withdrawal (s) from a commercial thermal radiant heat stimulus device (IITC Life Science Inc., Plantar Analgesia Meter for Thermal Paw, Woodland Hills, CA, USA) [48,49]. Rats were acclimated to the experimenter and testing room for at least 3 days prior. On the day of testing, rats were habituated to the stimulus device for 15 min with a constant 30 °C base. The focused radiant heat (light) stimulus was applied to the plantar surface of each hind paw (4 × 6 mm), and the time until the paw was lifted was recorded (latency interval, s). Stimulus intensity (100%) and rate of heating was kept constant across the study. Maximal time of

heat exposure for all measurements was cut off at 20 s. Each rat was tested four times, alternating left and right hind paw, with at least 5 min between trials and the mean latency response for all measurements was calculated. This pattern allowed sufficient time between measurements to prevent learned responses or develop hyperalgesia to repeated stimuli [50,51]. All testing occurred in white light, but during the dark phase of the rat's cycle [52]. The average latency to withdrawal was calculated for each individual at each testing timepoint.

2.6. Ultrasonic vocalization

Rats use ultrasonic vocalizations (USVs) in a variety of social environments to communicate [53–57]. Ultrasonic vocalizations are often studied in the context of sexual encounters, as both partners tend to vocalize frequently throughout the experience. During the acclimation period, rats were re-introduced to experimental conditions prior to testing in order to reduce any novelty effects. Acclimation to testing procedures lasted 10 days and was followed by testing that lasted 5 days in order to maximize the recording of female vocalizations in a variety of estrous stages. During acclimation and testing, a female rat in her home cage was placed under the microphone (see below) and a stimulus male was placed in the cage with her. The male was removed after mounting the female or after three minutes of disinterest to avoid confusion concerning the origin of the calls. Following the removal of the male, female-only ultrasonic vocalizations were recorded for 90 s. Recordings were made in a sound-proof room using an ultrasonic microphone with high directional properties for recording (CM16 Avisoft, Germany), a flat frequency range of up to 150-kHz, and a working frequency response range of 10–180-kHz.

For acoustic analysis, a Fast Fourier Transform of 512 points with a frame size of 100%, flat top window, and temporal resolution of 75% overlap was used to build spectrograms from each waveform. A high pass filter was used to eliminate noise below 25-kHz. Calls were then categorized (simple, harmonic, frequency modulated (FM)) by independent raters masked to condition (see [30,58,59] for details). Because few-to-no harmonic calls existed for both genotypes over time, statistical analysis is focused on simple and FM call types. Total number of calls and percent complex calls were collected and analyzed. The average was calculated for simple and FM (ultrasonic vocalizations in the following parameters: duration (s), bandwidth (Hz), intensity (loudness, dB), and peak frequency (Hz). Analysis of peak frequency revealed no additional significant information and subsequently is presented in the supplementary tables. Maximum and average of the top 10 calls were also analyzed; they did not significantly differ from the average and thus are presented in the supplementary tables; the results section and corresponding graphs are focused on the average simple and average FM duration, bandwidth, and intensity. Interrater reliability was performed on a subset of the data to confirm accurate results between experimenters; reliability was over 0.85 between raters.

2.7. Anhedonia

2.7.1. Sucrose preference curve—Sucrose preference testing is often used as a measure of depression and anhedonia in animal experiments and has been found to be a valid measure in numerous antidepressant study paradigms [60,61]. Optimal concentration

of sucrose was chosen based on development of a sucrose curve. Briefly, sucrose solutions were prepared at concentrations of 0.5, 1, 2, 8, 15, or 30% (weight/volume) and put into 50 ml conical tubes (filled to 45 mL) fitted with sipper caps (steel sipper tubes inserted through a rubber stopper). Concentration order was randomized using a Latin Squares design, with a 24 h delay between tests. Rats were allowed free access to water for three hr after testing and then resumed water restriction. To eliminate side-bias, the order of the centrifuge tubes rotated daily. These data were used to plot a sucrose curve to determine the optimal sucrose concentration.

2.7.2. Sucrose preference testing—For three days prior to each testing timepoint, all rats were introduced or re-introduced to experimental conditions to reduce any novelty effects. Rats were singly housed in an empty cage and given a pair of 50 ml conical tubes fitted with sipper caps. On the day of testing, following overnight water restriction, rats were individually placed in an empty cage and given one 50 ml conical tube containing 8% sucrose solution (in reverse osmosis (RO) water) and one containing 100% RO water. Rats were then left to drink from either conical tube *ad libitum* for 2 h. The position of the tubes was switched regularly to reduce any influence of side bias. Sucrose preference was calculated as a percentage of the volume of sucrose intake over the total volume of fluid intake and averaged over the testing timepoint for each rat.

2.8. Anxiety

2.8.1. Elevated plus maze—The elevated plus maze (EPM) is used to study signs of anxiety in a neurobiological context [62]. Within this assay closed arm activity is associated with increased anxiety and open arm activity is associated with decreased anxiety. Prior to the beginning of testing, the rats were habituated to the room to decrease the confound of novel surroundings. Each rat was then placed in the center of the maze and allowed to explore for 5 min while a Basler ac1300–06 (Basler GenIcam Exton, PA) camera monitored the rat's movements around the arena. The maze consists of two closed arms with high walls but no roof, and two open arms, crossed in an "X" shape and elevated 50 cm off the ground. Variables analyzed included number of entries into open and closed arms, and time (s) spent in open and closed arms for each rat. Data was processed and analyzed with Ethovision (Noldus Ethovision XT (Wageningen, Netherlands)).

2.8.2. Light/dark box—In order to avoid confounds in testing (*i.e.* habituation) all rats were tested with a 4 week washout period between EPM and light/dark box; testing for this assay occurred at 3, 5 and 7 mo [63]. The Light/Dark Box assay is based on the tendency of rats to spontaneously explore in response to mild stressors combined with the aversion to bright light. The light chamber (30 cm × 40 cm × 44 cm) is brightly illuminated with white fluorescent light from above, whereas the dark chamber (20 cm × 40 cm × 44 cm) receives no illumination. The two chambers are connected by a 10 cm × 15 cm door. Rats were placed in the center of the light chamber and data was video recorded with a Basler ac1300–06 (Basler GenIcam Exton, PA) camera positioned 120 cm above the apparatus for 5 min. Measures collected include total occurrences of rat in light chamber, total transitions, total time spent in light chamber (s), percent time spent in light chamber (%, time spent in light chamber/total time × 100), total time in dark chamber (s), percent time spent in dark

chamber (%, time in dark chamber/total time × 100), number of nose pokes and total duration of nose pokes (s). Nose pokes were operationally defined as presence of nose through mid-body in the light chamber with mid-body through tail simultaneously in dark chamber. Total time in dark chamber was calculated by subtracting time in light chamber and duration of nose pokes from total time. Time in the light chamber and total number of transitions between chambers are inversely correlated with measures of anxiety. Nose pokes into the light chamber represent an exploratory and/or potential risk assessment behavior [64]. Video and tracking data was automatically analyzed by Ethovision (Noldus Ethovision XT (Wageningen, Netherlands)) and simultaneously reviewed by two independent raters to ensure accuracy of computer-based measurements (rater reliability above 0.90).

2.9. Tissue harvest, processing, and high-performance liquid chromatography

After testing at 8 mo of age, rats were deeply anesthetized with isoflurane and rapidly decapitated. The brains were dissected and immediately frozen and stored at −80 °C. Brains were sliced coronally on a cryostat at 250 μm thickness at −15 °C and mounted on gelatin-coated glass slides. A 2 mm tissue punch was collected bilaterally within the striatum (SR; 6 total punches) and substantia nigra (SN; 4 total punches) using the Brain Punch Set (FST 18035–02, Foster City, CA, USA) under a dissection microscope over dry ice (Fig. 1). Tissue samples were transferred to microcentrifuge tubes and stored at −80 °C. Anatomically equivalent sections were used from each rat.

Tissue samples from the SR, SN were homogenized using 0.5 ml of 0.2 M perchloric acid and 100 mg isoproterenol in 0.1 M acetic acid including 2 mg/mL EDTA-2Na per 100 mg wet tissue weight. Homogenates were sonicated on ice for 5–10 s, incubated on ice for 30 min, and centrifuged at 13,000 rpm for 15 min at 4 °C to pellet the cell debris. The supernate was collected, and the pH modified with 1 M sodium acetate to become a pH of 3.0. Samples were filtered through a spin column and stored at −80 °C for downstream High-Performance Liquid Chromatography (HPLC) analysis.

HPLC was used to determine the concentration of the catecholamines and their metabolites (Table 1). All samples were analyzed by Amuza Inc. of San Diego, CA, using an Eicom HTEC-510 HPLC-ECD equipped with a graphite electrode, an Eicom SC-3ODS reverse phase C18 column, and an AS-700 autosampler. The analysis was performed at 25 °C and the detector set at +750 mV *vs.* Ag/AgCl. The mobile phase was 0.1 M citrate-acetate buffer (pH 3.5), 15% methanol, 190 mg/l sodium decane sulfonate, and 5 mg/l EDTA. The flow rate was 0.4 ml/min. The pg/uL for each metabolite was analyzed by brain region and genotype.

2.10. Statistical analysis

All statistical analyses were conducted with SAS 9.4 Software (SAS Institute, Cary, NC), SigmaPlot® 12.5 (Sys-tat Software, Inc., San Jose, CA, USA) or GraphPad Prism™ 7 (GraphPad Software, Inc., La Jolla, CA, USA). Data is presented as means and standard error of the means (SEM). Results are reported as: interaction data, main effect data, and post-hoc analysis. Changes to degrees of freedom correlate to lost data points.

A two-way repeated measure analysis of variance (ANOVA) was used to analyze changes in body weight over time (2–8 mo) and between genotypes (*Pink1−/−* vs wildtype). Fishers Least Significant Difference (LSD) was used for post-hoc comparisons. Assumptions of ANOVA (normality and variance were assessed. Critical level of significance was set a priori at 0.05.

A three-way, repeated measures analysis of variance (ANOVA) was used to evaluate the behavioral variables (limb sensorimotor, acoustic, anhedonia, and anxiety) between genotype (*Pink1−/−* and wildtype and estrous phase (Yes, No) over time (2, 4, 6, 8 mo). Linear mixed-effects models with a random intercept for rat were fit with genotype, estrous stage, and time as fixed effects, and adjusted for body weight. This module was assessed for homogeneity of covariance matrix and normally distributed residuals. Type III tests were used to assess predictor significance. The Bonferroni method was used to correct for multiple comparisons with analyzes conducted at the 0.05/3 = 0.017 significant level (to account of the fitting of models for each outcome).

Concentrations of brain neurotransmitters and metabolites quantified with HPLC and genotype were analyzed with one-tailed student's t-tests within each brain region (SR and SN), respectively. Norepinephrine and epinephrine were unable to deconvolute within several samples; two separate animals (one per brain region) did not have samples that fell within the range of detection and were omitted from the statistical analysis.

3. Results

Means (SEM) for behavioral data are presented in Table 2; means (SEM) for ultrasonic vocalization acoustic parameters are in Table 3 (FM vocalizations) and Table 4 (Simple vocalizations). Body weight was not found to be a significant co-variable in any of the analyses, and subsequently is presented in a separate section below. For clarity, this section reports significant findings; for transparency, all other data can be found in the supplementary data (Appendix Tables A.1–A.5) including interaction/main effect F statistics (df) or t-statistics (df) (as appropriate), and p values.

3.1. Body weight

There was no interaction between genotype (*Pink1−/−*, wildtype) and testing timepoint (2, 4, 6, 8 mo) ($F(3, 54) = 0.99$, $p = 0.41$). However, there was a main effect of genotype, where all *Pink1−/−* rats were significantly heavier at each timepoint compared to wildtype controls ($F(1, 54) = 7.86$, $p = 0.012$) (Fig. 2). Additionally, there was a significant main effect of time, as rats from both genotypes, as expected, gained weight over time ($F(3, 54) = 125.61$, $p < 0.001$). Post hoc analyses showed significant differences between 2 and 4, 2 and 6, 2 and 8 mo as well as between 4 and 8 and 6 and 8 mo ($p < 0.001$ for all comparisons).

3.2. Limb sensorimotor control

3.2.1. Tapered balance beam—There was a significant interaction between genotype and timepoint for the total time to traverse the whole balance beam ($F(3, 46) = 4.25$, $p = 0.0099$; Fig. 3A). In general, over time, rats from both genotypes traversed the whole beam more quickly. However, post hoc analysis showed that *Pink1−/−* rats were significantly

slower than wildtype at 2 mo of age ($p = 0.0004$). Neither estrous stage (Yes or No) and timepoint or genotype and estrous stage produced significant interaction effects for any of the parameters measured.

There were no significant interactions for genotype × timepoint × estrous for the time to traverse the last 1/3 of the beam (Fig. 3B) or for the number of foot-faults (Fig. 3C). There was a significant main effect of time for time to traverse the last 1/3 of the beam ($F(3, 46) = 5.18$, $p = 0.0036$), and number of foot-faults ($F(3, 44) = 6.20$, $p = 0.0013$). Post hoc tests for time to traverse the last 1/3 of the beam revealed significant differences between performance at 2 and 6 mo ($p = 0.026$), 2 and 8 mo ($p = 0.0007$) and 4 and 8 mo ($p = 0.0014$). For foot-faults, post hoc analysis showed there were significant differences between 2 mo and 4 ($p = 0.0001$), 6 ($p = 0.0091$), and 8 mo ($p = 0.0133$).

3.2.2. Cylinder—For the cylinder test of limb motor movements, there were no significant interactions between genotype × timepoint × estrous. There were no significant main effects of genotype for hindlimb movements. There were main effects of time for the number of hindlimb movements ($F(3, 44) = 10.88$, $p < 0.001$) where all rats had a decrease in the number of hindlimb movements in the cylinder over time (Fig. 4A). Specifically, there were significant differences between 2 and 4 mo ($p = 0.0010$), 2 and 6 mo ($p < 0.0001$), 2 and 8 mo ($p < 0.001$), 4 and 6 mo ($p = 0.0031$) and 4 and 8 mo ($p = 0.0027$). Additionally, there were no significant main effects of time or genotype for the number of total forelimb movements (Fig. 4B) or total number of rears and lands (Fig. 4C).

3.2.3. Nociception—There was a significant interaction between genotype × timepoint ($F(3, 47) = 7.03$, $p = 0.0005$; Fig. 5) for the average latency to withdraw from the thermal stimulus. Specifically, there were significant differences at 2 mo compared to all other timepoints (4, 6, 8 mo) ($p < 0.001$ for all comparisons). At 2 mo both *Pink1*−/− and wildtype rats had significantly longer latencies to withdrawal compared to other timepoints. There were no other genotype differences at 4, 6, or 8 mo of age.

3.3. Vocalizations

3.3.1. Number of calls—When examining the total number of calls produced, there was a significant interaction between genotype and timepoint ($F(3, 104) = 3.14$, $p = 0.028$; Fig. 6A). Post hoc analysis showed significant differences between genotypes occurred at 2 mo of age ($p = 0.034$). For both genotypes, there was a significant difference in the number of calls at 2 mo compared to 4 ($p = 0.0062$), 6 ($p = 0.011$), and 8 mo ($p = 0.026$). In general, both genotypes called approximately 1.4 times more when in estrus than when not in estrus (Fig. 6B).

3.3.2. Percent complex calls—There were no significant interactions for percent complex calls. There was a significant main effect of time for the percent of calls designated as complex ($F(3, 99.5) = 9.62$, $p < 0.0001$; Fig. 7), but no other significant main effects. In general, it is noted that all rats produced a lower percentage of complex calls over time. Post hoc analysis revealed significant differences between 2 and 4 mo ($p = 0.022$), 2 and 8 mo ($p = 0.0012$), 4 and 6 mo ($p = 0.0070$), 4 and 8 mo ($p < 0.0001$), and 6 and 8 mo ($p = 0.0087$).

3.3.3. Duration—There were no significant interaction effects (genotype × timepoint × estrous) for duration. There was a main effect of timepoint for both simple and FM calls, respectively ($F(3, 99.2) = 10.18$, $p = < 0.0001$; Fig. 8A) ($F(3, 83.9) = 4.04$, $p = 0.0099$; Fig. 8B). Post hoc analysis showed that for average duration, simple calls were significantly different at 4 mo than at 2, 6, or 8 mo ($p < 0.0001$ for all timepoints). FM calls were significantly different between 8 and 2 mo ($p = 0.0028$) as well as 8 and 4 mo ($p = 0.0032$). The 4 mo timepoint was the driving factor in the significant reduction in simple duration. However, at 8 mo the FM calls showed the opposite finding where there was an increase in duration compared to earlier timepoints.

3.3.4. Bandwidth—There were no significant interactions for average bandwidth (genotype × timepoint × estrous). There was a main effect of timepoint for the average bandwidth of simple calls ($F(3, 98.1) = 22.84$, $p < 0.0001$; Fig. 9A); post hoc tests revealed a significant difference when comparing simple calls at 4 mo with 2 ($p < 0.0001$), 6 ($p < 0.0001$), or 8 mo ($p = 0.0005$) timepoints. There were no significant main effects for the average bandwidth of FM calls ($F(3, 86 = 1.22$, $p = 0.31$; Fig. 9B).

3.3.5. Intensity—There were no significant interaction effects on any measures of intensity (genotype × timepoint × estrous). There was a main effect of genotype for FM calls for average intensity ($F(1, 27.1) = 8.43$, $p = 0.0073$; Fig. 10). Post hoc tests revealed that in general, *Pink1−/−* rats produced FM vocalizations with less average intensity than wildtype controls. A representative spectrogram can be found in Fig. 11.

3.4. Anhedonia

A sucrose preference curve generated prior to study initiation demonstrated that 8% sucrose yielded detectable effect (Fig. 12A). Statistical analysis revealed no significant interaction effects. There was a main effect of genotype, ($F(1, 18) = 5.34$, $p = 0.033$), with *Pink1−/−*rats consuming on average a lower percentage of sucrose when compared to wildtype controls (Fig. 12B). Estrous stage ($F(1, 13) = 0.72$, $p = 0.41$) and timepoint ($F(3, 48) = 0.95$, $p = 0.426$) did not show significant main effects.

3.5. Anxiety

3.5.1. Elevated plus maze—EPM analysis indicated there were significant interaction effects between genotype and timepoint for number of entries into open arms ($F(3, 47) = 5.10$, $p = 0.0039$) and time spent in open and closed arms ($F(3, 47) = 8.37$, $p = 0.0001$) (Fig. 13A–D). Post hoc analyses showed that at 4 mo, wildtype rats spent significantly more time in closed arms and at 6 mo made significantly fewer entries into open arms than *Pink1−/−* rats. At 8 mo, *Pink1−/−* rats made fewer entries into open arms and spent significantly less time in open arms than wildtype controls.

There were no significant interaction effects for number of entries into closed arms. However, there was a significant main effect of timepoint for time spent in closed arms ($F(3, 47) = 5.17$, $p = 0.0036$).

3.5.2. Light/dark box—There was no significant interaction between genotype (wildtype, *Pink1−/−*) and testing timepoint (3, 5, 7 mo) ($F(2, 36) = 1.98$, $p = 0.15$) on the Light/Dark Box assay. There was a significant main effect of timepoint ($F(2, 36) = 15.08$, $p < 0.001$) and a significant main effect of genotype ($F(1, 36) = 13.20$, $p = 0.002$) (Fig. 14). Specifically, at each timepoint all rats spent more time in dark (s); post hoc analysis demonstrated differences between 3 and 5mo ($p < 0.001$), 3 and 7 mo ($p = 0.002$) and 5 and 7 mo ($p = 0.043$). Furthermore, all *Pink1−/−* female rats spent less time in the dark compared to wildtype control rats ($p = 0.002$).

3.6. HPLC

There were significant differences between genotypes (*Pink1−/−*, wildtype) for the concentration of MHPG in the striatum ($t(17) = 2.008$, $p = 0.030$), where *Pink1−/−* rats had significant increased concentrations compared to wildtype. This also corresponds to the trend for a decrease in NE in the striatum ($t(11) = 1.51$, $p = 0.08$). Additionally, there was a trend for an increase in 3MT in the striatum of *Pink1−/−* rats ($t(17) = 1.62$, $p = 0.06$) compared to wildtype. There were no other significant differences between genotypes and neurotransmitters/metabolites (DA, EPI, 5HT, DOPAC, HVA, or HIAA) in the striatum (Fig. 15A, C, E; Table 1/**8**). There were no significant differences between genotypes and any of the HPLC variables in the substantia nigra (Fig. 15 B, D, F; Table 1).

4. Discussion

PD is a progressive, degenerative disorder that results in deficits in limb and cranial sensorimotor control as well as changes to anxiety and affective state that negatively impact an individual's health and quality of life. However, most basic mechanistic research in rodents has been conducted exclusively in males. The purpose of this study was to assay limb sensorimotor function, ultrasonic vocalization, anxiety, and anhedonia in the Pink1−/− female rat model of early-onset PD. We tested the specific hypotheses that *Pink−/−* female rats would demonstrate impaired limb motor and vocal performance with increased anxiety and anhedonia, and that changes which would become more pronounced with disease progression. The statistical analysis was designed to account for body weight; *Pink1−/−*rats are consistently heavier compared to controls [30,31]. Finally, estrous phase appeared to be an important variable for the number of calls produced within a testing session, but did not interact with other limb motor, acoustic, anhedonia, or anxiety behaviors. Associated estrus behaviors including darting or freezing did not appear to influence behavioral performance. Concurrent with the male literature, the female *Pink1−/−* rat shows several early-onset signs that may be useful for researchers studying the onset of cranial sensorimotor dysfunction as well as changes to affective state in the absence of significant nigrostriatal catecholamine dysfunction.

4.1. Pink1−/− female rats do not show a consistent decline in limb motor performance with disease progression

There were no observed significant differences in the female *Pink1−/−* rat compared to wildtype in both the tapered balance beam and the cylinder tests of limb motor function. This finding suggests that disease progression may follow a different time-course in females

compared to what has been previously noted in male *Pink1−/−* rats [30]. This sex difference in motor deficit onset is analogous to what has been noted in humans, where the average age of onset of clinical features is on average 2.1 years later in females than in males and in the early stages is characterized as being more "benign" in females [65]. Applying the rat-human aging comparison presented by Sengupta, 2013 where one human year is the equivalent of two rat weeks, we calculated the age of the rat in human years. Considering the slightly later onset and more benign nature of early clinical features in human females, we postulate that limb deficits may begin to appear in the female *Pink1−/−* model at around 9–10 mo of age, highlighting an area for future research.

We did observe significant differences in limb motor performance at 2 mo of age, where *Pink1−/−* rats were slower to traverse the whole tapered balance beam, suggesting the *Pink1−/−* females demonstrated more difficulty on the task at a younger adult age and made a more drastic improvement in performance than wildtype rats with age. It is possible that this finding could be attributed to the acclimation period, the fact that this was the first timepoint tested, or alternative neurological factors such as cognition; however, we did not directly test cognition in this study. All animals were first tested at 2 mo of age, it is possible that *Pink1−/−* rats could not compensate in the first exposure to the task which is reflected in the longer transit times. Similar to previous work, all rats, regardless of genotype, took longer to cross the last 1/3 of the beam, increased the number of errors (foot-faults), and performed fewer hindlimb movements in the cylinder with age [30]. Additionally, because rats can make compensatory adjustments during behavioral testing, we retrospectively analyzed the first trial at 8 mo of age (time to cross whole beam, time to cross last 1/3, and foot faults with the hypothesis that the first trial may be an more sensitive measure of deficit. Those results also show that at the 8 mo timepoint there were no genotype differences for these measures ($p > 0.05$ for all t-tests). Moreover, there were no significant genotype differences in the latency to remove the foot from a thermal source, which is consistent with the non-significant findings on the beam and cylinder test. Interestingly, the 2 mo timepoint did show significant increases in latency compared to other timepoints.

Our lab and others have reported that male *Pink1−/−* rats exhibit an unexpected hindlimb deficit (dragging of hindlimbs, but without overt flaccid or spastic paralyses or sensory deficit) at 6 mo of age, disappearing by 8 mo of age [29,30]. We used detailed qualitative observations during this period in females and only one female exhibited any signs of hindlimb issues, which resolved within a week of onset. This finding is important in description of the model and is another significant difference between previously reported studies in males.

4.2. Pink1−/− female rats show reduced loudness of ultrasonic vocalizations

Pink1−/− female rats demonstrated reduced average intensity (loudness) of the FM calls as compared to wildtypes; however, there were no differences in intensity for simple calls. We posit that FM calls may have more translational relevance to human speech due to the presence of rapid pitch variation. Likewise, human speech deficits in PD often include impairments in loudness and pitch variation, leading to a soft and monotone voice [10,19,66,67]. Additionally, similar to work done in the male *Pink1−/−* rat, there were no

main effects of time, suggesting that the intensity deficits were stable over time. Interestingly, the previous work in *Pink1−/−* male rats show significant deficits encompassing multiple acoustic variables including bandwidth, intensity, call complexity, and call rate between 2 and 8 mo of age [30]. By contrast, female *Pink1−/−* rats do not show the same progressive vocalization deficits.

Both genotypes produced significantly more calls when they were in estrus than when they were not in estrus, which may be related to how vocalizations are elicited using mating behaviors. Females often vocalize within mating scenarios [53] and do not produce, or produce few vocalizations when they are ovariectomized [68]. Thus, it is likely that significantly increased vocalization in estrous serve a biologically relevant communicative purpose. At the 2 mo timepoint, *Pink1−/−* rats had a significantly higher number of calls, however this trend was reversed by 8 mo. All female rats, regardless of genotype, produced significantly fewer complex calls with age.

For most of the acoustic variables, there were main effects of time, but there were no obvious trends when examining which timepoints were significantly different among parameters and call types; for example, duration of FM calls increased from 2 to 8 mo but there were no differences in FM bandwidth. A proposed reason for the scarcity of vocal deficits is a possible slower progression of vocal degradation in females. The 2–8 mo timepoint range may have been too early in the manifestation of PD to observe subtle acoustic differences. Prior research in animal models has posited that estrogen and progesterone may be neuroprotective and play a role in ameliorating the early effects of PD neuropathology [13,39,69]. Overall, these data suggest vocal dysfunction at an early-mid stage onset in females similar to the deficits identified in males [30].

4.3. Anhedonia is increased in Pink1−/− female rats at 2 mo

Pink1−/− female rats ingested a significantly lower percentage of sucrose solution than wildtype controls, suggesting the presence of anhedonia in the *Pink1−/−* females beginning as early as 2 mo; however, this disproportion did not increase over time. In general, research investigating anhedonia in PD is sparse, owing to challenges in defining and assessing the disorder in patients [22]. There are even fewer studies examining mood disorders in females with PD [23], and studies often produce mixed results due to study population differences, retrospective versus prospective data collection, and differences in methodological design.

4.4. Anxiety increases in Pink1−/− female rats

In general, *Pink1−/−* female rats display behavior that is consistent with the onset of anxiety in the EPM. *Pink1−/−* rats did not spend significantly more time in closed arms or make more entries into closed arms; however, the abrupt decrease in time spent in open arms suggests *Pink1−/−* rats experienced a heightened anxiety at 8 mo of age. At 8 mo, wildtype rats demonstrated less anxiety and were more willing to explore open arms than *Pink1−/−* rats, the inverse of what occurred at previous timepoints. Given that other changes we expected to appear prior to 8 mo (vocalization deficits) and at 8 mo (limb deficits) did not occur, it may be that the timeline for female brain pathology/parkinsonian phenotype is different than previously hypothesized. The method of analysis used in this study described

the overall time spent in each arm and number of entries, as a whole documenting general locomotor activity. It did not take into consideration the time spent in transition. Long Evans rats, phenotypically, are an exploratory strain(i.e. compared to Sprague Dawley {Turner, 2014 #2026}) which may account for increases in number of movements from arm to arm.

Although wildtype rats demonstrated more anxiety in the Light/Dark Box test, the difference in magnitude between genotypes decreased dramatically between the 5 mo timepoint and the 7 mo timepoint. When compared to the EPM results, the *Pink1−/−* rats did not start to display more anxiety than wildtype rats until 8 mo of age. Thus, this gradual progression of anxiety in *Pink1−/−* rats in the Light/Dark Box up until 7 mo corresponds with the trajectory of anxiety as measured by the EPM as well. Further, the overall results of the Light/Dark Box test, the tendency for the *Pink1−/−* to spend more time in open arms, is similar to findings in 12 mo old A53 T alpha-synuclein mice {Graham, 2010 #2027, {George, 2008 #2028}}. In other words, *Pink1−/−* anxiety may manifest at later timepoints; therefore, this hypothesis should be tested in future longitudinal studies.

4.5. Pink1−/− female rats do not show significant catecholamine reductions at 8 mo of age

Consistent with a preclinical timepoint, there were no significant genotype differences in dopamine or its metabolites (DOPAC, HVA, 3MT) in the striatum or the substantia nigra. There were also no significant differences in norepinephrine, epinephrine, or serotonin (and HIAA) between genotypes in either brain region. There was a significant difference in MHPG in the striatum, where *Pink1−/−* rats had increases in this metabolite (but not the substantia nigra). There was a trend ($p = 0.08$) for a decrease in striatal norepinephrine, consistent with an increase in MHPG. Previous work in male *Pink1−/−* rats by Grant et al., showed no significant differences in TH immunolabeling (rate limiting step in catecholamine synthesis) in the striatum or substantia nigra. However, other work (in males) has shown that brainstem (locus coeruleus) catecholamine concentrations are disrupted [59]; and, noradrenergic cells in the locus coeruleus are positively correlated to vocalization intensity. Norepinephrine is hypothesized to be degraded earlier in the disease process [70], and further dysfunction is hypothesized to ascend rostrally from brainstem to cortex [71–73]. The increases in striatal MHPG reported here may suggest early-stage noradrenergic dysfunction that should be examined at later timepoints in this model; likewise, further evaluation of the brainstem nuclei is warranted. Work by Dave et al. (2014) has shown that male 8 mo *Pink1−/−* rats exhibit increased dopamine and serotonin in the striatum that correspond to gait and motor features. This, along with the absence of significant limb motor and striatal changes in female rats is suggestive of a sex-specific difference in this PD model. Additionally, Grant et al. (2015) showed insoluble alpha-synuclein in the brainstem; this study did not assay for the presence of abnormal alpha synuclein; however, this is an area for future study.

4.6. General conclusions

This work expanded on the *Pink1−/−* rat model of PD by examining sensorimotor and affect changes in female *Pink1−/−* rats, while addressing the challenging task of assaying behavioral variation due to rapid estrous cycling. Results of behavioral testing support that

changes in affect as well as possible deficits in some vocalization performance, in particular with regard to intensity, preceded the development of any limb motor deficits in the female *Pink1−/−* model. Our results support findings in human populations that changes in affect often appear before clinical features (*e.g.* limb motor), and that these affect changes are widely experienced by women with PD. Overall, these findings suggest the female *Pink1−/−* rat may be a good translational model for human PD populations with regard to anhedonia and anxiety. More in-depth research is needed to fully examine the complexities of male-female differences in the manifestation of PD in translational animal models and in humans.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

The authors acknowledge the valuable contributions of John Szot, Sarah Lechner, and Jesse Hoffmeister as well as the statistical expertise of Nicholas Marka.

Funding

This work was supported by the National Institutes of Health (R21 DC016135 (Kelm-Nelson)).

Appendix

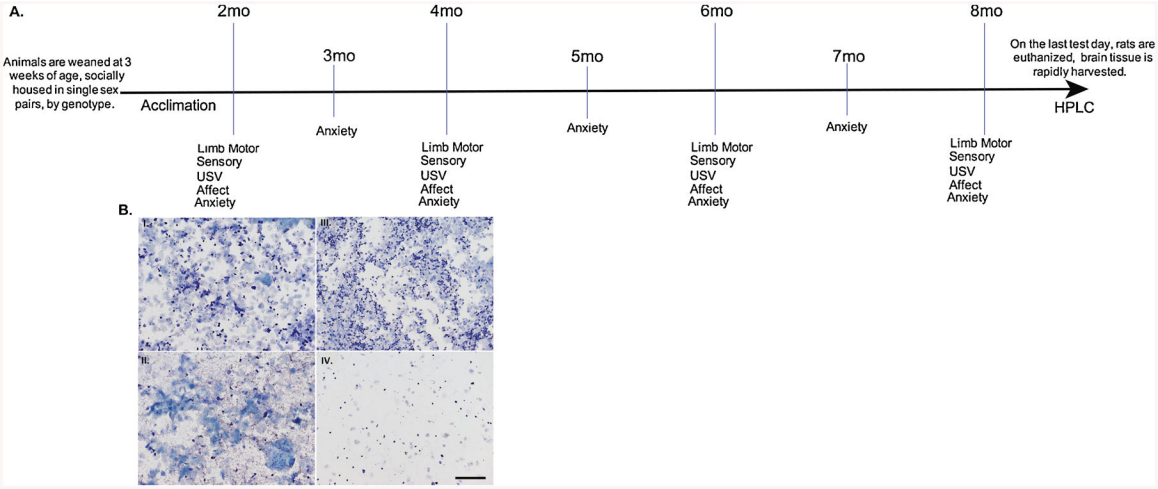

Fig. A1.

Behavioral assay timeline and estrous stage determination. (A) timeline showing initial animals and housing, behavioral testing at each timepoint, and analysis of brain tissue. (B) Estrous stage determination: (I) Estrus (II) Diestrus (III) Metestrus (IV) Proestrus.

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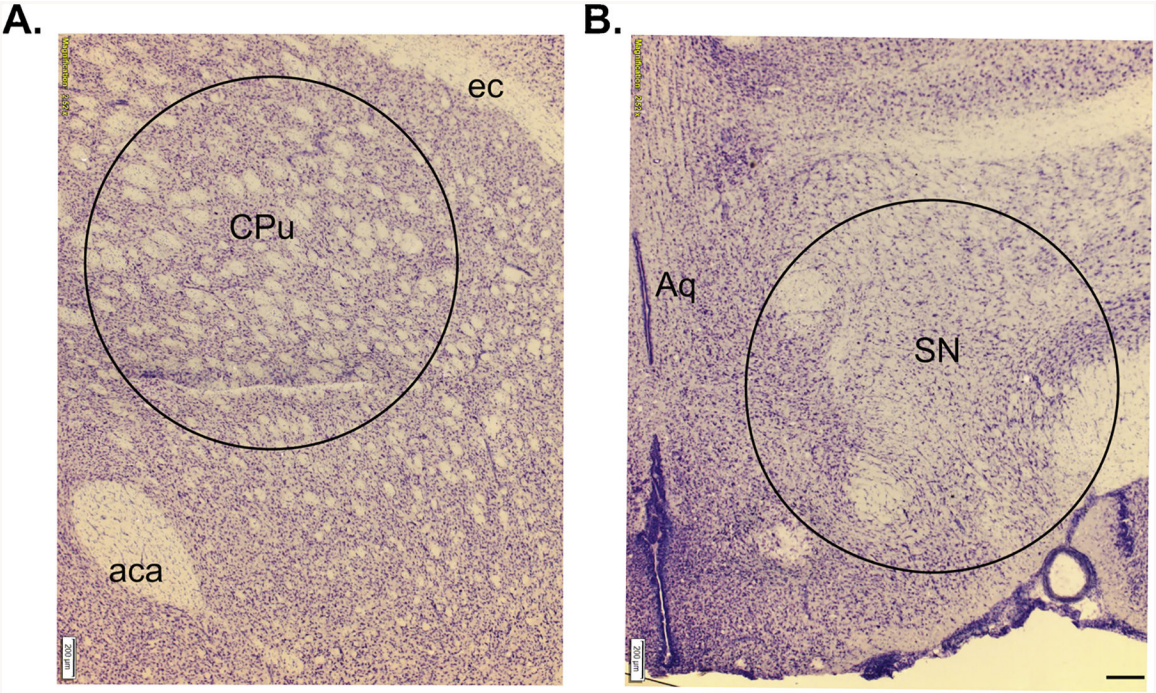

Fig. 1. Brain tissue figure.

Approximate location and size (2 mm) of tissue samples collected for HPLC tissue processing. Representative Nissl stained brain section (50 u M thickness) with circles demonstrating approximate dissections of (A) striatum and (B) substantia nigra. Scale bar indicates 200um thickness; magnification2.52 ×. Abbreviations: aca = anterior commissure, Aq: aquaduct, CPu: caudate putamen (*i.e.* striatum), ec = external capsule, SN = substantia nigra (Paxinos and Watson, 2005).

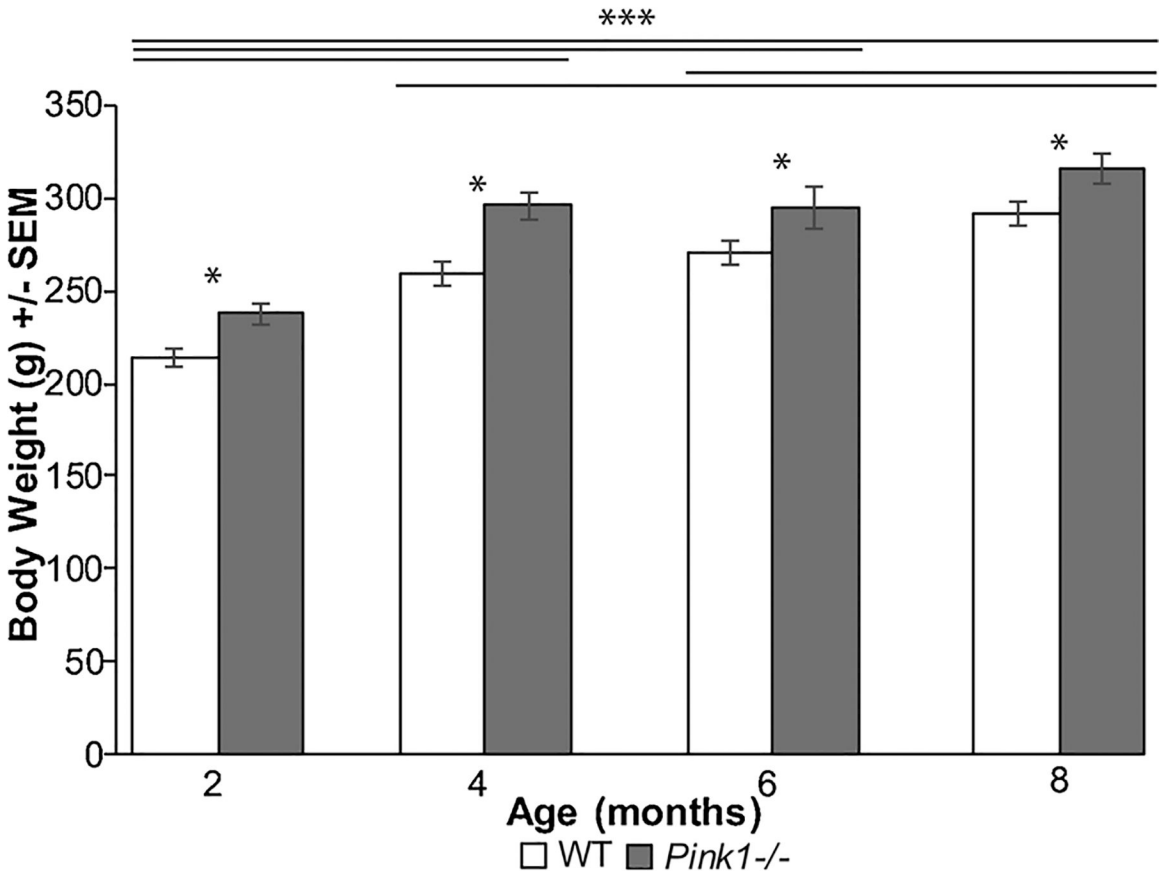

Fig. 2. Body weight.

Means (± SEM) in wildtype (white bar) compared to Pink1 −/− (dark gray bar). Pink1−/− rats were significantly heavier at each timepoint. Asterisks represent statistical significance between genotypes (* $p < 0.05$; ** $p < 0.01$, ***$p < 0.001$). Bars indicate significance between timepoints with asterisks showing levels of significance (* $p < 0.05$; ** $p < 0.01$, ***$p < 0.001$).

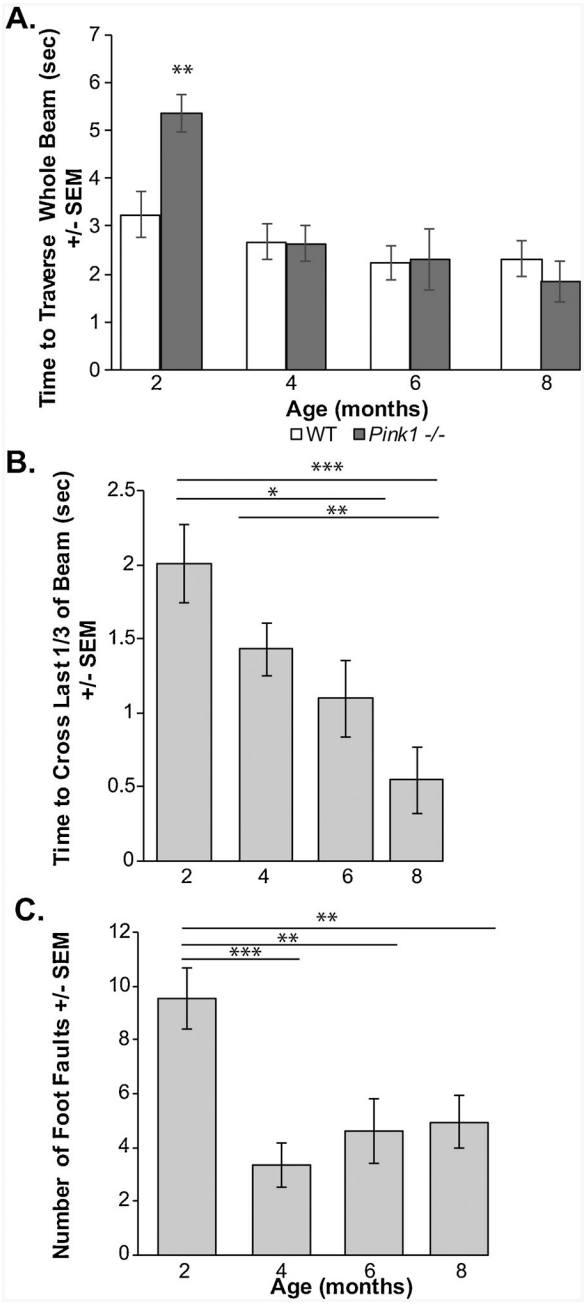

Fig. 3. A-C: Tapered balance beam traversal.

(A) Average time (± SEM) of both wildtype and Pink1 −/− rats to traverse the entire tapered balance beam. ** denotes $p < 0.01$. Difference in traversal time represented at each timepoint for wildtype (dark gray bar) and Pink1 −/− (light gray bar) rats. Pink1−/− rats were significantly slower at 2 mo. (B) Average time (± SEM) for all rats to traverse the last 1/3 of the beam at each timepoint. Bars indicate significance between timepoints with asterisks showing levels of significance (* $p < 0.05$; ** $p < 0.01$, ***$p < 0.001$). (C) Average number of foot-faults made by all rats at each timepoint. Bars indicate significance between

timepoints with asterisks showing levels of significance (* $p < 0.05$; ** $p < 0.01$, ***$p < 0.001$).

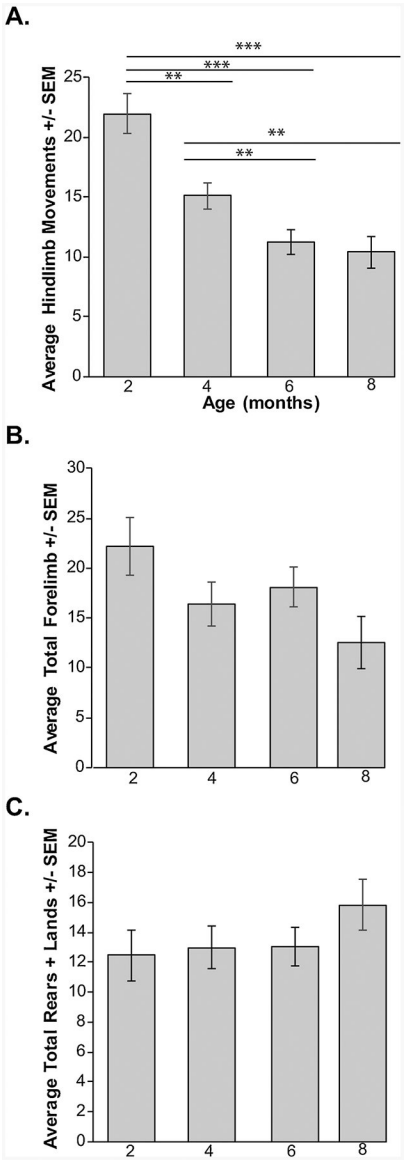

Fig. 4. A-C: Cylinder movement.

*(*A) Average number of hindlimb movements by all rats at each timepoint (± SEM). Both Pink1−/− and wildtype rats moved less as they aged. Bars indicate significance between timepoints with asterisks showing levels of significance (* $p < 0.05$; ** $p < 0.01$, ***$p < 0.001$). (B) Average number of forelimb movements by all rats at each timepoint (± SEM). (C) Average total rears + lands by all rats at each timepoint (± SEM).

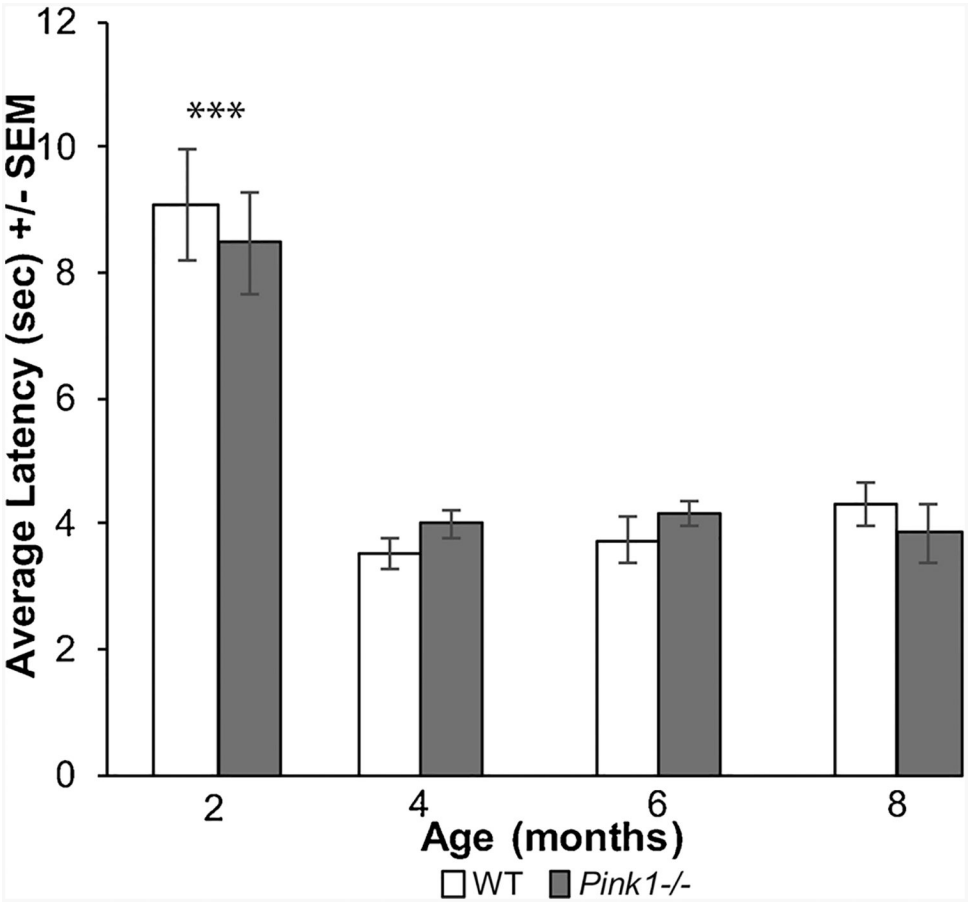

Fig. 5. Average latency to withdraw from hot stimulus.

Difference in average latency represented at each timepoint for Pink1−/− and wildtype rats. Pink1−/− rats had a significantly lower latency than wildtype rats at 2 mo. Means (± SEM) in wildtype (white bar) compared to Pink1 −/− (dark gray bar) Asterisks represent statistical significance between genotypes (* $p < 0.05$; ** $p < 0.01$, ***$p < 0.001$).

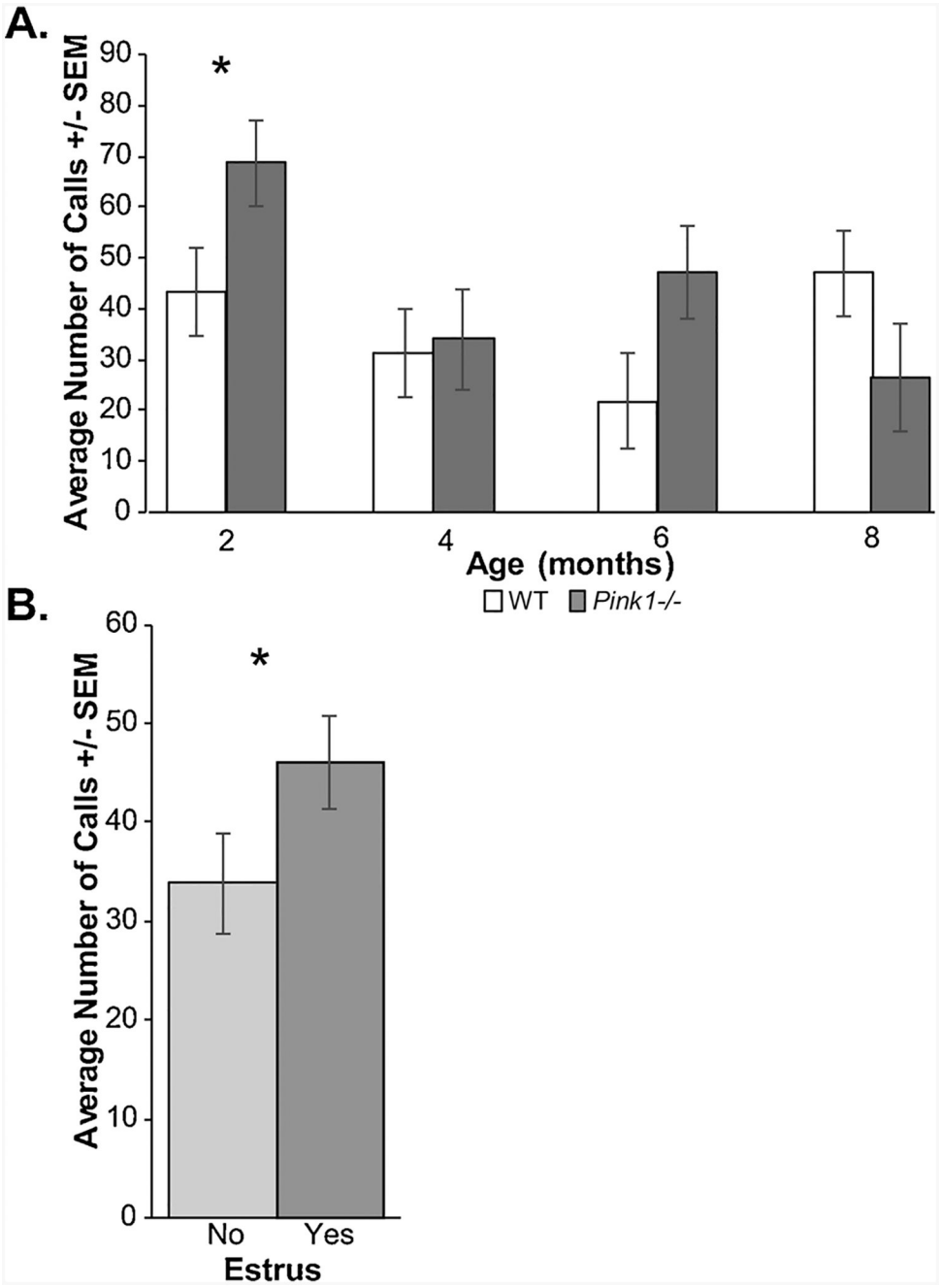

Fig. 6. A-B: Number of calls (ultrasonic vocalizations).

*(*A) Average number of calls (± SEM) made by wildtype (white bar) compared to Pink1−/− (dark gray bar) rats at each timepoint. Pink1−/− rats produced significantly more calls than wildtype rats at 2 mo. Statistical significance between genotypes indicated by *p < 0.05. (B) Average number of calls (± SEM) made by all rats in estrous (dark gray bar) and out of estrus (light gray bar). All rats produced more calls in estrus than when not in estrus. Asterisks represent statistical significance between stages (* p < 0.05; ** p < 0.01, ***p < 0.001).

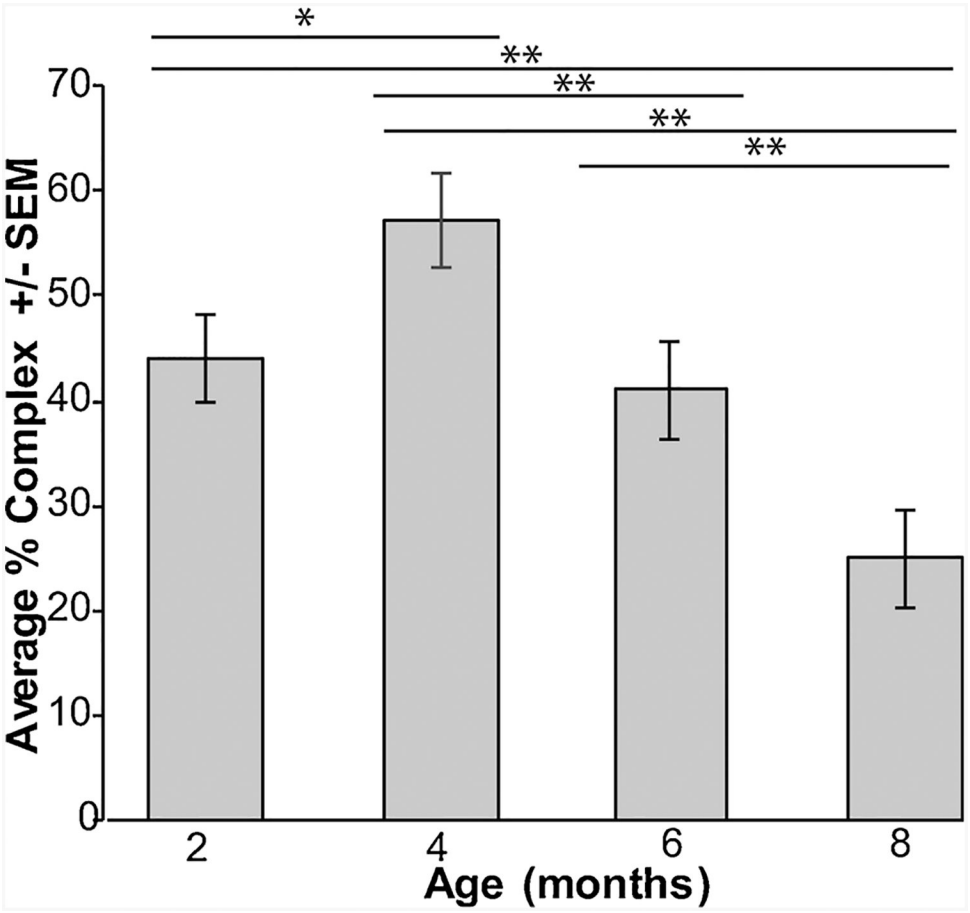

Fig. 7. Percent complex calls (ultrasonic vocalizations).

Average percent of complex calls made by all rats at each timepoint (± SEM). Bars indicate significance between timepoints with asterisks showing levels of significance (* $p < 0.05$; ** $p < 0.01$, ***$p < 0.001$).

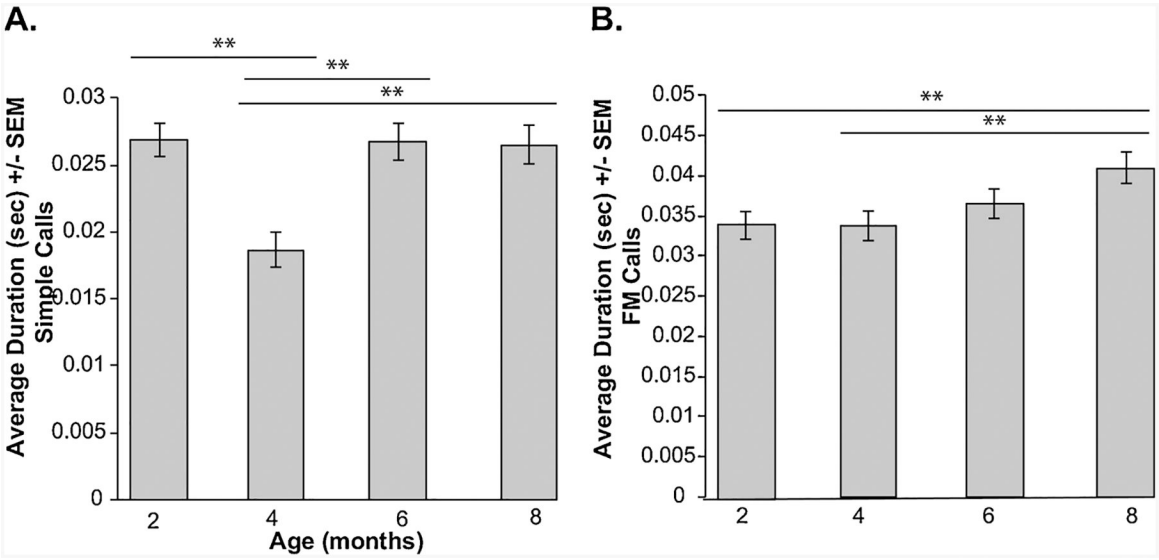

Fig. 8. A-B: Ultrasonic vocalization duration.

*(*A) Average duration of simple calls produced by all rats at each timepoint (± SEM). (B) Average duration of frequency modulated (FM) calls produced by all rats at each timepoint (± SEM). Bars indicate significance between timepoints with asterisks showing levels of significance (* $p < 0.05$; ** $p < 0.01$, ***$p < 0.001$).

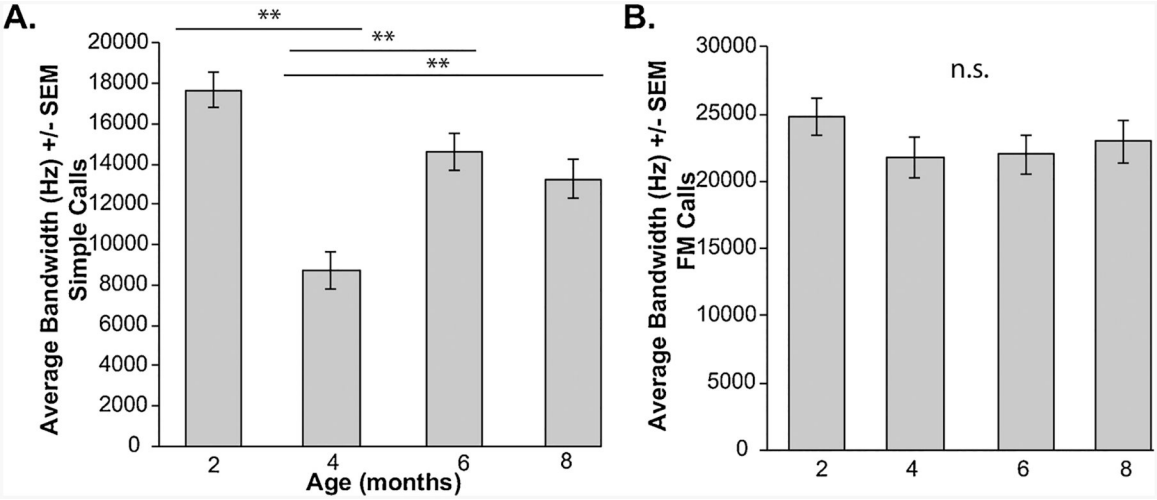

Fig. 9. A-B: Ultrasonic vocalization bandwidth.

*(*A) Average bandwidth of simple calls produced by all rats at each timepoint (± SEM). (B) Average bandwidth of FM calls produced by all rats at each timepoint (± SEM). Bars indicate significance between timepoints with asterisks showing levels of significance (* $p < 0.05$; ** $p < 0.01$, ***$p < 0.001$).

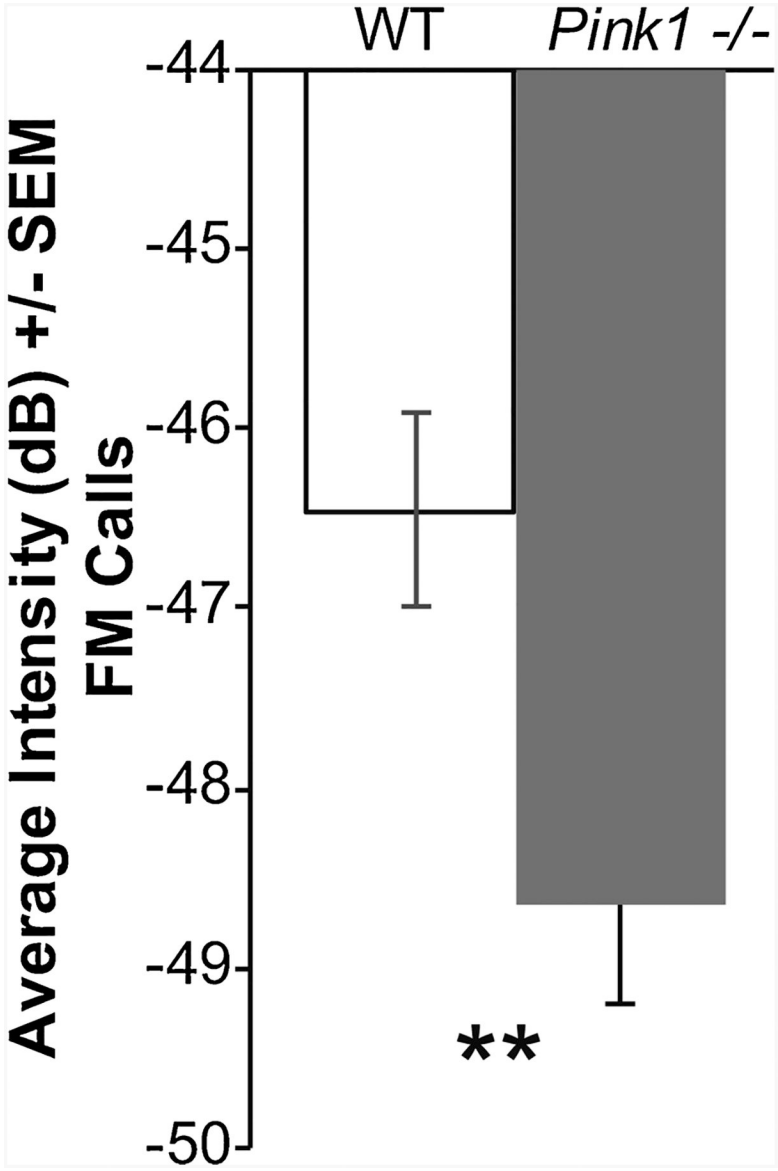

Fig. 10. Ultrasonic vocalization intensity.

Average intensity of frequency modulated (FM) calls (± SEM) produced by wildtype (white bar) compared to Pink1−/− (dark gray bar). Pink1−/− rats produced calls with significantly lower intensity than wildtype rats at all timepoints. Statistical significance between genotypes denoted by ** $p < 0.01$.

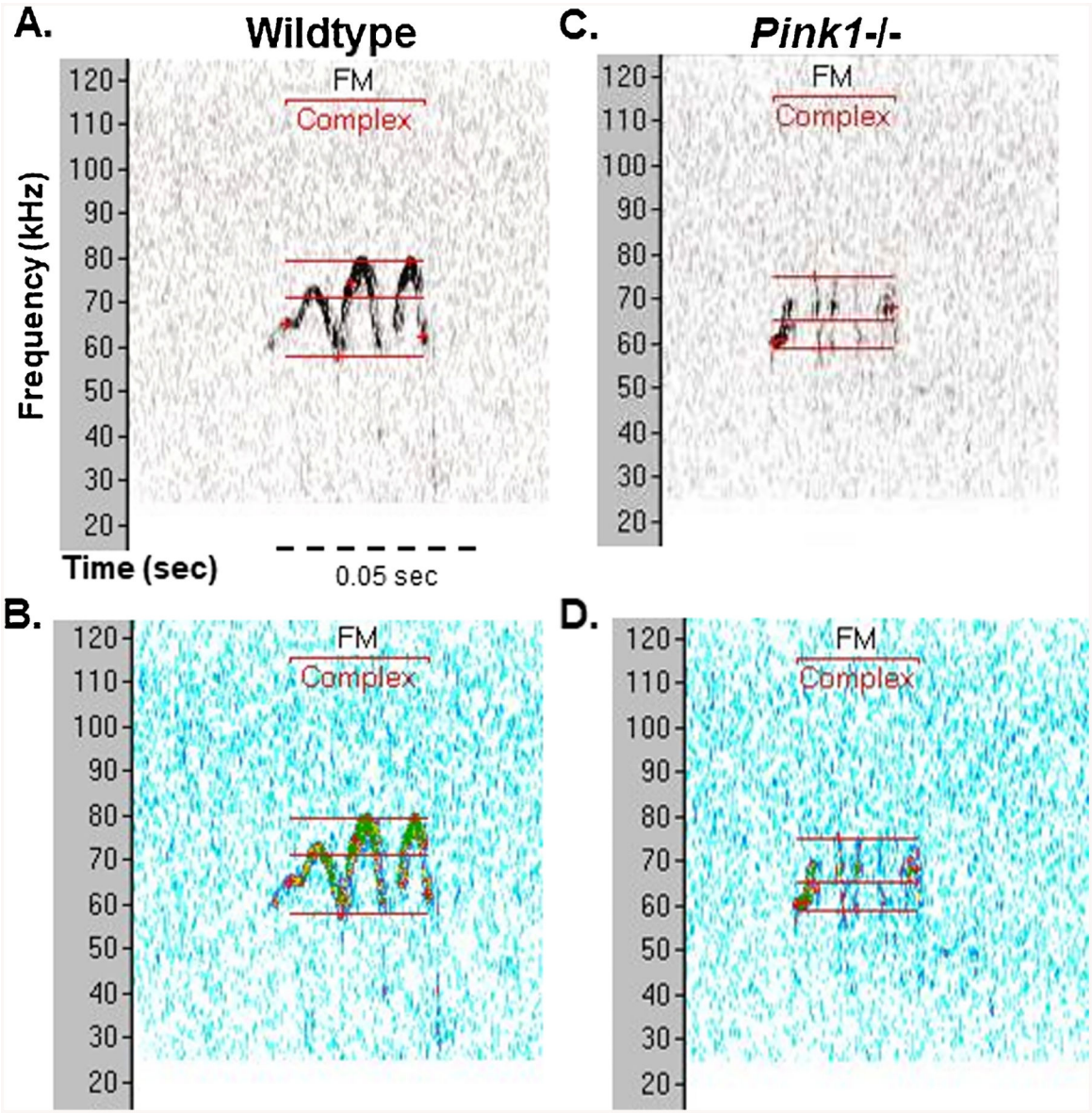

Fig. 11. Ultrasonic vocalization spectrogram representative of intensity differences in frequency modulated (FM) calls.

Demonstrative spectrogram of frequency modulated (FM) calls produced by wildtype (left, A–B) compared to Pink1−/− (right, C–D) rats. Stronger visible color denotes higher intensity.

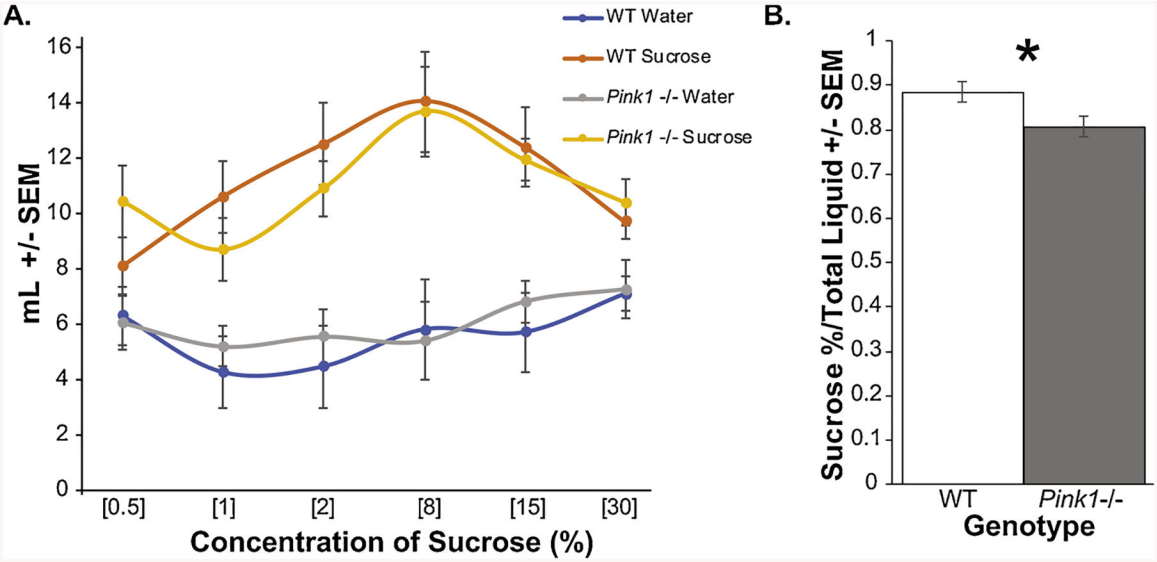

Fig. 12. A-B: Sucrose preference test dose response curve and % sucrose solution consumed.

(A) Sucrose dose response curve showing wildtype and Pink1−/− sucrose and water consumption (mL) at increasing concentrations of sucrose. A concentration of 8% yielded the maximum difference in water versus sucrose solution consumption. (B) Means (± SEM) % sucrose solution consumed in wildtype (white bar) compared to Pink1 −/− (dark gray bar). Pink1−/− rats consumed a lower % of sucrose solution than wildtype rats at all timepoints. Statistical significance between genotypes indicated by *$p < 0.05$.

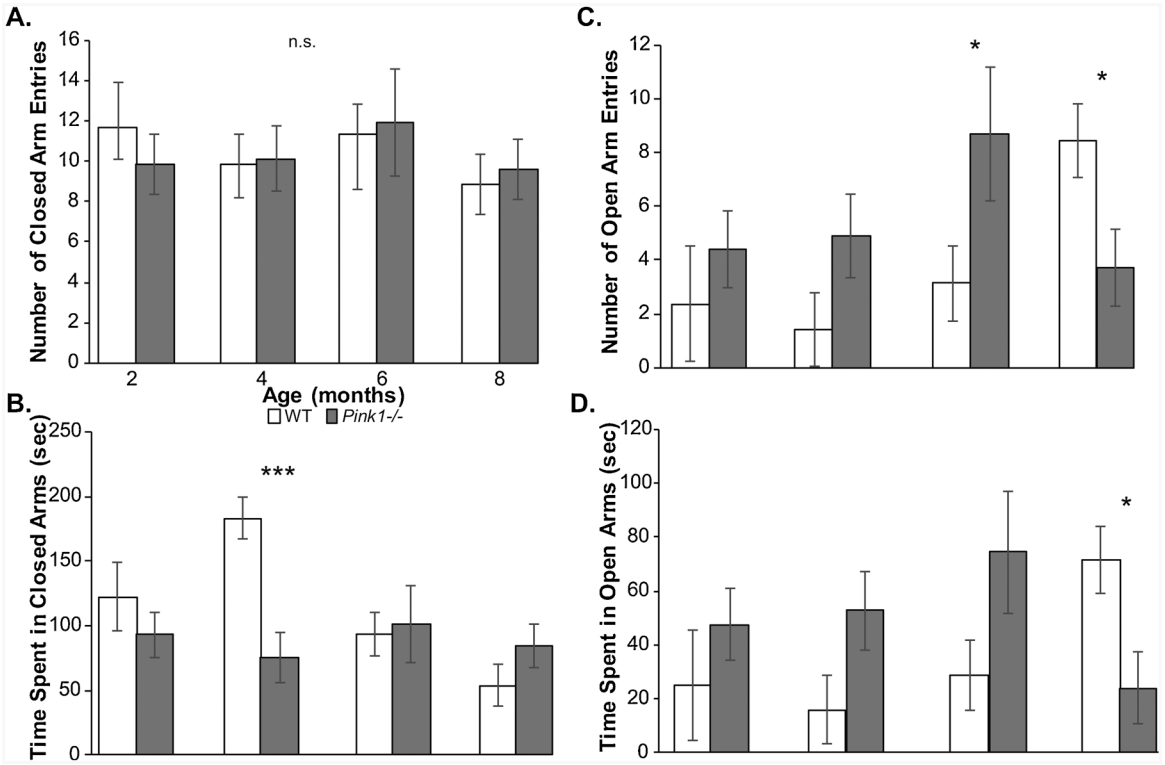

Fig. 13. A-D: Average number of entries and time spent in closed arms versus open arms over time in the Elevated Plus Maze (EPM).

(A) Average number of entries (± SEM) of both wildtype (light gray bars) and Pink1−/− (dark gray bars) rats into closed arms. (B) Average time (± SEM) in seconds spent in closed arms by each genotype. Wildtype rats spent significantly more time in closed arms than Pink1−/− rats at 4 mo. (C) Average number of entries (± SEM) of both wildtype and Pink1−/− rats into open arms. Pink1−/− rats made significantly more entries onto open arms than wildtype rats at 6 mo and made significantly fewer entries onto open arms at 8 mo. (D) Average time (± SEM) in seconds spent in open arms by each genotype. Time in open and closed arms does not account for time in transition. Wildtype rats spent significantly more time on open arms than Pink1−/− rats at 8 mo. Asterisks represents statistical significance between genotypes (* $p < 0.05$; ** $p < 0.01$, ***$p < 0.001$). n.s. indicates no significance.

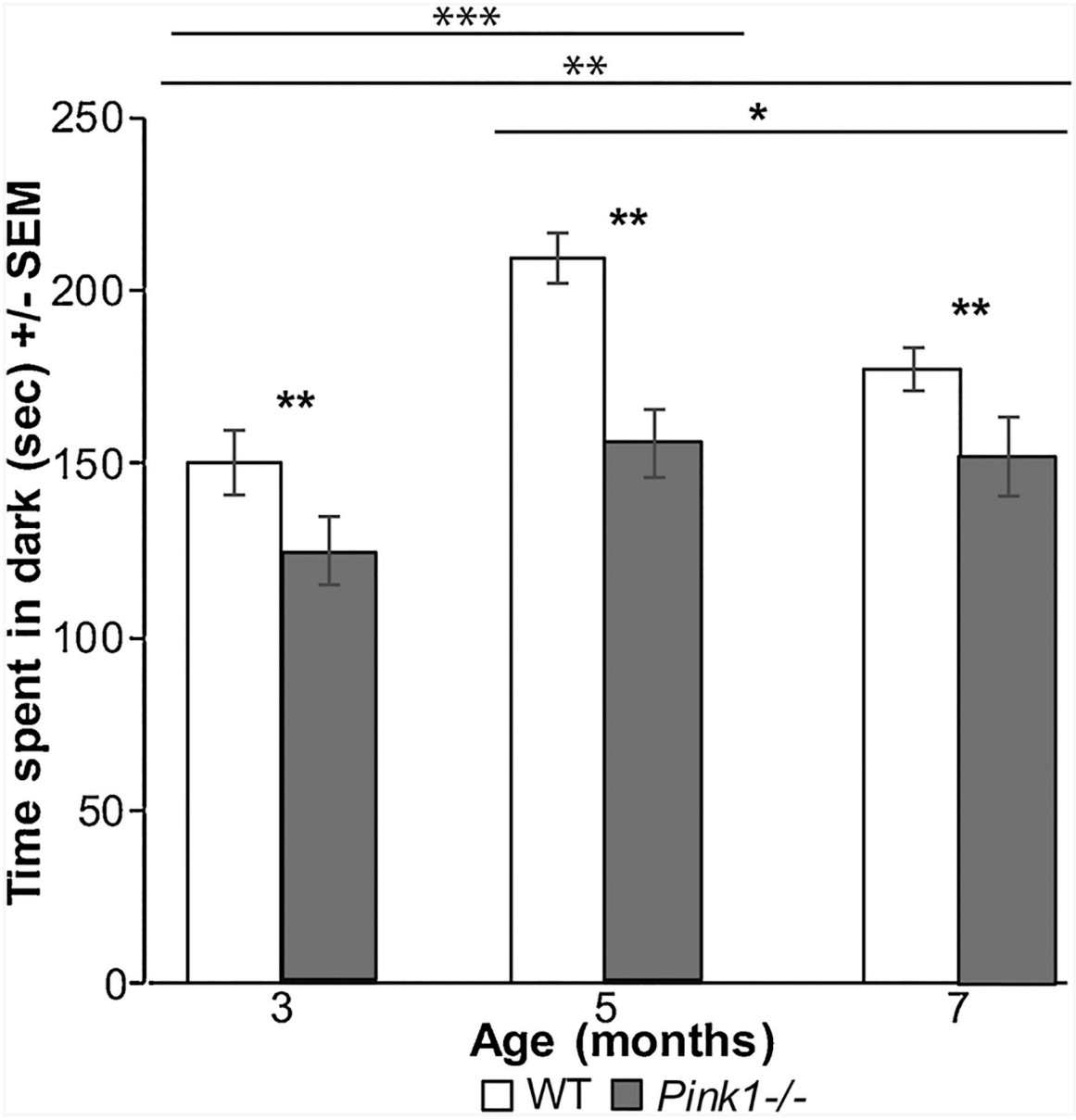

Fig. 14. Average time spent in the dark side of the Light/Dark Box.

Mean amount of time (± SEM) spent in the dark by each genotype across timepoints. Pink1−/− rats spent significantly more time in the dark than wildtype rats at each timepoint. Asterisks represents statistical significance between genotypes (* $p < 0.05$; ** $p < 0.01$, ***$p < 0.001$). Bars indicate significance between timepoints with asterisks showing levels of significance (* $p < 0.05$; ** $p < 0.01$, ***$p < 0.001$).

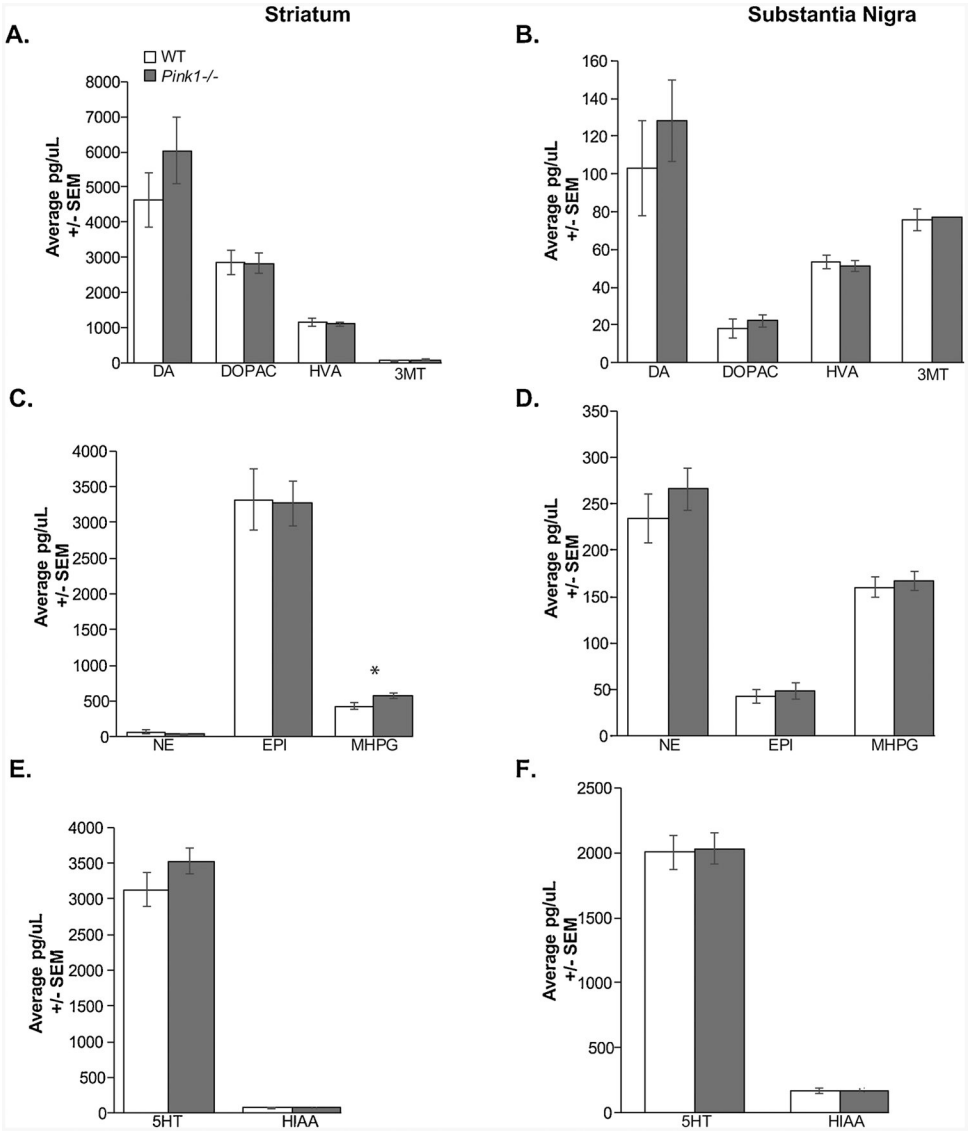

Fig. 15. A-F: Average pg/uL HPLC data in the striatum and substantia nigra.

(A, B) Average (pg/uL) +/− SEM for wildtype (white bars) and Pink1−/− (gray bars) for DA and metabolites (DOPAC, HVA, 3MT). (C,D) Average +/− SEM for NE, EPI, and metabolites (MHPG). (E, F) Average +/− SEM for 5HT and its metabolites (HIAA). Asterisks represents statistical significance between genotypes (* $p < 0.05$; ** $p < 0.01$, ***$p < 0.001$). Abbreviations: DA = dopamine, DOPAC = 3,4-Dihydroxyphenylacetic Acid, HVA= Homovanillic Acid, 3MT = 3-Methoxy-4-Hydroxyphenethylamine, NE = norepinephrine, EPI = epinephrine, MHPG = 3-Methoxy-4-Hydroxyphenylglycol, 5HT = serotonin, HIAA = 5-Hydroxyindoleacetic Acid.

Table 1

Summary of HPLC Data.

HPLC Metabolite	Abbreviation	Definition	SR Wildtype Mean (SEM)	SR Pink1−/− Mean (SEM)	SN Wildtype Mean (SEM)	SN Pink1−/− Mean (SEM)
Dopamine	DA	Neurotransmitter	4638.003 (767.29)	6038.65 (960.60)	103.08 (24.98)	128.13 (21.66)
Norepinephrine	NE	Neurotransmitter	70.512 (20.39)	35.305 (12.84)	233.85 (26.54)	265.79 (22.90)
Epinephrine	Epi	Neurotransmitter	3317.42 (431.44)	3267.80 (312.26)	42.504 (6.84)	48.31 (8.58)
Serotonin	5 HT	Neurotransmitter	3131.56 (233.01)	3525.10 (181.28)	2005.05 (132.56)	2032.70 (123.90)
3,4-Dihydroxyphenylacetic Acid	DOPAC	Metabolite of dopamine	2846.35 (355.80)	2829.05 (286.08)	18.33 (5.00)	22.15 (3.14)
Homovanillic Acid	HVA	Metabolite of dopamine catabolism	1161.17 (106.23)	1103.34 (48.99)	53.62 (3.46)	51.16 (2.93)
3-Methoxy-4-Hydroxyphenethylamine	3 MT	Metabolite of dopamine	56.76 (7.85)	83.71 (15.28)	75.87 (5.56)	77.16 (6.59)
3-Methoxy-4-Hydroxyphenylglycol	MHPG	Metabolite of norepinephrine	430.74 (53.72)	570.15 (42.20)	160.08 (10.84)	166.85 (10.38)
5-Hydroxyindoleacetic Acid	HIAA	Primary metabolite of serotonin	73.05 (11.65)	76.50 (12.91)	170.52 (22.62)	170.62 (14.54)

HPLC neurotransmitter and metabolite data (means (SEM)) in striatum (SR) and substantia nigra (SN).

Table 2

Behavioral means (SEM).

Behavioral Variable	Units	Wildtype				Pink1−/−			
		2mo	4mo	6mo	8mo	2mo	4mo	6mo	8mo
Bodyweight	g	213.73 (4.59)	259.30 (6.48)	270.3 (7.16)	290.8 (6.74)	237.25 (5.1819)	295.4 (6.84)	295.1 (11.30)	315.8 (8.28)
% Sucrose	mL	0.91 (0.044)	0.85 (0.042)	0.87 (0.03)	0.9086 (0.032)	0.74 (0.03540)	0.81 (0.04)	0.83 (0.04)	0.85 (0.04)
Time to Cross Beam	s	3.23 (0.48)	2.67 (0.37)	2.22 (0.36)	2.32 (0.37)	5.35 (0.3935)	2.64 (0.37)	2.31 (0.63)	1.83 (0.43)
Time to cross last 1/3 of beam	s	1.99 (0.37)	1.58 (0.26)	1.34 (0.25)	0.57 (0.26)	2.03 (0.29)	1.28 (0.26)	0.87 (0.43)	0.53 (0.32)
Number of foot faults	#	10.34 (1.60)	2.63 (1.18)	3.67 (1.17)	5.21 (1.18)	8.78 (1.27)	4.04 (1.19)	5.52 (2.07)	4.69 (1.41)
Cylinder Hindlimb	#	22.14 (2.25)	17.34 (1.60)	12.51 (1.42)	10.85 (1.61)	21.77 (1.87)	12.95 (1.52)	9.95 (1.53)	9.97 (1.83)
Cylinder Forelimb	#	24.06 (3.84)	17.93 (3.10)	19.43 (2.74)	13.64 (3.17)	20.29 (3.17)	14.84 (2.95)	16.75 (2.95)	11.41 (3.53)
Rears and Lands	#	12.39 (2.36)	13.17 (2.03)	14.28 (1.81)	16.83 (2.09)	12.52 (2.01)	12.76 (1.91)	11.84 (1.90)	14.86 (2.22)
USV Total Calls	#	43.38 (8.76)	31.26 (8.73)	21.83 (9.30)	46.94 (8.51)	68.62 (8.31)	33.94 (9.74)	47.02 (9.20)	26.38 (10.56)
USV % Complex	%	0.44 (0.06)	0.57 (0.06)	0.38 (0.06)	0.24 (0.06)	0.45 (0.057)	0.57 (0.07)	0.45 (0.06)	0.26 (0.07)
EPM Time in closed arms	s	122.59 (26.18)	183.11 (16.29)	93.15 (16.71)	53.75 (16.20)	93.21 (17.39)	75.35 (18.86)	100.79 (29.82)	84.40 (17.13)
EPM Time in open arms	s	25.07 (20.44)	15.92 (12.64)	28.94 (12.93)	71.71 (12.55)	47.66 (13.58)	52.71 (14.67)	74.53 (22.67)	24.13 (13.42)
EPM Entries in closed arms	#	11.65 (2.22)	9.88 (1.46)	11.30 (1.50)	8.82 (1.50)	9.86 (1.52)	10.13 (1.65)	11.93 (2.67)	9.62 (1.49)
EPM Entries in open arms	#	2.37 (2.14)	1.42 (1.36)	3.14 (1.39)	8.43 (1.35)	4.41 (1.43)	4.88 (1.56)	8.69 (2.51)	3.715 (1.41)
		3mo	*5mo*	*7mo*		*3mo*	*5mo*	*7mo*	
Light Dark Box Time Spent in Dark Side	s	150.76 (9.25)	209.43 (7.49)	177.29 (6.60)		123.77 (9.67)	155.14 (9.60)	152.59 (11.44)	

Mean (standard error of the mean (SEM)) for behavioral variables at each testing timepoint for each genotype. Abbreviations: EPM = elevated plus maze, g = grams, USV = ultrasonic vocalization, s = second.

Table 3

Frequency modulated calls – Means (SEM).

Age	Acoustic parameter/unit	Average		Maximum		Top 10	
		Pink1−/−	Wildtype	Pink1−/−	Wildtype	Pink1−/−	Wildtype
2 mo	Duration (s)	0.03 (0.002)	0.035 (0.002)	0.06 (0.06)	0.06 (0.01)	0.05 (0.01)	0.04 (0.004)
	Bandwidth (kHz)_	24082 (1939.04)	25479 (1863.33)	48695 (4182.53)	44403 (4020.31)	34683 (2968.42)	32601 (2853.09)
	Intensity (dB)	−46.77 (0.88)	−45.47 (0.85)	−35.88 (1.69)	−36.28 (1.61)	−41.15 (1.30)	−41.93 (1.24)
	Peak Frequency (kHz)	66907 (1578.61)	69431 (1511.27)	66907 (1578.61)	69431 (1511.27)	63009 (1265.92)	63952 (1216.47)
4 mo	Duration	0.03 (0.003)	0.035 (0.002)	0.06 (0.01)	0.07 (0.01)	0.04 (0.01)	0.05 (0.01)
	Bandwidth	19821 (2052.71)	23676 (2023.43)	34409 (4425.69)	46767 (4363.03)	24032 (3141.38)	31654 (3096.81)
	Intensity	−49.71 (0.94)	−46.90 (0.92)	−40.90 (1.82)	−33.51 (1.79)	−46.64 (1.39)	−41.70 (1.36)
	Peak Frequency	65765 (1681.78)	69270 (1654.92)	65765 (1681.78)	69270 (1654.92)	61919 (1340.16)	63947 (1321.04)
6 mo	Duration	0.04 (0.003)	0.04 (0.003)	0.07 (0.01)	0.05 (0.01)	0.05 (0.01)	0.038 (0.01)
	Bandwidth	21345 (2058.59)	22616 (2080.35)	50071 (4436.89)	38958 (4485.23)	31116 (3149.61)	24236 (3183.65)
	Intensity	−49.27 (0.94)	−48.14 (0.95)	−39.70 (1.85)	−39.04 (1.85)	−45.16 (1.40)	−47.25 (1.40)
	Peak Frequency	66464 (1694.08)	67528 (1704.45)	66464 (1694.08)	67528 (1704.45)	62421 (1344.03)	61560 (1358.21)
8 mo	Duration	0.04 (0.003)	0.04 (0.002)	0.06 (0.01)	0.08 (0.01)	0.04 (0.01)	0.05 (0.01)
	Bandwidth	21286 (2446.60)	24617 (1947.64)	35543 (5269.76)	35228 (4199.30)	24001 (3741.48)	27802 (2980.66)
	Intensity	−48.77 (1.13)	−45.32 (0.89)	−44.46 (2.22)	−38.20 (1.72)	−47.34 (1.68)	−43.90 (1.31)
	Peak Frequency	66619 (2028.46)	65367 (1593.94)	66619 (2028.46)	65367 (1593.94)	62612 (1597.42)	62212 (1271.57)

Mean (standard error of the mean) for each acoustic parameter of frequency modulated (FM) ultrasonic vocalizations at each testing timepoint for each genotype. Abbreviations: dB = decibel, kHz = kilohertz, s = second.

Table 4

Simple calls – Means (SEM).

Age	Acoustic parameter/unit	Average		Maximum		Top 10	
		Pink1−/−	Wildtype	Pink1−/−	Wildtype	Pink1−/−	Wildtype
2 mo	Duration (s)	0.03 (0.002)	0.03 (0.002)	0.06 (0.01)	0.05 (0.01)	0.04 (0.004)	0.04 (0.004)
	Bandwidth (kHz)_	15815 (1201.65)	19530 (1231.56)	43989 (3261.89)	44620 (3344.79)	28057 (2607.23)	28885 (2664.12)
	Intensity (dB)	−48.55 (0.75)	−47.98 (0.76)	−37.17 (1.65)	−35.76 (1.69)	−42.61 (1.34)	−43.68 (1.37)
	Peak Frequency (kHz)	53515 (1289.96)	58698 (1312.78)	65487 (1832.08)	68169 (1867.54)	59802 (1643.82)	62965 (1674.85)
4 mo	Duration	0.02 (0.002)	0.02 (0.002)	0.03 (0.01)	0.03 (0.01)	0.02 (0.004)	0.02 (0.004)
	Bandwidth	9377.09 (1370.77)	8018.24 (1227.38)	17812 (3732.27)	16861 (3335.03)	11857 (2935.87)	8689.11 (2651.61)
	Intensity	−49.57 (0.86)	−49.71 (0.76)	−41.52 (1.92)	−43.19 (1.69)	−47.47 (1.53)	−48.83 (1.37)
	Peak Frequency	56742 (1431.88)	57702 (1305.81)	63617 (2045.06)	63515 (1857.93)	58514 (1831.92)	58814 (1666.14)
6 mo	Duration	0.03 (0.002)	0.03 (0.002)	0.06 (0.01)	0.05 (0.01)	0.04 (0.004)	0.03 (0.004)
	Bandwidth	14252 (1294.56)	14976 (1308.41)	35514 (3523.75)	32665 (3561.84)	22450 (2777.70)	18532 (2806.38)
	Intensity	−49.87 (0.81)	−49.14 (0.82)	−36.14 (1.81)	−39.33 (1.83)	−44.35 (1.44)	−46.85 (1.46)
	Peak Frequency	56061 (1358.60)	56419 (1372.16)	63958 (2203.48)	64750 (1957.66)	60812 (1736.66)	58542 (1754.16)
8 mo	Duration	0.03 (0.002)	0.03 (0.002)	0.05 (0.01)	0.06 (0.01)	0.03 (0.004)	0.04 (0.004)
	Bandwidth	13358 (1486.17)	13171 (1197.34)	34004 (4049.32)	33278 (3253.89)	19074 (3171.95)	21593 (2583.56)
	Intensity	−51.25 (0.94)	−49.03 (0.75)	−42.13 (2.08)	−37.34 (1.65)	−48.57 (1.65)	−44.25 (1.33)
	Peak Frequency	54027 (1539.89)	55553 (1270.04)	63958 (2203.48)	65927 (1808.30)	57025 (1972.76)	59963 (1621.30)

Mean (standard error of the mean) for each of simple ultrasonic vocalization acoustic parameter at each testing timepoint for each genotype. Abbreviations: dB = decibel, kHz = kilohertz, s = second.