USV DIFFERENCES BETWEEN HAD-1 AND LAD-1 RATS

Alcohol-Naïve USVs Distinguish Male HAD-1 from LAD-1 Rat Strains

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Abstract

Ultrasonic vocalizations (USVs) are mediated through specific dopaminergic and cholinergic neural pathways and serve as real-time measures of positive and negative emotional status in rodents. Although most USV studies focus primarily on USV counts, each USV possesses a number of characteristics shown to reflect activity in the associated neurotransmitter system. In the present study, we recorded spontaneously emitted USVs from alcohol-naïve high alcohol drinking (HAD-1) and low alcohol drinking (LAD-1) rats. Using our recently developed WAAVES algorithm we quantified four acoustic characteristics (mean frequency, duration, power and bandwidth) from each 22 – 28 kHz and 50 – 55 kHz frequency modulated (FM) USV. This rich USV representation allowed us to apply advanced statistical techniques to identify the USV acoustic characteristics that distinguished HAD-1 from LAD-1 rats. Linear mixed models (LMM) examined the predictability of each USV characteristic in isolation and linear discriminant analysis (LDA) and binomial logistic regression examined the predictability of linear combinations of the USV characteristics as a group. Results revealed significant differences in acoustic characteristics between HAD-1 and LAD-1 rats in both 22 – 28 kHz and 50 – 55 kHz FM USVs. In other words, these rats selectively bred for high- and low-alcohol consumption can be identified as HAD-1 or LAD-1 rats with high classification accuracy (approx. 92-100%) exclusively on the basis of their emitted 22-28 kHz and 50-55 kHz FM USV acoustic characteristics. In addition, acoustic characteristics of 22 – 28 kHz and 50 – 55 kHz FM USVs emitted by alcohol-naïve HAD-1 and LAD-1 rats significantly correlate with their future alcohol consumption. Our current findings provide novel evidence that USV acoustic characteristics can be used to discriminate between alcohol-naïve HAD-1 and LAD-1 rats, and

may serve as biomarkers in rodents with a predisposition for, or against, excessive alcohol intake.

Key words: Linear Mixed Model; Linear Discriminant Analysis; Binomial Logistic Regression; Selectively bred Rats; Alcoholism Biomarker

Introduction

Drug addiction is a chronic relapsing disorder with a strong emotional component. During initial use, drugs of abuse hijack the midbrain reward system to produce euphoria and heightened positive emotional states (Wise & Koob, 2014). Persistent or chronic use of these drugs results in a shift in the baseline homeostatic activity of these systems and results in the emergence of a negative affective or withdrawal state when the drug is no longer present (Koob & Volkow, 2016). The onset of this negative state is an important aspect of the transition from recreational drug use to drug dependence. Moreover, individuals with pre-existing negative affective states either due to depression (Conner, Pinquart, & Gamble, 2009; Schuckit, Smith, & Chacko, 2006), posttraumatic stress (Gilpin & Weiner, 2016), or early life adversity (Cornelius, De Genna, Goldschmidt, Larkby, & Day, 2016) are likely to engage in relapse like behaviors (Watkins, Franz, DiLillo, Gratz, & Messman-Moore, 2015) which can further increase their risk of developing a substance use disorder (SUD). Furthermore, strategies aimed at improving emotional regulation have shown promise in reducing drug abuse behaviors (Tang, Tang, & Posner, 2016). Together these studies highlight an important need for understanding the role of emotion in promoting hazardous drug use.

Emotion has been described as a complex psychological state with three components: i) a subjective experience, ii) an underlying neural substrate, and iii) an expressive/behavioral and/or autonomic response (Chiurchiù & Maccarrone, 2016). The clinical studies described above show a clear relationship between the subjective experience (internal and/or external) produced by alcohol and other drugs of abuse and the resulting emotional response. However, since there are few reliable pre-clinical models of emotion, the neural substrates that underlie these phenomena are not well understood.

USV DIFFERENCES BETWEEN HAD-1 AND LAD-1 RATS

Ultrasonic vocalizations (USVs) have been identified as real-time functional measure of emotional status in rodents (Brudzynski, 2009, 2013). Converging evidence from ethological, pharmacological, and neuroanatomical studies has shown that 22 – 28 kHz USVs occur in response to alarm, punishment, or avoidance behaviors and typically represent negative affective status; while 50 – 55 kHz frequency modulated (FM) USVs, directly evoked by dopamine release (Scardochio, Trujillo-Pisanty, Conover, Shizgal, & Clarke, 2015) and produced in response to rewarding stimuli including food, drugs, or sex are thought to represent positive affective states (Knutson, Burgdorf, & Panksepp, 2002). Moreover, each USV is multidimensional and is characterized by a rich set of acoustical properties, including frequency (kHz), duration, bandwidth and power. 22 – 28 kHz USV counts and acoustic characteristics can be directly regulated by cholinergic agonists and antagonists (Brudzynski, 2001; Brudzynski & Bihari, 1990) and 50 – 55 kHz FM USVs can be directly regulated by activating (Ahrens et al., 2013; Maier, Abdalla, Ahrens, Schallert, & Duvauchelle, 2012) or inhibiting (Williams & Undieh, 2010; Wintink & Brudzynski, 2001) the dopaminergic system. Other neurotransmitter systems also shown to modulate USV activity include the neurotensin (Prus, Hillhouse, & LaCrosse, 2014; Steele, Whitehouse, Aday, & Prus, 2017), 5-HT (Beis et al., 2015; Wöhr, van Gaalen, & Schwarting, 2015) and adenosine (Simola, Costa, & Morelli, 2016) systems. Therefore, spontaneous baseline USV activity may relay important information about underlying neurotransmission.

Animal models of high alcohol consumption reveal an intimate relationship between USVs and propensity for excessive drinking. For example, the selectively bred alcohol preferring (P) and alcohol non-preferring (NP) rats are established rodent models of high alcohol drinking and alcohol avoidance, respectively. The high-alcohol-drinking (HAD-1) and low-alcohol-

drinking (LAD-1) rats are another such model that satisfy many of the criteria for an animal model of alcoholism, such as high levels of alcohol consumption during adolescence and adulthood, pronounced alcohol seeking behaviors, and an alcohol deprivation effect under relapse conditions (Bell, Rodd, Engleman, Toalston, & McBride, 2014; McBride, Rodd, Bell, Lumeng, & Li, 2014). In two studies from our laboratory we found that P and HAD-1 rats spontaneously emit significant numbers of negative affect USVs even in the alcohol-naïve state (Reno et al., 2015; Thakore et al., 2016).

Our recent development of a MATLAB-based algorithm (WAAVES) (Reno & Duvauchelle, 2014; Reno, Marker, Cormack, Schallert, & Duvauchelle, 2013) automates the tabulation of USV counts and acoustic characteristics, thereby allowing us to conduct long term studies exploring counts and acoustic characteristics of spontaneously emitted USVs over multiple recording sessions. Using this tool, we conducted a study focused just on P and NP rats (Reno et al., 2017) and found that alcohol-naïve P and NP rats can be distinguished based solely on the acoustic properties associated with 22 – 28 kHz USVs. The ability to distinguish between high- and low-drinking lines according to USV profiles suggests that drinking propensity and USV emissions may be regulated by common neural substrates. The present study aims to extend our previous findings and examine whether either positive or negative affect-associated USV acoustic properties can similarly be used to distinguish between alcohol-naïve HAD-1 and LAD-1 rats.

This work embraces the multidimensional nature of each USV and subjects these USVs to multivariate statistical procedures including linear mixed modeling, linear discriminant analysis and binomial logistic regression. Linear mixed modeling has a number of advantages over more traditional ANOVA based approaches. For example, rather than using individual or

group means, each and every USV acoustic characteristic is input into the analyses, resulting in a full representation of all data. The goal of linear discriminant analysis is to estimate the linear "discriminant" that best separates the multidimensional USVs associated with two groups of rats. In essence, multivariate data is linearly combined to produce a univariate variable aimed at separating groups. We use a 10,000-iteration training-test bootstrapping procedure to fit the model and to determine whether the percent of animals correctly classified by linear discriminant analysis is significantly above chance. Binomial logistic regression is similar in spirit to linear discriminant analysis in that the goal is to discriminate two groups of rats from a linear combination of the USV acoustic characteristics. The difference is that binomial logistic regression makes fewer assumptions regarding the nature of the underlying distributions. By including both linear discriminant analysis and binomial logistic regression we can look for convergence in the conclusions drawn.

Using these powerful analytic tools, the goal of this study was to determine whether HAD-1 and LAD-1 rats could be distinguished solely from the acoustic characteristics associated with spontaneous USVs emitted in the alcohol-naïve state. In this study, linear mixed modeling was used to assess whether the mean frequency, duration, bandwidth, or power of 22 – 28 kHz and 50 – 55 kHz FM calls differed significantly between the HAD-1 and LAD-1 rat lines. Next, we used linear discriminant analysis to determine whether a linear combination of these four acoustic characteristics could be used to distinguish HAD-1 from LAD-1 rats. Lastly, we cross-validated the LDA results using binomial logistic regression.

Methods

Animals

Male high alcohol drinking rats (n = 6; HAD-1 rats: generation 65) and low alcohol drinking rats (n = 6; LAD-1 generation 64) were obtained from Indiana University School of Medicine, Indianapolis, IN, at approximately 32 days old. Animals were handled 5 days per week for 4 weeks to habituate them to experimenters. The University of Texas Institutional Animal Care and Use Committee (IACUC) approved all housing and experimental procedures.

Ultrasonic Vocalization Recordings

HAD-1 and LAD-1 rats were recorded under alcohol-naïve conditions. Following the habituation period, USVs were recorded in 4 hour sessions for 3 days/week for 4 weeks. CM16 microphones were used with an UltraSound Gate interface (Avisoft Bioacoustics) to record USVs at a 250-kHz sampling rate and a 16-bit resolution. On recording days, animals were weighed at the beginning of the dark cycle, transported to a test room, and placed into recording cages (which were identical to their home cage but only used for USV recordings) for 4h test sessions. Each animal was assigned its own recording cage in order to prevent any non-specific behaviors related to novelty or conspecific scents (Wöhr, Houx, Schwarting, & Spruijt, 2008). Based on rat and chamber size, we approximate the distance between the animal's head and the centered microphone to range from 5 cm to 28.4 cm. After the recording session, the animals were transported back to the vivarium and returned to their home cage.

Analysis of USVs

Ultrasonic vocalization recordings were analyzed using the WAAVES program (Reno & Duvauchelle, 2014; Reno et al., 2013). This program reads audio files and produces a frequency spectrogram. The spectrogram is then scanned for sound objects using MATLAB's *Image*

Processing Toolbox (MathWorks, Inc. Natick, MA). For 50 – 55 kHz FM USVs, WAAVES identifies sound objects with a minimum duration of 5 ms occurring in a range of 30 – 120 kHz. An inter-call interval of 10 ms was used to discriminate between individual calls and avoid counting call fragments as separate calls. FM USVs were defined as calls that varied more than 5 kHz over the entire duration of the call. 22 – 28 kHz calls were identified as sound objects occurring in a frequency range of 20 to 30 kHz with a minimum duration of 200 ms. An inter-call interval of 100 ms was used to separate individual calls. These call parameters were derived from the existing literature as well as extensive trial-and-error testing in the laboratory. Some preliminary tests of the robustness of these parameters were undertaken during development of the WAAVES algorithm. Generally speaking, the results were robust to small changes in the WAAVES parameters. Once the calls are identified, several measurements of interest are extracted from each USV call and stored for subsequent analysis. The mean frequency, duration, bandwidth, and power for both 50-55 kHz FM and 22-28 kHz calls were used for statistical analysis.

Validation Process for WAAVES Automation. Validation of WAAVES-generated USV data requires correspondence with human-derived analyses. Experimenters manually analyzed subsets of USV data recorded during the experiment to compare human assessment with WAAVES output. USV data subsets used for manual validation consisted of 80 (out of 3456) 10-min USV files recorded from HAD and LAD rats. The total number of calls identified via manual analysis was correlated with the total number of calls identified by the automated WAAVES program. Separate correlations were also conducted for each group (i.e. HAD and LAD) in order to confirm comparable findings across rat lines. The correlation coefficients are reported in the results.

EtOH Drinking Sessions

Following the USV recording experiments the rats received chronic intermittent access to three bottle choice alcohol (water, 15% EtOH, 30% EtOH) in the home cage, 24 hrs/day, 3 days/week (e.g. Monday, Wednesday, Friday) for 4 weeks in order to validate high vs low alcohol consumption between the selectively bred HAD-1 and LAD-1 rats.

Statistical Approach

A standard statistical approach would utilize repeated measures ANOVA to analyze the USV data. In this approach, all calls emitted by a rat are used to calculate an average, and then any potential group differences in these averages are assessed. Thus, this method results in loss of important information pertaining to the inter-individual variability in USV calls for each rat, which, in turn, reduces power. To overcome these problems, we used linear mixed models to examine the effect of selective breeding (e.g. HAD-1 vs LAD-1) on total USV counts and the pattern of USV acoustic characteristics (e.g. mean frequency, duration, bandwidth, or power). Linear mixed models allow us to use the data from all the calls emitted by each rat, and can also assess for random day-to-day variation due to repeated measurements even in the event of missing data at any of the time points measured. If a significant group effect was observed, its impact on the model's goodness of fit was tested by creating a reduced null model without the group, and then by comparing the reduced model with the full model using an ANOVA. The p-values resulting from the ANOVA are also reported.

Linear Mixed Models. We assessed differences in total USV counts and each of the four USV characteristics as a function of rat line using a linear mixed model in R (R Core Team, 2015) using the package "lmerTest" (Kuznetsova, Brockhoff, & Christensen, 2016). The linear models were generated for each experimental group for each of the 4 acoustic characteristics of

interest. The models were used to assess the effect of time, rat line, or an interaction of these factors on each of these characteristics. Whenever a significant effect was observed a new reduced model was generated by removing the significant factor and compared with the original model using an ANOVA in order to assess the impact of the respective factor on the goodness-of-fit for the model. The resulting model is a regression equation where the intercept and slope is allowed to vary for each rat:

$$Y_{Acoustic\ Characteristic} = \beta_0 + \beta_{Rat\ Line} X_{Rat\ Line} * \beta_{Set\ Day} X_{Set\ Day} + W_{Rat} + U_{Rat*Set\ Day}$$

where $Y_{Acoustic\ Characteristic}$ is the acoustic characteristic being modeled (e.g. mean frequency, duration, bandwidth, or power), each predictor variable is represented by its subscripted X, W_{Rat} represents the random effect of each individual rat, and $U_{Rat*Set\ Day}$ represents the random effect of day to day variation for each rat. A random slope coefficient was included to protect against potential noise introduced by random day-to-day variation in call parameters for each rat. The coefficients (β) are estimated and assessed for significance, as if so, the contribution to the goodness of fit of the model was assessed.

Linear Discriminant Analysis. LMM focuses on each acoustic property in isolation. To assess the combined interactive effect of all four USV characteristics we applied linear discriminant analysis (LDA) using the R package "MASS" (Venables & Ripley, 2002) to determine if a linear combination of these data was capable of distinguishing the rat lines (e.g., HAD-1 vs LAD-1). A linear combination of the multivariate data is used to calculate a univariate (discriminant) value that represents the maximum separation between the groups. Thus, the LDA can be used to determine whether USVs, across acoustic characteristics, emitted by alcohol-naïve HAD-1 rats differ from those emitted by alcohol-naïve LAD-1 rats. Because we were interested

in examining the ability of these acoustic characteristics to distinguish rat lines, we assessed all USVs emitted by each group (e.g. HAD-1 rats and LAD-1 rats) without reference to time.

Since the data are used in building the model, it is possible that the best fitting model would be specific to the data used and may not necessarily generalize to the population as a whole. To address this issue and ensure the generalizability of the model, we split the data into a training and testing subset; where one half of the animals are used to train the model and the remaining half are used to test it. When dividing the groups into training and testing subsets, it is possible that certain combinations of animals within each subset may be more (or less) representative of the entire dataset and, in turn, bias the ability of the model to accurately separate the groups. Thus, in order to produce an accurate assessment, we repeated the LDA 10,000 times, each time randomly selecting half of the data as our training set and using the remaining half to test the model. We then computed the percent of animals correctly assigned to their group[1] for each of the 10,000 iterations. The resulting distribution allows us to estimate the average percent correct and standard error for each iteration, thereby allowing us to compute 95% confidence intervals around the mean percent correct for the 10,000 trials. If the model performs no better than chance alone, we would expect 50% of the animals to be correctly categorized. Therefore, if the 95% confidence interval around the average percent correct includes 50% we cannot conclude that the model is performing better than chance at an alpha level of 0.05.

[1] To compute the percentage of animals correctly assigned to their groups by the LDA, we first computed the average LDA value across all USVs emitted by each animal. Next, we combined the average USV LDA values for each animal to compute the group averages for HAD-1 and LAD-1 rats. We then calculated the midpoint between these two means and used this midpoint as the decision boundary for separation. The animals were then classified as HAD-1 or LAD-1 based on the side of the decision boundary on which their LDA values clustered.

Binomial Logistic Regression. Binomial logistic regression was performed similar to the LDA. The data were randomly split into two groups; one group was used to train the model and the other group was used to test the classification accuracy of the model. Unlike the LDA that produces linear discriminant coefficients, the logistic regression provides probability values for whether a call belongs to a HAD-1 or LAD-1 rat. Similar to the LDA, we then averaged the call probabilities to obtain the average probability for each rat. These rat probabilities were then grouped by HAD-1 or LAD-1 and the midpoint between the probabilities was used as the decision boundary for separation. This process was also repeated 10,000 times and the mean classification accuracy and 95% confidence interval for these iterations are reported.

Results

Linear Mixed Models

22 – 28 kHz USVs. We began by examining differences in USV counts. Alcohol-naïve HAD-1 (total call counts = 854.83 ± 259.71) and LAD-1 (total call counts = 613.33 ± 289.52) rats spontaneously emitted 22 – 28 kHz USVs during the 4-hour recording sessions. However, no significant effect of rat line was observed on the total number of calls emitted. Next, we examined the USV acoustic properties. We observed significant group*day interactions on the mean frequency ($p < 0.0001$, $t_{4576} = 3.974$; Figure 1a), duration ($p < 0.0001$, $t_{165.4} = 4.591$; Figure 1b) and power ($p < 0.001$, $t_{6656} = -3.319$; Figure 1d), but not the bandwidth ($p = 0.146$, $t_{133.6} = -1.461$; Figure 1c) of 22 – 228 kHz USVs. Removal of the interaction significantly reduced the goodness-of-fit for the model for each of the three parameters: mean frequency ($p < 0.0001$, $\chi^2 = 15.397$), duration ($p < 0.0001$, $\chi^2 = 16.333$), and power ($p < 0.01$, $\chi^2 = 10.751$). However, visual analysis did not reveal any clear group*day trends. It is possible that theses test may be too sensitive to the within-subject day-to-day variability observed in USV calls. Therefore, the main

effect of group was also analyzed for each acoustic characteristic whether or not a group*day interaction was observed. We found a significant effect of rat line on the duration ($p < 0.0001$, $t_{2222} = -25.02$; Figure 1b), and power ($p < 0.0001$, $t_{7888} = 4.946$; Figure 1d) of 22-28 kHz USVs of alcohol-naïve HAD-1 and LAD-1 rats. No such effect of rat line was observed on the mean frequency ($p = 0.303$, $t_{6894} = 1.03$; Figure 1a) or bandwidth ($p = 0.151$, $t_{4142} = 1.438$; Figure 1c). Removal of the group effect resulted in a significant reduction in the goodness-of-fit of the linear mixed model for both the duration ($p < 0.0001$, $\chi^2 = 592.38$) and power ($p < 0.0001$, $\chi^2 = 24.06$).

In summary, there were statistically significant group*day interactions in the mean frequency, duration and power, but not the bandwidth of 22 – 28 kHz USVs, although the directionality of these interactions was not apparent through visual analysis. We also observed a significant effect of rat line between alcohol-naïve HAD-1 and LAD-1 rats on the duration and power of 22 – 28 kHz calls. Post-hoc analyses did not reveal any clear differences in the call power of 22-28 kHz USVs between HAD-1 and LAD-1 rats, the LAD-1 rats made longer calls than HAD-1 rats on all recording days over the 4-week period. No further effects were observed on the mean frequency or bandwidth of 22 – 28 kHz USVs.

Next, we used regression analyses to determine whether the USV acoustic properties of 22 – 28 kHz calls corresponded with future alcohol consumption in these rats. We found a significant negative correlation between the duration of 22 – 28 kHz calls and future EtOH consumption ($R = -0.866$, $p < 0.01$) in the combined HAD-1 and LAD-1 sample. No further correlations were observed between EtOH consumption and the mean frequency, bandwidth or power of 22 – 28 kHz calls.

50 – 55 kHz USVs. We again began with an examination of USV counts and followed with analyses of the USV acoustic characteristics. Both HAD-1 (total call counts = 272 ± 44.84)

and LAD-1 (total call counts = 168.17 ± 34.38) rats emitted spontaneous 50 – 55 kHz FM USVs. There was a significant effect of rat line on the total number of USVs emitted ($p < 0.05$, $t_{102.05} = 2.396$). Removing the effect of rat line resulted in a significant reduction in the goodness-of-fit of the model ($p < 0.05$, $\chi^2 = 5.3354$). There were no significant group*day interactions for the mean frequency ($p = 0.212$, $t_{68.270} = -1.259$), duration ($p = 0.872$, $t_{14,722} = -0.164$), bandwidth ($p = 0.602$, $t_{19.906} = -0.529$) or power ($p = 0.108$, $t_{27.270} = -1.663$) of 50 – 55 kHz FM calls. However, significant effects of rat line were observed in the mean frequency ($p < 0.0001$, $t_{289.31} = 9.896$; Figure 2a), duration ($p < 0.0001$, $t_{18.779} = 14.72$; Figure 2b), bandwidth ($p < 0.0001$, $t_{79.790} = -5.248$; Figure 2c), and power ($p < 0.01$, $t_{204.19} = -3.18$; Figure 2d) of 50 – 55 kHz FM USVs of alcohol-naïve HAD-1 and LAD-1 rats. Removing this effect significantly reduced the goodness-of-fit for the model regarding each of the four parameters: mean frequency ($p < 0.0001$, $\chi^2 = 89.31$), duration ($p < 0.0001$, $\chi^2 = 117.48$), bandwidth ($p < 0.0001$, $\chi^2 = 25.963$), and power ($p < 0.01$, $\chi^2 = 8.3959$).

In summary, although we did not see any group*day interaction in any of the characteristics measured, there were statistically significant group differences between alcohol-naïve HAD-1 and LAD-1 rats in USV counts, mean frequency, duration, bandwidth and power of 50 – 55 kHz FM calls. The HAD-1 rats made calls with a higher mean frequency and longer duration than the LAD-1 rats, while the LAD-1 rats made calls with a wider bandwidth. The effect of rat line on USV dB levels (e.g., power) of these calls was not clear.

Regression analysis was used to determine whether the USV acoustic properties of 50 – 55 kHz FM calls corresponded with future alcohol consumption in these rats. We found a significant positive correlation between future EtOH consumption and the mean frequency ($R = 0.690$, $p < 0.05$) and duration ($R = 0.899$, $p < 0.001$) of 50 – 55 kHz FM USVs in the combined

sample. In addition, a significant negative correlation was observed between EtOH consumption and the bandwidth ($R = -0.815$, $p < 0.01$) of 50 – 55 kHz FM calls in the combined sample. There was no significant correlation between EtOH consumption and the power of 50 – 55 kHz FM calls emitted by these rats.

Linear Discriminant Analysis

After assessing the differences between HAD-1 and LAD-1 rats on total emitted calls and each individual acoustic characteristic using linear mixed models, we sought to examine whether it was possible to discriminate these groups by using a combination of the mean frequency, duration, bandwidth, and power of USV calls. One way to achieve this is to use a linear discriminant analysis, a statistical and machine-learning method used to separate two or more classes of objects (e.g. HAD-1 vs. LAD-1) based on a linear combination of explanatory variables. To achieve this aim, we split our data into "testing and "training" subsets and used the bootstrapping approach described in the statistical methods above. Once we were confident that the LDA model could accurately classify the two strains we generated a new equation using the entire data set in order to calculate the coefficients associated with each acoustic characteristic.

22 – 28 kHz USVs. The LDA equation calculated using the mean frequency, duration, bandwidth, and power of 22 – 28 kHz USVs from alcohol-naïve rats resulted in perfect characterization of HAD-1 and LAD-1 rats in 3,674 of the 10,000 iterations. The mean classification accuracy was 81.96%, and the 95% confidence interval was 50% - 100%. Though it should be noted that 9,283 out 10,000 iterations produced classification accuracy greater than 66.66%. For the LDA equation the order of the degree of separation contributed by each of the acoustic characteristics was as follows: call duration, power, bandwidth, and mean frequency. With call duration contributing the most to the separation and mean frequency contributing the

least. Applying LDA to the full complement of the data resulted in a maximum separation accuracy of 91.66% (Figure 3a). The corresponding equation coefficients are listed in the table below (Table 1).

50 – 55 kHz USVs. The LDA equation calculated using the mean frequency, duration, bandwidth, and power of 50 – 55 kHz USVs from alcohol-naïve rats resulted in perfect characterization of HAD-1 and LAD-1 rats for all 10,000 iterations. Therefore, the mean classification accuracy was 100% and the 95% confidence interval was the same (Figure 3a). The order of contribution to the separation capacity of the model was: call duration, mean frequency, power, and bandwidth. Applying the LDA to the full complement of data also resulted in complete separation of the two rat lines. The corresponding equation coefficients are listed in the table below (see Table 1).

Binomial Logistic Regression

While linear discriminant analysis is a well-established method of classifying a binary data set (such as the HAD-1 vs. LAD-1 data) using independent predictor variables (such as USV acoustic characteristics), it relies on the assumption that these predictor variables are normally distributed. In order to test the distribution of our data we performed a Shapiro-Wilk normality test and found that none of the four variables of interest had a normal distribution. Although small deviations in normality are not thought to significantly impact the outcome of LDA, the lack of normality in our data highlighted the need to further validate the results achieved with the LDA approach using a second method. Binomial Logistic Regression is another such technique that can be used to develop linear classification models, which relies on fewer assumptions than the LDA method (Pohar, Blas, & Turk, 2004). Thus, the logistic regression approach might be better suited in instances where the assumptions of the LDA are

violated. We performed logistic regression in a manner similar to the LDA method described above. The data were split into testing and training subsets and the classification accuracy was measured. The process was repeated 10,000 times, and the mean and its 95% confidence interval for classification accuracy are reported.

22 – 28 kHz USVs. The binomial logistic regression equation calculated using the mean frequency, duration, bandwidth, and power of 22 – 28 kHz USVs from alcohol-naïve rats resulted in perfect characterization of HAD-1 and LAD-1 rats in 5,044 of the 10,000 iterations. The mean classification accuracy was 91.74%, and the 95% confidence interval was 83.33% - 100%. Similar to the LDA results when logistic regression was applied to the full complement of data, a separation accuracy of 91.66% was achieved (Figure 3b). The corresponding logistic equation coefficients are reported in the table below (see Table 2).

50 – 55 kHz USVs. The binomial logistic regression equation calculated using the mean frequency, duration, bandwidth, and power of 50 – 55 kHz USVs from alcohol-naïve rats also resulted in perfect characterization of HAD-1 and LAD-1 rats for all 10,000 iterations. As such, the mean classification accuracy was also 100% and the 95% confidence interval was the same (Figure 3b). The corresponding logistic equation coefficients are reported in the table below (Table 2).

Together, these results show that the logistic regression approach is indeed more robust than the LDA approach in classifying “unseen” data. However, when applied to the complete dataset, the LDA provides similar accuracy. Thus, the BLR provides strong confirmatory support for the present LDA results.

Alcohol Consumption

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Repeated-measures ANOVA revealed a significant group by time interaction for EtOH consumption levels ($p < 0.001$, $F_{11,110} = 3.72$; Figure 4a). Pearson's correlation analysis was used as a post-hoc measure to further explore the group by time interaction. As expected, escalation in alcohol intake was observed over time in HAD-1 rats ($r = 0.387$, $p < 0.001$; Figure 4b), but not in LAD-1 rats ($r = -0.153$, $p = 0.20$).

Validation of USV Analysis

WAAVES-automated analysis and manual analysis results were highly correlated for both the 22 – 28 kHz calls ($r = 0.996$) and 50 – 55 kHz ($r = 0.936$). Correlation analyses run separately for HAD and LAD 22-28 and 50-55 kHz USVs showed comparable high correspondence across lines between WAAVES and human-derived counts (HAD: 22 – 28 kHz: $r = 0.997$; 50 – 55 kHz: $r = 0.940$; LAD: 22 – 28 kHz: $r = 0.999$; 50 – 55 kHz: $r = 0.946$).

Discussion

Ultrasonic vocalizations are established markers of positive and negative affective states in rodents. A plethora of studies have shown that different types of USV calls can be elicited by a wide variety of behavioral and pharmacological manipulations. These calls are especially sensitive to modulation by dopaminergic, as well as, cholinergic agonists and antagonists (Brudzynski, 1994; Brudzynski et al., 2011; Simola, 2015). In the present study, we explored whether USV acoustic characteristics from alcohol-naïve rats can be used to discriminate between selectively bred high- and low-alcohol drinking rats. We found clear differences in the acoustic characteristics of 50 – 55 kHz FM and 22 – 28 kHz USV calls between alcohol-naïve HAD-1 and LAD-1 rats. Moreover, we were able to use machine-learning algorithms to

accurately identify rats as HAD-1 vs LAD-1 exclusively on the basis of USV acoustic parameter data.

Frequency modulated 50 – 55 kHz USVs may serve as biomarkers of activity in the mesolimbic dopaminergic system. This activity is associated with positive affective states (i.e., reward and positive reinforcement). Studies have shown that these calls can be directly evoked by dopamine release (Scardochio et al., 2015) and modulated by pharmacological manipulations of dopaminergic transmission. For instance, administration of psychostimulants such as cocaine, amphetamine, and methylphenidate, which are known to increase mesolimbic dopaminergic activity, dose dependently increases the total number of 50 – 55 kHz FM USV calls in rodents (Ahrens, Ma, Maier, Duvauchelle, & Schallert, 2009; Burgdorf, Knutson, Panksepp, & Ikemoto, 2001; Maier et al., 2012). In addition to the increased call counts, amphetamine administration has also been shown to increase in the mean frequency and bandwidth of 50 – 55 kHz calls (Brudzynski et al., 2011; Simola, 2015). Furthermore, these changes could be reversed via D1 and D2 receptor antagonism, or through experimental degradation of the nigrostriatal dopaminergic pathway (Ciucci et al., 2009; Wintink & Brudzynski, 2001; Wright, Dobosiewicz, & Clarke, 2013).

In the present study, we showed that alcohol-naïve HAD-1 rats not only emitted more spontaneous 50 – 55 kHz FM USVs than the LAD-1 rats, but the calls emitted by the HAD-1 rats also had a higher mean frequency, narrower bandwidth, and longer duration than the calls emitted by the LAD-1 rats. These results, in combination with the previous findings about the neural substrates underlying 50 – 55 kHz USVs, suggest that HAD-1 rats may have enhanced basal dopaminergic activity as compared to the LAD-1 rats. Interestingly, alcohol-preferring HAD and P rat lines display 10 – 30% lower tissue levels of dopamine and its metabolites

(dihydroxyphenylacetic acid and homovanillic acid) in the nucleus accumbens (Acb) and the anterior striatum when compared to their LAD and NP counterparts, respectively (Gongwer, Murphy, McBride, Lumeng, & Li, 1989; Murphy et al., 2002). Though these results may seem paradoxical at first, lower in vitro basal tissue dopamine levels are thought to mediate increased dopaminergic activity as a compensatory mechanism. Indeed, ventral tegmental area (VTA) dopamine neurons were found to have increased burst firing in P rats (Morzorati & Marunde, 2006). Although a similar increase in burst firing was not seen in HAD-1 rats, other in vivo studies have shown increased levels of extracellular dopamine in the HAD-1 rats when compared with the LAD-1 rats (Katner & Weiss, 2001). This is consistent with increased dopaminergic activity. More recent research has shown that HAD-1 rats have elevated catechol-O-methyl transferase (COMT) mRNA in the posterior VTA, Acb shell, and central amygdala compared with LAD-1 rats (McBride et al., 2012, 2013). COMT enzymatically breaks down dopamine, and other catecholamines, which provides further support for increased dopaminergic activity in the extended amygdala of HAD-1 vs LAD-1 rats. Although we did not directly measure dopaminergic activity in the present study, known differences in dopaminergic transmission between HAD-1 and LAD-1 rats are consistent with our findings that these rat lines can be identified exclusively according to the acoustic characteristics of their emitted 50 – 55 kHz FM USVs.

Emission of 22 – 28 kHz USVs, on the other hand, is associated with medial cholinergic transmission. Contrary to 50 - 55 kHz FM USVs, 22 - 28 kHz USVs are associated with anxiety and other negative affective states (Brudzynski, 2009, 2013). These types of calls can be directly induced with cholinergic activation of the medial hypothalamic/preoptic region in rodents, via carbachol (Brudzynski & Bihari, 1990), and conversely, can be antagonized with application of

cholinergic antagonists, such as atropine and scopolamine (Brudzynski, 2001). Furthermore, the acoustic characteristics, such as call duration, power, and bandwidth, of 22 – 28 kHz USVs were also modulated by carbachol administration in a dose dependent manner (Brudzynski, 1994).

In this study, we found that the significant differences in the acoustic characteristics of spontaneously emitted 22 – 28 kHz USV call duration and power between HAD-1 and LAD-1 rats varied enough to develop a linear discriminant model that could discriminate between HAD-1 and LAD-1 rats with high accuracy. Unfortunately, no study to date has explored potential differences in cholinergic transmission between HAD-1 and LAD-1 rats. Therefore, future studies will need to investigate the cholinergic system in HAD-1 and LAD-1 rats, in order to determine whether the differences in 22 – 28 kHz USV acoustic characteristics between HAD-1 and LAD-1 rats are associated with corresponding differences in cholinergic transmission in these rat lines.

Previous work conducted in our lab showed that 22 – 28 kHz USV acoustic features could be used to accurately discriminate between calls emitted by P vs NP rats (Reno et al., 2017). Moreover, these differences were in line with published literature on differences in cholinergic transmission between P and NP rats (Bell et al., 2016). Here we show that similar to the P/NP rat lines, alcohol-naïve HAD-1 and LAD-1 rats also have differences in the acoustic characteristics of spontaneously emitted 22 – 28 kHz USVs. However, as indicated above, unlike the P/NP rats, HAD-1 and LAD-1 rats also have measurable differences in the acoustic characteristics of spontaneous 50 – 55 kHz FM USVs.

Finally, we show that acoustic characteristics of 22 – 28 kHz and 50 – 55 kHz FM USVs spontaneously emitted by alcohol-naïve HAD-1 and LAD-1 rats correlate with future alcohol consumption of these rats. Our current findings provide novel evidence that USV acoustic

characteristics can be used to discriminate between alcohol-naïve HAD-1 and LAD-1 rats, and may serve as biomarkers in rodents with a predisposition for, or against, excessive alcohol intake.

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Table 1

COEFFICIENTS FOR LINEAR DISCRIMINANT ANALYSIS.

USV Subtype	$\beta_{\text{Mean Frequency}}$	β_{Duration}	$\beta_{\text{Bandwidth}}$	β_{Power}
22 – 28 kHz	-0.3676575	-0.8221113	0.4190684	-0.7509814
50 – 55 kHz FM	0.6065341	0.7826930	-0.2455201	-0.2847963

Note. The coefficients represent the β values associated with each acoustic characteristic used to calculate the linear discriminant values for each 22 – 28 kHz or 50 – 55 kHz frequency modulated (FM) call. The magnitude of these coefficients represents the contribution of the respective acoustic characteristic to the total separation achieved by the LDA model.

Table 2

COEFFICIENTS FOR BINOMIAL LOGISTIC REGRESSION.

USV Subtype	β_0	$\beta_{\text{Mean Frequency}}$	β_{Duration}	$\beta_{\text{Bandwidth}}$	β_{Power}
22 – 28 kHz	0.51509 ± 0.03061	-0.62074 ± 0.03114	-1.46758 ± 0.04180	0.94335 ± 0.04741	-1.17084 ± 0.03414
50 – 55 kHz FM	0.61281 ± 0.04622	0.51768 ± 0.04557	0.80332 ± 0.05774	-0.20861 ± 0.04334	-0.27239 ± 0.04560

Note. The coefficients represent the β values associated with the intercept (β_0) and each acoustic characteristic used to calculate the log odds ratio for each 22 – 28 kHz or 50 – 55 kHz frequency modulated (FM) call. The magnitude of these coefficients represents the contribution of the respective acoustic characteristic to the total separation achieved by the logistic regression model.

USV DIFFERENCES BETWEEN HAD-1 AND LAD-1 RATS

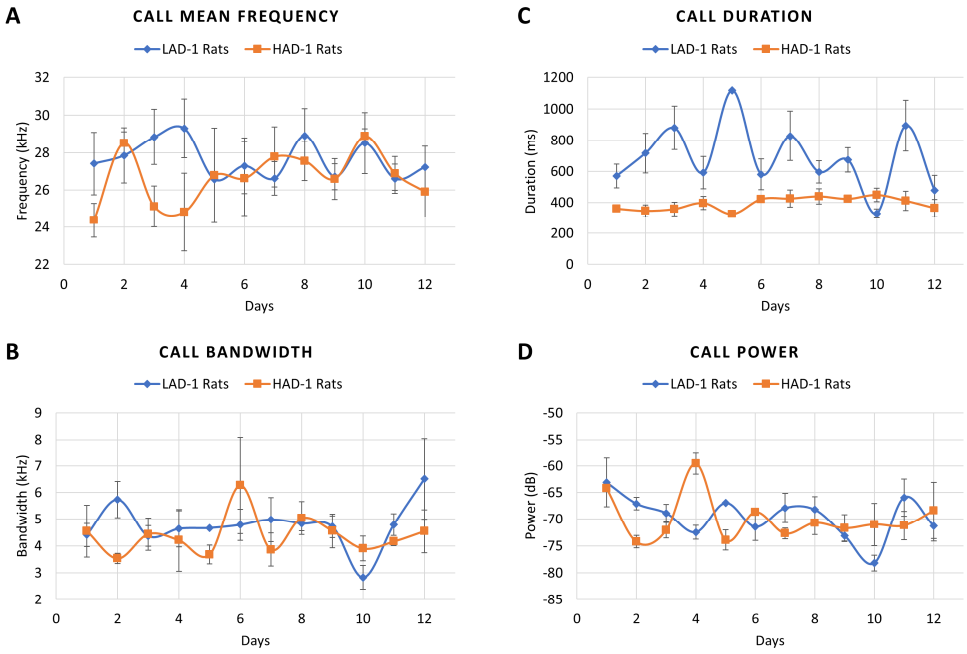

Figure 1. 22 – 28 kHz USV acoustic characteristics of HAD-1 vs. LAD-1 rats. Linear mixed models were used to assess the effect of selective breeding (HAD-1 vs. LAD-1) on the acoustic characteristics of spontaneously emitted 22 – 28 kHz USVs. **A)** Mean Frequency of individual calls did not differ between HAD-1 and LAD-1 rats ($p = 0.303$). **B)** Duration of the calls emitted by LAD-1 rats was significantly higher than those emitted by HAD-1 rats ($p < 0.0001$). **C)** Bandwidth of calls did not differ between HAD-1 and LAD-1 rats ($p = 0.151$). **D)** Power of each call was significantly different between HAD-1 and LAD-1 rats ($p<0.0001$), though no clear direction of this effect was observed.

USV DIFFERENCES BETWEEN HAD-1 AND LAD-1 RATS

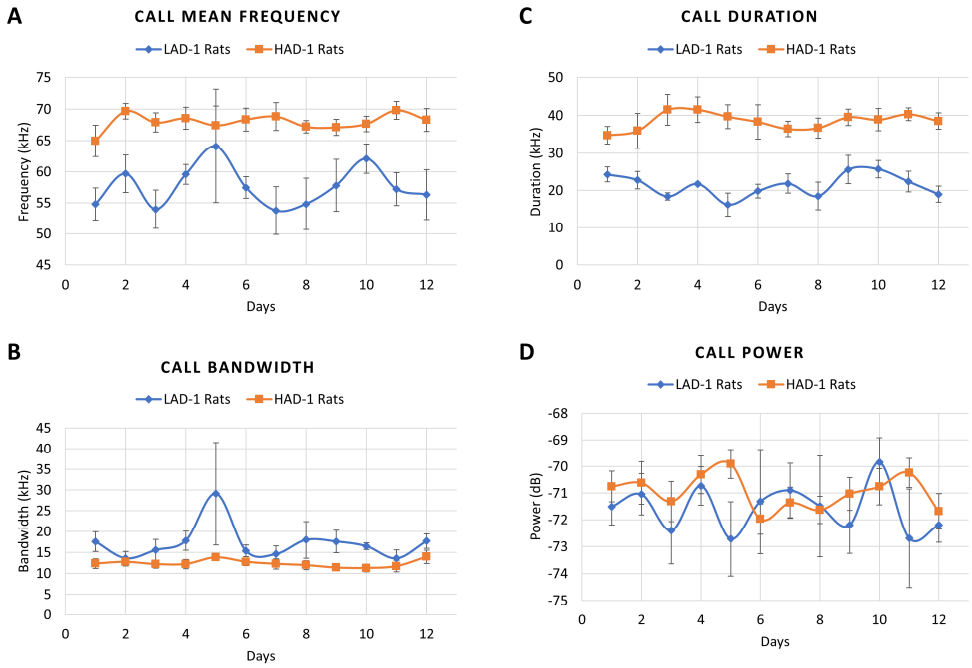

Figure 2. 50 – 55 kHz FM USV acoustic characteristics of HAD-1 vs. LAD-1 rats. Linear mixed models were used to assess the effect of selective breeding (HAD-1 vs. LAD-1) on the acoustic characteristics of spontaneously emitted 50 – 55 kHz Frequency Modulated USVs. **A)** Mean Frequency of the calls emitted by HAD-1 rats was higher than those emitted by the LAD-1 rats ($p < 0.0001$). **B)** Duration of the calls emitted by HAD-1 rats was significantly higher than those emitted by LAD-1 rats ($p < 0.0001$). **C)** Bandwidth of calls made by LAD-1 rats was wider than those made by HAD-1 rats ($p < 0.0001$). **D)** Power of each call was significantly different between HAD-1 and LAD-1 rats ($p<0.01$), though once again no clear direction of this effect was observed.

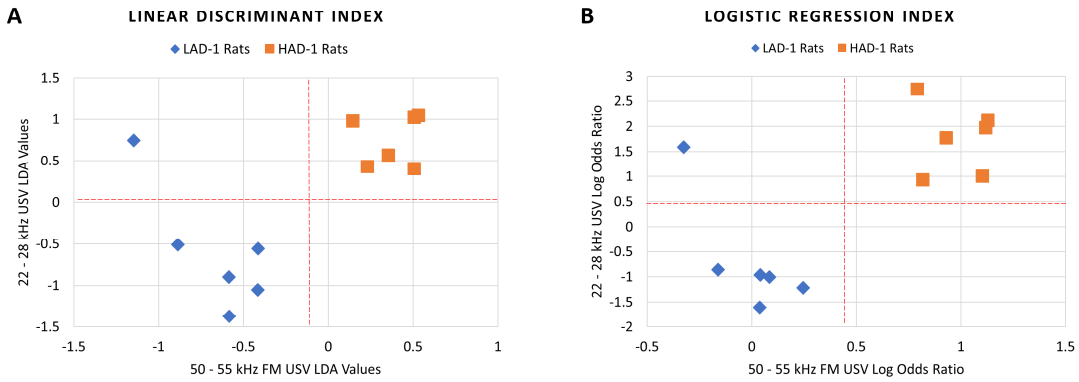

Figure 3. Maximal separation between HAD-1 and LAD-1 rats achieved via LDA and Binomial Logistic Regression Analyses using 22 – 28 kHz and 50 – 55 kHz FM USV data.

A) Linear Discriminant Analysis provided accurate discrimination of 11/12 rats based on 22 – 28 kHz USV data and a complete discrimination of 12/12 rats based on 50 – 55 kHz FM USV data. Horizontal line represents the discrimination threshold for 22 – 28 kHz calls, vertical line represents the discrimination threshold for 50 – 55 kHz FM calls. **B)** Binomial Logistic Regression applied to the complete data set matched the maximal separation achieved by the LDA.

USV DIFFERENCES BETWEEN HAD-1 AND LAD-1 RATS

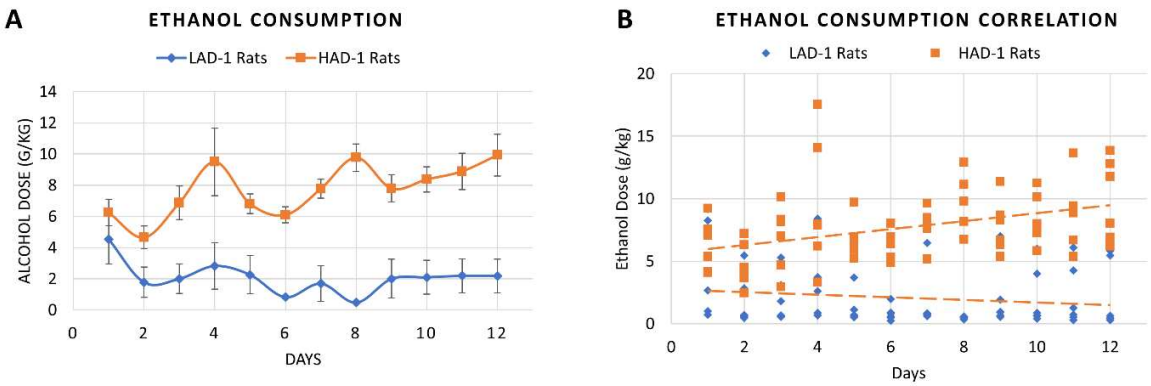

Figure 4. Total alcohol consumption during 4 weeks of 24-hour chronic intermittent ethanol availability sessions. A) HAD-1 rats consumed significantly more alcohol than the LAD-1 rats ($p < 0.001$). **B)** Pearson's correlation analysis revealed an escalation in alcohol intake over time in HAD-1 rats ($r = 0.387$, $p < 0.001$), but not in LAD-1 rats ($r = -0.153$, $p = 0.20$).

Highlights

- Rats selectively bred for high- and low-alcohol consumption can be identified as HAD-1 or LAD-1 rats with high classification accuracy (approx. 92-100%) exclusively on the basis of 22-28 kHz and 50-55 kHz FM USV acoustic characteristics
- Acoustic characteristics of 50 – 55 kHz FM and 22 – 28 kHz USVs in alcohol-naïve HAD-1 and LAD-1 rats significantly correlate with future alcohol consumption
- Findings provide novel evidence that USV acoustic characteristics can be used as biomarkers in rodents with a predisposition for, or against, excessive alcohol intake