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Alcohol-preferring P rats emit spontaneous 22–28kHz ultrasonic vocalizations that are altered by acute and chronic alcohol experience

James M. Reno, B.A.[b,c], **Neha Thakore, PharmD**[a,c], **Rueben Gonzales, PhD**[a,c], **Timothy Schallert, PhD**[b,c], **Richard L. Bell, PhD**[d], **W. Todd Maddox, PhD**[b], and **Christine L. Duvauchelle, PhD.**[a,c,*]

[a]The University of Texas at Austin, College of Pharmacy, Division of Pharmacology and Toxicology, 2409 University Avenue, Stop A1915, Austin, TX 78712, USA

[b]The University of Texas at Austin, Department of Psychology, 108 E. Dean Keeton Avenue, Stop A8000, Austin, TX 78712, USA

[c]Waggoner Center for Alcohol and Addiction Research, The University of Texas at Austin, 2500 Speedway, Stop A4800, Austin, TX 78712, USA

[d]Institute of Psychiatric Research, Department of Psychiatry, Indiana University School of Medicine, Indianapolis, IN 46202, USA

Abstract

Background—Emotional states are often thought to drive excessive alcohol intake and influence the development of alcohol use disorders (AUDs). To gain insight into affective properties associated with excessive alcohol intake, we utilized ultrasonic vocalization (USV) detection and analyses to characterize the emotional phenotype of selectively bred alcohol-preferring “P rats”; an established animal model of excessive alcohol intake. USVs emitted by rodents have been convincingly associated with positive (50–55 kHz) and negative (22–28 kHz) affective states. Therefore, we hypothesized that 50–55 and 22–28 kHz USV emission patterns in alcohol-preferring P rats would reveal a unique emotional phenotype sensitive to alcohol experience.

Methods—50–55 kHz frequency-modulated (FM) and 22–28 kHz USVs elicited from male alcohol-preferring P rats were assessed during access to water, 15% and 30% EtOH (v/v). (EtOH; n=12) or water only (Control; n=4) across 8 weeks of daily drinking-in-the-dark (DID) sessions.

Results—Spontaneous 22–28 kHz USVs are emitted by alcohol-naïve P rats and are enhanced by alcohol experience. During DID sessions when alcohol was not available (e.g., “EtOH OFF” intervals), significantly more 22–28 kHz than 50–55 kHz USVs were elicited, while significantly more 50–55 kHz than 22–28 kHz USVs were emitted when alcohol was available (e.g., “EtOH ON” intervals). In addition, USV acoustic property analyses revealed chronic effects of alcohol experience on 22–28 kHz USV mean frequency, indicative of lasting alcohol-mediated alterations to neural substrates underlying emotional response.

*Correspondence: Christine L. Duvauchelle, PhD, Associate Professor, The University of Texas at Austin, College of Pharmacy, Division of Pharmacology and Toxicology, 2409 University Avenue, Austin, TX 78712, Tel: 512-471-1090, FAX: 512-475-6088, duvauchelle@mail.utexas.edu.

Conclusions—Our findings demonstrate that acute and chronic effects of alcohol exposure are reflected in changes in 22–28 and 50–55 kHz USV counts and acoustic patterns. These data support the notion that initiation and maintenance of alcohol intake in P rats may be due to a unique, alcohol-responsive emotional phenotype and further suggest that spontaneous 22–28 kHz USVs serve as behavioral markers for excessive drinking vulnerability.

Keywords

Alcohol-Preferring Rats; Drinking-In-The-Dark; Emotional Status; Negative Affect; Excessive Alcohol Intake

Introduction

Affect plays an important role in alcohol use disorders (AUDs). For instance, increased negative emotions correspond with increased urges to use alcohol and positive emotional status is associated with alcohol avoidance in alcohol-abstinent individuals with AUDs (Dermody et al., 2013, Schlauch et al., 2013).

To better understand interactions between neurobehavioral/affective states and AUDs, developing relevant animal models is essential. Selectively bred alcohol-preferring P rats display a behavioral phenotype akin to the diagnostic criteria for AUDs and thus provide an excellent model of alcoholism (Bell et al., 2006, Bell et al., 2011, Bell et al., 2012, Bell et al., 2014). Ultrasonic vocalizations (USVs) emitted by rodents are social signals revealing affective states and are typically categorized into two main types: 50–55 kHz frequency-modulated (FM; positive-affect related) and 22–28 kHz (negative-affect related) USVs (Burgdorf et al., 2008, Burgdorf et al., 2011, Panksepp, 2011). Ascending mesolimbic cholinergic (Brudzynski, 1994) and dopaminergic pathways (Burgdorf et al., 2007) mediate 22–28 and 50–55 kHz USV emissions, respectively, and are hypothesized to concurrently initiate positive and negative emotional states (Bihari et al., 2003, Brudzynski, 2009, Brudzynski, 2013). In addition, 50–55 FM and 22–28 kHz USVs can provoke social approach or behavioral inhibition in conspecific receivers in conjunction with brain activation in regions of reward and aversion processing, respectively (Burgdorf et al., 2008, Wohr and Schwarting, 2012, Brudzynski, 2013, Wohr and Schwarting, 2013). Both cholinergic and dopaminergic neural systems are activated during ethanol (EtOH) administration (Larsson et al., 2005, Valenta et al., 2013) and undergo synaptic changes as a result of alcohol exposure (Zhang et al., 2006, Bernier et al., 2011). Therefore, by studying animal models of excessive alcohol intake in conjunction with USV recordings, neurobiological substrates common to emotionality and high alcohol intake may be revealed.

50–55 kHz FM USV counts are significantly increased during cocaine (Maier et al., 2012) and amphetamine administration (Ahrens et al., 2009) and in drug-associated environments (Ma et al., 2010, Maier et al., 2010). Tolerance to cocaine's rewarding effects is demonstrated by the attenuation of cocaine-induced 50–55 kHz USVs after chronic self-administration, even while cocaine responding remains high (Maier et al., 2012). In alcohol withdrawal models, wherein researchers expose animals to a passive route of alcohol

administration (vapor inhalation), the inducement of 22–28 kHz USVs by mild aversive stimuli (air puffs) has been interpreted as a demonstration of negative affect during alcohol withdrawal (Knapp and Pohorecky, 1995, Williams et al., 2012, Berger et al., 2013). While these studies are useful in showing the negative emotional states that accompany the malaise of alcohol withdrawal, the role of affect in the initiation and maintenance of high alcohol consumption remains unclear.

With overriding aims to characterize the emotional phenotype of alcohol-naïve and alcohol-experienced P rats and to determine the role of emotional status in association with alcohol motivation and alcohol experience, we recorded ultrasonic vocalizations of alcohol-preferring P rats in EtOH (three bottle choice of water, 15% and 30% EtOH) and Control (water only) treatment groups across 8 weeks of *Drinking in the Dark* (DID; a voluntary alcohol consumption model) sessions (Bell et al., 2006, Bell et al., 2011). We test the reasonable prediction, derived from our prior work with cocaine (Maier et al., 2012) that the effects of alcohol intake on USV counts will emerge during early alcohol exposure and will attenuate with extended experience. Second, we take the novel approach of examining the acoustic properties of USV mean and peak frequency (e.g., in kHz) and duration within each call type (e.g., 22–28 and 50–55 kHz USVs) that may be altered by alcohol experience. A few studies have examined USV acoustic properties after acute drug treatments (Bihari et al., 2003, Brudzynski and Holland, 2005, Thompson et al., 2006) and found that mean frequency and duration of 22–28 kHz USVs provoked by aversive stimuli were altered under different drug treatments, but 50–55 kHz USV acoustic patterns remained remarkably stable. Taking into account cumulative effects of repeated alcohol exposure on neural pathways common to 50–55 and 22–28 kHz USV production in the current experiment, we predicted that alterations in USV acoustic properties would emerge as a result of chronic, voluntary alcohol exposure.

MATERIALS AND METHODS

Subjects

For the DID experiment, sixteen male alcohol-preferring rats P-rats (P generation = 73) originating from selectively bred rats from a closed colony of Wistar rats (Bell, et al. 2014) were obtained from the Alcohol Research Resource Center at the Indiana University School of Medicine at approximately 7 weeks of age. In order to maximize use of USV recording equipment and sample size during the course of the experiment, eight of the rats were housed under a reverse light/dark (lights out at 0700) and eight of the rats on a traditional light/dark cycle (lights out at 1900) so that DID experiments would commence at the start of the dark cycle for all animals (e.g., sessions conducted at 7 a.m. - 2 p.m. and 7 p.m. - 2 a.m.). Animals were handled daily for 4 weeks prior to the start of the experiment and were group-housed in wire-topped plastic cages (22x44x20 cm) until 1 week prior to the start of the experiment when they were singly housed, but in proximity to former cage mates. Rats were handled daily and received food and water *ad libitum* throughout the entire experiment. Animals assigned to the Control condition were distributed across all three litters and housing groups to guard against the possibility of littermate or cage effects in the small-n group.

Procedures

Apparatus—The DID procedure was conducted in a testing room apart from the vivarium home cages. USV recording sessions were conducted in test chambers identical to home cages (22X44X20 cm) with the addition of an affixed ultrasonic microphone (Avisoft Bioacoustics, Berlin, Germany) on the top center of a sealed Plexiglas cover and the presence of an olfactory cue (coffee scent) to designate test context. Each animal was consistently assigned to a specific test chamber to control for non-specific USV emissions induced by novel environments and conspecific odors (Wohr et al., 2008). In addition, to maintain a clean environment while minimizing potential 50–55 kHz USV responses to fresh bedding (Natusch and Schwarting, 2010) half of the previous day's bedding mixed was retained and half was replaced on a daily basis.

USV Recording—Ultrasonic vocalizations (USVs) were recorded in a range of 10–250 kHz on a PC using CM16 microphones and an UltraSoundGate interface (Avisoft Bioacoustics, Berlin, Germany) at a sampling rate of 250 kHz and a 16-bit resolution. Considering rat and test chamber size, the approximate distance between the centered microphone and the animal's head during test sessions could range from 5 cm to 28.4 cm.

USV Analyses and Validation Procedures—Frequency-modulated (FM) 50–55 kHz and 22–28 kHz USV counts were quantified using the WAAVES algorithm as previously described (Reno et al., 2013). Briefly, the WAAVES algorithm applies a set of conditions to define 50–55 kHz FM and 22–28 kHz USVs and to filter out noise elements prior to tabulation. Some of the conditions specify USV acoustic parameters, such as frequency range and variation (e.g., in kHz), USV call duration and inter-call intervals (e.g., in milliseconds). Acoustic parameters and sound conditions were defined largely in accordance with existing USV literature (Burgdorf et al., 2008, Brudzynski, 2009, Yee et al., 2012). Certain settings (e.g., minimum call duration and inter-call intervals) were based on our experience with noise filtering during USV data collection.

50–55 kHz FM USVs—The WAAVES algorithm defines 50–55 kHz USVs as sound units occurring within a frequency range of 30–120 kHz with a minimum duration of 5 ms. Inter-call intervals of 10 ms or greater were discriminating factors used to avoid counting fragments of calls and to separate single USVs from multiple USVs. The 50–55 kHz USVs were further divided into flat or frequency-modulated (FM) based on the frequency variation within each call. FM calls were defined as USVs that varied 5 kHz or more over the entire duration of the USV. All other USVs of the 50–55 kHz type were designated as flat calls. In the current report, all data analyses (counts and USV parameters) of the 50–55 kHz USVs include only FM USVs, as this type has been shown to be specifically responsive to positive reinforcing events (Ahrens et al 2009, Burgdorf et al 2011, Brudzynski 2013). Counts and parameters of flat 50–55 kHz USVs are not used in these analyses.

22–28 kHz USVs—The 22–28 kHz USV calls were defined as those occurring within the frequency range of 20–30 kHz with a minimum duration of 200 ms. An inter-call interval of at least 100 ms was the criterion set for differentiation between successive 22–28 kHz USVs in order avoid multiple counts of a single, long duration 22–28 kHz USV.

Acoustic Parameters—Acoustic parameter data, including frequency (kHz) and duration measures of each USV are generated during the WAAVES tabulation process. Mean frequency was defined as the grand mean of frequencies determined at every half millisecond of each call. USV duration was simply the duration (ms) of each call. Peak frequency was determined by identifying the frequency at which maximum power occurred within each USV.

Validation Process for WAAVES automation—Validation of WAAVES-generated USV counts requires high correspondence with human-derived counts obtained through visual and auditory means. For this experiment, subsets of USV data recorded during the DID procedure were closely analyzed by research assistants blind to experimental conditions. USV data subsets included experimental and control animals, both light/dark conditions and a full range of USV counts (e.g., high to low quantities). For the 50–55 kHz FM USVs, the data subsets consisted of 55 1-min files. Due to longer duration characteristics of 22–28 kHz USVs, the data subset comparison included 50 10-minute files.

Experimental Sessions: P Rat Drinking-in-the-Dark

Drinking-in-the-Dark (DID) sessions commenced at the start of the dark cycle for all P rats. Animals were weighed 5 days/week just prior to lights out (e.g., 0700 or 1900, depending on light/dark cycle of home cages). Animals were transported to a DID testing room separate from vivarium housing. DID sessions, conducted in the dark with only red illumination, were 7-h in duration and consisted of three 1-h drinking intervals (e.g., “EtOH ON”) interspersed with two 2-h water only intervals (e.g., “EtOH OFF”). During “ON” drinking intervals, rats had access to three 50 ml sipper tubes. For the EtOH group (n=12) tubes were filled with water, 15% ethanol and 30% ethanol (v/v; Pharmco-Aaper) while the Control group (n=4) had access to three tubes of water. During the “OFF” intervals, a single tube of water was available for each animal in both groups. Fluid intake was recorded after each drinking interval. USVs were recorded for the entire 7-h session in each rat three days/week (first, third and fifth day of each week).

Validation of Alcohol Intake Measurements: Blood Alcohol Level Determination

One week after the completion of the DID experiment, P rats from the EtOH group (n=8) were given 30 minutes of ethanol access and immediately anesthetized with isoflurane. The saphenous vein was punctured and blood was collected for determination of blood alcohol level. Triplicate 10 ul samples of blood were pipetted into glass vials containing 90 ul of supersaturated sodium chloride and sealed with a septum. Gas chromatography was conducted as previously described (Valenta et al., 2013). This test was performed specifically to confirm the accuracy of previous ethanol intake measurements, not to confirm blood alcohol levels during DID sessions.

Statistical Analyses

Daily EtOH and Water Intake—Total fluid intake (ml) across the 40 7-h DID sessions was compared between the EtOH and Control groups using 2 x 40 (group X session) mixed design ANOVA.

USV Counts and Acoustic Parameters—To test our predictions of differential effects of acute and chronic alcohol experience on USVs, Weeks 1–4 and Weeks 5–8 were designated as acute and chronic intervals, respectively. Two (group) x 4 (week) mixed-design ANOVAs were conducted on weekly 50–55 kHz FM and 22–28 kHz USV counts (totals of three 7-h sessions/week), USV mean and peak frequencies (kHz) and USV duration (ms) of both call types.

To compare 22–28 and 50–55 kHz FM USV counts elicited specifically during intervals of alcohol availability (e.g., "EtOH ON"; 3-h totals) and alcohol unavailability (e.g., "EtOH OFF"; 4-h totals), within-subject analyses of EtOH and Control groups were performed using two (USV call type) x 4 (week) mixed-design ANOVAs

USV Count and Blood Alcohol Concentration Validation—Pearson's correlation was used to examine the relationship between WAAVES tabulation and human-derived counts and to examine the relationship between amount of ethanol consumed (grams of ethanol per kilogram/30-min access period) and blood alcohol concentration (milligram percent).

RESULTS

P Rat Drinking-in-the-Dark Sessions

USVs during standard and reverse light/dark cycles—Animals housed under standard (lights out at 1900) and reversed (lights out at 0700) light cycles did not display group differences in ethanol intake ($F(1, 11)=3.18$; n.s.), emission of frequency-modulated 50–55 kHz USVs ($F(1, 11)=2.15$; n.s) or emission of 22 kHz USVs ($F(1, 11)=3.28$; n.s.). Therefore, these groups were combined for all subsequent analyses.

Total fluid intake during DID sessions (EtOH ON and OFF Intervals – 7- h total) —EtOH and Control P rats drank comparable amounts of fluid per 7-h test period over the course of 40 daily DID sessions. A comparison of the total amount (ml) of fluid consumption (e.g., EtOH and/or H2O) during each 7-h DID session between EtOH and Control groups was performed using a 2 group x 40 session mixed design ANOVA. Similar levels of consumption between groups ($F(1, 14)=3.01$; n.s.), were observed, but significant day ($F(39, 546)=4.772$; $p<0.001$) and interaction effects ($F(39, 546)=3.29$; $p<0.001$) were also revealed. These effects were likely the result of Control group drinking activity, which, as depicted in Fig 1, show variable patterns of fluid intake, including increasing intake levels over time. Indeed, within-subject ANOVAs of fluid intake show significant day effects for the Control group ($F(39, 117)=3.278$, $p<0.0001$) but not the EtOH group ($F(39,429)=1.319$, n.s.).

Ethanol intake during DID sessions ("EtOH ON" Intervals – 3-h total)—P rats rapidly acquire EtOH drinking to pharmacologically relevant levels. Within-subject repeated measures ANOVA showed significant changes in total ethanol intake per day over 40 DID sessions ($F(39, 429)=2.43$; $p<0.001$), reflecting a rapid increase in ethanol consumption after Week 1 (see Fig 1 Inset).

USV Counts

Between subject analyses: EtOH vs Control Conditions

22–28 kHz USVs - Weeks 1–4 and Weeks 5–8: Significant enhancement of 22–28 kHz USV counts in the EtOH group during Weeks 1–4 indicates acute effects of alcohol experience (see Fig 2A). A 2 group x 4 week mixed-design ANOVA conducted on the weekly average of spontaneously emitted 22–28 kHz USVs by EtOH and Control P rats during Weeks 1–4 of DID sessions showed a significant main effect of group ($F(1,14)=7.277$, $p=.017$), but no week or interaction effects ($p>0.05$). No significant effects emerged during Weeks 5–8 ($p>0.05$).

50–55 kHz FM USVs - Weeks 1–4 and Weeks 5–8: No significant group differences were detected in 50–55 kHz USV counts (see Fig 2B). A significant effect of week was detected during Weeks 1–4 ($F(3,42)=8.547$, $p=.001$) but not during Weeks 5–8 ($F(3,42)=.669$, n.s.) likely reflecting adaptation to the novel DID test environment during initial exposure. No other significant group or interaction effects were observed during Weeks 1–4 or 5–8 ($p>0.05$).

Within-subject analyses: 22–28 kHz vs 50–55 kHz FM USVs: Two (USV call type) x 4 (week) mixed-design ANOVAs were conducted to compare the proportion of 22–28 and 50–55 kHz FM USVs elicited specifically during intervals of alcohol access (e.g., “EtOH or Control ON”; 3-h daily totals) and alcohol absence (e.g., “EtOH or Control OFF”; 4-h daily totals) during Weeks 1–4 and 5–8.

EtOH Group - Weeks 1–4 and Weeks 5–8: Significant shifts in USV call types occurred in response to alcohol access conditions. Within-subject analyses focusing on the effects of alcohol presence and absence during DID sessions revealed that 50–55 kHz FM USV counts were significantly greater than 22–28 kHz USVs during “EtOH ON” intervals, (Weeks 5–8: $F(1,11)=11.453$, $p=.006$; Weeks 1–4, marginal significance: $F(1,11)=4.569$, $p=.056$; see Fig 3A). Significant week ($F(3, 33) = 7.053$, $p=.001$) and interaction ($F(3,33)=4.037$, $p=.015$) effects were also observed in Weeks 1–4 during EtOH ON intervals, but not Weeks 5–8 ($p>0.05$). During “EtOH OFF” intervals, 22–28 kHz USVs were significantly higher than 50–55 kHz FM USVs during Weeks 1–4 ($F(1,11)=13.170$, $p=0.004$) and Weeks 5–8 ($F(1,11)=8.891$, $p=0.012$; see Fig 3B). No additional main or interaction effects during OFF intervals were observed in Weeks 1–4 or 5–8 ($p<0.05$).

Control Group – Weeks 1–4 and Weeks 5–8: In Weeks 1–4, significantly more 50–55 kHz FM USVs than 22–28 kHz USVs were elicited during ON ($F(1,3)=11.790$, $p=0.041$) intervals, but not during OFF intervals or ON or OFF intervals during Weeks 5–8 ($p>0.05$; see Fig 3C).

USV Acoustic Parameters

Mean Frequency

22–28 kHz USVs – Weeks 1–4 and 5–8: Chronic alcohol experience suppressed mean frequency of 22–28 kHz USVs compared to Controls (see Fig 4A). Two group x 4 week

mixed-design ANOVAs conducted on weekly mean frequency of 22–28 kHz USVs elicited by EtOH and Control P rats revealed significant group effects during Weeks 5–8 ($F(1,14)=10.219$, $p=0.006$) and marginal significance across Weeks 1–4 ($F(1,14)=4.062$, $p=0.063$). No significant week or interaction effects were detected during Weeks 1–4 or 5–8 ($p>0.05$).

50–55 kHz FM USVs- Weeks 1–4 and Weeks 5–8: No significant group or interaction effects were revealed on weekly mean frequency of the 50–55 kHz FM USVs in the EtOH and Control P rats during Weeks 1–4 and 5–8 ($p>0.05$) (see Fig 4B). Significant week effects were detected in Weeks 1–4 ($F(3,42)=4.093$, $p=0.012$), but not Weeks 5–8 ($p>0.05$).

Peak Frequency

22–28 kHz USVs – Weeks 1–4 and 5–8: No significant group, week or interaction effects were revealed on weekly peak frequency of the 22–28 kHz USVs in the EtOH and Control P rats during Weeks 1–4 and 5–8 ($p>0.05$).

50–55 kHz FM USVs- Weeks 1–4 and Weeks 5–8: No significant group or interaction effects were revealed on weekly peak frequency of the 50–55 kHz FM USVs in the EtOH and Control P rats during Weeks 1–4 and 5–8 ($p>0.05$). Significant week effects were detected in Weeks 1–4 ($F(3,42)=3.146$, $p=0.012$), but not Weeks 5–8 ($p>0.05$).

Mean Duration

22–28 kHz USVs - Weeks 1–4 and 5–8: Mean duration 22–28 kHz USVs did not differ significantly between EtOH and Control P rats during Weeks 1–4 or 5–8 of DID sessions (see Fig 4C), such that no significant main or interaction effects were observed ($p>0.05$).

50–55 kHz FM USVs - Weeks 1–4 and 5–8: No significant group, week or interaction effects were revealed during Weeks 1–4 or 5–8 ($p>0.05$, see Fig 4D).

Validation Tests: Alcohol consumption assessments: Blood alcohol concentration tests were performed to confirm the accuracy of alcohol consumption assessments during DID sessions. Pearson's correlation was used to examine the relationship between amount of ethanol consumed (grams of ethanol per kilogram/30-min access period) and blood alcohol concentration (milligram percent). After 30 minutes of alcohol access, the amount of ethanol consumed (g/kg/30-min) was significantly correlated with blood alcohol concentration (mg %, $r(6)=0.71$; $p<0.05$, see Fig 5).

Validation Tests: Correspondence between WAAVES-generated and manual USV assessments: WAAVES automated analysis and manual human analysis techniques (visual and auditory confirmation) were highly correlated for both 22–28 kHz USVs ($r(48)= 0.985$; $p<0.001$; Fig 6A) and 50–55 kHz FM USVs ($r(53)=0.97$; $p<0.001$; Fig 6B).

DISCUSSION

The present study revealed that selectively bred alcohol-preferring P rats; both alcohol naïve and alcohol experienced, elicit numerous spontaneous 22–28 kHz USVs. A number of

studies have reported the ability to evoke 22–28 kHz USV responses through acute or conditioned aversive treatments in a variety of rat strains (Knapp and Pohorecky, 1995, Knapp et al., 1997, Williams et al., 2012, Berger et al., 2013), yet this is the first report of non-conditioned and unprovoked emission of long-duration 22–28 kHz USVs in any rat line. In addition, these 22–28 kHz USVs showed immediate and long-term responses to alcohol experience and comprised an atypically high proportion of total USVs elicited by P rats.

Average alcohol intake in the EtOH group over 8 weeks of DID sessions was 2.12 (+/− 0.03) g/kg/session. Though a pharmacologically relevant dose (as confirmed by blood alcohol concentration via gas chromatography), it is considerably lower than previously reported for P rats during similar scheduled alcohol access sessions (e.g., ≈ 5–7 g/kg) (Bell et al., 2006, Bell et al., 2011, Bell et al., 2012, Bell et al., 2014). We suggest that methodological differences employed in our studies; such as extended pre-experiment handling procedures may account for this difference. For instance, the present study employed 4 weeks of daily handling sessions prior to DID sessions to increase familiarity with human interaction and decrease anxiogenic responses. However, standard DID methodologies, as cited above, do not report this practice. If innate negative emotional responses to the environment contribute to alcohol motivation in P rats, lower alcohol intake levels would be a reasonable prediction for well-handled P rats. Nevertheless, even though P rats were noticeably more docile after extensive handling, subsequent alcohol intake reached pharmacologically relevant levels throughout the duration of the study.

Our hypothesis that voluntary alcohol consumption would influence USV counts during early alcohol experience was confirmed by data showing significantly enhanced spontaneous 22–28 kHz USVs during Weeks 1–4 in the EtOH group compared to Controls. These alcohol effects were followed by diminished USV emissions despite continued ingestion of high alcohol intake; a behavioral response pattern consistent with other reports of “tolerance” to alcohol-induced behavioral effects after repeated alcohol experience (Tampier et al., 2000, Batista et al., 2005, Ginsburg et al., 2008, Bell et al., 2011).

The human literature has provided strong evidence linking negative emotional states to alcohol drinking (Schlauch et al., 2013). In rodents, 22–28 kHz USVs are elicited in response to a number of unfavorable conditions, including external (e.g., predator threat) (Litvin et al., 2007) and adverse internal states (e.g., fear, pain, anxiety) (Bihari et al., 2003, Brudzynski, 2009, Brudzynski, 2013). Though the specific circumstances prompting spontaneous emissions of 22–28 kHz USVs by P rats are yet to be determined, the current findings of enhanced 22–28 kHz USVs in the EtOH group indicate these calls are closely associated with alcohol ingestion.

An unexpected finding in the current study was the atypically high proportion of spontaneous 22–28 kHz elicited from P rats compared to their 50–55 kHz FM USVs. Indeed, as revealed by within group analyses, total USV counts of 22–28 kHz and 50–55 kHz FM USVs were (usually) statistically comparable in Controls, unlike findings from our previous studies with Sprague Dawley rats showing 50–55 kHz USVs at close to 100% (Ahrens et al., 2009, Ma et al., 2010, Maier et al., 2010, Maier et al., 2012, Ahrens et al., 2013). However, if negative affective status increases the propensity for ethanol intake in P

rats as well as humans (e.g., Dermody, et al 2013), enhanced 22–28 kHz USV activity could be an unintentional artifact of selective breeding for high ethanol intake.

P rats in the EtOH group elicited significantly more 22–28 kHz than 50–55 kHz FM USVs in the absence of alcohol (e.g., EtOH OFF) and significantly more 50–55 kHz FM USV counts during alcohol availability (e.g., EtOH ON). These significant differences endured throughout acute and chronic phases of the DID sessions (e.g., Weeks 1–4 and 5–8). In addition to demonstrating the high responsiveness of 22–28 kHz and 50–55 kHz FM USVs to scheduled alcohol availability conditions, these findings suggest that alcohol-experienced rats experience a negative reinforcement state in the absence of alcohol and immediate positive effects during alcohol access. However, since DID sessions begin with “EtOH ON” intervals (e.g., first hour of all DID sessions), it could be argued that the apparent positive response to EtOH availability could reflect novelty effects to the test environment. Indeed, this appears to be the case with Controls, as they elicited enhanced 50–55 kHz FM USVs compared to 22–28 kHz USVs during initial (Weeks 1–4) ON intervals. On the other hand, the EtOH group demonstrated enhanced proportion of 50–55 kHz FM USVs during EtOH ON intervals at marginal significance levels during Weeks 1–4, but statistical significance with continued EtOH experience (e.g., Weeks 5–8). In contrast, no other significant differences between USV types were observed over weeks or session intervals in Controls after Weeks 1–4.

The existence of overlapping pathways of neural activation between ethanol (Larsson et al., 2005) and USV emissions (Bihari et al., 2003, Brudzynski, 2009, Brudzynski, 2013) led to our prediction that changes in neural pathways sub-serving alcohol intake would be revealed in USV acoustic pattern characteristics after chronic alcohol exposure. Although this seems to be a reasonable prediction, acoustic parameters such as USV duration, mean and peak frequency are rarely reported because of the difficulty in accurately calculating these parameters using manual assessment techniques. These problems are circumvented with the use an automated MATLAB-based USV analyses program, such as our WAAVES algorithm (Reno et al., 2013, Reno and Duvauchelle, 2014) because the duration of each USV and all frequencies contributing to each USV are tabulated in the course of identifying each USV. In addition, the WAAVES algorithm can be programmed to identify the frequency at which the maximum power occurs within each USV. As a result, acoustic parameters can be easily calculated using a MATLAB-based algorithm such as WAAVES. Indeed, our findings of significant group differences in mean frequency of 22–28 kHz USVs support our hypothesis that USV acoustic parameters may reflect alcohol-induced neural alterations.

On the other hand, chronic alcohol intake did not significantly alter mean frequency of 50–55 kHz FM USVs. However, it is possible that decreased dopaminergic tone in ethanol-naive P rats may account for the absence of modified 50–55 kHz FM USV acoustic properties. For instance, ethanol-naïve P rats have lower levels of dopamine (DA) and its metabolites in the nucleus accumbens (NAcc) and anterior striatum (Murphy et al., 1982, Murphy et al., 1987) and fewer DA projections from the ventral tegmental area (VTA) to the NAcc (Zhou et al., 1995) compared with non-preferring (NP) rats, the selectively-bred, alcohol-avoiding counterparts (Bell et al., 2006, Bell et al., 2012).

Previous research showed that cocaine self-administration activates cholinergic interneurons within the NAcc (Berlanga et al., 2003). In similar work, it has been shown that chronic ethanol drinking by P rats significantly up-regulated (~50%) cyclin-dependent kinase 5 (Cdk5, a marker of neuronal plasticity) immunoreactive cholinergic interneurons in the NAcc while significantly down-regulating (~50%) Cdk5 immunoreactive cholinergic interneurons in the infralimbic and prelimbic regions of the prefrontal cortex (Camp et al., 2006). Dopamine controls acetylcholine release and D1- and D2-like dopamine receptors have been identified on cholinergic interneurons of the NAcc (Alcantara et al., 2003, Berlanga et al., 2005). Regarding plasticity of the mesolimbic DA system, evidence with Wistar rats indicates that intracranial self-administration of ethanol into the VTA is mediated by dopaminergic activity within this system (Rodd et al., 2004c). When assessing ethanol-naive P and Wistar control rats, it was found that the posterior VTA of P rats is more sensitive to the reinforcing effects of intracranial self-administration ethanol, such that lower concentrations of ethanol are self-administered, than that of Wistar rats (Rodd et al., 2004a). Subsequent work with the P rat indicated that chronic ethanol consumption shifted the dose-response curve of intracranial self-administration ethanol into the posterior VTA to the left and interspersed periods of ethanol deprivation further enhanced the reinforcing effects of intracranial self-administration ethanol into the posterior VTA (Rodd et al., 2005a, Rodd et al., 2005b). The present findings of altered emotional responses, as measured by USV calls, combined with the above discussion on enhanced sensitivity to the reinforcing effects of ethanol as well as evidence for a highly plastic mesocorticolimbic dopamine system in P rats suggests that the P rat has an innate imbalance between the mesocorticolimbic dopamine system, its modulation by acetylcholine and vice versa. However, further studies are needed to evaluate this hypothesis.

Findings from the current study support the notion that 22–28 kHz and 50–55 kHz FM USVs reflect emotional responses to the presence and absence of alcohol, and that positive and negative affect influence voluntary alcohol intake behavior. A unique, alcohol-sensitive emotional phenotype, exemplified by prominent representation of both 22–28 and 50–55 kHz FM USVs, adds a relevant component to previous characterizations of the P rat as a reliable animal model of human alcoholism (Rodd et al., 2004b, Bell et al., 2006, Bell et al., 2011, Bell et al., 2012, Bell et al., 2014, McBride et al., 2014).

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Abbreviations

AUDs	Alcohol Use Disorders
DID	Drinking in the Dark
EtOH	Ethanol
FM	Frequency-modulated

P rat	Selectively-bred alcohol-preferring rat
USVs	ultrasonic vocalizations
NAcc	Nucleus accumbens
VTA	Ventral tegmental area

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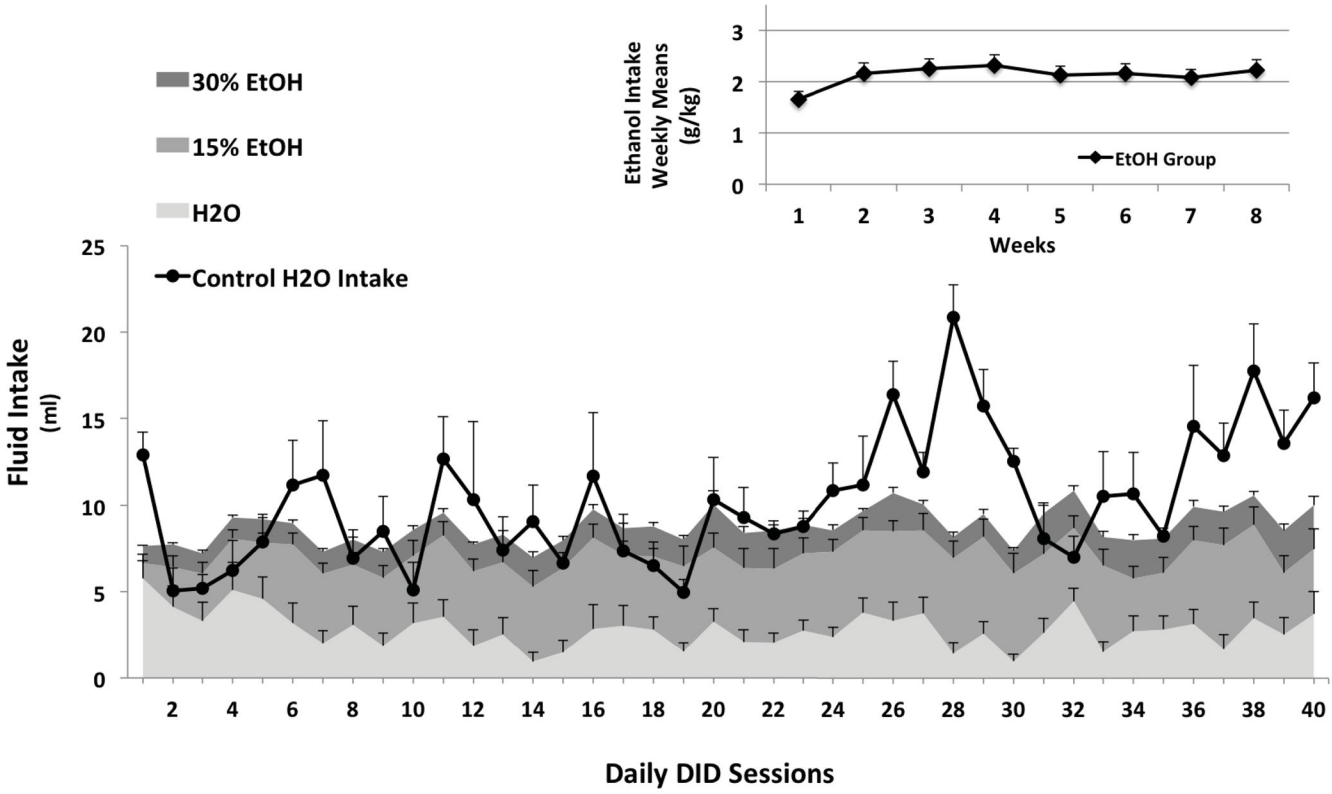

Fig 1. Total fluid intake during DID sessions (ml; mean +/− SEM)

Shaded areas represent fluid intake (water, 15% and 30% EtOH) in EtOH group (n=12) during DID session drinking bouts. Control water intake is represented by overlapping line graph. Total fluid intake was comparable between EtOH and Control groups, though weekly differences indicated an upward trend in Control water intake and stable fluid intake in the EtOH group over time. *Inset:* EtOH Intake (weekly means +/− SEM). Daily EtOH (mean +/− SEM) intake was 2.12 g/kg (+/− 0.03).

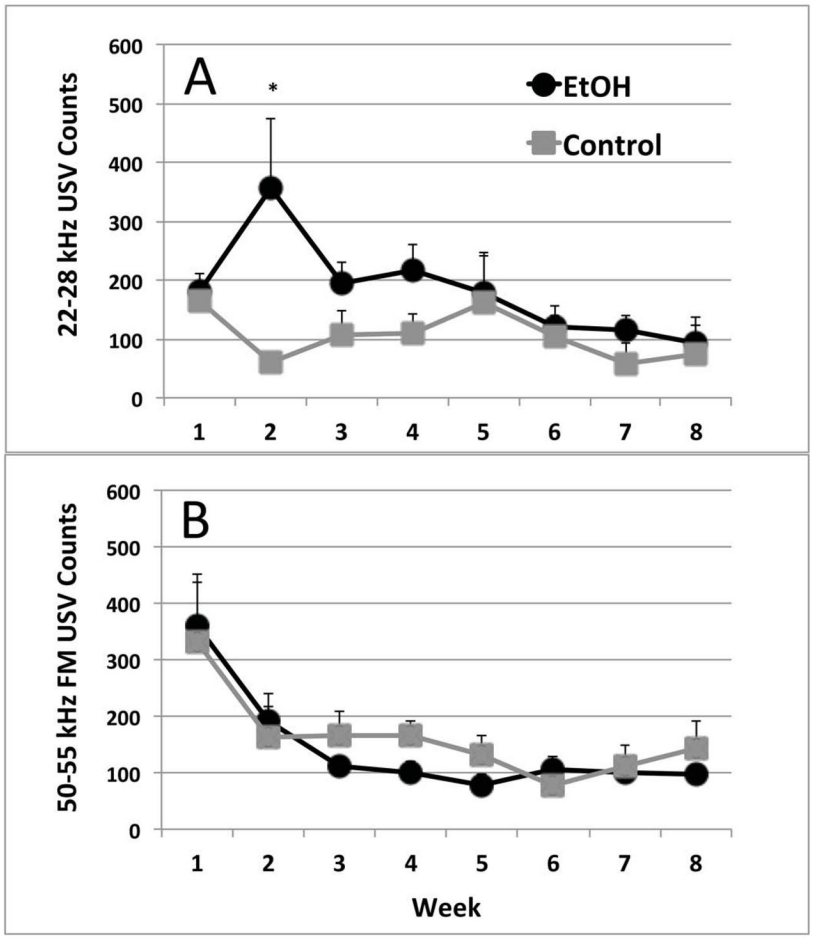

Fig 2. USV Counts in EtOH and Controls during DID sessions (weekly mean +/− SEM)

A. The EtOH group (n=12) spontaneously elicited significantly more 22–28 kHz USVs than Controls (n=4) during Weeks 1–4 ($p<0.05$), but not Weeks 5–8. Post hoc tests showed significant differences between EtOH and Controls during Week 2 (*= $p<0.05$). **B.** 50–55 kHz FM USV counts did not vary significantly between EtOH and Control (n=4) groups, but a significant week effect likely reflects the downward trend across 8 weeks of DID sessions.

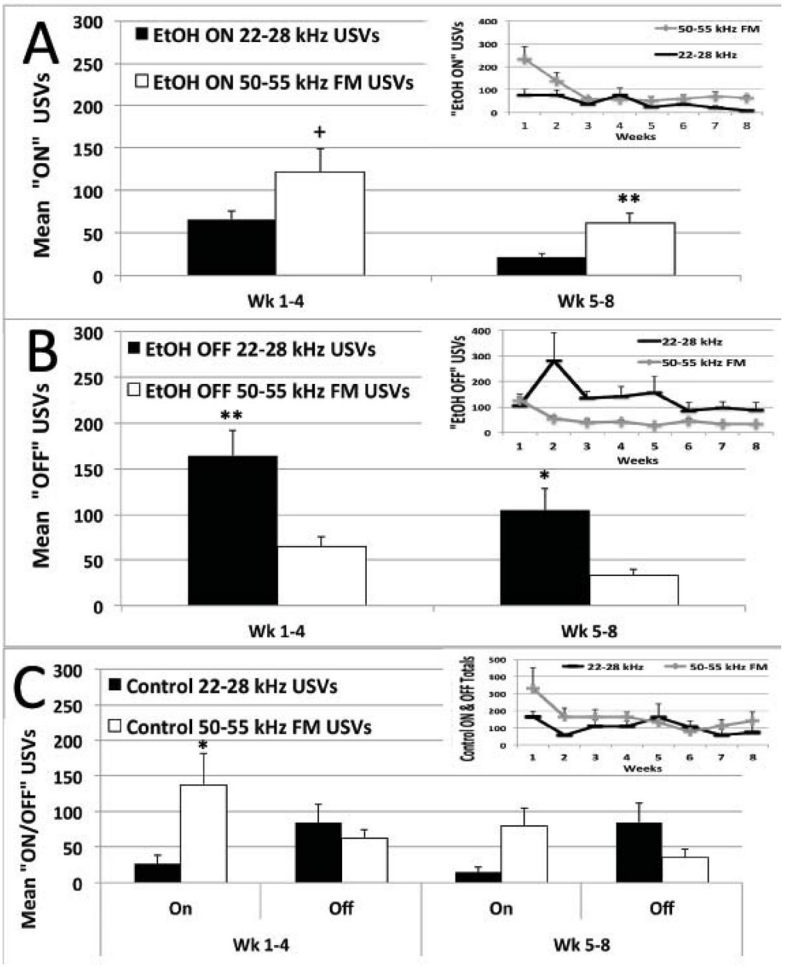

Fig 3. 22–28 kHz and 50–55 kHz FM USV Counts during "EtOH ON" and "EtOH OFF" Intervals: Weeks 1–4 and 5–8 (mean +/− SEM)

A. <u>EtOH Condition:</u> P rats (n=12) elicited significantly more 50–55 kHz FM USV counts than 22–28 kHz USVs during Weeks 1–4 and 5–8 (+ = p @ 0.056 (*marginal*); ** = p < 0.01) during alcohol access (e.g., EtOH "ON" intervals; 3-h total). *Inset:* Weekly USV totals during "EtOH ON" intervals (3-h/DID session, 3 days/week). **B. <u>EtOH Condition:</u>** Significantly more 22–28 kHz USVs than 50–55 kHz FM USVs were elicited while EtOH was not available (e.g., "EtOH"OFF" intervals (4-h total) during Weeks 1–4 and 5–8 (*, ** = p<0.05 and 0.01, respectively). *Inset:* Weekly USV totals during "EtOH OFF" intervals (4-h/DID session, 3 days/week). **C. <u>Control Condition:</u>** Significantly more 50–55 kHz FM USVs were elicited by Controls (n=4) during initial "ON" intervals of Weeks 1–4 (*= p <0.05), but not during Weeks 5–8. No significant differences between the number of 22–28 kHz and 50–55 kHz FM USVs elicited by Controls were detected during "OFF" intervals during Weeks 1–4 or 5–8. *Inset:* Weekly USV session totals (e.g., including both "ON" and "OFF" intervals).

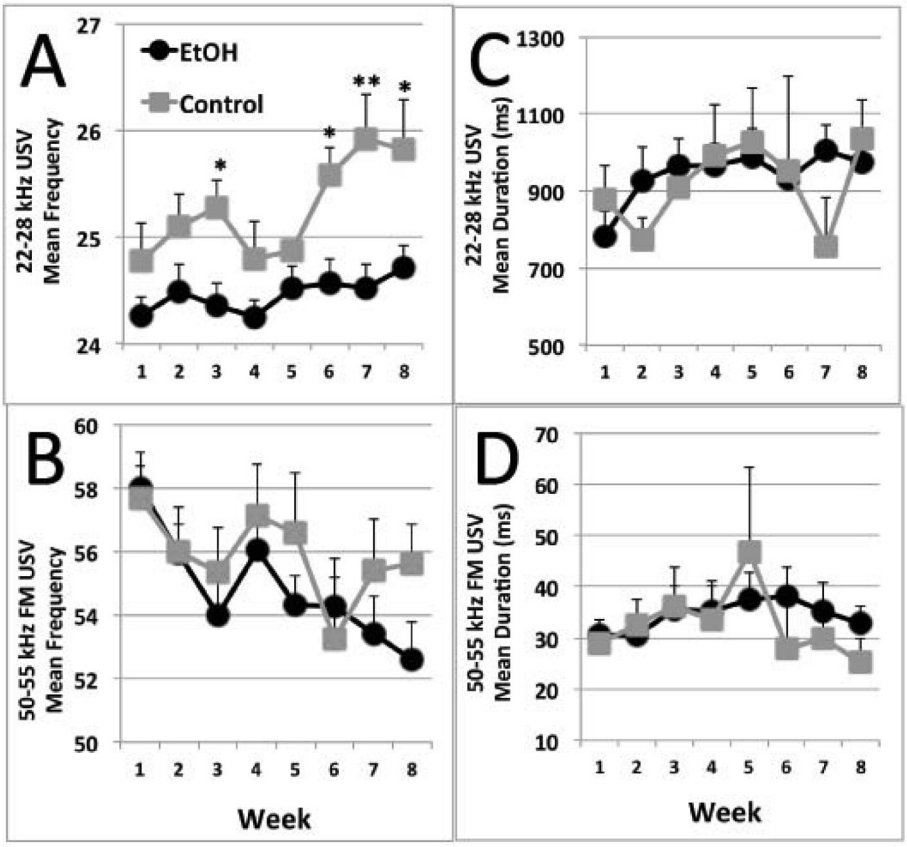

Fig 4. USV Acoustic Parameters: EtOH and Control groups. A & B. USV Frequency (weekly means +/− SEM)

A. 22–28 kHz USVs: Significant differences between EtOH (n=12) and Control (n=4) groups were detected in 22–28 kHz USV frequency particularly during Weeks 5–8 (*, ** = p <0.05, 0.01, respectively). **B. 50–55 kHz FM USVs:** No significant overall or weekly group differences were observed in USV frequency. **C & D. USV Duration (weekly means +/− SEM).** No significant overall or weekly group differences were observed in **C. 22–28 kHz or D. 50–55 kHz FM** USV duration across 8 weeks of DID Sessions.

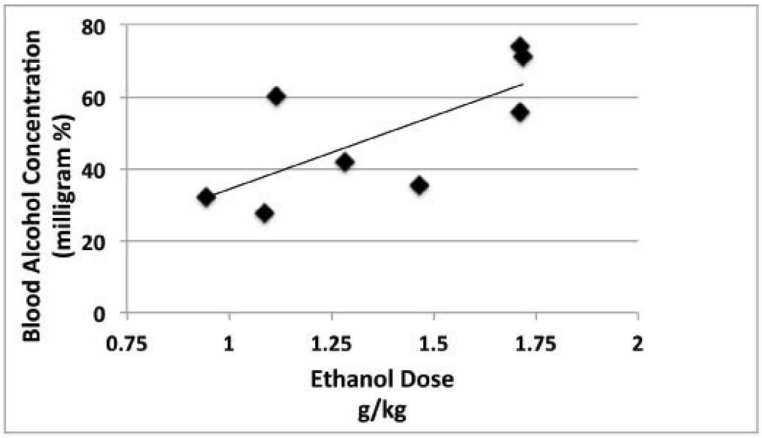

Fig 5. Alcohol Intake Validation: Correlation between blood alcohol concentration (mg%) and assessed ethanol dose

One week after the completion of the DID experiment, blood alcohol concentrations were calculated in alcohol-experienced P rats (n=8) after a single 30-min EtOH access session. This session was conducted to confirm accuracy of EtOH intake measurements during the previous DID experiment, not to simulate blood alcohol concentrations during the actual DID sessions. Significant positive linear relationship ($r= 0.71$, $p<0.05$) between blood alcohol concentrations and assessed EtOH intake confirmed measurement accuracy. It is important to note that this dose range did not reach the average EtOH intake during DID sessions because of the abbreviated 30-min EtOH access interval during this test session compared to 3-h total EtOH access during DID sessions.

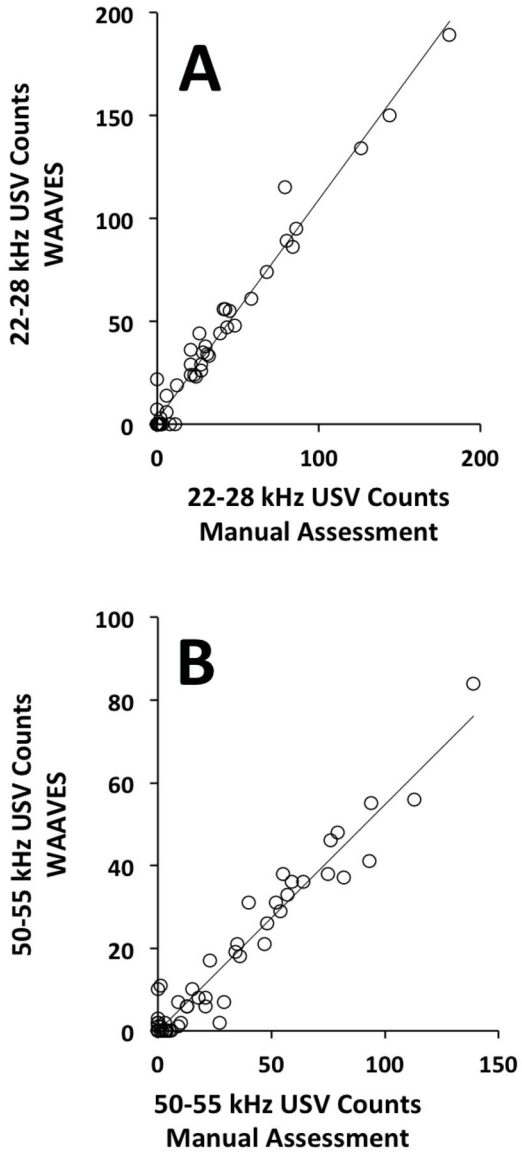

Fig 6. WAAVES USV Count Validation: Correlation between WAAVES and human USV assessments

A. 22–28 kHz USV Counts: From a sample of fifty 10-min USV files, a highly significant linear relationship ($r(48)= 0.985$; $p<0.001$) was revealed between USV counts assessed by WAAVES and manual (visual confirmation) techniques. **B. 50–55 kHz FM USV Counts:** Fifty-five 1-min USV files were used to verify WAAVES analyses by comparison to manual assessment (visual and auditory confirmation). A highly significantly positive linear relationship exists between automated and manual USV assessments ($r(53)=0.97$; $p<0.001$). However, it should be noted that the higher number of 50–55 kHz FM counts tabulated through manual techniques also includes USVs that would not be counted using the WAAVES algorithm (e.g., USVs in which duration is less than the 5 ms criteria).