Sequential organization and circadian effects on ultrasonic vocalizations in the social interactions of male WT and zQ175 mice with WT females

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Introduction

Preclinical research investigating solutions to Huntington's Disease (HD) frequently relies on the use of transgenic rodent models that recapitulate symptoms of the disease at varying stages of their development. The zQ175 KI Het transgenic mouse model, developed at Psychogenics, Inc, expresses ~175 CAG repeats at the HTT locus and exhibits robust behavioral deficits consistent with HD at approximately 6 months of age. Previous phenotyping studies have not examined ultrasonic vocalizations (USVs) in this or any other HD model, potentially ignoring a potentially useful phenotyping tool, which is prominently impaired in models of other neurodegenerative diseases, including Parkinson's and Alzheimer's disease. This pilot investigated the acoustic properties and sequential organization of USVs in young (9 week) WT and zQ175 mice. To capture potential phenotypic differences, a single female WT mouse was paired with either a male WT or zQ175 mouse in the **Phenocube®** testing environment, allowing continuous analysis of USVs along with parallel measurement of behavioral activity and social interaction in a courtship setting.

Methods

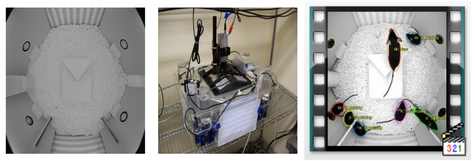
Subjects

Subjects were 32 mice (16 male, 16 female) obtained from Jackson Laboratories (Bar Harbor, ME). Male mice included zQ175 KI Het (n=8) and WT (n=8) mice on a C57BL/6J background; females were exclusively WT. Mice arrived in colony facilities at 7 weeks of age, were isolated in OptiMICE cages (Animal Care Systems, CO), and were maintained on a 12:12 light/dark cycle with *ad libitum* access to food (5001 rodent diet, Harla-Tekland) and water. Cages were enriched with the standard PGI enrichment regime of two play tunnels, plastic bone, and Envirodry. Following a one-week acclimation period, mice were subcutaneously implanted with RFID transponder chips.

Apparatus and Testing Procedure

Mice were tested in single male-female pairs in the **Phenocube®** (PC) testing environment (described below), with pairs consisting of a zQ175_WT female and a zQ175_WT or zQ175_Het males. Pairs were naïve to each other when introduced to PC. Mice freely interacted in the PC environment and microphones captured their vocalizations over a 2-day period. Food and water were freely available during this period.

Phenocube® is a high-throughput platform that assesses circadian, cognitive, social and motor behavior exhibited by group-housed mice. Experiments are conducted using modified Intellicage® units (New Behavior AG), each with a camera mounted on top of the cage for computer vision analysis. IntelliCages have 4 corners with small doors that contain antennas to pick up the ID from the electronic chips. Inside the corners, two small gates give access to water bottles and allow measurement of nosepoking and cognitive performance.



*At left, a top-down shot of the internal **Phenocube®** testing environment. In middle, an exterior shot of a single **Phenocube®** testing chamber. At right, Automated computer vision algorithms provide continuous measurement of behaviors over a 48 hour period. An ultrasonic microphone affixed to the chamber ceiling captured vocalizations over this period.*

USV measurement and Classification

Acoustic analysis software (Sound Analysis Pro) was used for semi-automated analyses of vocalizations captured during social interaction experiments. The acoustic parameters of USVs, including pitch, duration, modulation, etc, were then extracted for statistical analysis (see middle panel, right). Additionally, these parameters were used to classify USVs into discrete syllable types. Different syllable types were classified by applying a threshold-based criterion to cumulative frequency distributions of USV pitch, frequency modulation, and entropy. The parameters used to classify syllables according to these criterion, yielding a total of 42 possible syllable types, are enumerated below. Of these, we identified 12 syllable types that were reliably observed, whereas other potential syllable types appeared only rarely (<0.3% of total USVs).

Frequency component	Frequency range	Entropy Component	Entropy Range	Modulation component	Modulation range
P1	>79kHz	E1	< -5.8	M1	$> 10^0$
P2	62-73 kHz	E2	-5.8 - -4.8	M2	$< 10^0$
P3	60-62 kHz	E3	> -4.8		
P4	53-60 kHz				
P5	33-53 kHz				
P6	>31 kHz				
P7	25-31 kHz				

At left, syllables were *classified by pitch (7 possible categories), entropy (three categories), and frequency modulation (two categories), based on criterion applied to corresponding cumulative frequency distributions for each measure*

Data analysis

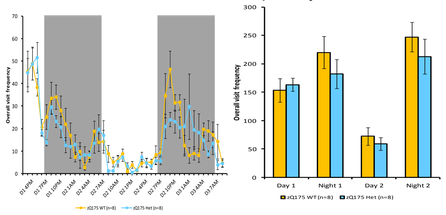
Behavioral data compiled with the **Phenocube®** computer vision tracking system (top panel at right) and USV parameters (middle panel at right) were analyzed with general linear mixed-models (GLMM) implemented in SAS (v9.4) with the PROC MIXED procedure. These and all other statistical tests were conducted with α=0.05. T

o analyze the sequential organization of syllable types (see above on syllable classification), we calculated the probability of each syllable type transitioning to another syllable type, to repeat itself, or to terminate in silence. Transition matrices calculated as described were initially analyzed with chi-square goodness-of-fit tests to identify non-random organizations in syllable transitions, and to compare transition matrices in WT and WT-zQ175 pairings. Further analysis revealed that the chi-square analysis was vulnerable to false-positives given the size of transition matrices, involving approximately 45,000 transitions. To account for this we calculated the sum of squared deviations (SS) from observed distributions and distributions expected by chance, and used random simulation methods to identify appropriate critical values at α=0.05

Results

Behavioral measures in the **Phenocube®** testing Environment

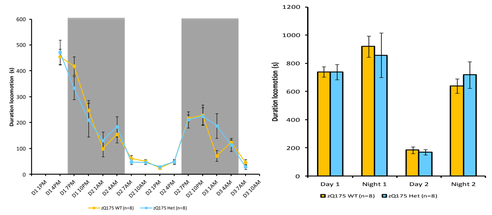
Corner visit frequency



At left, mean (± SEM) overall visit frequency in WT and zQ175 mice are plotted across 3 hour bins. Shaded areas indicate dark cycle. At right, mean (± SEM) overall visit frequency in WT and zQ175 USVs are plotted by 12 hour (day/night) bins for each genotype.

Overall visit frequency (total count) was not affected by male genotype (F(1,14)=0.5, p=0.4902), bu0
t overall visits in WT and WT-zQ175 pairings were driven by circadian cycle (F(3,42)=55.72, p<0.0001

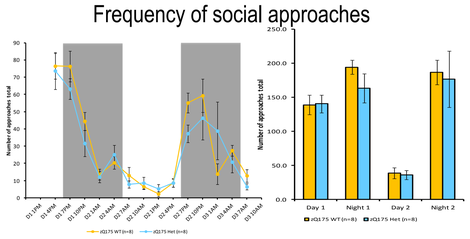
Duration of Locomotion



At left, mean (± SEM) duration of locomotion is plotted for WT and zQ175 mice across 3 hours bins. At right, mean (± SEM) duration of locomotion is plotted by 12 hour (day/night) bins for each genotype.

Circadian cycle was a significant determinant of locomotion in both WT and WT-zQ175 pairings (F(3,38)=55.26, p<0.0001), but there were no significant effect associated with heterozygosis at the HTT locus (F(1,14)=0.04, p=0.8414)

Frequency of social approaches

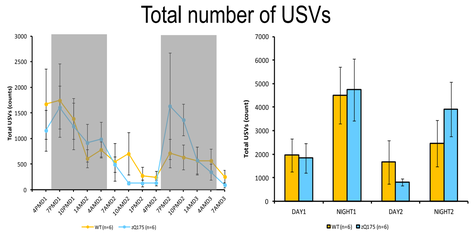


At left, mean (± SEM) total number (counts) of USVs sampled in WT and WT-zQ175 pairings are plotted across 3 hour bins. Shaded areas indicate dark cycle. At right, mean (± SEM) number of USVs are plotted by 12 hour (day/night) bins

Social approaches were not affected by genotypic differences (F(1,14)=0.59, p=0.4537), but these behaviors were sensitive to circadian cycle (F(3,38)=54.87, p<0.0001), with more approaches happening at night.

Parameters of Ultrasonic Vocalizations (USVs) observed in WT-WT and WT – zQ175 pairings

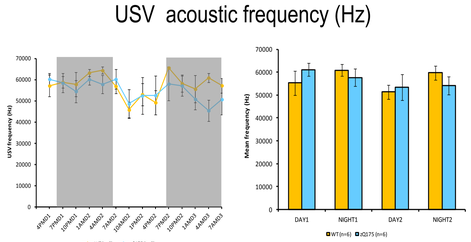
Total number of USVs



At left, mean (± SEM) total number (counts) of USVs sampled in WT and WT-zQ175 pairings are plotted across 3 hour bins. Shaded areas indicate dark cycle. At right, mean (± SEM) number of USVs are plotted by 12 hour (day/night) bins

There was no overall effect of heterozygosis at the HTT locus (F(1,10)=0.0, p=0.9584) on the number of USVs observed, but the effect of circadian cycle was significant (F (3, 26)=7.43, p=0.0012). The interaction of these factors was not significant (F(3,26)=0.42, p=0.7436).

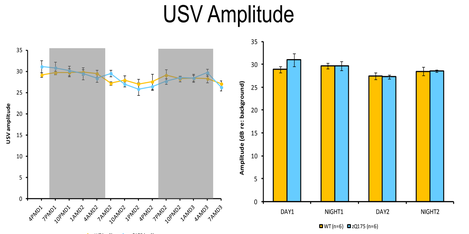
USV acoustic frequency (Hz)



At left, the mean (± SEM) acoustic frequency(i.e., pitch) of WT and zQ175 USVs are plotted across 3 hour bins. Shaded areas indicate dark cycle. At right, mean (± SEM) acoustic frequency of USVs in WT and WT-zQ175 pairings are plotted by 12 hour bins

Heterozygosis at the *HTT* locus had no significant overall effect on the mean acoustic frequency (i.e., pitch) of USVs (F(1,10)=0.01, p=0.9683), nor were there any significant effects of circadian cycle (F(3,26)=2.01, p=0.1378), or interaction among these factors (F(3,26)=0.7, p=0.5586).

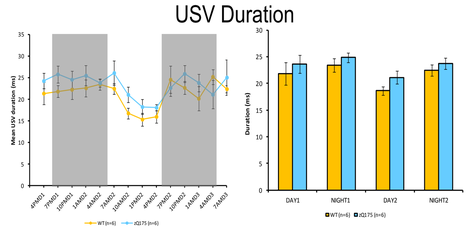
USV Amplitude



At left, the mean (± SEM) amplitude of USVs in WT and WT-zQ175 pairings are plotted across 3 hour bins. Shaded areas indicate dark cycle. At right, mean (± SEM) USV amplitude are plotted by 12 hour (day/night) bins for WT and WT-zQ175 pairings .

Heterozygosis at the *HTT* locus had no significant overall effect on the mean acoustic frequency (i.e., pitch) of USVs (F(1,10)=0.01, p=0.9683), nor were there any significant effects of circadian cycle (F(3,26)=2.01, p=0.1378), or interaction among these factors (F(3,26)=0.7, p=0.5586).

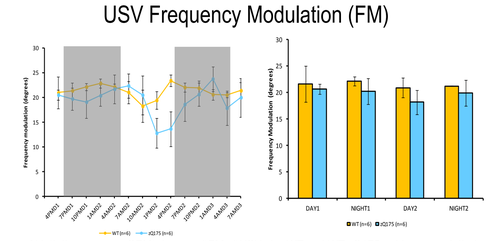
USV Duration



At left, the mean (± SEM) duration of USVs in WT and WT-zQ175 pairings are plotted across 3 hour bins. Shaded areas indicate dark cycle. At right, mean (± SEM) duration of USVs are plotted by 12 hour (day/night) bins for WT and WT-zQ175 pairings.

USV duration varied with circadian cycle (F(3,26)=3.99, p=0.0184), with USVs being shorter in day relative to night, but there was no effect of male heterozygosis at the HTT locus (F(1,10)=2.9, p=0.1193)

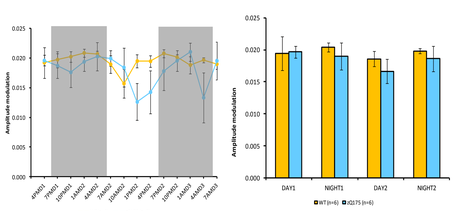
USV Frequency Modulation (FM)



At left, the mean (± SEM) degree of FM of USVs for WT and WT-zQ175 pairings are plotted across 3 hour bins. Shaded areas indicate dark cycle. At right, mean (± SEM) FM is plotted by 12 hour (day/night) bins for WT and WT-zQ175 pairings.

Frequency modulation in USVs was not affected by male genotype (F(1,10)=0.64, p=0.4439) or by circadian cycle (F (3,26)=0.77, p=0.5201), nor by the interaction of these factors (F(3,26)=0.16, p=0.9254).

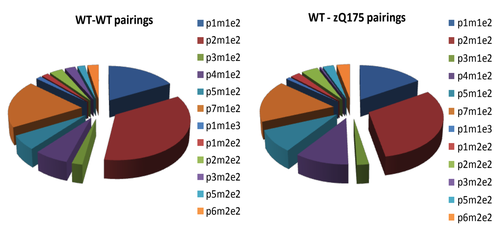
USV Amplitude Modulation (AM)



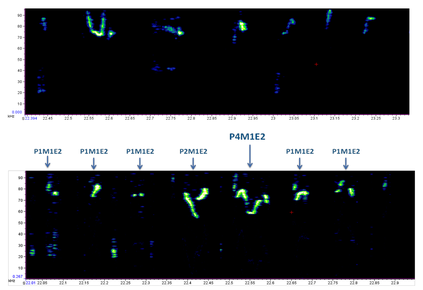
At left, the mean (± SEM) AM of USVs in WT and WT-zQ175 pairings are plotted across 3 hour bins. Shaded areas indicate dark cycle. At right, mean (± SEM) AM is plotted by 12 hour (day/night) bins for WT and WT-zQ175 pairings .

AM in USVs was not affected by male genotype (F(1,10) =0.36, p=0.5619) or by circadian cycle (F(3,26)=1.97, p=0.1436), nor by the interaction of these factors (F(3,26) =0.34, p=0.7934).

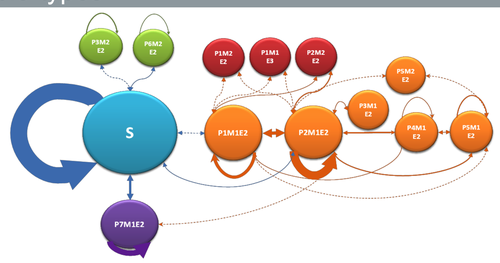
Classification and sequential organization of USV syllable-types



Relative proportion of syllable types observed in the interactions of WT-WT pairs (left panel) and zQ175-WT pairs (right panel). Data reflect observed number of syllable types / total syllable types. We found no significant differences in relative proportions of specific syllable types in WT and WT-zQ175 pairings (ps>0.05)



At top, a spectrogram showing a sequence of P1M1E2 syllables. USV frequency (KHz) is plotted on the y-axis and time (s) is plotted on the x-axis. These short high-frequency syllable types typically occurred in repetitive sequences that were preceded and followed by silence. At bottom, a similar sequence of repetitive syllables is punctuated by the mergence of a more-complex vocalization



Transition state diagram of syllable sequences observed in WT-WT social interactions. The frequency of each syllable type (and silence, represented by S) is represented by the relative size of each circle, and the relative likelihood of transitioning to other syllable types is represented by the thickness of corresponding arrows.

Our analysis detected non-random patterns in transition probabilities in WT-WT interactions (SS=80503, p<0.05) and zQ175-WT interactions (SS = 62528.3, p<0.05). These results confirm non-random structures in transition matrices in both genotype conditions. Further analysis directly comparing WT-WT vs zQ175-WT indicated transition matrices were statistically similar (SS=5292.1, p>0.05).

Conclusions

- ❖ Replicating prior results, here we find that heterozygosity at the HTT locus has little effect on behavioral activity and sociality in young (9 week) zQ175 Het KI mice, but these behaviors are strongly circadian-bound. We found a consistent pattern in the number of USVs and their duration, which differed across day/night cycles but not between pairing types
- ❖We identified 12 discrete USV syllable types that varied in pitch, frequency modulation, and entropy. The sequence of syllable types, captured in transition matrices, exhibited non-random patterns likely reflecting an underlying organization. Whereas the present study focused on heterogeneous pairings, future studies may find more robust phenotypic differences by comparing homogenous pairings, or testing subjects at a later age, where behavioral deficits are more pronounced
- ❖This pilot study demonstrates the utility of the **Phenocube®** system for continuous measurement of USVs in a testing environment that simultaneously captures social, cognitive, and behavioral activity