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Voluntary ethanol consumption changes anticipatory ultrasonic vocalizations but not novelty response

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Abstract

Novelty and sensation seeking (NSS) and affective disorders are correlated with earlier ethanol (ETOH) consumption, and sustained drinking into adulthood. Understanding the NSS response and affective response before and after voluntary ETOH consumption could elucidate important individual differences promoting sustained ETOH consumption. This study determined that NSS and affective response to rewarding stimulation—measured by ultrasonic vocalizations (USVs)—change after adolescent ETOH voluntary drinking. Rats were tested for their NSS response using the inescapable novelty test. Then rats were tested for their affective response to a natural reward and USVs were measured. The natural reward was experimenter-induced play behavior. Rats were exposed to ETOH for 8 weeks using an intermittent two bottle paradigm. After 8 weeks of voluntary consumption, rats were retested for their response to NSS and affective response to natural reward. Results indicate that voluntary ETOH consumption did not change the response to novelty. Control and ETOH exposed rats decreased their novelty response equally after ETOH consumption, suggesting the decrease was due to age. Importantly, voluntary ETOH consumption changed affective USVs. Compared to water-drinking control rats, ETOH-consuming rats elicited greater anticipatory trill USVs to a natural reward-associated context during a post-drinking probe test. Tickle-induced trill USVs did not change differently between ETOH and control rats. These results provide evidence that voluntary intermittent ETOH exposure increases the anticipation of reward and may represent a form of incentive salience. We postulate these diverging effects could be due to differences in incentive salience or reward processing. Together, these results suggest that voluntary ETOH consumption changes the affective response to conditioned and unconditioned natural rewards and offers a behavioral mechanism for studying affective reward processing after ETOH consumption.

Keywords

Novelty seeking; Intermittent access; Ethanol; Ultrasonic vocalizations; Reward processing

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1. Introduction

Alcoholism affects over 16.6 million adults and over 650,000 youth, ages 12–17 in the United States, while only 10–15% are treated for alcohol use disorder (AUD) (National Institute on Alcohol Abuse and Alcoholism, 2013). AUDs are widespread, however, the specific traits contributing to these addictive behaviors vary between individuals. Individual differences moderate the development of AUD [1], but what individual difference traits change after chronic alcohol use is not clearly understood. Determining initial individual differences that promote the development of AUD is important, but determining what individual differences change after chronic ethanol (ETOH) exposure could aid in determining key traits that promote the development of AUDs.

Animal and clinical studies have reported a strong comorbidity between alcohol abuse and anxiety disorders. People who binge drink alcohol excessively are more likely to display depressive behaviors even after consumption had ceased [2]. In order to measure the effect of binge drinking on affect and other behaviors rodent models of binge drinking have been developed. Previous literature indicates successful preclinical binge drinking models incorporate an intermittent access two bottle choice paradigm (IAE). Rats drink significantly more within the first 30 min and in a 24 h period when alcohol is available on intervening days compared to continuous access [3,4]. The IAE successfully models the binge and withdrawal cycles that contributes to dysregulation of hypothalamic-pituitary-adrenal axis, which may result in emotional or affective disorders [5]. The choice to drink ETOH is important in modeling binge drinking and AUD in rodents. In humans, the development of AUD occurs after voluntary drinking, suggesting that individual differences in consumption or response to ETOH play a significant role in AUD. Therefore, a rodent model of voluntary binge ETOH consumption is important for determining what individual differences promote high levels of drinking. Specifically, determining how novelty and sensation seeking (NSS) and emotional stability are related to the development of AUD or determining how they change after chronic ETOH exposure is paramount to understand individual vulnerabilities to AUD contributing to addictive behavior in humans.

In humans, high NSS individuals report earlier ETOH experimentation, greater overall consumption, and sustained drinking behavior into adulthood [6,7]. Sustained levels of escalated ETOH consumption can lead to depressive behavior, and a decrease in positive affect in response to once hedonic stimuli [7,8]. To date, rodent models of NSS have determined that rodent NSS is complex and may be multidimensional as evidenced by unique uncorrelated behavioral responses [9–12]. Novelty *choice* is measured with the novelty place preference test. Higher novelty *preferring* rats show a propensity to transition to compulsive cocaine self-administration and are resistant to aversive consequences associated with drug reinforcement [1,11,13]. Novelty *response* is tested in the inescapable novelty test. Higher novelty *responders* show faster rates of stimulant self-administration acquisition [9,14]. Therefore, data indicate that novelty choice is important for compulsive drug taking and forced novelty response is important for experimentation and acquisition of drug taking [13]. However, stimulant and depressant addiction show separate neurobiological adaptations and behaviors [15]. Despite both of these novelty tests' relation to addiction, the tests are uncorrelated, suggesting that the inescapable novelty test and

novelty place preference test are measuring different aspects of the NSS response. One research group hypothesizes that unlike the novelty place preference test, the inescapable novelty test is measuring the stress response, as evidenced by elevated corticosterone levels [14]. Higher ETOH drinking on an intermittent access schedule results in greater changes to plasma corticosterone levels, and is highly correlated with dysfunctional corticotropin-releasing factor regulation. [5]. Since the IEN test could be used to determine rats' stress response, we aimed to understand how the IEN response changed after intermittent access to ETOH. Alternatively, other research has found that high novelty responders show less preference for ETOH, drink less ETOH, and show a greater ethanol-induced response in locomotor activity when compared to low novelty responders [16,17].

Given that there is a relationship between the IEN and ETOH responses, we focused on determining if the IEN response changes after ETOH consumption [1,11,13,14,18,19]. While the relationship between NSS and drug reward has been researched extensively, determining how natural rewards change after chronic ETOH drinking has not been established. Our lab recently determined that subjective drug value may change differently as a function of the IEN response, such that higher and lower IEN responding rats show different rates of ultrasonic vocalizations (USVs) after repeated non-contingent amphetamine injections [20]. Given that USVs change as a function of amphetamine dose and IEN response, we aimed to understand if 50 kHz USV in response to natural reward change differently after voluntary ETOH consumption.

USVs show large individual differences across rats but remain stable across time, indicating they can be used to study trait-like characteristics in affective and motivational states in rodents [21,22]. Fifty kHz USVs are emitted in response to appetitive stimuli and are indicative of positive affect [23–27]. Trill and frequency modulated (FM) USVs are types of 50 kHz USV that is highly correlated with reward [10,23,28–31]. Twenty-two kHz USVs are emitted when presented with aversive stimuli, during drug withdrawal, and are indicative of negative affect [32–35]. Experimenter-induced tickling is designed to mimic juvenile rodent play behavior. Rats emit high rates of 50 kHz USVs when being tickled; lending support that rats find experimenter tickling rewarding [36]. When USVs are recorded to measure reward that results from psychomotor stimulants or food reinforcement, different USVs are emitted in either optimal or suboptimal reinforcement conditions [32,37]. Previous research has determined that 50 kHz USVs are emitted not only in response to self-administration of psychostimulants, but in anticipation of receiving amphetamine [30], cocaine [28] and ETOH reinforcement [38]. In summary, USVs can be used to interpret positive and negative states in rodents in response to various rewards and affective states.

Our previous research shows that anticipatory USVs change differently in the context associated with natural reward (experimenter-induced tickling), and with actually receiving natural rewards [10]. The present study aimed to determine if voluntary ETOH drinking changes the IEN and USV responses differently. To determine how these individual difference traits change, we measured the IEN response and USV response before and after the intermittent ETOH exposure phase. Since 50 kHz USVs are a reliable measure of rodent positive affect [21,26,39,40], we designed the USV test to probe for 50 kHz USVs because our objective was to determine how the response to naturally rewarding stimulation changes

after ETOH drinking. We hypothesized that rats would decrease their locomotor activity in the IEN test after ETOH exposure. Lastly, given that anticipatory USVs increase after associative reward learning, we hypothesized that higher ETOH consumption would result in greater anticipatory 50 kHz trill USVs, while 50 kHz trill USVs in response to receiving a natural reward would decrease.

2. Methods

2.1. Animals

Thirty-eight male Long Evans rats arrived in the lab from Charles River Laboratories at 30 days old. Rats were housed individually in an opaque shoebox cage with pine chip bedding in a temperature and humidity controlled colony room on a reverse 12:12-h light dark cycle with lights off at 10:00A.M. All experimentation occurred in the dark cycle. Food and water were available ad libitum throughout the experiment. Rats habituated to their homecages for one week. During that first week, rats were handled for approximately one minute daily to facilitate experiment handling procedures. Experimentation began 10 days after arrival. All behavioral procedures were approved by Kansas State University Institutional Animal Care and Use Committee and were in accordance with the National Institute of Health guidelines for the Human Use and Care of Laboratory Animals (National Research Council, 2011).

2.2. Apparatus

2.2.1. Inescapable novelty test—Six locomotor activity chambers measuring $46.6 \times 46.6 \times 46.6$ cm were used to measure locomotor activity in response to a novel environment. Each locomotor chamber was constructed of transparent plexiglass walls and plastic flooring that was covered with pine shavings bedding. A photobeam sensor surrounded the locomotor chamber and was approximately 2.54 cm above the plastic flooring. The photobeam was comprised of a 16×16 (x-axis) photocell array. Each photocell was spaced 2.54 cm apart (Coulbourn Instruments, TruScan 2.01) and measured the amount of horizontal movement in centimeters. The amount of horizontal movement (cm) was also recorded in 5-min blocks of time for each session. A white noise generator (70 dB) was used to create background noise to mask sounds from other chambers and from experimenter generated sounds. Rats were weighed then subsequently placed inside of the activity chamber for 30 min.

2.2.2. Heterospecific test and ultrasonic equipment—A standard transparent shoebox cage with black geometric shapes taped to the side and paperchip bedding was used as the apparatus. The shapes and bedding were used to provide a contextual cue. All USVs were recorded in a separate room using an ultrasonic microphone (Ultramic 200 K) purchased from dodotronic.com and SEAwave recording software. The microphone was positioned above the shoebox cage and held in place and did not move during recording. Both the microphone and software were connected to a separate computer that automatically saved each sound file. Analysis of each sound file was done using Avisoft SASLab Pro Bioacoustics sound analysis software. Potential USVs of all call types and frequencies were counted automatically and manually scored by a trained researcher similar to previous literature [10,20,41,42]. Briefly, a spectrogram was created for each rat that displayed all sounds recorded in the possible 1–100 kHz sound range. SASLab Pro automatically marked

all potential audible sounds and USVs, and parameter estimates were measured. A low-pass filter removed audible sounds below 18 kHz. A trained researcher then identified the shape of the USV. USV shapes were identified as either: short, fixed frequency (FF), frequency modulated (FM) or trill. During this step, all USVs were verified using the playback function in SASLab Pro. The playback function reduces the speed of the playback and allows the USVs to become audible. To ensure all sounds were USVs, all USV calls were played and verified to have a whistle-like sound during playback. Parameter estimates were then copied in Microsoft Excel. Using Excel's data functions, ultrasonic vocalizations were categorized into different call types. Twenty-two kHz USVs were operationally defined as occurring in the frequency of 20–28 kHz. In addition, they were required to be flat in shape with no visual fluctuations and have a duration of more than 10 ms. Fifty kHz USVs were operationally defined as occurring in a range of 35–90 kHz and having a duration of greater than 10 ms. Fixed frequency (FF) 50 kHz USVs were flat in shape with no visual fluctuations. Frequency modulated (FM) 50 kHz USVs were operationally defined as having one fluctuation/change in frequency and occurring for at least 10 ms. Trill 50 kHz USVs were operationally defined as have two or more fluctuations, a change in frequency, and/or complex shape that was not flat or frequency modulated (FM). Short 50 kHz USVs were very short in duration and operationally defined as having a visual appearance of a dot with no fluctuations. These calls were again verified to be USVs by the playback function in the software. All fluctuations were operationally defined as having a greater than 3 kHz change in frequency.

2.3. Procedure

2.3.1. Individual difference tests—All rats received the individual difference tests in the same order. The inescapable novelty test was first followed by the heterospecific test (experimenter tickling to mimic juvenile play). The order was determined on level of intrusiveness, and by previous research [9,10,20]. A schematic of the experimental procedures outlines the timeline of events (Fig. 1).

2.3.2. Inescapable novelty screen—Rats were weighed then transported into a separate testing room. Rats were subsequently placed inside of the novel open apparatus environment for 30 min and locomotor activity was measured. Distance traveled was recorded every 5 min and summed at the end of the session to yield a total distance traveled (cm). Rats were tested again in the IEN test after the 8 week voluntary ETOH drink phase. To ensure the testing apparatus was novel, the transparent walls were covered with non-transparent white plastic and the pine shavings bedding was replaced with paper chip bedding.

2.3.3. Heterospecific USV test—Rats were weighed and transported into a separate room where ultrasonic recordings took place. The room was free of other rats and external noise that would interfere with ultrasonic recording. Ultrasonic recording occurred on the first and fourth test days. The *initial* heterospecific test was designed to probe for 50 kHz USV response to a novel environment and the first response to experimenter-induced tickling. For this initial test (Day 1), individual rats were allowed to explore the novel environment for two minutes and were not tickled during this two minute period, and all types of USVs were recorded. After two minutes of recording, rats were wrestled and

‘tickled’ using gentle, fast-finger movements along the back and neck and ventral side [24,36], similar to conspecific play for two additional minutes. During this tickling phase, tickling occurred for 15 s intervals. These 15 s tickling intervals repeated for a total of two minutes. The total duration of the initial heterospecific test (Day 1) was 4 min (2 min of exploration of novel environment recording + 2 min of tickling recording). The rats were always allowed to explore the tickle-associated context for two minutes before any experimenter tickle stimulation. On Day 2 and Day 3, each rat experienced 2 additional days of the 4 min procedure, but USVs were not recorded. On Day 4, rats underwent the same 4 min procedure, and the ultrasonic microphone (Ultramic 200 K) recorded potential anticipatory USVs (context-induced) and USVs in response to being tickled (2 min of anticipatory recording + 2 min of tickling recording). USVs were recorded again after the 8 week voluntary oral ETOH drink phase using the same procedures and time periods (2 min of anticipatory recording + 2 min of tickling recording). The post drink test was completed on one day. This would determine if anticipatory USVs or tickle-induced USVs change differently in ETOH and control rats. All tickling was performed by the same research assistant to ensure consistency.

2.3.4. Intermittent access, 2-bottle choice drinking—Following the IEN and heterospecific tests, the intermittent access phase began. Drinking sessions began 2 h into the dark cycle. The ETOH rats were exposed to increasing concentrations of ETOH over three weeks (5%, 10%, and 20% w/v), and had ETOH access on Monday, Wednesday, and Friday to 20% ETOH for 6 weeks after gradual exposure [3,4]. ETOH was prepared in tap water. The placement of the ETOH and water bottles was randomized to control for side preference. For the 8 weeks of intermittent ETOH access, ETOH consumption for each animal was measured as grams of ETOH consumed per kilogram of body weight per day (g/kg). ETOH measurements were made by subtracting the mass of the bottle from its previous mass. Both ETOH and water measurements were taken at the beginning, 1 h, and 24 h marks for each ETOH day. On water days, measurements were taken only at the beginning and 24 h marks. For control purposes, a group of eight rats were not exposed to ETOH and were given access to water for the entirety of the 8-week drink phase. Previous research examining the effects of excessive ETOH consumption excludes rats that do not consume greater than 4 g in a 24 h period [3], but the goal of this was to determine if NSS or 50 kHz USVs change after ETOH exposure. Therefore all rats, regardless of the amount of ETOH consumed, were included in statistical analyses and none were excluded.

2.4. Analyses

The purpose of this research study was to determine how NSS and USVs are changed after chronic voluntary oral ETOH exposure. In addition, we aimed to determine how USV types changed in response to a tickle-paired environment. The current design has unequal cell sizes and violates the assumptions of the ANOVA, therefore linear mixed effects models were used to test the main effects and interactions to determine, how drinking changed across days, if IEN response changed after drinking, and how types of USVs changed after drinking. Each mixed effects model had a fixed effect of group (ETOH or control), day, and interaction of group×day. In addition, each model entered individual rats as a random effect, which allows variability within each rat to vary across days. Estimates of effects were

estimated with restricted maximum likelihood estimation (REML). Linear mixed effects models over several advantages over ANOVA, including the ability to provide acceptable reliability across multiple observations within a single animal [43]. When interactions were significant simple effects were probed using Bonferroni corrected comparisons. We compared responses before the drink phase to post drink period test responses. Our lab has recently published that in Sprague-Dawley rats different types of USVs reveal differences in reward processing [10]. Therefore, another aim of this study was to determine whether conditioned and unconditioned reward processing changed differently in Long-Evans rats after ETOH exposure.

3. Results

3.1. Voluntary ETOH consumption

Overall, rats showed an increase in ETOH (g/kg) consumption when given 20% ETOH (w/v) when compared to the 5% and 10% ETOH (w/v) concentrations ($F(23, 662) = 3.49$, $p < 0.001$; Fig. 2). On average, when given 20% ETOH, rats consumed 2.3 g/kg and 2.4 g/kg more when compared to 5% and 10% ETOH respectively.

3.2. Change in forced novelty response

Rats were pseudorandomly assigned to ETOH or control groups, such that higher and lower novelty responders were equally distributed across the drinking groups. Therefore, there was no difference in the inescapable novelty test (IEN) before the voluntary drink phase ($t(36) = 0.56$, $p = 0.58$). After the voluntary drink phase, the rats were tested in the IEN test again. Analyses indicated that the ETOH IEN response decreased ($t(29) = 4.22$, $p < 0.001$), and the control IEN response decreased ($t(7) = 6.06$, $p < 0.001$), but ETOH and control rats' IEN responses decreased equally and were not different ($t(36) = 1.53$, $p = 0.16$), suggesting that the decrease in IEN was likely due to age and not ETOH consumption (Fig. 3). Finally, to fully determine that ETOH drinking was not preferentially changing an anxiety-like response in the IEN test, we analyzed the margin distance and the center distance during the inescapable novelty test, and analyses indicated no differences between ETOH and control rats (all p 's $> .05$). Results clearly demonstrate that the ETOH and control rats were not different, indicating voluntary ETOH consumption did not change the response to novelty or anxiety-like behavior.

3.3. Anticipatory USVs in response to conditioned and unconditioned environments

Linear mixed effects modeling was used to determine how different types of USVs changed across recording days because ETOH and control rats had unequal sample sizes; which is a violation of repeated measures ANOVA. In this section, the USVs being analyzed are in response to an environment and not in response to being tickled. Thus, these USVs are the anticipatory USVs. The mixed effects model determined that fixed frequency (FF) 50 kHz USVs did not change across day, indicating no difference between ETOH and control rats, and there was no significant interaction (all p 's $> .05$). Frequency modulated (FM) 50 kHz USVs increased from baseline responding ($\beta = 1.82$, $t(74) = 3.91$, $p < 0.001$). There was no main effect of group, and no significant interaction of group $\times$ day (all p 's $> .05$), suggesting that ETOH and control rats did not emit different rates of FM 50 kHz USVs across day. For

short 50 kHz USVs, there was a main effect of day ($\beta = 0.73$, $t(74) = 2.03$, $p < 0.05$), indicating that short 50 kHz USVs increased from the baseline measurement. However, there was no main effect of group and no significant interaction on the rates of short USVs. Short 50 kHz USVs did not increase differently between ETOH and control rats across day.

Notably, trill 50 kHz USVs, a type of USV that have been suggested to increase in response to rewarding stimulation [29] also showed an increase across day, ($\beta = 22.46$, $t(74) = 5.21$, $p < 0.001$). ETOH rats emitted more trill USVs as evidenced by a statistical trend of group, ($\beta = 10.73$, $t(36) = 1.90$, $p = 0.06$). Importantly, there was a significant interaction of group and day, ($\beta = 9.21$, $t(74) = 2.14$, $p < 0.05$), indicating that ETOH and control rats emitted different rates of trill 50 kHz USVs across days. Simple effects tests revealed that ETOH rats and control rats did not have different rates of trill USVs measured on Day 1 or Day 4, indicating that prior to the drinking phase the ETOH and control rats had similar rates of trill USVs. After the drinking phase, ETOH had greater anticipatory trill 50 kHz USVs, when compared to the control rats (Fig. 4).

The last type of USV evaluated was 22 kHz USV. The linear mixed effects revealed no effect of group, ($\beta = 0.15$, $t(36) = 0.78$, $p > 0.05$), suggesting that ETOH and control rats had similar rates of 22 kHz USVs. There was a main effect of day, ($\beta = 0.98$, $t(74) = 4.19$, $p < 0.001$), and significant interaction of group $\times$ day, ($\beta = 0.65$, $t(74) = 2.76$, $p < 0.01$). This result indicates that across the three recording days, the ETOH and control rats changed differently in the rates they vocalized. However, these results should be interpreted with caution because of the low number of 22 kHz USV observed across the recording sessions (for all recording sessions, $M = 3.24$, $SD = 4.46$). Many rats did not emit any 22 kHz USV.

In response to the context, anticipatory 22 kHz USVs were not correlated with total anticipatory 50 kHz USVs (all p 's $> .05$) on any recording day, and 22 kHz USV were not correlated with reward trill 50 kHz USVs (all p 's $> .05$) on any recording day, suggesting that rats are not vocalizing at high rates indiscriminately. A complete summary of means and standard deviations of each type of anticipatory USV can be viewed in Table 1.

3.4. USVs in response to experimenter tickling: probing for 50 kHz USVs

Similar to the analyses above, linear mixed effects models were used to understand differences in USV type in response to experimenter tickling. Again, this test was designed to probe for 50 kHz USVs, but we recorded all USVs of all call types: the first tickling session and fourth tickling session, which occurred prior to the ETOH exposure phase. The final recording was completed after the exposure phase. During each USV recording test, rats were tickled as described in the methods.

In response to being tickled, FF 50 kHz USVs were not different between ETOH and control rats. ETOH and control rats did not change their rate of FF USVs calling across days, and there was no significant interaction of group and day (all p 's $> .05$), providing strong support that FF 50 kHz USVs do not change in response to repeated tickle stimulation. Frequency modulated (FM) 50 kHz USVs increased across repeated tickling tests ($\beta = 3.49$, $t(74) = 3.19$, $p < 0.005$). There was no main effect of group, ($\beta = -1.14$, $t(36) = 0.78$, $p > .05$), indicating that ETOH and control had the same number of FM USVs. There was also no

interaction between group $\times$ day ($\beta = 1.07$, $t(74) = 0.98$, $p > 0.05$). Short 50 kHz USVs showed a similar pattern as FF USVs, such that there was no main effect of day, no effect of group, and no significant interaction (all p 's $> .05$). This indicates that short 50 USVs are not changed by repeated tickling or evoked by rewarding stimulation.

Analysis revealed that when the rats were repeatedly tickled the trill 50 kHz USVs increased from baseline responding ($\beta = 59.58$, $t(74) = 5.33$, $p < 0.05$). There was a statistical trend of group ($\beta = 22.08$, $t(36) = 1.98$, $p = 0.055$), such that on average ETOH rats vocalized more than control rats. There was not a significant interaction of group $\times$ day, meaning that ETOH and control rats vocalized at similar rates across the recording sessions (Fig. 5). After the voluntary drinking phase, ETOH and control rats decreased trill USVs in response to tickling, but the rate of the decrease was not different across the groups ($t(36) = 1.66$, $p = 0.10$). A complete summary of means and standard deviations of each type of USV that resulted from tickling can be viewed in Table 2.

Analysis of 22 kHz USVs revealed that there was no effect of day, group, or group $\times$ day interaction (all p 's $> .05$). This suggests that 22 kHz USVs did not change across days and that ETOH and control rats did not differ in their 22 kHz USV calling. Again, our test was designed to probe for 50 kHz USVs and, therefore, low rates of 22 kHz calling were observed.

4. Discussion

The aim of the present study was to determine if novelty response and USVs change after chronic voluntary ETOH consumption. The current experiment determined that anticipatory trill 50 kHz USVs increase in response to a reward-associated context and in response to receipt of rewarding stimulation. Most importantly, voluntary ETOH consumption changes anticipatory 50 kHz USVs, suggesting that chronic ETOH exposure changes how natural rewards are processed or alters the retrieval of the memory of reward-associated contexts. When rats received rewarding tickle stimulation the ETOH and control rats did not change differently across any type of 50 kHz or 22 kHz USVs. Importantly, voluntary ETOH did not change the forced novelty response when compared to the control rats. Both the ETOH and the control rats decreased their locomotor response to novelty, which likely indicates that the decrease was due to age and not ETOH consumption.

Novelty and sensation seeking has been studied in rodents using the locomotor response to a novel environment, novelty place preference, and novel object preference tests. Collectively, the forced and choice novelty responses in rodents have been positively correlated with abuse vulnerability. The choice to engage a novel environment is positively correlated with higher conditioned place preference to amphetamine and morphine, and greater resistance to adverse consequences in a cocaine self-administration paradigm [11]. However, neurobiological and behavioral discrepancies emerge when examining depressive and stimulant drugs of abuse [15]. Preclinical studies examining whether the forced novelty response predicts ETOH consumption have resulted in contradicting findings. Some studies report higher forced novelty responders drink more ETOH and other research reports no relationship [16,44]. Therefore, our aim was to determine if novelty response *changes* as a

result of voluntary drinking. We thought this finding would enlighten why there may be discrepancies between published literature. If the forced novelty response is changed after ETOH consumption it would suggest that there is an interaction of when individual differences in novelty response are critical to understanding ETOH consumption. Our results are in agreement with previous research that suggests that forced novelty response is not changed by voluntary ETOH consumption [44]. We also extend this previous research by measuring a baseline novelty response and show that the novelty response decreases into adulthood but not as a function of ETOH exposure.

Other research has suggested that the inescapable novelty test is more reflective of a stress test because it is known to increase corticosterone for 120 min after completion of the test [14]. Mild stress and low doses of ETOH administered non-contingently increase locomotor activity in high novelty responders but not low novelty responders [17]. Voluntary ETOH consumption changes the corticosterone and locomotor responses, but only when challenged with amphetamine and not under drug-free conditions [44]. This indicates that voluntary ETOH consumption, especially in high novelty responders, induces long lasting changes in the mesolimbic reward pathway. Notably this effect was only observed with high drinking Wistar rats. Therefore, it is possible that strain differences in voluntary ETOH consumption affect later locomotor response to a novel unavoidable environment. This is supported by other literature that suggests differences in metabolism across strains [4], suggesting Long-Evans rats obtain higher blood ETOH concentrations with less g/kg consumed. Therefore, our failure to observe an effect of voluntary ETOH drinking on IEN response could be attributed to differences in strain or because corticosterone was not elevated enough to observe differences between ETOH and control rats.

Our lab recently published that 50 kHz USVs emitted by Sprague-Dawley rats are sensitive to conditioned and unconditioned reward, such that the type of the 50 kHz USV changes in response to a context-associated with natural reward and receipt of natural reward (tickling) [10]. For the current experiment, we hypothesized that Long-Evans rats would show a similar pattern in the change in 50 kHz USVs, but we also aimed to understand how natural rewards are changed after chronic voluntary ETOH consumption. Our results indicate that Long-Evans rats show a similar pattern in 50 kHz USV in response to conditioned and unconditioned reward, such that FM and trill USVs increase in response to an environment paired with natural reward (tickling). Most interestingly, after the drinking phase was complete trill USV *increased* in the ETOH rats, suggesting contextual sensitization which is a hallmark of the incentive sensitization theory of addiction [45,46]. However, when receiving the actual natural reward (tickling) both the ETOH exposed rats, and water-drinking control rats showed a *decrease* in trill USVs. The current results provided support that trill 50 kHz USVs can be used to understand different types reward processing. We and others suggest that FM and trill USVs could be used to understand incentive salience by disentangling wanting and liking. Future research should focus on understanding USVs subtypes in response to both conditioned and unconditioned reward. The large body of evidence supports our hypothesis that rodent USVs are reliable [21], and capable of elucidating these separate reward systems (wanting and liking) [47–49]. For example, in a conditioned place preference experiment, conditioned place preference magnitude is positively correlated with 50 kHz USVs in the drug paired side [27,30]. In further support of

anticipatory 50 kHz USVs being representative of incentive salience, Buck et al. (2014) determined that in a subgroup of high drinking rats, 50 kHz USVs were increased. Their interpretation of these results was that this subgroup of rats had an increased alcohol seeking or motivational salience (i.e. craving), although these anticipatory USVs may also represent a form of negative reinforcement because they were only observed in dependent rats [38].

Furthermore, the results also suggest that USVs can be used to understand how natural rewards change value after chronic drug exposure. Understanding how natural rewards change is an area of drug addiction that is understudied but is potentially rich in elucidating the development of compulsive drug taking and relapse. Specifically, we suggest that USVs are a possible research tool to understand the inverted-U function that pertains to the interest in natural rewards following chronic drug administration [8].

All of the rats recalled the reward-paired context and one interpretation is that anticipatory 50 kHz USV could represent a behavioral output to a reward memory resistant to change. This is particularly exciting because it represents a form of long-term memory that can be behaviorally measured. In a previous manuscript, we hypothesized that reward USVs were resistant to extinction, because in that experiment, the saline-treated animals never decreased responding to a tickle-paired environment after seven extinction trials and a 14-day rest [20]. In the current experiment, approximately nine weeks elapsed between the last heterospecific test session and the post drinking phase heterospecific test. Rats from both the ETOH and control groups recalled the environment paired with tickling with ETOH rats showing an elevated response. Adolescent ETOH drinking has been shown to decrease cognitive and memory function. These studies have determined that voluntary drinking affects hippocampal and amygdala structures, suggesting rodent models of voluntary drinking may affect limbic structures more than cortical [50–52]. Taken together, because the Pavlovian association made between the environment and tickling was left intact after chronic ETOH intake, it could suggest other brain areas are implicated in learning, processing, and remembering natural rewards or more simply, the voluntary ETOH consumption was not large enough to significantly alter reward memory.

In summary, our results provide evidence that voluntary ETOH consumption in adolescence and early adulthood changes processing of natural reward, but not the forced novelty response. Moving forward, trill 50 kHz USVs are a valuable research tool that can be used to elucidate the complexities of reward processing before and after chronic ETOH self-administration. Interestingly, trill 50 kHz USVs displayed two distinct patterns of responding. The first, a wanting pattern that sensitized and became hyper-responsive after ETOH self-administration. The second, a liking pattern that showed an inverted U shape function, indicating decreased liking of natural rewards. Taken together, trill 50 kHz USVs can be used to study conditioned and unconditioned reward, but also how reward processing changes following chronic drug administration.

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HIGHLIGHTS

- Response to novelty decreases with age.
- Anticipatory ultrasonic vocalizations are changed after voluntary ethanol exposure.
- Natural reward-associated contexts are recalled after prolonged ethanol exposure.
- Contexts associated with natural reward increase anticipatory ultrasonic vocalization after ethanol exposure.
- Ethanol exposure does not affect tickling ultrasonic vocalizations.

Fig. 1. Timeline of experimental procedures. Inescapable novelty test (IEN). Heterospecific test (H-USV). ETOH exposure from weeks 2–9. Retested in the IEN and H-USV tests following ETOH exposure. ETOH $n = 30$; Control $n = 8$.

Fig. 2. Mean $\pm$ SEM ETOH consumed (g/kg) after 24 h access across sessions. ETOH consumption significantly increased across time and with the introduction of 20% ETOH (*).

Fig. 3.

Mean $\pm$ SEM distance traveled (cm) decreased from the first to second IEN test. “*” indicates a significant decrease in both groups suggesting the effect is likely due to age. There is no significant effect on IEN novelty response due to ETOH consumption.

Fig. 4.

Mean $\pm$ SEM trill 50 kHz USVs in response to environment. ETOH rats vocalized more after ETOH exposure. For ETOH rats, * indicates a significant difference between ETOH and Control rats. Before voluntary ETOH consumption there were no baseline differences between ETOH and Control rats on Day 1 or Day 4. Lower left panel shows individual difference responses for trill USV from Day 4 to Post test for ETOH rats. Lower right panel shows individual difference response for trill USV from Day 4 to Post test for Control rats.

Tickle-Induced USV

Fig. 5.

Mean $\pm$ SEM trill 50 kHz USV in response to experimenter induced play behavior. For ETOH rats, * indicates that with repeated tickling rats increased trill USVs on Day 4 and on the Post test session in ETOH rats. ^ indicates that repeated tickling increased trill USV on Day 4 and on the Post test session in Control rats. There were no differences in ETOH or Control rats in rates of USV calling across sessions ($p = 0.055$).

Table 1

Means and standard deviations of subtypes of anticipatory USVs and anticipatory total USVs across the three recording days. Day 1 and Day 4 were recorded prior to the voluntary consumption phase. The post recording day was measured after the drinking phase of the experiment. Fixed frequency (FF), frequency modulated (FM).

USV Type	Day 1			Day 4			Post		
	Control	ETOH		Control	ETOH		Control	ETOH	
FF	0.00 ± 0.00	0.00 ± 0.0		0.13 ± 0.35	0.07 ± 0.25		0.13 ± 0.35	0.30 ± 0.70	
FM	1.38 ± 3.11	0.80 ± 1.61		4.50 ± 6.07	4.77 ± 4.49		4.00 ± 3.07	5.47 ± 4.38	
Short	0.63 ± 1.06	0.90 ± 1.30		2.75 ± 2.77	3.33 ± 4.13		2.13 ± 3.31	2.33 ± 2.48	
Trill	1.88 ± 4.22	4.90 ± 11.26		29.13 ± 9.60	50.63 ± 42.38		28.38 ± 30.34	68.23 ± 51.87	
Total 50 kHz	3.87 ± 8.32	6.63 ± 13.27		36.50 ± 47.92	58.80 ± 48.95		34.63 ± 35.50	76.37 ± 56.35	

* note values are expressed as M ± SD.

Table 2

Means and standard deviations of subtypes of tickle-induced USVs and tickle-induced total USVs across the three recording days. Day 1 and Day 4 were recorded prior to the voluntary consumption phase. The post recording day was measured after the drinking phase of the experiment. Fixed frequency (FF), frequency modulated (FM).

USV Type	Day 1			Day 4			Post		
	Control	ETOH		Control	ETOH		Control	ETOH	
FF	0.38 ± 0.52	0.60 ± 0.89		0.50 ± 0.54	0.50 ± 0.63		0.38 ± 0.52	0.978 ± 1.19	
FM	5.50 ± 5.63	9.83 ± 7.27		16.25 ± 7.74	18.70 ± 8.66		14.63 ± 11.14	14.67 ± 11.71	
Short	5.63 ± 6.19	5.43 ± 4.65		9.00 ± 3.42	9.00 ± 5.53		4.75 ± 4.20	4.20 ± 2.61	
Trill	28.00 ± 28.13	62.50 ± 43.81		174.75 ± 82.63	224.90 ± 85.22		140.50 ± 66.90	188.83 ± 85.77	
Total 50 kHz	39.62 ± 37.91	78.60 ± 51.99		200.50 ± 87.57	253.13 ± 88.52		161.00 ± 75.83	208.40 ± 89.22	

* note values are expressed as M ± SD.