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Effects of acute ethanol administration and chronic stress exposure on social investigation and 50 kHz ultrasonic vocalizations in adolescent and adult male Sprague–Dawley rats

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Abstract

Adolescents drink largely in social situations, likely in an attempt to facilitate social interactions. This study sought to examine alterations in the incentive salience of a social stimulus following repeated stress exposure and acute ethanol administration in adolescent and adult male Sprague–Dawley rats. Subjects were either exposed to 5 days of restraint stress, chronic variable stress (CVS), which consisted of a different stressor every day, or non-stressed. On test day, the animals were injected with 0, 0.25, 0.5, or 0.75 g/kg ethanol and placed in a social approach test in which they could see, hear, and smell a social conspecific, but could not physically interact with it. All the animals showed an interest in the social stimulus, with adolescents engaging in more social investigation than adults. Restraint stressed adults showed ethanol-induced increases in social investigation, while ethanol effects were not seen in any other group. An ethanol-associated increase in 50 kHz ultrasonic vocalization (USV) production was only evident in restraint stressed adolescents following 0.75 g/kg ethanol. 50 kHz USVs were not correlated with time spent investigating the social stimulus in any test condition. These results show that age differences in the facilitatory effects of ethanol on incentive salience of social stimuli are moderated by stress, with the facilitation of social approach by ethanol only evident in restraint stressed adults.

Keywords

Adolescence; Ultrasonic vocalization; Incentive salience; Social; Alcohol; Stress

1. Introduction

Adolescence has been shown to be a period of particular vulnerability for alcohol use, with alcohol intake during this period contributing to future alcohol use disorders (Crews et al., 2007). One of the factors contributing to alcohol consumption during adolescence is alcohol's ability to facilitate social interactions (Beck and Treiman, 1996; Smith et al., 1995). Interactions with peers during the adolescent period provide a substantial source of positive experiences for humans (La Greca et al., 2001; Larson and Richards, 1991) and rats (Douglas et al., 2004). Acute administration of relatively low doses of ethanol (0.5–0.75 g/kg) has been shown to facilitate social interactions in socially-housed adolescent rats placed with a novel conspecific under familiar, non-anxiogenic circumstances — social facilitation which is not normally seen under these conditions in adults (Spear and Varlinskaya, 2005;

Varlinskaya and Spear, 2002, 2006b; Willey et al., 2009). Low doses of ethanol have also been shown to increase social approach/investigation in a testing situation in which the adolescents could not physically interact with the stimulus animal, suggesting an ethanol-related increase in the incentive salience of social stimuli among adolescents (Willey and Spear, under revision-a).

Berridge and Robinson (1998) have proposed the separation of reinforcement into the hedonic properties (“liking”) and craving properties, or incentive salience (“wanting”). Approach towards a reward is thought to reflect incentive salience, while direct interaction with the reward (e.g., consumption or social contact) is thought to involve a hedonic component (although incentive salience may also play a role). The social approach paradigm utilized in the present study attempts to measure age related differences in the incentive salience of social stimuli by measuring approach towards, and investigation of, a social stimulus without the ability to physically interact with (‘consume’) the social stimuli.

Exposure to stressors typically decreases social interactions, although under some circumstances stress has been found to enhance the social facilitatory properties of ethanol in both adolescents and adults. For example, following repeated restraint stress, adolescent rats exhibit ethanol-induced social facilitation at a lower dose than non-stressed adolescents, whereas adults express social facilitation at doses of 0.25 or 0.5 g/kg ethanol — social facilitation that is not typically evident in adulthood (Varlinskaya et al., 2010). These findings indicate a stress-induced increase in sensitivity to ethanol's social facilitating effects in both adolescent and adult animals. To date, it is unknown whether this stress-induced enhancement of ethanol's social facilitating effects may be due to an ethanol-induced increase in the incentive value of social stimuli in stressed animals and if this might differ by age.

Given that animals may habituate to a repeated stressor (Heinrichs and Koob, 2006; Lucas et al., 2007), whereas they are less likely to habituate to unexpected stressors (Griffiths et al., 1992; Muscat and Willner, 1992; Tonissaa et al., 2008), this study sought to examine differences between repeated restraint stress and chronic variable stress (CVS) on ethanol-induced changes in the incentive value of a social stimulus. To determine whether changes in ethanol sensitivity seen after repeated stress are a consequence of the stressor itself, or rather the adaptations induced by the repeated stress exposure, this experiment compared the effects of repeated predictable and unpredictable stressors for influencing ethanol's effects on the incentive value of social stimuli, with the expectation that predictable restraint stress will have less impact than CVS due to stressor habituation.

Given that low doses of ethanol have been shown to increase the incentive value of a social stimulus in adolescent animals using a reward approach task (Willey and Spear, under revision-a) adapted from Nocjar and Panksepp (2002), the same reward approach task was used to parse out the effects of stress and ethanol on social incentives in adolescent and adult rats. In addition, ultrasonic vocalizations (USVs) were recorded as they have been used as a measure of affect (Knutson et al., 1998, 1999; Panksepp and Burgdorf, 2000). USVs in the 50 kHz range are thought to reflect positive affective states as they are increased by electrical stimulation of the reward pathway (Burgdorf et al., 2007), and are emitted not only during interactions with rewarding stimuli, including social interactions (play fighting — Knutson et al., 1998; Willey et al., 2009), social exploration (Blanchard et al., 1993), experimenter tickling (Burgdorf and Panksepp, 2001; Mallo et al., 2007; Panksepp and Burgdorf, 2000), and amphetamine administration (Knutson and Panksepp, 1997; Thompson et al., 2006), as well as to contexts and cues associated with rewards such as morphine administration (Burgdorf et al., 2001) and social interactions (Knutson et al., 1998). Hence these 50 kHz USVs may reflect in part not only the hedonic value of rewarding stimuli, but

also under some circumstances perhaps the incentive salience of rewards predicted by contexts or cues that elicit approach towards these rewards. By measuring both time spent investigating a social stimulus and 50 kHz USVs produced in the presence of a social stimulus, this study sought to determine the effects of repeated stress and acute challenge with ethanol on the incentive motivation for a social stimulus in adolescent and adult rats.

2. Methods

2.1. Subjects

A total of 480 Sprague–Dawley male rats bred and reared in our colony at Binghamton University were used in these studies (240 used as experimental subjects and 240 as stimulus animals [see below]). The day after birth all litters were culled to 8–10 pups (6 males and 4 females when possible) and housed with their mother in standard breeding cages. At weaning on postnatal day (P) 21 animals were housed with a same-sex littermate. All the animals were maintained in a temperature-controlled (20–22 °C) vivarium on a 12-/12-h light/dark cycle (lights on at 0700) with ad libitum access to food (Purina Rat Chow, Lowell, MA) and tap water. The animals were treated in accordance with guidelines established by the National Institutes of Health using protocols approved by the Binghamton University Institutional Animal Care and Use Committee.

2.2. Apparatus

Each reward approach testing apparatus consisted of a custom-built open arena (45×45×30 cm for adolescents and 60×60×30 cm for adults) with triangular reward proximity boxes (13.5×13.5×18.75 cm for adolescents and 18×18×25 cm for adults) placed diagonally across opposite corners of the arena. The apparatuses were made of black Lexan with wire mesh screens (17.8×6.4 cm for both ages) installed in the wall of the reward proximity boxes abutting the open arena that prevented physical contact but still allowed visual and olfactory access of the test animal to a social stimulus within the proximity box. Clean aspen shavings were used to coat the floor of the arena given that it has been shown that 50 kHz USV production is greatly decreased in the absence of bedding (Natusch and Schwarting, 2010). Shavings were not used in the reward proximity chambers to decrease the production of 50 kHz USVs by stimulus animals. During testing sessions, behaviors were recorded by a video camera for later behavioral scoring. After each testing session the shavings were removed and the apparatus was cleaned with a 6% hydrogen peroxide solution and allowed to dry before further testing.

Ultrasonic vocalizations were measured using Avisoft UltraSoundGate CM16 microphones, recorded by an UltraSoundGate 416–200 recording device, digitized by Avisoft-Recorder USG, and stored as .wav-files for later analysis (Avisoft Bioacoustics, Berlin, Germany). The microphones were placed on the top edge of the chambers and pointed downward into the open arena. The microphones are sensitive to frequency ranges between 10 and 125 kHz with a high sensitivity at 50 kHz and moderate directional properties. Sound analysis was performed using Avisoft-SASLab Pro software (Avisoft Bioacoustics, Berlin, Germany) and sound files were converted to spectrograms for manual visual counts and labeling of calls for automated analysis of peak frequency, mean call duration, and total call duration. A sound was considered to be a 50 kHz USV if it fell between 35 and 70 kHz with a duration of 30–80 ms. Within the 50 kHz range, calls were further divided into frequency modulated (FM) or flat calls as described by Burgdorf and Panksepp (2006). Calls were classified as FM if they contained any frequency changes (increases, decreases, or “trills”) whereas calls with a near-constant frequency were classified as flat calls. Any calls containing both flat and FM components were classified as FM. Calls were considered in the 22 kHz category if they fell between 18 and 32 kHz with a duration of 300–3000 ms. Very few animals in this

experiment emitted 22 kHz USVs, and as such these calls will not be discussed further. Juvenile rats have been shown to spend more time with a high vocalizing partner than a low vocalizing partner (Panksepp et al., 2002), and hence stimulus animals were not devocalized. Consequently, 50 kHz USVs recorded during testing reflect both the stimulus and experimental animals.

2.3. Procedure

The present experiment analyzed the effects of age (adolescent, adult), repeated stress exposure (non-stressed, repeated restraint stress, chronic variable stress [CVS]), and low doses of ethanol (0, 0.25, 0.5, 0.75 g/kg) on social approach and investigation. The design was a 2 (age)×3 (stress exposure)×4 (ethanol dose) factorial, with 10 subjects per group. All testing was conducted between 1300 h and 1700 h under low light conditions (8–10 lx). Animals used as social stimuli were of the same age and sex but from a different litter as the experimental subjects, and were not manipulated prior to being placed in the testing apparatus. The subjects were exposed to stressors (or non-stressed) on days P30–34 in adolescents or P70–74 in adults. On the day prior to testing (P35 or P75) all experimental animals were placed individually in the testing arena with both stimulus cages empty for 30 min to habituate them to the testing situation, given that rats typically do not direct social behavior towards a social stimulus until they have investigated a new environment (Vanderschuren et al., 1995). On test day (P36 or P76), each experimental animal was injected with a dose of ethanol and placed individually in a holding cage for 25 min prior to being placed for 10 min in the testing arena which contained a non-familiar conspecific in one stimulus cage, while the other cage remained empty. The test sessions were videotaped and USVs were recorded for later analysis. Immediately following testing, experimental animals were rapidly decapitated and trunk blood collected for analysis of blood ethanol concentration (BEC), corticosterone (CORT), and progesterone levels to determine if there were age- or stress-induced alterations in these physiological measures.

Videotapes were analyzed for latency to approach the social stimulus chamber and time spent investigating (nose within 1 cm of the mesh screen) the social and empty chambers.

2.4. Stress exposure

Subjects in the repeated stress groups were exposed to stressors for 5 days prior to testing (P30–34 or P70–74). Non-stressed animals were weighed on P30 and P34 or P70 and P74 but were not otherwise manipulated. The animals in the repeated restraint stress group were weighed daily and placed for 90 min each day in Plexiglas restraint tubes with a sliding plug to allow for adjustment of the tube length for each animal's size (Braintree Scientific, Braintree, MA). Cylinders measured 18.0×4.7 cm for adolescents and 23.0×8.0 cm for adults (length×diameter). The stressors administered to subjects in the CVS group varied across day. Stress days 1 and 5 consisted of 90 min of restraint stress. Day 2 of stress exposure for the CVS group consisted of 16 h of social isolation and water deprivation (1700–0900) after which the animals were returned to their home-cage with cage-mate and ad lib water availability. Day 3 of stress exposure consisted of a 10 min forced swim in a cylindrical container measuring 35.5×14 cm for adolescents and 46×20 cm for adults (height×diameter) filled 2/3 with 24–26 °C tap water. On day 4 of stress exposure, the animals were subjected for 7 h with their home-cage tilted at a 45° angle.

2.5. Ethanol administration

Ethanol was administered intraperitoneally (i.p.) as a 12.6% (v/v) solution in physiological saline, a relatively low concentration of ethanol that induces little (if any) tissue irritation at the site of injection. Dose of ethanol (0.25, 0.5, and 0.75 g/kg) was varied by altering volume rather than concentration to avoid concentration-induced differences in ethanol

absorption rate (Linakis and Cunningham, 1979). Control subjects were injected with physiological saline isovolumetric to the highest ethanol dose. All solutions were administered at room temperature.

2.6. BEC, CORT, and progesterone determinations

Blood samples were collected in heparinized tubes and frozen at -80°C until analysis of BEC or centrifuged at 2°C for 20 min at 3000 rpm and plasma samples removed and stored at -80°C until the time of CORT or progesterone assay. The samples were assessed for BECs via headspace gas chromatography using a Hewlett Packard (HP) 5890 series II Gas Chromatograph (Wilmington, DE). At the time of assay, the blood samples were thawed and 25- μl aliquots were placed in airtight vials. The vials were placed in a HP 7694E Auto-Sampler, which heated each individual vial for 8 min, and then extracted and injected a 1.0 ml sample of the gas headspace into the HP 5890 series Gas Chromatograph. Ethanol concentrations in each sample were determined using HP Chemstation software which compared the peak area under the curve in each sample with those of standard curves derived from reference standard solutions.

Plasma CORT levels were determined by radioimmunoassay (RIA) using competitive binding tritium based RIA kits obtained from MP Biomedicals, Inc. (Solon, OH) with 100% specificity for rat CORT. The sensitivity of the competitive binding assay was 25 pg/tube, with inter-assay coefficients of 10.4% and intra-assay coefficients of 6.1%. The standards and samples were assayed in duplicate using a Packard Tricarb 2100TR liquid scintillation analyzer, with disintegrations per minute averaged against a standard curve plotted with each assay and input into GraphPad Prism 5.0 software for calculation of concentration in each sample. For analysis of progesterone, plasma samples were thawed, and progesterone levels assessed via RIA using a ^{125}I RIA double antibody kit from MP Biomedicals (Solon, OH), with a specificity of 100% for progesterone. Progesterone assay sensitivity was 0.11 ng/ml, with inter-assay coefficients of 7.9% and intra-assay coefficients of 5.9%. The samples and standards for the assay were run in duplicate, using a Packard Cobra II Autogamma Counter (PerkinElmer, Boston, MA), with disintegrations per minute averaged against a standard curve.

2.7. Data analyses

Prior to analyses, all data were subjected to Levene's test for homogeneity of variance and were assessed for outliers. All measures satisfied homogeneity of variance assumptions. The subjects were removed as outliers if any of their behavioral scores were more than 2 standard deviations above or below the group mean. No more than 2 animals were removed from any group resulting in final group sizes of 8–10.

In order to analyze specific effects of age, stress, and ethanol, a series of targeted analyses of variance (ANOVA) were conducted. Baseline differences in social investigation and 50 kHz USV production across age were first analyzed, focusing on data from non-stressed, saline-injected animals. Assessment of ethanol and stress effects were then assessed at each age through 3-way analyses of dose $\times$ stress $\times$ either chamber (in analyses of social investigation) or USV type (in the USV analyses) followed by targeted ANOVAs assessing dose effects within each stressor condition. Pairwise planned comparisons were made using relatively liberal Fisher's LSD tests to determine the locus of significant main effects and interactions of ANOVAs where 3 orthogonal factors were analyzed to avoid inflating the possibility of type 2 errors (Carmer and Swanson, 1973). 2 factor ANOVAs were further analyzed with Dunnett's test for between-subjects measures and Bonferroni's test for within-subjects measures to minimize inflating type 1 errors. Spearman R correlations were

conducted to compare 50 kHz USV production and social investigation. All findings were considered significant at a $p < 0.05$.

3. Results

3.1. Age effects

In order to determine whether there were baseline differences across age in social investigation and 50 kHz USV production, ANOVAs were conducted that focused on the data from non-stressed, saline-injected animals. The 2 (age) $\times$ 2 (chamber) ANOVA of time spent investigating each chamber revealed main effects of age, $F(1,18) = 14.02$, $p < 0.01$, and chamber, $F(1,18) = 412.30$, $p < 0.001$, with a significant interaction of these variables, $F(1,18) = 13.06$, $p < 0.01$. Further analysis revealed that all animals spent significantly more time investigating the social chamber than the empty chamber, and that adolescents spent significantly more time investigating the social chamber than adults whereas there were no age differences in time spent investigating the empty chamber (Fig. 1, top left panel). Due to baseline age differences in social investigation, effects of stress and ethanol were explored using separate analyses at each age. The 2 (age) $\times$ 2 (call type) ANOVA of 50 kHz USV production revealed no significant main effect or interaction involving age (Fig. 1, top right panel).

To analyze the effects of age and repeated stress exposure on BECs, a 2 (age) $\times$ 3 (stress) $\times$ 3 (dose) ANOVA was conducted. The main effects of age, $F(1,155) = 160.03$, $p < 0.001$, and dose, $F(2,155) = 2518.57$, $p < 0.001$, were tempered by an interaction of these variables, $F(2,155) = 5.60$, $p < 0.01$, with adolescents having lower BECs than adults at all doses and this dissociation increasing as dose increased (see Table 1). There were no main effects or interactions involving stress exposure on BECs.

To analyze the effects of age and repeated stress on hormone levels, data from saline-injected animals were analyzed with a 2 (age) $\times$ 3 (stress) ANOVA. There was a main effect of stressor on CORT levels, $F(2,54) = 3.44$, $p < 0.05$, with CVS animals exhibiting lower CORT levels than non-stressed animals (Fig. 1, bottom left panel). There was no significant main effect or interaction with age. Progesterone levels were significantly affected by stress, $F(2,54) = 4.97$, $p < 0.05$, with both repeatedly stressed groups having lower progesterone levels than non-stressed animals, regardless of age (Fig. 1, bottom right panel).

3.2. Adolescents

The 3 (stress) $\times$ 4 (dose) $\times$ 2 (chamber) ANOVA of the time adolescents spent investigating each chamber revealed main effects of dose, $F(3,104) = 4.64$, $p < 0.01$, and chamber, $F(1,104) = 2296.96$, $p < 0.001$, with an interaction of these factors, $F(3,104) = 4.27$, $p < 0.01$. The separate 4 (dose) $\times$ 2 (chamber) ANOVAs conducted within each stress group revealed dose effect only in the non-stressed adolescents, with significant main effects of dose, $F(3,31) = 6.47$, $p < 0.01$, and chamber, $F(1,31) = 924.22$, $p < 0.001$, and their interaction, $F(3,31) = 5.92$, $p < 0.01$, in this group. Further testing revealed no differences in time spent investigating the empty chamber in the non-stressed adolescents; however, non-stressed adolescents injected with 0.75 g/kg ethanol spent significantly less time investigating the social chamber than their saline-injected counterparts. Both the restraint stress and CVS treated adolescents, like their non-stressed counterparts, spent significantly more time investigating the social chamber than the empty chamber, $F(1,36) = 649.79$, $p < 0.001$, and $F(1,37) = 856.73$, $p < 0.001$, respectively, but there were no main effects or interactions involving dose (Fig. 2, top panels).

Adolescent 50 kHz USV production data were first analyzed with a 3 (stress) $\times$ 4 (dose) $\times$ 2 (call type) ANOVA, which revealed main effects of each variable. Both stressed groups

emitted significantly more 50 kHz USVs than non-stressed adolescents, $F(2,99) = 5.67$, $p < 0.01$. Adolescents injected with 0.75 g/kg ethanol emitted significantly more USVs than saline-injected adolescents, $F(3,99) = 3.06$, $p < 0.05$. All adolescents emitted more FM 50 kHz USVs than flat USVs, $F(1,99) = 13.64$, $p < 0.001$. Exploring dose effects within each stressor condition, the 4 (dose) $\times$ 2 (call type) ANOVA of non-stressed adolescents revealed greater expression of FM than flat USVs as indexed by a main effect of call type, $F(1,30) = 7.45$, $p < 0.05$, but no main effect or interaction involving dose. Restraint stressed adolescents also emitted more FM than flat USVs, $F(1,35) = 7.39$, $p < 0.05$, with restraint stressed adolescents injected with 0.75 g/kg ethanol emitting more 50 kHz USVs than their saline-injected counterparts (main effect of dose, $F(3,35) = 2.89$, $p < 0.05$). Analysis of CVS exposed adolescents revealed no significant effects of dose or call type on 50 kHz USV production (Fig. 2, bottom panels).

3.3. Adults

In the analysis of time spent investigating the chambers, there was a significant main effect of chamber, $F(1,105) = 1495.80$, $p < 0.001$, with adults spending more time investigating the social chamber than the empty chamber (Fig. 4, top panel). There was also a main effect of stress exposure, $F(2,105) = 3.82$, $p < 0.05$, in which CVS exposed adults spent slightly but significantly more time overall investigating both the chambers than non-stressed adults (see inset in top panel of Fig. 4). When these data were analyzed separately by stress group, the 4 (dose) $\times$ 2 (chamber) ANOVA of investigation time again revealed a significant main effect of chamber for all stressor conditions, with adults spending more time investigating the social than empty chamber. The restraint stressed group was the only group to have a significant interaction of chamber and ethanol dose, $F(3,38) = 3.47$, $p < 0.05$, with 0.5 and 0.75 g/kg ethanol injected adults spending more time investigating the social chamber (but not the empty chamber) than their saline-injected counterparts (Fig. 4, top panels).

There was a main effect of call type on 50 kHz USV production in adults, $F(1,101) = 44.62$, $p < 0.001$, with all adults emitting significantly more FM than flat calls (Fig. 4, bottom panel). When the 50 kHz USV data were analyzed separately by stress group, main effects of call type again were evident, with no significant main effects or interactions involving dose evident in any stressor group (Fig. 4, bottom panels).

3.4. BEC and hormone data

In the analysis of BECs there was a main effect of dose in both adolescents, $F(2,77) = 1288.79$, $p < 0.001$, and adults, $F(2,78) = 1255.83$, $p < 0.001$, with each dose being significantly different from the others but no main effect or interaction involving the stress condition (see Table 1).

Analysis of CORT levels in adolescents revealed a main effect of ethanol dose, $F(3,104) = 2.96$, $p < 0.05$, with adolescents injected with 0.25 g/kg ethanol having lower CORT levels than saline-injected adolescents (see Fig. 3). There were no effects of ethanol or stress on CORT levels in adults.

In the analysis of progesterone levels in adolescents, there were main effects of stress, $F(2,104) = 13.28$, $p < 0.001$, and dose, $F(3,104) = 3.59$, $p < 0.05$. Post-hoc tests revealed that non-stressed adolescents had higher progesterone levels than adolescents exposed to either stress condition, and that adolescents injected with 0.75 g/kg ethanol had significantly higher progesterone levels than those injected with 0.25 g/kg; however, none of the ethanol groups were significantly different from their saline-injected counterparts (Fig. 3 bottom left panel).

Analysis of progesterone levels in adults revealed a main effect of ethanol dose, $F(3,105) = 5.45$, $p < 0.01$, with adults injected with 0.75 g/kg ethanol having higher progesterone levels than saline-injected adults (Fig. 3, bottom right panel).

3.5. Correlations

There were no significant correlations between time spent investigating the social chamber and FM or flat 50 kHz USV production in either age group.

4. Discussion

Overall, the social stimulus was effective in engaging interest of the test animals, with animals at both ages and across all conditions generally spending more time investigating the social chamber than the empty chamber. Such social motivation was particularly strong among adolescents, with non-stressed, saline-injected adolescents spending more time investigating the social chamber (but not the empty chamber) than their adult counterparts. The only significant effect of ethanol on social investigation in adolescents was seen in the non-stressed group; with 0.75 g/kg ethanol decreasing investigation of the social chamber compared to saline-injected controls. In contrast to adolescents, ethanol increased social investigation in restraint stressed adults at the 0.5 and 0.75 g/kg doses, an effect not seen with any other dose/stress combination. CVS exposure increased overall investigation in adults, with CVS-exposed adults spending more time investigating both chambers collectively than did their non-stressed counterparts.

In contrast to the behavioral data, there were no age differences in 50 kHz USV production among control animals. Adolescents and adults both emitted significantly more FM than flat 50 kHz USVs collapsed across all stress and dose groups. Stress exposure increased 50 kHz USV production in adolescents, with both restraint and CVS exposed adolescents emitting more 50 kHz USVs than non-stressed adolescents. An increase in 50 kHz USV production following 0.75 g/kg ethanol administration was seen in restraint stressed adolescents — the only effect of ethanol on 50 kHz USV production that was evident.

As has been shown previously, animals spent more time investigating the social chamber than the empty chamber, indicating interest in this stimulus (Willey and Spear, under revision-a,b). Non-stressed, saline-injected adolescents spent more time investigating the social stimulus than adults, a finding reminiscent of conditioned place preference data where socially housed adolescents were found to spend more time than adults in a context previously paired with access to a conspecific (Douglas et al., 2004) and increased social interactions in adolescents compared to adults (Spear and Varlinskaya, 2005; Varlinskaya and Spear, 2002, 2006b, 2008; Willey et al., 2009). Together these findings suggest that adolescents place more incentive value on social stimuli than adults.

Repeated stress exposure did not affect social investigation in saline-injected animals. This finding is in contrast to the stress-induced decreases in social interactions previously seen using a modified social interaction test (Varlinskaya et al., 2010). These different findings are likely due to differences in the procedures used. In Varlinskaya et al.'s (2010) study, subjects received their final stressor exposure 30 min prior to social interaction testing, whereas in the present experiment animals were tested 2 days after their final exposure to the stressor. When testing animals 30 min following the last restraint period, it is unclear whether observed effects are due to repeated restraint exposure, consequences of the more proximal acute restraint exposure, or, more likely, a combination of the two.

Although not reaching significance ($p < .06$) at the 0.5 g/kg dose in non-stressed adolescents in the present study, a reliable increase in social investigation has previously been seen

following low doses of ethanol in this test among adolescents (Willey and Spear, under revision-a); findings were consistent with the low dose ethanol stimulation of social interactions frequently reported in adolescent rats (Spear and Varlinskaya, 2005; Varlinskaya et al., 2010; Varlinskaya and Spear, 2002, 2006b, 2008; Willey et al., 2009). In the present study, a decrease in social investigation emerged in non-stressed adolescents following 0.75 g/kg ethanol which is not typically seen in tests of social interactions (Spear and Varlinskaya, 2005; Varlinskaya and Spear, 2002, 2006a, 2008, 2010) or approach (Willey and Spear, under revision-a). One possible explanation of these disparate results could be that without the physical contact that is experienced in tests of social interactions, animals may have lost interest in the social stimulus in the present study.

When adolescents were exposed to repeated stress in the present study, no sign of an ethanol facilitation of social behavior was evident. These data contrast with the stress-induced sensitization to ethanol's social facilitating effects seen previously in the social interaction test following chronic restraint in adolescents (Varlinskaya et al., 2010). As repeated restraint decreased baseline levels of social interactions in the social interaction test (Doremus-Fitzwater et al., 2009; Varlinskaya et al., 2010), ethanol may have simply restored social interactions due to its anxiolytic properties. Given that repeated stress did not alter baseline levels of social investigation in saline-injected adolescents in the present study, stressed adolescents may have been resistant to ethanol effects because in this test there was no social suppression for ethanol to reverse.

Among adults, non-stressed animals did not show ethanol-induced changes in social investigation, whereas facilitatory effects of ethanol on social investigation emerged in adults after repeated restraint — findings were reminiscent of the increase in ethanol-induced social facilitation that emerges in adults following repeated restraint but not in their non-stressed counterparts (Varlinskaya et al., 2010). The present study is the first to suggest that the ethanol-induced increase in social interactions which emerges in adults following repeated stress (Varlinskaya et al., 2010) may be related to a stress-induced increase in the incentive salience of the social stimulus. These effects, however, were not seen in CVS exposed adults, a possible indication that the increased sensitivity to ethanol's social facilitating effects in restraint stressed adults may be related to neural alterations associated with habituation to a repeated stressor (e.g., increased dopamine levels in the nucleus accumbens — Di Chiara et al., 1999) — habituation that would likely be prevented or at least slowed by the exposure of adults in the CVS group to various stressors across days (Griffiths et al., 1992; Muscat and Willner, 1992; Tonissaar et al., 2008).

Ethanol had no effect on USV production at either age in any stress condition, with the exception of an increase in 50 kHz USV production in restraint stressed adolescents after injection of 0.75 g/kg ethanol. The lack of ethanol effects on 50 kHz USV production in non-stressed animals is reminiscent of prior studies (Willey and Spear, under revision-a; Willey et al., 2009). Additionally, 50 kHz USVs and behaviors were not correlated at either age, suggesting that they each may reflect different underlying causes. It should be noted, however, that the USV data were collected from a pair of animals whereas the behavioral data reflect the experimental animal only — a difference that could potentially contribute to this dissociation. Yet, stimulus animals used for all groups were treated identically, and hence seemingly would have provided similar contributions to USV production across groups (although it nevertheless is conceivable that experimental animals from different groups may have provoked alterations in vocalization among the stimulus animals). While 50 kHz USVs have been suggested to represent a measure of positive affect or hedonics (Knutson et al., 1998, 1999; Panksepp and Burgdorf, 2000), several recent studies have shown a disconnect under some circumstances between behavioral measures of incentive salience or consummatory behavior and 50 kHz USV production when both measures are

assessed separately in each animal (Hamed et al., 2012; Sadananda et al., 2012; Simola et al., 2012; Wright et al., 2012). In the present study, alterations in time spent investigating the social stimulus were not accompanied by alterations in 50 kHz USV production and vice versa. Likewise, although adolescents spent more time investigating the social stimulus than adults, there were no age differences in emission of 50 kHz USVs. Collectively, these results extend prior findings to document further that 50 kHz USV production may not ubiquitously represent a measure of positive affect under all circumstances.

Both repeated stressor paradigms increased 50 kHz USV production in adolescent rats when compared to non-stressed controls. This may have been due to an attenuated stress response of repeatedly stressed animals to the handling and general manipulation associated with the stressor exposure. Indeed, the hormone data provide some evidence that repeatedly stressed adolescents show some habituation to these manipulations, as indexed by decreased progesterone levels in restraint-exposed and CVS adolescents relative to their non-stressed counterparts. It has been previously reported that rats which had undergone experimental manipulations for several days prior to testing emitted more 50 kHz USVs than experimentally naive rats (Wohr et al., 2008). Alternatively, neural alterations induced by repeated stress exposure may overlap with the neural control of 50 kHz USV production. For instance, activity in dopaminergic systems, especially in the nucleus accumbens has been found to play an important role in 50 kHz USV production (Burgdorf et al., 2001; Thompson et al., 2006) and to be increased following repeated stress exposure (Di Chiara et al., 1999; Patterson et al., 2010; Yi et al., 2008); hence, chronic stress-induced increases in dopamine activity may be at least partly responsible for the increased 50 kHz USV production seen in the stressed rats.

The CORT levels measured immediately following testing are relatively high, similar to levels seen during stress exposure. These high levels are likely due to a number of factors. The subjects received an i.p. injection 25 min prior to testing, a procedure that has been consistently shown to increase CORT levels (ex: Willey et al., 2012). The subjects were then socially isolated in a holding cage for 25 min which likely further increased CORT levels (Ferland and Schrader, 2011). Although not intended to be stressful, some components of the social approach task may have also contributed to elevations in CORT. Novelty exposure has been shown to increase CORT levels (Beerling et al., 2011; Terranova et al., 1999), and while the test subjects had been previously habituated to the testing apparatus, they had never experienced a conspecific separated by a screen, a novel situation that could have contributed to elevated CORT levels. Exposure to an unfamiliar social stimulus following social isolation likewise has also been reported to elevate levels of CORT (Cirulli et al., 1996; Terranova et al., 1999). Lastly, CORT levels have been shown to increase in situations in which an expected reward is not delivered (extinction) (de Boer et al., 1990). The inability of the experimental subject to interact with the social stimulus in the present study may have a similar effect as the reward of physically interacting with the stimulus is omitted. Thus, multiple factors inherent in our testing situation may have collectively contributed to the relatively high post-test CORT levels seen in the animals that may have partially masked effects of the dependent variables on this measure.

Social interaction tests have shown that adolescents engage in more social behaviors than adults (Varlinskaya and Spear, 2002, 2006a, 2008), but it was unclear whether the adolescent engagement in social behavior reflects an increase in the incentive salience of social stimuli per se. Using the social approach test that is thought to measure the incentive value of social stimuli, given that the experimental animal is unable to physically interact with, or 'consume', this rewarding stimulus (Nocjar and Panksepp, 2002, 2007), the present experiment found adolescents to place greater incentive value on social stimuli than adults. This is the first study to show that repeated stress in adults can induce an increased incentive

salience of a social stimulus following low dose ethanol exposure, similar to that previously seen in non-stressed adolescents (Willey and Spear, under revision-a). Thus, with both ethanol-induced social facilitation (Varlinskaya et al., 2010) and the social approach task examined here, following repeated stressful experiences, adults exhibit more adolescent-typical social responding following alcohol. To the extent that these data are applicable to humans, they support the suggestion that adolescents may drink in social situations due in part to ethanol-induced enhanced salience of peers and that this adolescent-like state of enhanced social motivation may be reinstated by ethanol following repeated stress or its adaptations in adults.

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Fig. 1. Top panels: Data from non-stressed, saline-injected animals. Top left panel — Mean seconds spent investigating the social and empty chambers. * indicates significant age difference in time spent investigating the social chamber. Top right panel — Mean number of FM and flat 50 kHz USVs during the 5 min test. Bottom panels: data from saline-injected animals. Bottom left panel — Mean CORT levels immediately following testing. Bottom right panel — Mean progesterone levels immediately following testing. + indicates significant difference from non-stressed animals, collapsed across age. Vertical bars indicate SEMs.

Fig. 2.

Data from adolescent animals. Top panels — Mean seconds spent investigating the social and empty chambers. * indicates 0.75 g/kg ethanol group spent less time investigating the social chamber than saline-injected adolescents of the same stress condition. Bottom panels — Mean 50 kHz USV production during the 5 min test. + indicates 0.75 g/kg ethanol groups emitted more 50 kHz USVs than their saline-injected counterparts within the same stress condition. Inset shows 50 kHz USV production collapsed across call type and dose. # indicates both stress groups emitted significantly more USVs than non-stressed animals. Vertical bars indicate SEMs.

Fig. 3. Top panels — Mean CORT levels immediately following testing in adolescents and adults. Bottom panels — Mean progesterone levels immediately following testing in adolescents and adults. * indicates a significant difference from saline-injected group when collapsed across stress conditions. + indicates significant effect of stress exposure collapsed across ethanol dose. Vertical bars indicate SEMs.

Fig. 4.

Data from adult animals. Top panels — Mean seconds spent investigating the social and empty chambers. * indicates more time investigating the social chamber than the saline-injected group within stress condition. Inset reflects mean investigation collapsed across chamber and dose. + indicates CVS group spent significantly more time investigating than non-stressed group when collapsed across chamber and ethanol dose. Bottom panels—Mean 50 kHz USV production during the 5 min test. Inset reflects mean 50 kHz USV production collapsed across call type and dose. Vertical bars indicate SEMs. Note Y-axis scaling differences in the insets.

Table 1

Mean blood ethanol concentrations (ng/ml) $\pm$ SEM at each age and dose of sample collected immediately after testing.

	0.25 g/kg	0.5 g/kg	0.75 g/kg
Adolescent	0.27 $\pm$ 0.09	23.01 $\pm$ 0.58	51.60 $\pm$ 1.08
Adult	5.50 $\pm$ 0.70	31.07 $\pm$ 0.89	62.03 $\pm$ 0.86