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ORIGINAL INVESTIGATION

Relationship between voluntary ethanol drinking and approach-avoidance biases in the face of motivational conflict: novel sex-dependent associations in rats

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Abstract

Rationale Aberrant approach-avoidance conflict processing may contribute to compulsive seeking that characterizes addiction. Exploration of the relationship between drugs of abuse and approach-avoidance behavior remains limited, especially with ethanol.

Objectives To investigate the effects of voluntary ethanol consumption on approach-avoidance conflict behavior and to examine the potential approach/avoidance bias to predict drinking in male and female rats.

Methods Long-Evans rats consumed ethanol for 5 weeks under the intermittent access two-bottle choice (IA2BC) paradigm. Approach-avoidance tendencies were assessed before and after IA2BC drinking using a previously established cued approach-avoidance conflict maze task and the elevated plus maze (EPM).

Results Female rats displayed higher consumption of and preference for ethanol than males. In the conflict task, males showed greater approach bias towards cues predicting conflict than females. In females only, a median split and regression analysis of cued-conflict preference scores revealed that the more conflict-avoidant group displayed higher intake and preference for ethanol in the first few weeks of drinking. In both sexes, ethanol drinking did not affect cued-conflict preference, but ethanol exposure led to increased time spent in the central hub in the males only. Finally, anxiety levels in EPM predicted subsequent onset of ethanol drinking in males only.

Conclusions Our results highlight sex and individual differences in both drinking and approach-avoidance bias in the face of cued conflict and further suggest that cued-conflict preference should be examined as a potential predictor of ethanol drinking. Ethanol exposure may also affect the timing of decision-making in the face of conflict.

Keywords Alcohol · Sex differences · Females · Anxiety · Elevated plus maze · Approach-avoidance conflict

Introduction

An approach-avoidance conflict occurs when an animal encounters a stimulus imbued with competing positive and negative valences, and accumulating evidence points to a dysregulation in approach-avoidance conflict resolution as a contributing factor to addiction (Fricke and Vogel 2020; Nguyen et al. 2015). Drugs of abuse in and of themselves are well documented to have rewarding and aversive properties (see Verendeev and Riley 2012, 2013 for reviews). For instance, the administration of ethanol can produce conditioned place preference or aversion in animals, with the suggestion that it carries short-term aversive properties and delayed, longer-lasting rewarding effects (Cunningham et al. 1997; Cunningham et al. 2002; Cunningham and Henderson 2000). It has also been proposed that reduced sensitivity to the aversive effects of ethanol is associated with heightened levels of consumption (Barkley-Levenson et al. 2015; Quintanilla et al. 2001; Risinger and Cunningham 1992), driving compulsive seeking that persists in the face of negative consequences. Cues associated with cocaine have also been shown to hold motivationally mixed valence for rats (Ettenberg 2004; Ettenberg and Geist 1991, 1993), and previous work from our laboratory has demonstrated that male rats with a history of subchronic cocaine exposure displayed

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greater approach tendencies towards bivalent, conflict-eliciting cues associated with both sucrose reward and shock as a result of attenuated learning of the aversive cue (Nguyen et al. 2015).

Although the rate of alcohol use disorder (AUD) is known to be higher in men than women (Grant et al. 2015), recent evidence points to a closing gap between the sexes with increasing alcohol consumption in women (Dawson et al. 2015; Grant et al. 2017). Compared to men, women tend to develop dependence more quickly (Mann et al. 2005) and are more vulnerable to the negative consequences resulting from excessive alcohol intake (Key et al. 2006; Klatsky et al. 1992; Mann et al. 2005; Urbano-Márquez et al.1995). Despite this, females continue to be underrepresented in preclinical research exploring the risk factors for excessive voluntary ethanol intake. Additionally, very few studies have explored sex differences in approach-avoidance conflict processing, with one recent exception, in which Xie et al. (2019) examined the effect of chronic intermittent ethanol (CIE) exposure on conflict behavior in male and female mice. Using a conditioned place preference paradigm in which a context was paired with three ethanol injections and then associated with a single footshock, it was found that ethanol history reduced the time that female mice, but not male mice, spent in the shock and ethanol-associated chamber compared to control mice (Xie et al. 2019), suggesting that CIE female mice had developed greater sensitivity to the aversive property of an ethanol-associated context.

A state of anxiety can be understood as an expression of approach-avoidance conflict (Aupperle et al. 2015; Bannerman et al. 2014), and anxiety disorders are highly comorbid with AUD (Ipser et al. 2015; Kessler et al. 2005; Kushner et al. 2000; Kranzler and Rosenthal 2003). Women are diagnosed with anxiety disorders at higher rates than men (Pigott 1999) and are more vulnerable to relapse driven by comorbidity (Hartwell and Ray 2013; Rubonis et al. 1994). However, preclinical research on the relationship between anxiety and ethanol consumption has yielded mixed results, again with an underrepresentation of females (Logrip et al. 2018). In male rodents, elevated anxiety, as measured by the elevated plus maze (EPM), predicts higher levels of future voluntary ethanol consumption (Jadhav et al. 2017; Peters et al. 2013; Spanagel et al. 1995, but see Stein et al. 2015). However, rats bred for low anxiety have been shown to drink more than those bred for high anxiety, and this effect is stronger in females than males (Henniger et al. 2002). Social stress in adolescence induced anxiety-like behavior that was associated with a subsequent increase in ethanol intake in male rats (Chappell et al. 2013; McCool and Chappell 2009; Roeckner et al. 2017). In females, the same social stress and drinking paradigms have not revealed such a relationship between anxiety-like behavior and ethanol consumption (Butler et al. 2014: Roeckner et al. 2017).

In this study, we sought to further investigate sex differences in the nature of relationships among voluntary ethanol consumption, approach-avoidance conflict resolution, and anxiety-like behavior. Male and female Long-Evans rats consumed ethanol under the intermittent access two-bottle choice paradigm for 5 weeks and were administered a Y-maze cued approach-avoidance task (adapted from Nguyen et al. 2015) and EPM test prior to, or after ethanol exposure, to examine (1) the sex difference in the effect of ethanol exposure on approach-avoidance conflict and (2) the relationship between approach/avoidance tendencies and voluntary ethanol drinking. Based on our previous work using cocaine pre-exposure, we hypothesized that ethanol exposure would bias rats towards approach of the conflict cue and that conflict approach tendencies would be associated with higher levels of ethanol consumption, regardless of when drinking occurred. We anticipated that females would drink more than males but that the same relationship between conflict resolution and drinking would exist independent of sex. Furthermore, we expected to see elevated anxiety associated with elevated ethanol consumption in male, but not female rats. We report important sex differences in approach-avoidance conflict processing and sex-dependent relationships between conflict resolution and voluntary ethanol consumption.

Materials and methods

Subjects

Fifty-six Long-Evans rats (28 male, 28 female, Charles River, QC, Canada) were used in this study. Rats arrived at approximately 225 g (males) or 150 g (females) and were habituated to the vivarium for a week before testing began. Animals were pair-housed, except when noted otherwise, in a temperature-controlled vivarium (22 °C) with a 12-h light/dark cycle, lights on at 07:00. Food and water were available ad libitum except during Y-maze testing, when rats were food-restricted and weights were maintained at a minimum of 85% of free-feeding weights. All experiments were conducted during the light cycle and with the approval of the University Animal Care Committee at the University of Toronto, and in accordance with the guidelines of the Canadian Council on Animal Care.

Behavioral procedures

Animals were divided into two groups: ethanol pre-exposure ($n = 10$ male, $n = 10$ female) and ethanol-naïve ($n = 18$ male, $n = 18$ female) groups (Fig. 1a). In order to assess the effects of ethanol pre-exposure on approach-avoidance conflict behavior, animals in the ethanol pre-exposure group underwent voluntary ethanol drinking under the intermittent access two-bottle choice (IA2BC) protocol prior to training in the

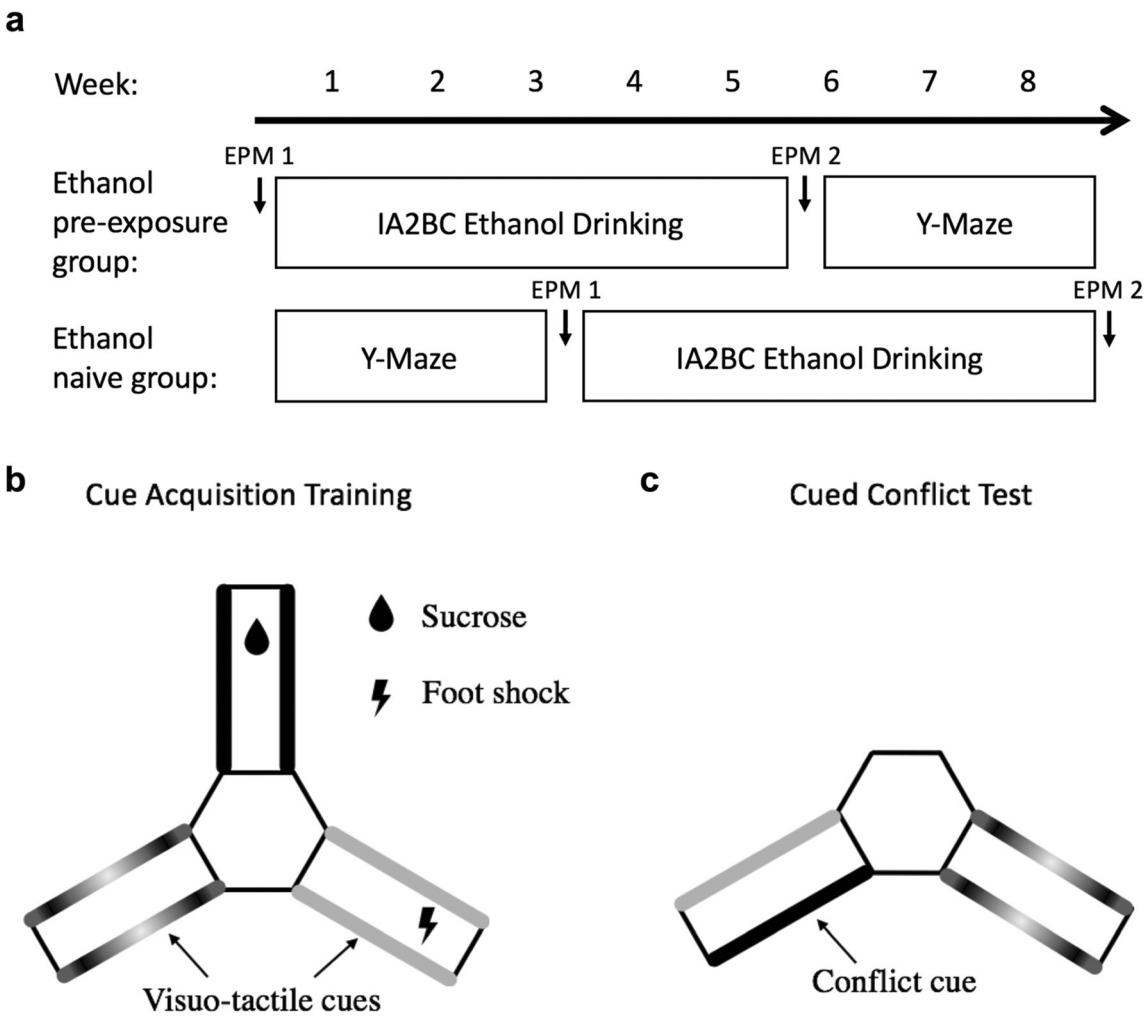


Fig. 1 **a** This timeline depicts the order of testing for the ethanol pre-exposure (n = 10 of each sex) and ethanol-naïve (n = 18 of each sex) groups, which both completed the same tasks across 8 weeks. EPM tests occurred before and after IA2BC drinking for both groups. Animals in the "ethanol pre-exposure" group completed the maze conflict task after exposure to ethanol, while the "ethanol-naïve" group completed the maze task first and then drank ethanol according to the IA2BC paradigm. **b**, **c** These schematic diagrams show the Y-maze configuration used to assess cued approach-avoidance conflict behavior. **b** This shows the arrangement of the maze and bar cues that lined the arms of the Y-maze during cue conditioning sessions. Forced time in the appetitive arm was accompanied by the delivery of 20% sucrose reward (×4), while forced time in the aversive arm was accompanied by .27 mA footshocks (×4). No outcome was delivered during forced time in the third (neutral) arm. **c** In the final conflict test, one sucrose-paired and one footshock-paired bar cue were presented together in a single arm to create the conflict configuration. The second available arm housed two neutral cues. No outcomes were delivered during this test

approach-avoidance conflict maze task and received two elevated plus maze (EPM) tests: one prior to and another following ethanol drinking. In the ethanol-naïve group, animals were trained on the approach-avoidance conflict maze task first, followed by ethanol drinking in order to investigate the ability of conflict-induced approach-avoidance bias to predict ethanol drinking levels. Additionally, as with the ethanol pre-exposure group, EPM tests were administered before and after ethanol drinking.

Intermittent access to ethanol two-bottle choice

Rats experienced 5 weeks of home cage intermittent access two-bottle choice (IA2BC) ethanol drinking sessions based on the paradigm developed by Wise (1973) and modified by Simms et al. (2008). Ethanol was available as a 20% v/v solution diluted from 95% v/v. Rats were single-housed with access to both ethanol and water bottles for three non-consecutive 24-h sessions each week. Bottle position was alternated each session to control for side preference. The rats' body weights, as well as water and ethanol bottle weights, were recorded at the beginning and end of each session. On non-drinking days, animals were returned to pair-housing with access to one water bottle. Furthermore, the ethanol and water bottles used on drinking days were placed in an empty drinking cage and weighed after 24 h to measure spillage, such that the amount recorded could be subtracted from the bottle weight changes recorded in the drinking sessions. For

all calculations, densities of .789 and 1 g/ml were used for ethanol and water, respectively. Ethanol preference was determined by taking the volume of the ethanol solution consumed and dividing by the total volume of fluid intake and then converted into a percentage.

Approach-avoidance conflict Y-maze

To examine approach-avoidance tendencies in a situation of conflict, a mixed-valence conditioning paradigm adapted from Nguyen et al. (2015) was used. Three arms (50 cm L × 11.5 cm W × 35 cm H) extended from a hexagonal central hub (11.5 cm W × 35 cm H) to form a Y shape. Stainless-steel guillotine doors, which could be operated manually, stood between the central hub and each arm. Arm floors consisted of stainless-steel bars connected to a shock scrambler (Lafayette Instrument Co., IN, USA), and stainless-steel inserts at the end of each arm contained a well for sucrose delivery. The entire apparatus was covered in red cellophane to limit visual access to extra-maze cues. Rats completed the maze task in equal-sex groups either prior to (n = 36) or following (n = 20) ethanol self-administration in the IA2BC paradigm (Fig. 1a).

Rats were first habituated to the Y-maze over four sessions. In the first, rats were confined to the central hub for 1 min, and then the doors were raised to allow exploration of all three arms for 5 min. The second session was identical to the first, except this time, three pairs of visuo-tactile denim, tape, or wood bar cues (46 cm L × 9.5 cm H) were placed along the lower half of the side walls in each of the arms. The time spent in each cued arm was recorded and used for cue assignment. For each individual rat, the set of cues that was most preferred was assigned to be paired with the aversive outcome, the least preferred cues were assigned with the appetitive valence, and the remaining cues with a neutral valence during subsequent conditioning sessions. In the third habituation session, rats were confined to the central hub for 1 min, and then the doors were raised to allow exploration of two arms: one containing the "neutral" cues and another containing the cue assigned as the appetitive cue on one side and the cue assigned as the aversive cue on the other wall. This session ensured that the configuration presented in the final conflict test would not be novel to the rats. In the final habituation, rats were confined to the central hub for 30 s and then allowed to explore each arm in turn; the doors were opened and closed to confine the rats to each of the three arms for 1 min, habituating them to the sequence of events that would occur during training.

Next, rats were administered nine identical daily conditioning sessions in the Y-maze (Fig. 1b). To begin each session, rats were confined to the central hub for 30 s and then guided into spending 2 min in each cued arm (with the door closed behind them), with 30 s in the center in between each arm exploration. While in the appetitively cued arm, 0.5 ml of sucrose solution (20%) was delivered 4 times over 2 min at a 30-s interval. In the aversively cued arm, animals received a 0.27 mA footshock (0.22 mA on the first session) delivered 4 times over the 2 min at random time points in each 30-s interval. In the neutral-cued arm, animals did not receive any outcomes. The maze was rotated every day, with each arm moving 60° clockwise 1 day and 60° counterclockwise the next. The cues were assigned to different arms each day, and the order of entry into the arms was altered each day. All these measures were in place to minimize the possibility of animals conditioning to other intra- or extra-maze cues.

To assess cue learning, a 5-min non-reinforced cue acquisition test, identical to the second habituation session, took place after every 4 conditioning sessions. In this session, rats were allowed to explore all cued arms, and the time spent exploring each of the arms was recorded. Rats were deemed to have learned the cue-outcome associations when they spent the most time in the appetitively cued arm on an acquisition test, followed by neutral-cued and aversively cued arms in that order. To account for rats with extreme baseline bias, difference scores were also calculated for each arm, which involved subtracting the time spent in each arm during habituation 2 from that recorded in the acquisition test. If rats displayed an increase in time spent in the appetitively cued arm, a decrease in time spent in the aversively cued arm and the change in neutral arm time fell in the middle; this was also taken as sufficient evidence of learning. Rats not demonstrating successful learning in either of the first two tests (n = 5) continued with four additional conditioning sessions and a third acquisition test.

Following the final acquisition test, rats had one refresher conditioning session, followed by a conflict test 24 h later. The conflict test was identical to the third habituation session, with only two cued arms available for the animals to explore for 5 min (Fig. 1c). One of the arms contained the neutral cues, and the other contained one appetitive cue on one wall and one aversive cue on the other wall to elicit an approach-avoidance conflict. These sessions were scored live and also video recorded for later scoring of additional measures. Time spent in both arms and the central hub was recorded, as well as the number of entries and latency to enter into each arm. Additionally, retreat behaviors, characterized by the animal backing out of an arm that it had partially or fully entered, as well as stay behaviors, defined as the animal turning back from a path out of an arm to remain in the arm, were recorded. From this final test, a cued-conflict preference measure was also calculated, taking the time spent in the arm containing the appetitively and aversively cued arms and dividing by the total time spent in both arms.

Elevated plus maze

To measure anxiety-like behavior, rats underwent two elevated plus maze (EPM) tests. The first test occurred 2 days before the first IA2BC drinking session, while the second was

administered 24 h following the conclusion of the final IA2BC drinking session in a novel testing room that was different to where the first test was administered. The two tests were therefore administered at least 5 weeks apart, in keeping previously reported conditions under which the one-time tolerance effect of EPM can be overcome (Schneider et al. 2011).

The maze was composed of gray Perspex, with a central platform (10 cm L × 10 cm W) connecting four arms (40 cm L × 10 cm W), two of which were enclosed in walls (22 cm H). Rats were placed in the center facing an open arm to begin, and tests were scored live over 10 min. An animal was considered to be in an open or closed arm once all four paws crossed into an arm and out of the central platform. Time spent in each region and the number of entries into open and closed arms were recorded.

Footshock sensitivity test

A subset of ethanol-naïve rats from the study (following IA2BC exposure, n = 16, n = 8/sex) and a new group of age-matched naïve rats (n = 16, n = 8/sex) were used to determine the effects of sex and ethanol history on sensitivity to footshock. The rats were placed in one of the arms of the Y-maze apparatus (50 cm L × 11.5 cm W × 35 cm H, without any cues) with a grid floor and shocks were delivered intermittently (1 s duration, 14 s inter-shock interval), beginning at 0.1 mA and increasing in 0.05 mA increments. The shock level at which each rat first displayed flinch, jump, and vocalization responses was recorded.

Statistical analysis

All data were analyzed using SPSS statistical package version 23.0 (IBM, ON, Canada). Repeated measures (RM) analyses of variance (ANOVA) were used to examine changes in ethanol drinking over the 5 weeks of drinking, with sex as a between-subjects factor. Cued-conflict test data (time spent, number of entries, latency to enter, retreats, and stays) and EPM data (time spent, number of entries) were analyzed with three-way RM-ANOVA, with arm serving as a within-subject factor and sex and ethanol history as the between-subjects factors. Where appropriate, Bonferroni-corrected independent sample two-tailed t-tests were used to examine group differences or significant interaction effects. To further examine the relationship between approach/avoidance tendencies and ethanol consumption, a preference score was calculated for both the cued conflict and EPM tasks by computing the discrimination ratio of the time spent in the conflict or open arms over the overall time spent in the conflict + neutral arms or open + closed arms. A median split of the cued-conflict preference scores or open arm preference scores were then conducted to generate two subgroups of animals: "low conflict preference" or "low anxiety" vs. "high conflict preference" or "high anxiety." For cued-conflict preference, we first established that ethanol history did not induce significant changes in the expression of approach-avoidance conflict preference. We then proceeded to pool the data across the ethanol-naïve and ethanol-exposure groups in both males and females to establish a correlation between conflict preference and ethanol drinking. For EPM, however, the ethanol-naïve and ethanol pre-exposed groups did not perform similarly, so we conducted a median split only on the data from 18 animals of each sex that completed the conflict Y-maze task before drinking (ethanol-naïve group). Regression analyses were also conducted to further examine the relationship between conflict preference or EPM performance and ethanol drinking. The α level was set to .05 for all tests.

Results

IA2BC ethanol drinking

Normalized to body weight, female rats drank more than male rats [sex: $F(1, 54) = 12.38, p < .001$], and drinking levels were stable across all 5 weeks [week: $F(3.49, 132.51) = .77, p = .53$] (Fig. 2a). Females had a significantly higher preference for ethanol than did males [sex: $F(1, 54) = 10.28, p < .01$], and a week by sex interaction [$F(3.69, 198.61) = 3.544, p < .01$] revealed that females, relative to males, demonstrated an increase in ethanol preference over time (Fig. 2b). Females and males drank similar levels of water [sex: $F(1, 54) = 1.32, p = .26$], with water consumption declining across the 5 weeks in both sexes [week: $F(3.35, 180.83) = 36.48, p < .0001$] (Fig. 2c). Males weighed more than females across all weeks [sex: $F(1, 54) = 425.01, p < .0001$], and while a main effect of week showed both sexes gained weight over time [week: $F(1.68, 90.75) = 934.24, p < .0001$], a sex by week interaction revealed that males gained weight at a faster rate than females [$F(1.68, 90.75) = 125.10, p < .0001$] (Fig. 2d).

Y-maze cue acquisition

All animals demonstrated successful learning in the Y-maze in the final acquisition test [arm: $F(2, 108) = 136.17, p < .0001$], with the appetitive arm most preferred, followed by the neutral, and then the aversive arms (all pairwise comparisons, $p < .0001$) (Fig. 3a and b). Similarly, difference scores capturing the change in time spent exploring the arms from habituation 2 to the acquisition test revealed a main effect of arm [arm: $F(2, 108) = 253.93, p < .0001$], with the greatest change in the appetitive arm, followed by neutral, and then aversive (all pairwise comparisons, $p < .0001$) (Fig. 3c and d). Importantly, there was no effect of ethanol history on the acquisition of cue-outcome associations (all effects for each sex, $p > .201$). Furthermore, there was no sex difference in cue

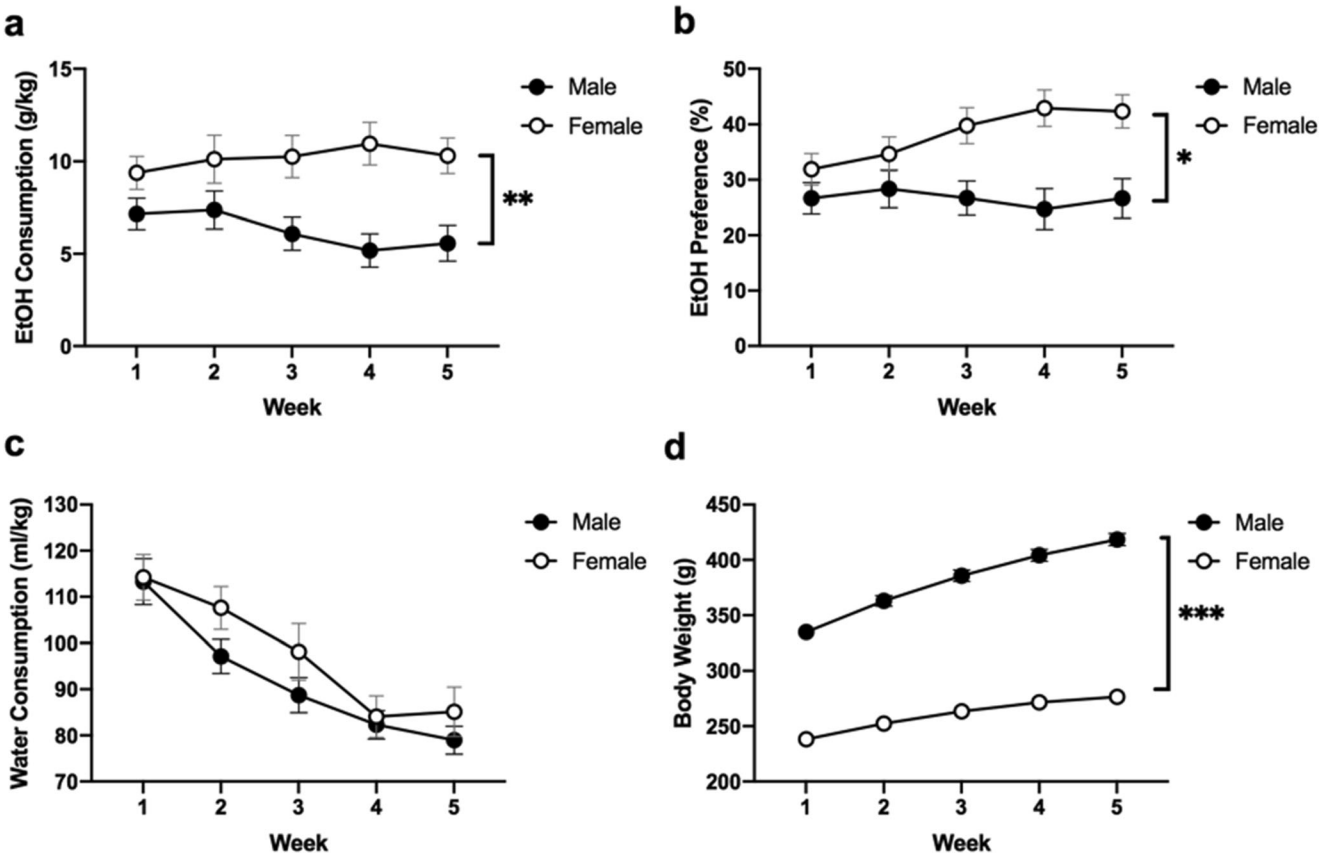


Fig. 2 Weekly averages of ethanol drinking (± SEM) across 5 weeks in the IA2BC paradigm for **a** ethanol consumption, **b** ethanol preference, **c** water consumption, and **d** body weight. Females (n = 28) drank more than males (n = 28) and displayed a higher preference for ethanol, whereas males weighed more. Water consumption declined over the 5 weeks in both sexes as body weights increased. *p < .05, **p < .01, ***p < .001

acquisition performance [sex by arm: F (2, 108) = 1.58, p = .21, sex: F (1, 54) = 2.62, p = .11]. Additionally, given that the acquisition test was conducted approximately 6 weeks later in the ethanol pre-exposed group than the ethanol-naïve group, we conducted a correlation analysis to examine the relationship between body weight variation and sensitivity to the aversive outcome. We report that the time that animals spent in the aversive arm did not correlate with body weight at the time of the test in males or females [male: r^2 = .01, p = .54, females: r^2 = .02, p = .48].

Detailed analysis of cued approach-avoidance conflict resolution behavior

Analysis of the time spent in the conflict vs. neutral arms in the conflict test revealed a significant effect of ethanol history in both males and females [F (1, 52) = 9.74, p < .01, no effect of arm or arm × history interaction, p > 0.87], with ethanol pre-exposed animals showing reduced exploration times of the arms overall compared to naïve animals (Fig. 4a, b). A separate analysis of the central hub time confirmed this effect with ethanol pre-exposed males spending more time in the central hub than ethanol-naïve males [t (26) = 3.68, p < .001], but this did not reach significance in the females [t (26) = 1.34, p = .19] (Fig. 4c). There were further sex differences in the time spent in the conflict and neutral arms irrespective of ethanol history [sex: F (1, 52) = 4.78, p < .05, sex × arm interaction: F (1, 52) = 7.83, p < .01] with the males spending significantly more time in the conflict arm over the neutral arm (p < .05) compared to females (p < .0001). In contrast, the females spent equal times exploring the conflict and neutral arms (p = .13).

The overall number of entries into both conflict and neutral arms was reduced in both males and females with ethanol history [F (1, 52) = 7.31, p < .01, but no effect of arm or arm × history, p > .18, Fig. 4e, f]. However, there were significant sex differences [sex × arm: F (1, 52) = 76.32, p < .05, sex × arm × history: F (1, 52) = 7.85, p < .01], with ethanol pre-exposed female rats making significantly fewer entries into the conflict arm compared to the neutral arm (p < .001) and compared to ethanol-naïve female rats (p < .01).

In contrast, the latency to enter the arms did not significantly differ by arm [F (1, 52) = .40, p = .53] or ethanol history [F (1, 52) = 2.69, p = .11] for either sex [sex: F (1, 52) = .004, p = 0.95, all interactions, p > .40, Fig. 4g, h]. Overall, animals emitted increased retreat behavior towards the conflict cue, compared to the neutral cue, as expected [arm: F (1, 26)

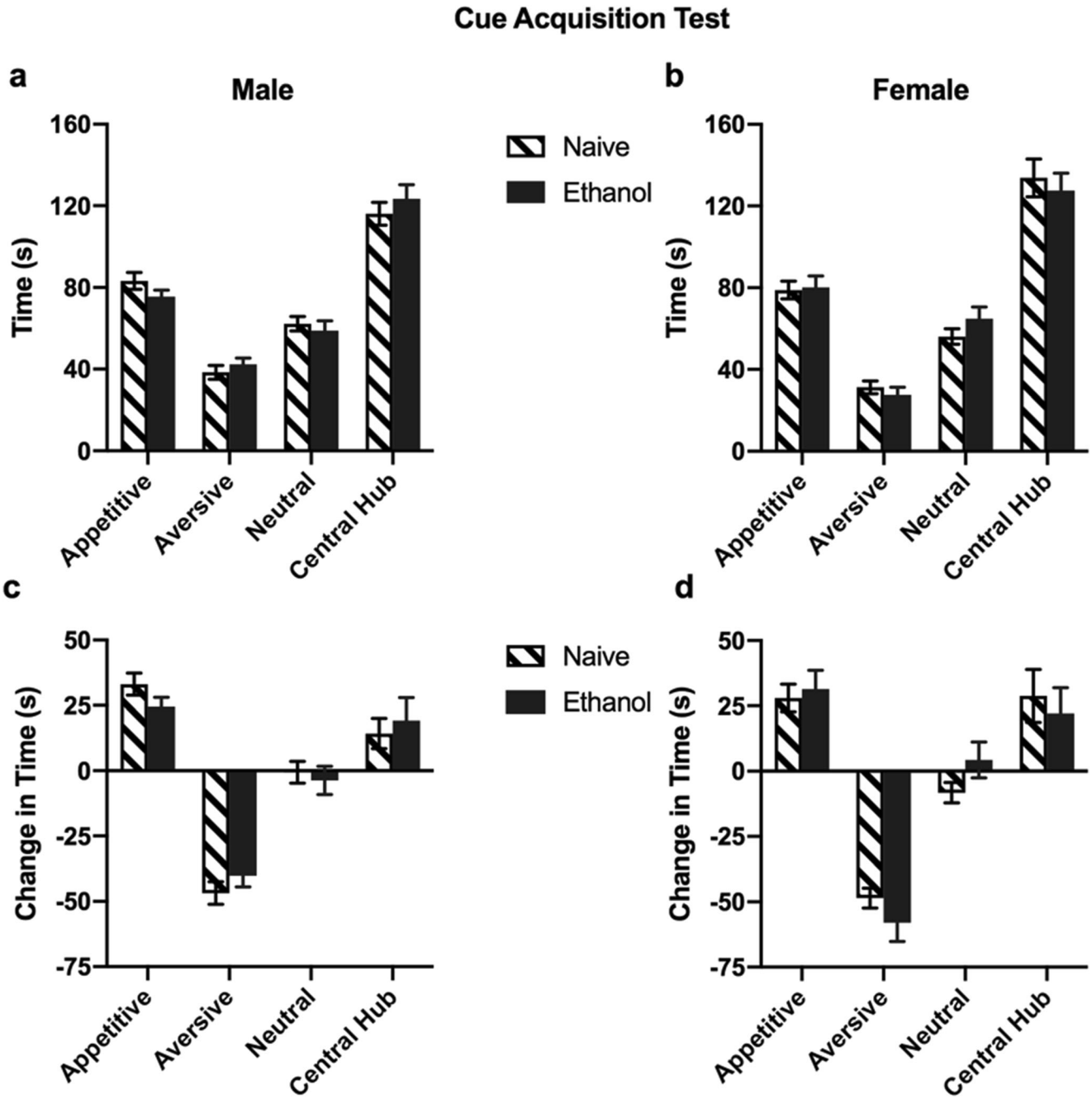


Fig. 3 Cue-outcome association learning as measured by average time spent (± SEM) in each arm on the acquisition test in **a** males ($n = 28$) and **b** females ($n = 28$) and as measured by the difference in time spent (± SEM) in each arm between the acquisition test and the second habituation session in **c** males and **d** females. Both sexes demonstrated successful learning of the cue-outcome associations independent of ethanol history.

=7.94, $p < .01$]. However, ethanol pre-exposure induced significant increases in the number of retreats emitted towards both conflict and neutral arms in both males and females [history: F (1, 26) = 4.81, $p < .05$, no significant effect of sex, or interactions, all $p > .16$, Fig. 4i, j]. Finally, animals in the ethanol pre-exposed group exhibited less stays in the conflict and neutral arms than ethanol-naïve animals [history; F (1, 52) = 4.58, $p < .05$, Fig. 4k, l], irrespective of sex [all sex effects: $p > .08$].

In summary, a history of ethanol exposure led to animals exhibiting a general reduction in the overall exploration of, and the number of entries into, the conflict and neutral arms. This was accompanied by increased retreat behavior from both conflict and neutral arms, as well as decreased stays in the ethanol pre-exposure group. Additionally, we found that the males spent significantly more time in the conflict than neutral arms compared to the females, and the ethanol pre-exposed females made significantly fewer entries into the

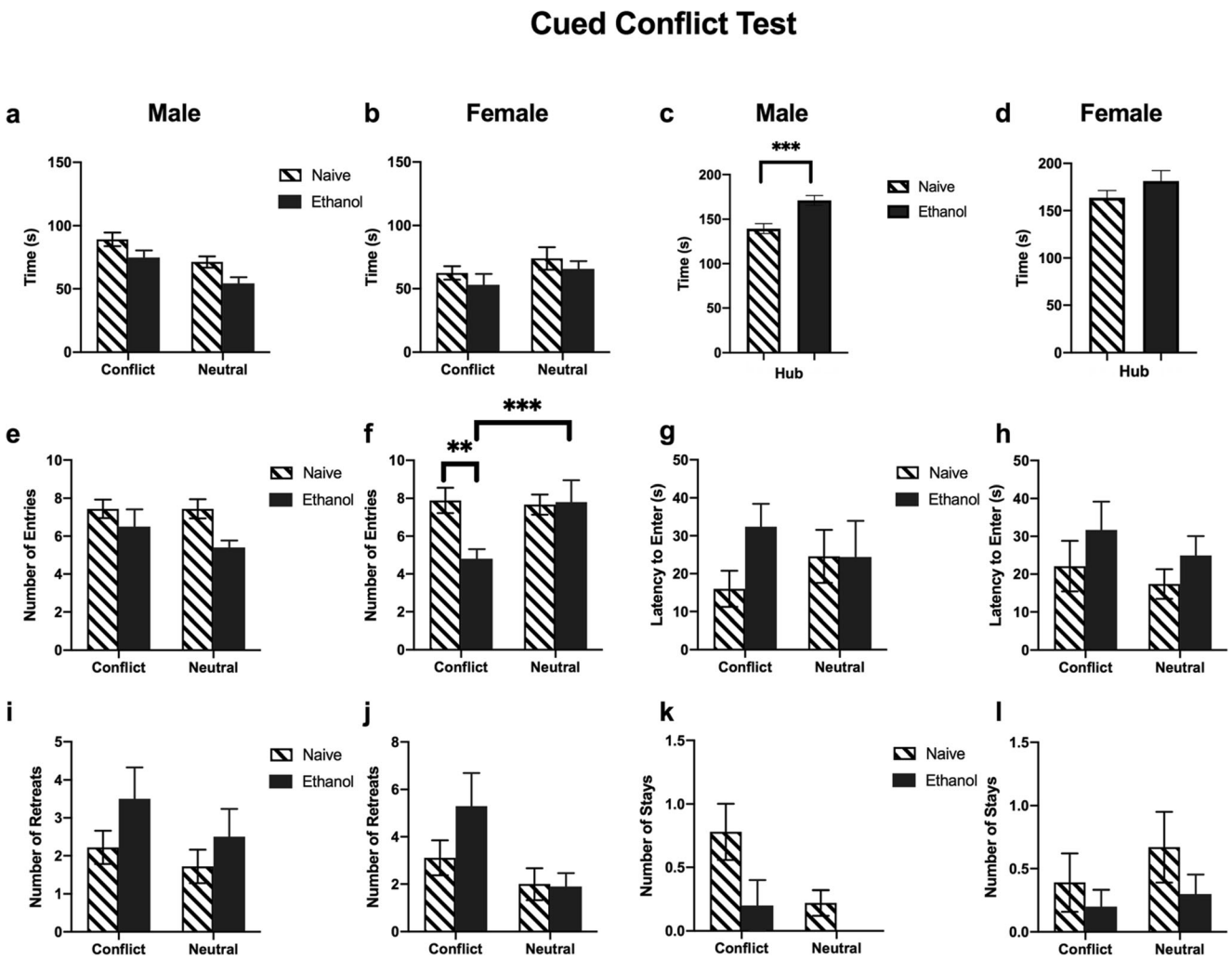


Fig. 4 Y-maze conflict test behaviors are shown here in groups based on ethanol history (naïve $n = 18$, ethanol $n = 10$ for each sex). Time spent in conflict and neutral arms (± SEM) in **a** males and **b** females. Time spent in the central hub (± SEM) in **c** males and **d** females. Number of entries (± SEM) into each arm in **e** males and **f** females. Latency to first entry (± SEM) into each arm in **g** males and **h** females. Number of retreats (± SEM) from each arm in **i** males and **j** females. Number of stays (± SEM) in each arm in **k** males and **l** females. Ethanol exposure reduced overall time spent exploring, and number of entries into the conflict and neutral arms in both males and females, but increased central hub time in the males and decreased the number of entries into the conflict arm in the females only. Males also displayed a preference for the conflict arm over the neutral arm irrespective of ethanol history. $^{*}p < .05$, $^{**}p < .01$, $^{***}p \leq .001$

conflict arm compared to the neutral arm, and ethanol-naïve animals.

Relationships among sex, cued-conflict preference, and drinking

Analysis of the cued-conflict preference score confirmed that males showed a higher cued-conflict preference score than the females [sex: $F(1, 52) = 9.36, p < .01$]. However, there was no significant effect of ethanol history for either sex [history: $F(1, 52) = .07$, $p = .79$, sex × history: $F(1, 52) = .82$, $p = .37$] (Fig. 5a). Given that ethanol history did not induce significant differences in the expression of approach-avoidance conflict preference behavior, we conducted a median split of the cued-conflict preference score, to generate two groups of animals with distinct conflict behavior [males: group: $t(26) = 6.27, p < .0001$, females: group: $t(26) = 6.64$, $p < .0001$] (Fig. 5b). These two groups did not differ in body weight across the drinking sessions, eliminating that as a possible explanation for any drinking differences [males, group: $F(1, 26) = .15$, $p = .70$ and females, group: $F(1, 26) = .17$, $p = .69$]. Analysis of ethanol drinking level across the 5 weeks of IA2BC revealed that in males, there was no effect of low vs. high cued-conflict preference on either measure [ethanol consumption: $F(1, 26) = .24$, $p = .63$ and ethanol preference: $F(1, 26) = 1.05$ $p = .32$] (Fig. 5c and i). In contrast, females in the low cued-conflict preference group showed a significantly higher consumption of ethanol overall [group: $F(1, 26) = 6.74$, $p < .05$] (Fig. 5f) and preference for ethanol [group: $F(1, 26) = 5.67$, $p < .05$] (Fig. 5l), compared to the high cued-conflict preference group.

Further regression analyses in females revealed that conflict preference was inversely correlated with ethanol drinking and preference in the first 3 weeks of IA2BC [consumption: r^2 = .19, p < .05, Fig. 5g, preference: r^2 = .15, p < .05, Fig. 5m] but not the latter 2 weeks [consumption: r^2 = .02, p = .45, Fig. 5h, preference: r^2 = .02, p = .44, Fig. 5n]. In males, conflict preference was not significantly correlated with either ethanol consumption or preference [all r^2 < .06, all p >.21, Fig. 5d–e, j–k].

EPM

Ethanol consumption (pre vs. post consumption tests) did not have a significant effect on the time spent in the open and closed arms of the EPM [pre-post: F (1, 52) = 1.88, p = .18], regardless of sex [pre-post × sex: F [1, 52) = .01, p = .94] or the timing of drinking exposure [pre-post × group: F (1, 52) = .48, p = .49]. However, there was a significant arm × sex interaction [F (1, 52) = 4.13, p < .05], which could be attributed to a significant increase in the time spent in the closed arms (p < .001), but not open arms (p = .81) in the females, compared to males, indicating that the females exhibited a more anxious phenotype. Furthermore, there was a significant arm × group interaction [F (1, 52) = 22.04, p < .0001], which was driven by the time spent exploring the open arms being higher and the time spent in the closed arms being lower in the male and female groups that were in the ethanol-naïve group (i.e., completed the Y-maze task first, both p <0.0001), compared to the ethanol pre-exposed group.

Analysis of the number of entries into the open and closed arms of the EPM (Fig. 6b, d, f, h) revealed a significant effect of ethanol consumption on the overall number of entries emitted in the open and closed arms [pre-post: F (1, 52) = 5.43, p < .03], as well as a significant interaction between pre-post × arm × sex [F (1, 52) = 4.83, p < .04]. Further post hoc analyses revealed this effect to be due to the males showing a reduced number of entries into the closed arms when the EPM test was administered following ethanol consumption (p < .0001, Fig. 6b, d). There was also a significant increase in the number of overall entries into the open and closed arms in the tests that were conducted in the ethanol-naïve group, following Y-maze testing [group: F (1, 52) = 17.11, p < .0001].

Relationship between anxiety-like behavior and drinking

Open arm preference scores in the ethanol-naïve groups in males and females were significantly higher than those of ethanol pre-exposed groups [group: F (1, 52) = 19.28, p < .0001]. Therefore, subsequent analyses of the relationship between anxiety-like behavior and ethanol drinking were conducted exclusively on data generated by the 36 animals (18 of each sex) in the ethanol-naïve group.

A median split of the open arm preference scores from the first EPM test resulted in two groups with distinct anxiety-like behavior in both males [group: t (16) = 5.07, p < .0001] and females [group: t (16) = 6.24, p < .0001] (Fig. 6f). This grouping, however, did not reveal any significant relationship between open arm preference and ethanol consumption or preference in males or females [male consumption: F (1, 16) = .01, p =.92, male preference: F (1, 26) = .01, p = .93, female consumption: F (1, 16) = .07, p = .80, female preference: F (1, 16) = .32, p = .58, data not shown]. Similarly, regression analyses in males and females failed to reveal any meaningful association between open arm preference on the first EPM and subsequent ethanol consumption or preference in the first (weeks 1–3) or second half (weeks 4–5) of IA2BC [all r^2 < .14, all p > .12]. However, when we examined the correlation between the initiation of ethanol drinking (first week only) and EPM open arm preference, we replicated prior research in finding a significant negative correlation between open arm preference and ethanol drinking in males (Chappell et al. 2013) [consumption: r^2 = .23, p < .05, Fig. 6g, preference: r^2 = .26, p < .05, Fig. 6h] but not females [Butler et al. 2014, consumption: r^2 = .01, p = .73, Fig. 6i, preference: r^2 = .01, p < .71, Fig. 6j].

Sensitivity to footshock

A 4-way ANOVA revealed no effect of sex or ethanol history, nor an interaction, on sensitivity to footshock for any of the flinch, jump, or vocalization measures (p > .07 for all): naïve males (in mA±SEM): flinch: 0.18 ± 0.02, jump: 0.39 ± 0.02, vocalization: 0.34 ± 0.02, ethanol males: flinch: 0.19 ± 0.02, jump: 0.43 ± 0.02, vocalization: 0.31 ± 0.03, naïve females: flinch: 0.18 ± 0.01, jump: 0.36 ± 0.02, vocalization: 0.37 ± 0.03, and ethanol females: flinch: 0.39 ± 0.02, jump: 0.43 ± 0.02, vocalization: 0.30 ± 0.02.

Discussion

In this study, we employed a mixed-sex sample of rats to investigate voluntary ethanol drinking behavior and its relationship with both approach-avoidance conflict behavior and anxiety-like behavior. We found sex differences, both dependent and independent of ethanol history, in the expression of approach-avoidance conflict behavior. Ethanol pre-exposure in both male and female rats induced decreased overall exploration time of and number of entries into the conflict and neutral arms and increased time in the central hub for males only. The females also exhibited reduced preference for the conflict arm, as well as the open arm in the EPM test than males irrespective of ethanol history, suggesting that they have a more avoidant phenotype in general. Furthermore, a significant association between reduced preference for

conflict cues and elevated ethanol consumption and preference was found in female rats only. Our results implicate individual and sex differences in approach-avoidance tendencies in the face of motivational conflict as potential predictors of drinking behavior.

Female rats showed elevated IA2BC ethanol drinking compared to males

Our rats reached levels of ethanol consumption that fell within the range of reported values in an extensive literature, with females having higher consumption and preference than males (Carnicella et al. 2014; Priddy et al. 2017; Simms et al. 2008), and with no evidence of escalation of ethanol drinking (Butler et al. 2014; Chappell et al. 2013; Priddy et al. 2017; Roeckner et al. 2017, but see Carnicella et al. 2014; Simms et al. 2008 for escalating male intake). However, drinking levels and patterns reported in the IA2BC literature are typically variable, and one measure that is not commonly reported is body weight. Here, we report that, as expected, both males and females gained body weight over time but that the increase for males occurred at a faster rate. The IA2BC paradigm facilitates ethanol drinking across a protracted period of time while allowing them to have unlimited access to food and water. Therefore, animals readily gain weight, and tracking body weight across drinking sessions is critical. As ethanol consumption is normalized to body weight, differences in the rate of increase in body weight are important factors for consideration and may be responsible for strain, sex, and other reported differences in ethanol drinking.

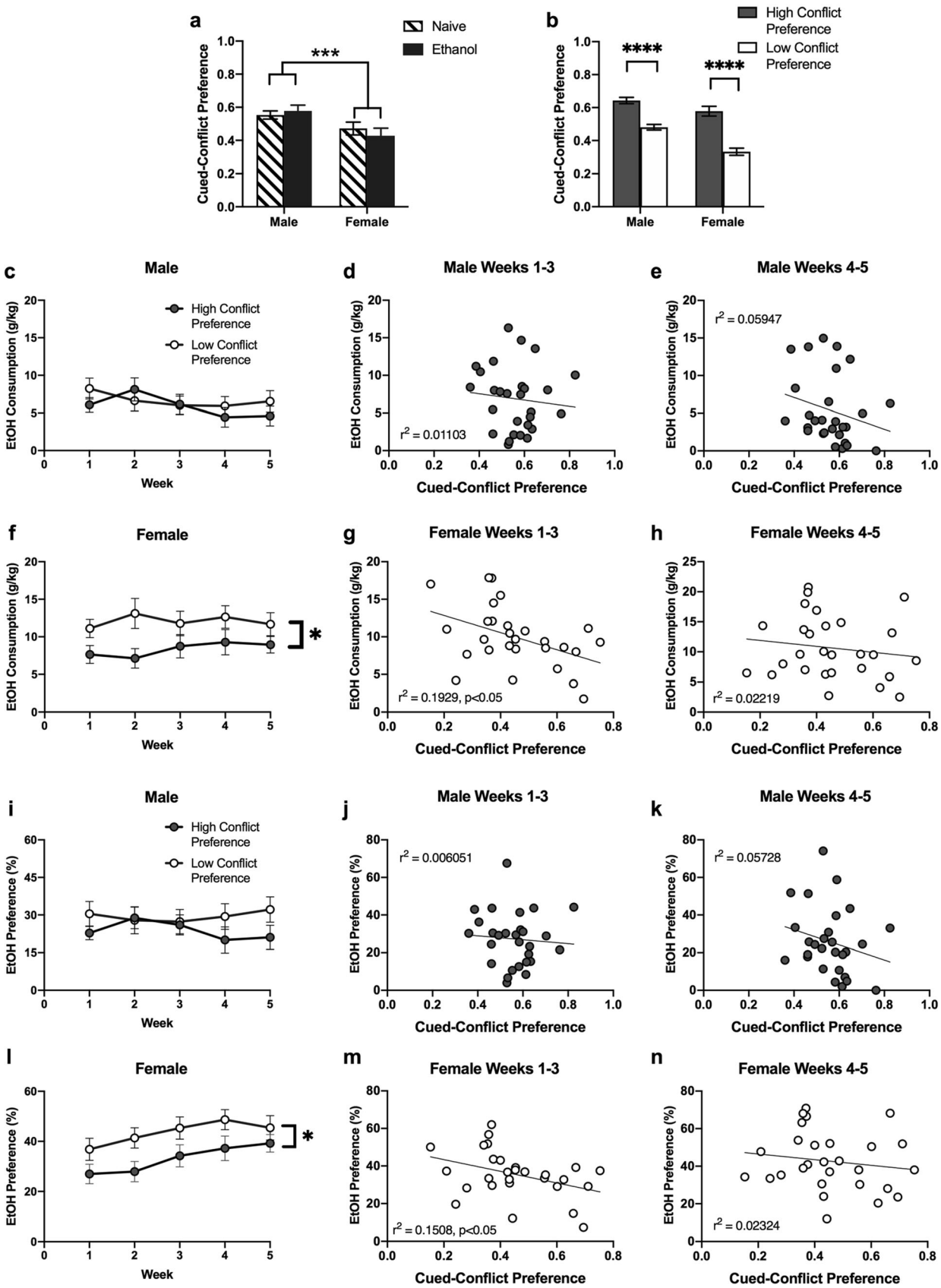
Females showed avoidance tendencies towards cues predicting conflict than males

Both males and females, irrespective of ethanol history, learned the cue-outcome associations successfully. Subsequently, however, we found a sex difference in conflict test performance, pointing towards lower cued-conflict approach tendencies in females compared to males. Additionally, we found that females made fewer entries into the conflict arm compared to the neutral arm, indicating that female rats exhibited greater avoidance tendencies under motivational conflict. One thought is that females were more sensitive to the level of shock, and this influenced their behavior in the conflict test. However, we did not observe any sex differences in the acquisition of aversive cue learning, or footshock sensitivity in the present study, which is consistent with previous reports (Day et al. 2016; Orsini et al. 2016). Instead, a more plausible explanation for the apparent avoidant tendencies in females is that females may be more risk averse than males. Indeed, in one study, Amodeo et al. (2018) placed a peanut butter-coated food pellet in a brightly lit center of an open field. Compared to males, females made fewer entries into and spent less time inside the center grid (Amodeo et al. 2018). In a risky decision-making task in which lever pressing for a small food reward was a safe option, but lever pressing for a large food reward came with the possibility of footshock, male rats showed a greater preference for the risky choice than females did (Orsini et al. 2016). Similarly, despite no differences in outcome sensitivity, female rats displayed less win-stay and more loss-shift behavior in a rat version of the Iowa Gambling Task (van den Bos et al. 2012). Moreover, it has been shown that gambling-related cues predicting uncertainty are more attractive to male rats than cues predicting certainty, whereas the cues do not differ in attraction for female rats (Hellberg et al. 2018). The conflict cue in our study may conceivably represent an uncertain outcome to the animals and, together with existing evidence, suggests that female rats have a more avoidant phenotype in the face of motivational conflict than do males.

Ethanol increased time spent in the central hub on the conflict test in males only

Ethanol history in male and female rats reduced the total exploration time of the conflict and neutral arms, compared to ethanol-naïve animals. This was mirrored by a significant increase in the time that the ethanol-exposed male rats spent in the central hub compartment in the males only. Together, these results could be indicative of a slowed decision-making or increased deliberation time before entering the arms. It is well known that ethanol use can cause cognitive deficits (Galaj et al. 2019; Gulick and Gould 2008; Stragier et al. 2015; West et al. 2018), but the effect of ethanol consumption on decision-making indices such as deliberation time is not well-studied. In a study on impulsivity, however, Spoelder et al. (2017) found that high-drinking males, relative

Fig. 5 Relationship between cued-conflict preference and EtOH consumption/preference. **a** Mean cued-conflict preference scores (± SEM), calculated as the ratio of time spent in the conflict arm to total arm exploration time on the conflict test, in ethanol pre-exposed ($n = 10$ for each sex) and ethanol-naïve ($n = 18$ for each sex) groups. Ethanol pre-exposure did not have an effect on the cued-conflict preference score, but the males showed higher preference for the conflict cue than the females. **b** A median split of the cued-conflict preference scores of both ethanol pre-exposed and ethanol-naïve groups revealed divergent cue-conflict preferences (± SEM) between the two resultant groups ($n = 14$ in each) within each sex. Ethanol consumption and preference (± SEM) were plotted across the 5 IA2BC weeks for low and high cued-conflict groups for males (**c**, **i**) and females (**f**, **l**). Low conflict cue preference scores in the females were associated with increased higher ethanol consumption and preference. Regression analyses were also conducted to assess the relationship between ethanol consumption and preference and cued-conflict preference scores in weeks 1–3 and weeks 4–5 of IA2BC drinking (males: **d**, **e**, **j**, **k**, females: **g**, **h**, **m**, **n**). A significant negative correlation was found between ethanol drinking/preference during weeks 1–3 and conflict cue preference in the females only. $^{*}p < .05$, $^{***}p \leq .001$, $^{****}p < 0.0001$


Relationship between Cued Conflict Preference and EtOH consumption/preference
a
Naive
Ethanol
Cued-Conflict Preference
***
Male
Female
b
High Conflict Preference
Low Conflict Preference
****
****
c
Male
High Conflict Preference
Low Conflict Preference
EtOH Consumption (g/kg)
Week
d
Male Weeks 1-3
r2 = 0.01103
Cued-Conflict Preference
e
Male Weeks 4-5
r2 = 0.05947
f
Female
*
g
Female Weeks 1-3
r2 = 0.1929, p<0.05
h
Female Weeks 4-5
r2 = 0.02219
i
Male
EtOH Preference (%)
j
Male Weeks 1-3
r2 = 0.006051
k
Male Weeks 4-5
r2 = 0.05728
l
Female
m
Female Weeks 1-3
r2 = 0.1508, p<0.05
n
Female Weeks 4-5
r2 = 0.02324

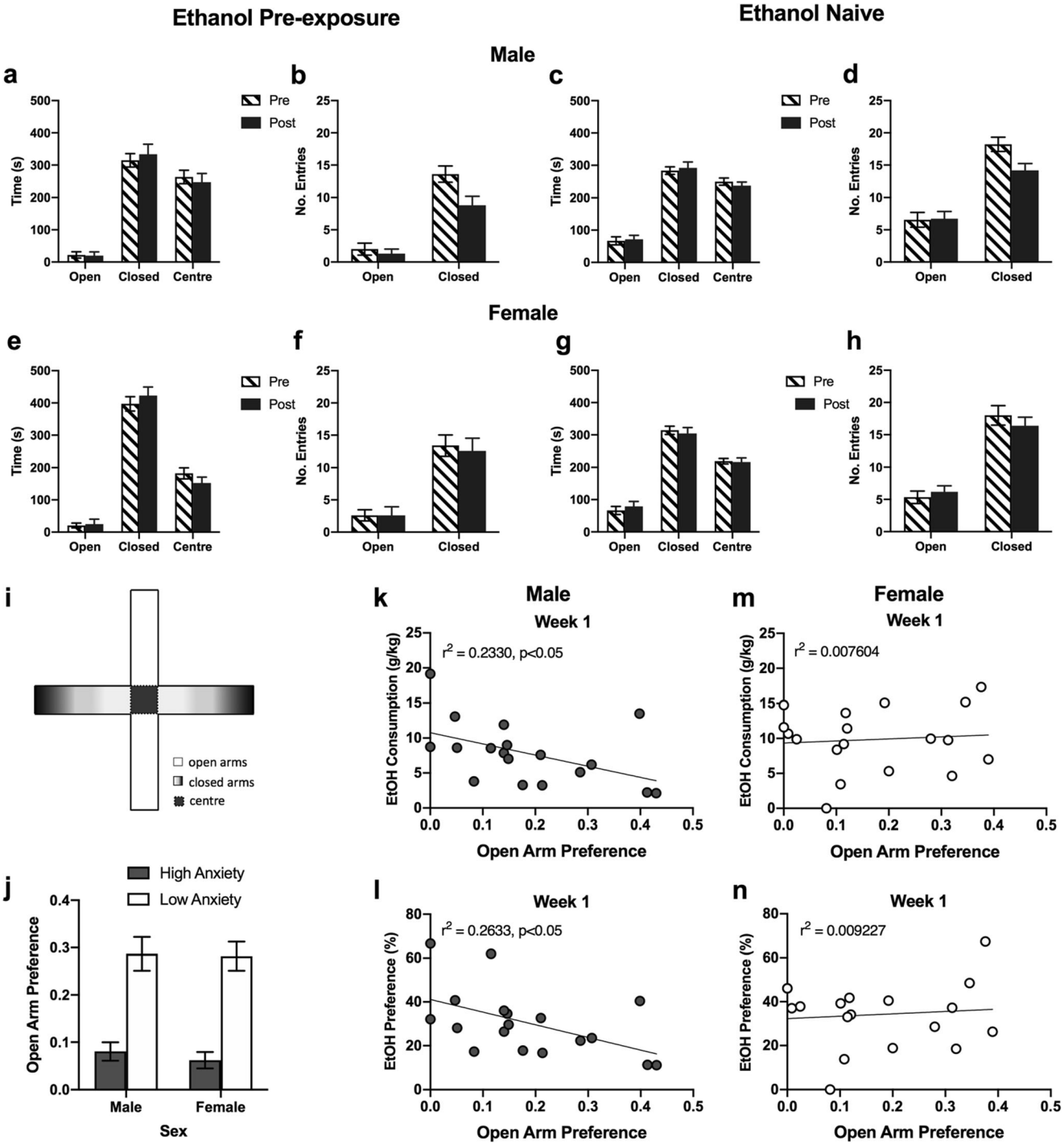


Fig. 6 **a** Elevated plus maze (EPM) performance (**i**), expressed as the mean time spent in (± SEM, **a**, **e**, **c**, **g**) and number of entries (± SEM, **b**, **f**, **d**, **h**) into the open and closed arms, and central compartment (for time spent measure only), in the ethanol pre-exposure (n = 10/sex) and ethanol-naïve groups (n = 18/sex). In all groups, two EPM tests were administered: one prior to and another after ethanol exposure. **j** A median split based on open arm preference scores generated from the first EPM test in the ethanol-naïve group created two distinct groups of animals (n = 9 in each) within each sex. Regression analyses were also conducted to assess the relationship between ethanol consumption and preference and open arm preference scores in week 1 of IA2BC drinking. We found a significant negative correlation between ethanol drinking and preference and open arm preference in the males, but not females. ***$p < .001$

to low-drinking males, showed a higher preference for a delayed reward during the training phase of a delayed reward task. Thus, it is possible that the effect of ethanol exposure we observed in our study represents a reduction in impulsive decision-making. However, the reduced impulsivity found by Spoelder et al. (2017) was a transiently observed effect that

disappeared with more time in withdrawal following intermittent ethanol access. Indeed, several effects of ethanol on decision-making have been shown to be dependent on the timing of testing. For example, ethanol enhances risky decision-making in males in adulthood when administered during adolescence, but not during adulthood (Schindler et al. 2014). Generally, there is lack of consensus on the effect of ethanol exposure in adults, with evidence that ethanol-exposed males, compared to naïve males, make less optimal decisions on a gambling task (Jeanblanc et al. 2018), exhibit increased optimal responding (Spoelder et al. 2017), or show no difference on an effort-based decision-making T-maze task with high- and low-reward options (Conte et al. 2019).

It should also be noted that the subtle difference in the effect of ethanol exposure on the performance of the conflict test in males and females, with the absence of a significant effect on the hub time in the latter, may be a reflection of the difference in the cumulative ethanol exposure that the females and males had experienced, with the females having been exposed to significantly more ethanol overall. While it is more intuitive to expect higher ethanol exposure to induce more drastic change in conflict-related behavior, the increased deliberation in the ethanol-exposed males, but not females, may point to certain indices of decision-making being vulnerable to disruption with different levels of ethanol exposure. Further investigation is therefore warranted to elucidate the exact conditions under which ethanol exposure impacts decision-making.

Ethanol has no effect on preference for cues predictive of conflict

The lack of influence of ethanol history on the conflict preference measure was contrary to our expectation, given that a history of subchronic cocaine exposure has previously been shown to increase preference for cues predictive of conflict in the same task (Nguyen et al. 2015). However, aside from these drugs having contrasting mechanisms of action and physiological effects, with cocaine being a psychostimulant and ethanol a depressant, there are important differences in the method of drug administration between the two studies, which may have given rise to the differential effects on cued-conflict behavior. Cocaine was delivered daily as an i.p. injection in Nguyen et al. (2015), whereas in the current study, consumption of ethanol was unforced (voluntary) and likely did not induce the same rise in blood ethanol levels as forced exposure can produce. In order to induce ethanol dependence, forced exposure methods are commonly used, relying on oral gavage administration, i.p. injections, or vapor chambers. Very few studies have directly examined the effects of forced ethanol administration on approach-avoidance conflict, but one recent study provided evidence that CIE vapor male mice did not differ in the expression of conflict elicited by a context associated with both ethanol and shock, compared to control male mice. This contrasted with the effect of CIE on females, which was to reduce approach of the conflict-eliciting context (Xie et al. 2019). While we observed similar avoidance tendencies towards a conflict cue in naïve females that subsequently exhibited high ethanol preference, we failed to see alterations in conflict cue-induced behavior in the rats (male or female) as a consequence of voluntary IA2BC ethanol drinking.

Rodents that have undergone forced exposure to ethanol have also been reported to show enhanced anxiety-like behavior on the EPM in early withdrawal (Ewin et al. 2019; Finn and Crabbe 1997; Kliethermes et al. 2004; Morales et al. 2015; Morales et al. 2018; Valdez et al. 2002; reviewed in Kliethermes 2005), but we failed to observe any increases in anxiety-like behavior on the EPM, in accord with other reports using a longer IA2BC paradigm with male rats (George et al. 2012), as well as with male and female mice tested for anxiety in the elevated zero maze (Bloch et al. 2020). Thus, it is possible that methods of ethanol administration that result in physiological dependence engender an anxious phenotype, and alteration of approach-avoidance conflict processing in ways that voluntary administration does not. Further studies are required to directly ascertain the effects of voluntary vs. forced ethanol administration on the expression of approach-avoidance conflict behavior.

Low preference for cues signaling conflict is associated with heightened ethanol consumption and preference in female rats

The present data showed that the group of females with low approach tendencies for cues predicting conflict exhibited greater consumption of and an elevated preference for ethanol compared to the group with higher approach tendencies. A lower conflict preference score is indicative of greater avoidance tendencies in the face of motivational conflict (Ito and Lee 2016; Schumacher et al. 2016), and this inverse relationship is highly consistent with the findings of a previous study that used rats bred to exhibit divergent behavior in a two-way active avoidance task; it was reported that Roman high-avoidant rats displayed a higher intake of and preference for ethanol than did Roman low-avoidant rats (Manzo et al. 2014). As discussed earlier, our results are also consistent with the avoidant tendencies of CIE female mice (Xie et al. 2019). Interestingly, the results of our regression analysis indicated that ethanol drinking behavior at the onset of drinking is best correlated with conflict-induced behavior, rather than drinking that has stabilized. Thus, the present findings suggest that avoidance of conflicting cues can be explored as a potential predisposing factor for ethanol consumption and preference, particularly in the early phases of developing the drinking habit.

A median split of the open arm preference scores from the first EPM test in the ethanol-naïve animals also resulted in groups with distinct anxiety-like behavior. However, this median split failed to reveal any significant relationship between anxiety-like behavior and ethanol consumption or preference in either males or females, suggesting that anxiety state is not a risk factor for elevated drinking in the present study, although as discussed earlier, a history of *forced* ethanol exposure can induce anxiety (Ewin et al. 2019; Finn and Crabbe 1997; Klietheremes et al. 2004; Morales et al. 2015; Morales et al. 2018; Valdez et al. 2002). Parallel regression analyses confirmed that there was no significant relationship between open arm preference and ethanol drinking in either females or males when we averaged ethanol drinking levels across 5 weeks. However, we found that decreased open arm preference scores (heightened anxiety) correlated significantly with higher ethanol consumption and preference in males (but not females) when we only considered drinking levels in the first week of IA2BC, replicating previous findings (Butler et al. 2014; Chappell et al. 2013). Thus, there is mounting evidence that a high anxiety phenotype may predict higher future ethanol intake and preference in males, particularly at the onset of drinking.

Conclusion

In this study, we identified novel sex-dependent associations between approach-avoidance conflict resolution and ethanol drinking behavior, highlighting the need for continued use of mixed-sex samples in alcohol research. In males, a history of voluntary ethanol drinking induced increased deliberation time in the face of motivational conflict, which is indicative of an alteration in decision-making processes. In females, low cued-conflict preference was associated with elevated ethanol intake and preference in the initial few weeks of drinkng, while in males, low open arm preference on the EPM was linked to higher ethanol drinking and preference at the onset of drinking, implicating individual differences in approach-avoidance conflict behavior as a potential predictor of future drinking. The relationship between ethanol and conflict preference may be further elucidated through the use of different ethanol exposure paradigms, including ones that induce dependence, as well as measuring addiction-related response outputs in operant paradigms.

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Declarations

Conflict of interest The authors declare no competing interests.

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