

Sex-dependent effects of chronic intermittent voluntary alcohol consumption on attentional, not motivational, measures during probabilistic learning and reversal

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Abstract (300/300 words)

Background: Forced alcohol (ethanol, EtOH) exposure has been shown to cause significant impairments on reversal learning, a widely-used assay of cognitive flexibility, specifically on fully-predictive, deterministic versions of this task. However, previous studies have not adequately considered voluntary EtOH consumption and sex effects on probabilistic reversal learning. The present study aimed to fill this gap in the literature. **Methods:** Male and female Long-Evans rats underwent either 10 weeks of voluntary intermittent 20% EtOH access or water only (H2O) access. Rats were then pretrained to initiate trials and learn stimulus-reward associations via touchscreen response, and subsequently required to select between two visual stimuli, rewarded with probability 0.70 or 0.30. In the final phase, reinforcement contingencies were reversed. **Results:** We found significant sex differences on several EtOH-drinking variables, with females reaching a higher maximum EtOH consumption, exhibiting more high-drinking days, and escalating their EtOH at a quicker rate compared to males. During early abstinence, EtOH drinkers made more initiation omissions and were slower to initiate trials than H2O drinking controls, particularly during pretraining. EtOH drinking females were most affected. A similar pattern in trial initiations was also observed in discrimination, but not in reversal learning. EtOH drinking rats were unaffected in their reward collection and stimulus response times, indicating intact motivation and motor responding. Although there were sex differences in discrimination and reversal phases, performance improved over time. We also observed sex-independent drinking group differences in win-stay and lose-shift strategies specific to the reversal phase. **Conclusions:** Females exhibit increased vulnerability to EtOH effects in early learning: there were sex-dependent EtOH effects on attentional measures during pretraining and discrimination phases. We also found sex-independent EtOH effects on exploration strategies during reversal. Future studies should aim to uncover the neural mechanisms for changes in attention and exploration in both acute and prolonged EtOH withdrawal.

Keywords: *reversal learning, probabilistic learning, win-stay, lose-switch, stimulus-reward, orbitofrontal cortex, sex differences*

Introduction

Individuals with Alcohol Use Disorder (AUD) show cognitive impairments, particularly in the domain of cognitive flexibility, broadly defined as the ability to adjust one's behavior in response to changes in the environment (1). Preclinical models that mimic alcohol (ethanol, EtOH) consumption in humans can elucidate underlying mechanisms related to such cognitive impairments in individuals with AUD (2).

Several groups have tested the effect of forced EtOH exposure on reversal learning, a robust measure of cognitive flexibility commonly used in experimental animals (3) that requires the remapping of reward contingencies. In deterministic (fully-predictive) reversal learning paradigms more frequently employed in behavioral pharmacology experiments, subjects first learn to discriminate and choose between two stimuli, one of which is rewarded and the other which is not. After successful discrimination, the associated outcomes of the two stimuli are reversed, forcing the subject to remap the associations (3–6). In probabilistic reversal learning (PRL) paradigms, each stimulus is associated with a probability of reward (e.g. .80/.20, .70/.30), with one stimulus associated with a higher probability of reward (the “better” option), and another with a lower probability of reward (the “worse” option) (3,7,8).

Voluntary alcohol consumption models (e.g. 2-Bottle Choice, EtOH gelatin) have been used to assess effects on deterministic reversal learning (6,9,10), resulting in no significant treatment group differences. However, it is worth noting that these studies only examined overall performance (i.e. trials to reach criterion, number of correct choices and errors) and did not report whether there were group differences on latency measures to initiate and commit trials and collect reward, or the types of strategies employed on a more fine-grained, trial-by-trial basis. Latency measures, for example, could be used to dissociate processing speed and decision-making from

motivational effects. Latency to collect reward along with number of initiated trials are commonly used as a measure of motivation, whereas response latency is often used as a measure of processing or decision-making speed in the Five-Choice Serial Reaction Time Task (5CSRTT) (11–16). Measures of attention vary, but can include number of omissions, as well as percentage of correct responses (i.e. accuracy) (11,13,14,17–19). Although omissions are thought to be indicative of inattentiveness, one must consider this measure in conjunction with response and reward latencies to rule out processing speed and motivational deficits, respectively, as potential confounds (19,20).

We also studied Win-Stay/Lose-Shift (WSLS) strategies commonly analyzed in PRL paradigms. WSLS strategies reflect an animal's tendency to select the same stimulus after being rewarded (i.e. Win-Stay) or switch to select a different stimulus after not being rewarded (i.e. Lose-shift) (21). Conversely, animals may use less advantageous strategies, such as selecting a different stimulus after being rewarded (i.e. Win-Shift), or choosing the same stimulus after not being rewarded (i.e. Lose-Stay). We probed these in the present study. Also noteworthy, most studies have exclusively used male animals, limiting the generalizability of the results given recent findings showing sex differences in consumption patterns, with female rodents showing higher EtOH intake levels and preference for EtOH (22–26), even exhibiting less aversion to EtOH compared to males (27–30).

The present study sought to address these gaps in the literature by probing the effects of a chronic intermittent voluntary alcohol consumption model on PRL in male and female rats. Rats were administered a 2-bottle choice procedure, during which they were given access to either both 20% EtOH and H₂O, or H₂O only, 3 days per week for a total of 10 weeks. Five days after their last day of EtOH access they underwent pretraining and advanced to PRL after meeting several training criteria. Given recent findings on sex differences in EtOH consumption, we hypothesized

that females would be more EtOH-preferring and reach higher EtOH consumption levels than males. Here, we corroborate previous findings of enhanced EtOH consumption and escalation in female rats compared to male rats. Surprisingly, we found sex-dependent treatment group differences in trial initiations, with the most robust effects in pretraining that carried through early discrimination. These results were in contrast to intact committed trials (i.e., stimulus response) and reward collection times throughout learning. We found changes in WSLs strategy specific to the reversal phase, with EtOH drinkers displaying more exploration (i.e. shift) strategies than H₂O drinkers. These effects, were not sex-dependent. Taken together, the present results suggest an enduring effect on attention and exploration-based strategies, but not motivational measures, in stimulus-reward association learning in prolonged abstinence from EtOH.

Methods

A timeline of all procedures is shown in **Figure 1A**. Subjects were adult male (n=16) and female (n=16) Long-Evans rats (Charles River Laboratories). All animals were between PND 60-70 upon start of EtOH or H₂O-only consumption and between PND 130-140 at the start of behavioral testing. All rats underwent a 3 day acclimation period, during which they were pair-housed and given food and water *ad libitum*, and remained in cages with no investigator interference. Following the 3-day acclimation period, animals were handled for 10 min per animal for 5 consecutive days. During the handling period, the animals were given unlimited food and water access and were tail marked. After the handling period, animals were singly-housed under standard housing conditions (room temperature 22–24° C) with a standard 12 h light/dark cycle (lights on at 6am). This study was conducted in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was

approved by the Chancellor's Animal Research Committee at the University of California, Los Angeles.

Rodent voluntary alcohol regimen: 2-bottle choice procedure

Rat home cages were modified to allow for the placement of 2 bottles. Rats (n=16; n=8 male, n=8 female) were given access to both water and 20% alcohol simultaneously, with placement of bottles counterbalanced, for a 24-hour period 3 days per week, and only water on the remaining days. Alcohol access was terminated at 10 weeks (29 days of access) after animals' EtOH consumption stopped escalating. An age-matched control cohort of water (H2O)-only drinking animals (n=16; n=8 male, n=8 female) was placed in modified home cages allowing for the placement of 2 bottles with water-only also for a total of 10 weeks. Weight of bottles was measured before and after alcohol and/or water-only access to measure daily consumption amounts with a control cage placed on the same rack to account for leakage.

Behavioral task: rodent probabilistic reversal learning (PRL) task

Immediately following the termination of the consumption period, all animals were placed on food restriction to 14 grams/day (females) or 18 grams/day (males) of chow for 5 days prior to behavioral testing. Animals were weighed every other day and monitored closely to not fall below 85% of their maximum, free-feeding weight. Behavioral testing was conducted in operant conditioning chambers outfitted with an LCD touchscreen opposing the sugar pellet dispenser (31,32) during the animals' inactive phase. All chamber equipment was controlled by customized ABET II TOUCH software. Following 5 days of forced abstinence (n=16; EtOH group) or a rest period (n=16; H2O group), animals began pretraining.

167 The pretraining protocol, adapted from established procedures described in Stolyarova et
168 al. (2017) (31), consisted of a series of phases: Habituation, Initiation Touch to Center Training
169 (ITCT), Immediate Reward Training (IMT), designed to train rats to nosepoke, initiate a trial, and
170 select a stimulus to obtain reward. During habituation, rats were required to eat five pellets out of
171 the pellet dispenser inside the chambers within 15 min before exposure to any stimuli on the
172 touchscreen. ITCT began with the display of white graphic stimuli on the black background of the
173 touchscreen. During this stage, a trial could be terminated for one of two reasons: if a rat touched
174 the displayed image (and received reward), or if the image display time (40 s) ended, after which
175 the stimulus disappeared, a black background was displayed, and a 10 s inter-trial interval (ITI)
176 ensued. If the rat did not touch within 40 s this was scored as an *initiation omission*. IMT began in
177 the same way as ITCT, but the disappearance of the white graphic stimulus was now paired with
178 the onset of a target image immediately to the left or right of the stimulus (i.e. forced-choice) that
179 the rat was required to nosepoke to obtain reward. During this stage, a trial could be terminated
180 for one of three reasons. First, if a rat touched the center display (i.e. white graphic stimulus) and
181 touched the image displayed on either side, after which there was a dispensation of one sucrose
182 pellet and illumination of the tray-light. Second, if the rat failed to touch the center white graphic
183 stimulus before the display time ended (40 s), the stimulus disappeared, a black background was
184 displayed, and a 10 s ITI ensued, scored as a *initiation omission*. Third, if the image display time
185 (60 s) ended, after which the stimulus disappeared, a black background was displayed, and a 10 s
186 ITI ensued, scored as a *choice omission*. Rats could also fail to respond to the center stimulus
187 within 40 s during this phase (i.e. initiation omission, as in the previous phase). For habituation
188 pretraining, the criterion for advancement was collection of all 5 sucrose pellets. For ITCT, the

criterion to the next stage was set to 60 rewards consumed in 45 min. The criterion for IMT was set to 60 rewards consumed in 45 min across two consecutive days.

After completion of all pretraining schedules, rats were advanced to the discrimination phase of the PRL task, in which they would initiate a trial by touching the white graphic stimulus in the center screen (displayed for 40 s), and choose between two visual stimuli presented on the left and right side of the screen (displayed for 60 s) counterbalanced between trials, assigned as the Better or Worse options, rewarded with a sucrose pellet, with probability $p_R(B)=0.70$ and $p_R(W)=0.30$, respectively. Assignment of the stimulus to better or worse reinforcement was counterbalanced across conditions. If a trial was not initiated within 40 s, it was scored as an initiation omission. If a stimulus was not chosen, it was scored as a choice omission, and a 10 s ITI ensued. If a trial was not rewarded, a 5 s time-out would follow, subsequently followed by a 10 s ITI. Finally, if a trial was rewarded, a 10 s ITI would follow after the reward was collected (**Fig 1B**). The criterion was set to 60 or more rewards consumed and selection of the better option in 80% of the trials or higher during a 60 min session across two consecutive days. After reaching criterion for the discrimination phase, the rats advanced to the reversal phase beginning on the next session. During the reversal phase, rats were required to remap stimulus-reward contingencies. The criterion for the reversal phase was the same as the discrimination phase.

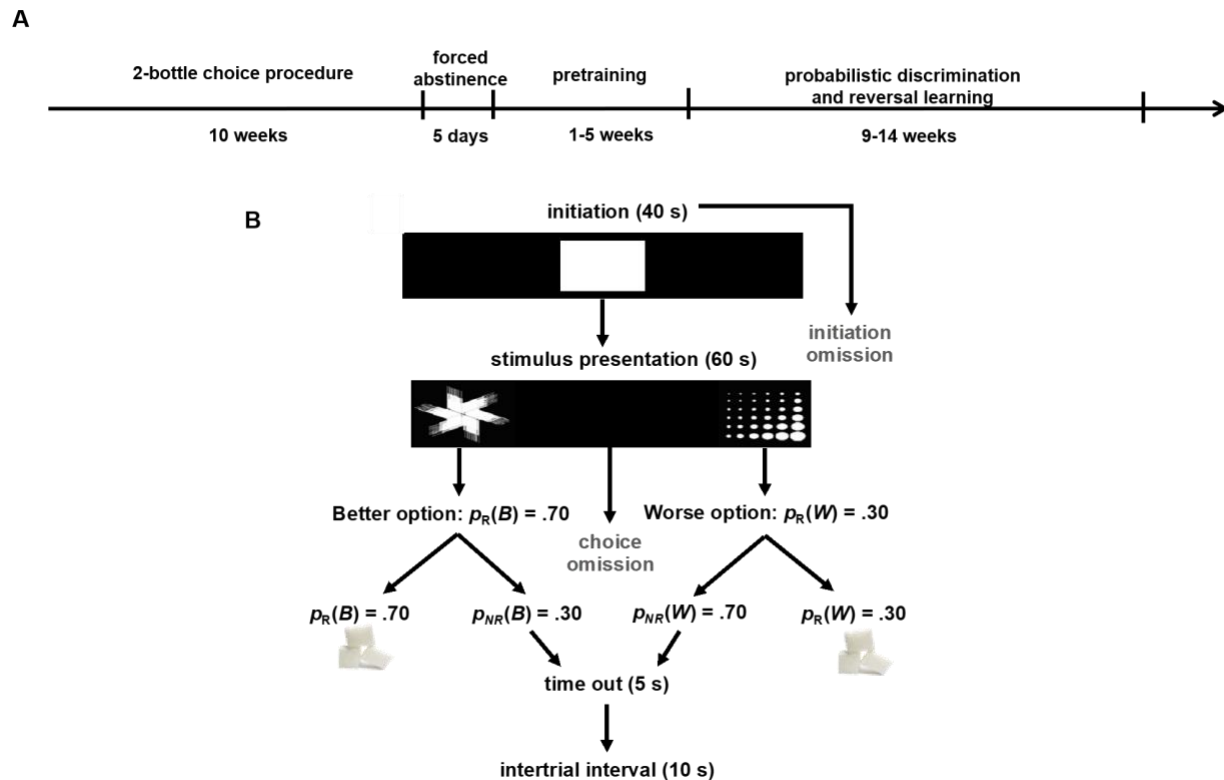


Fig 1. Experimental timeline and probabilistic discrimination and reversal learning paradigm. (A) Sequence of events during the voluntary EtOH drinking regimen (i.e. 2-bottle choice procedure), forced abstinence, and behavioral testing, depicted from left to right. **(B)** Task structure of probabilistic reversal learning (PRL), in which the animal initiated a trial by nose-poking the white graphic stimulus in the center screen (displayed for 40 s), and chose between two visual stimuli pseudorandomly presented on either the left and right side of the screen (displayed for 60 s), assigned as the Better or Worse options, rewarded with a sucrose pellet with probability $p_R(B)=0.70$ and $p_R(W)=0.30$, respectively. If a trial was not rewarded [$p_{NR}(B)$ or $p_{NR}(W)$], a 5 s time-out would commence. If a stimulus was not chosen, it was considered an omission and a 10 s ITI would commence. If a trial was rewarded, a 10 s ITI would follow reward collection.

Statistical analyses

To test the study hypotheses, a series of mixed-effects General Linear Models (GLM) and ANOVA analyses were conducted using MATLAB (MathWorks, Natick, Massachusetts; Version R2018b) (33) and SPSS (IBM SPSS Statistics, Version 25) (34). MATLAB was also used for graphing.

The EtOH consumption data were analyzed with ANOVAs with sex and drinking-group as between-subject factors, and EtOH consumption days (D1-D29) as a within-subject factor, with EtOH consumption (g/kg) as the primary outcome (35–37). Independent samples t-tests were

conducted for EtOH-drinking related variables, such as maximum EtOH consumption, calculated as the highest amount of EtOH consumption reached over the course of the 29 days of alcohol access averaged by sex, and the number of high-drinking days (i.e. 5+ g/kg/24 hrs) (38).

Learning data (sessions to criterion, percent correct), number of rewards collected, omission, and latency data were analyzed with GLM in MATLAB (*fitglm* function; Statistics and Machine Learning Toolbox; MathWorks, Natick, Massachusetts; Version R2017a). Percent correct, number of rewards, and initiation omissions, were analyzed using GLM across repeated days of testing per animal with drinking group (EtOH vs. H₂O) and sex (female vs male) as fixed effects and animal as a random effect. In Figures, we show the first 15-20 days of learning to avoid overweighting performance of increasingly fewer animals at the extremes. Omission (sums) and latency data (medians) included one observation per subject and were also analyzed with a GLM with drinking group and sex as fixed effects and animal as a random effect. All post-hoc tests were Bonferroni-corrected to account for the number of comparisons. Statistical significance was noted when p-values were less than 0.05, p-values between 0.05 and 0.06 are reported as marginally significant.

Trial-by-trial analyses were conducted to investigate potential sex and group differences in WSLS strategies used in the PRL task. WSLS are strategies commonly examined in decision-making tasks involving risk and reward that can reveal changes in sensitivity to surprising outcomes and feedback learning. Each trial was classified as a *win* if an animal received a sugar pellet, and as a *lose* trial if no reward was delivered. We classified decisions as Win-Stay when a rat chose the same stimulus on the subsequent trial after a win and as Lose-Shift when the rat switched to the alternative stimulus after a loss. We also studied less advantageous strategies: we classified selecting a different stimulus after being rewarded as Win-Shift, and choosing the same

stimulus after not being rewarded as Lose-Stay. For win-stay: $\text{sum}(\text{win-stay})/\text{sum}(\text{win})$; for lose-shift: $\text{sum}(\text{lose-shift})/\text{sum}(\text{lose})$; for win-shift: $1 - \text{sum}(\text{win-stay})/\text{sum}(\text{win})$; and for lose-stay: $1 - \text{sum}(\text{lose-shift})/\text{sum}(\text{lose})$. We compared the frequency of using advantageous strategies (i.e., win-stay, lose-shift) vs. less advantageous strategies (i.e., win-shift, lose-stay) by generating an adaptive score: $(\text{win-stay} + \text{lose-shift}) - (\text{win-shift} + \text{lose-stay})$. Ultimately, we compared the use of advantageous (i.e., win-stay, lose-shift) with less advantageous (i.e., win-shift, lose-stay) strategies by drinking group and sex.

Results

EtOH consumption

Independent samples t-tests showed that females reached a greater maximum level of EtOH consumption [$t(14)=3.46$, $p=0.004$] (**Fig 2A**) and exhibited more high-drinking days (i.e. days of EtOH consumption $5+ \text{ g/kg/24 hours}$) [$t(14)=3.00$, $p=0.01$] than males (**Fig 2B**). A repeated-measures ANOVA was used to assess the within-subject effect of EtOH drinking days and the between-subject factor of sex on EtOH consumption (g/kg). There was a within-subject effect of day [$F(28, 392)=6.68$, $p<0.0001$], suggesting an escalation of EtOH over the course of 29 EtOH drinking days (**Fig 2C**). A marginally-significant sex*day interaction was found, [$F(28,392)= 1.51$, $p=0.05$], with female animals escalating drinking more steeply than males over the 29 days. Although not significant, we found a trend for a main effect of sex [$F(1, 14)=3.42$, $p=0.09$]. Conversely, we saw a de-escalation of water (H₂O) consumption over the 29 days of alcohol access, [$F(28,392)=13.594$ $p<0.0001$; **Fig 2D**], but found no sex*day interaction, [$F(28,392)= 0.993$, $p=0.48$], yet a trend for an effect of sex [$F(1, 14)=3.25$, $p=0.09$]. It is important to note that H₂O-drinking animals reached a significantly higher body weight 413.44 ± 20.87 (M \pm SEM),

compared to EtOH-drinking animals 332.69 ± 24.09 , [$t(30)=2.53$, $p=0.02$], and that body weight was significantly negatively correlated with maximum EtOH consumption level [$r(16)=-0.67$, $p=0.005$], and number of high-drinking days [$r(16)=-0.61$, $p=0.01$]. Overall females exhibited heavier EtOH consumption patterns than males.

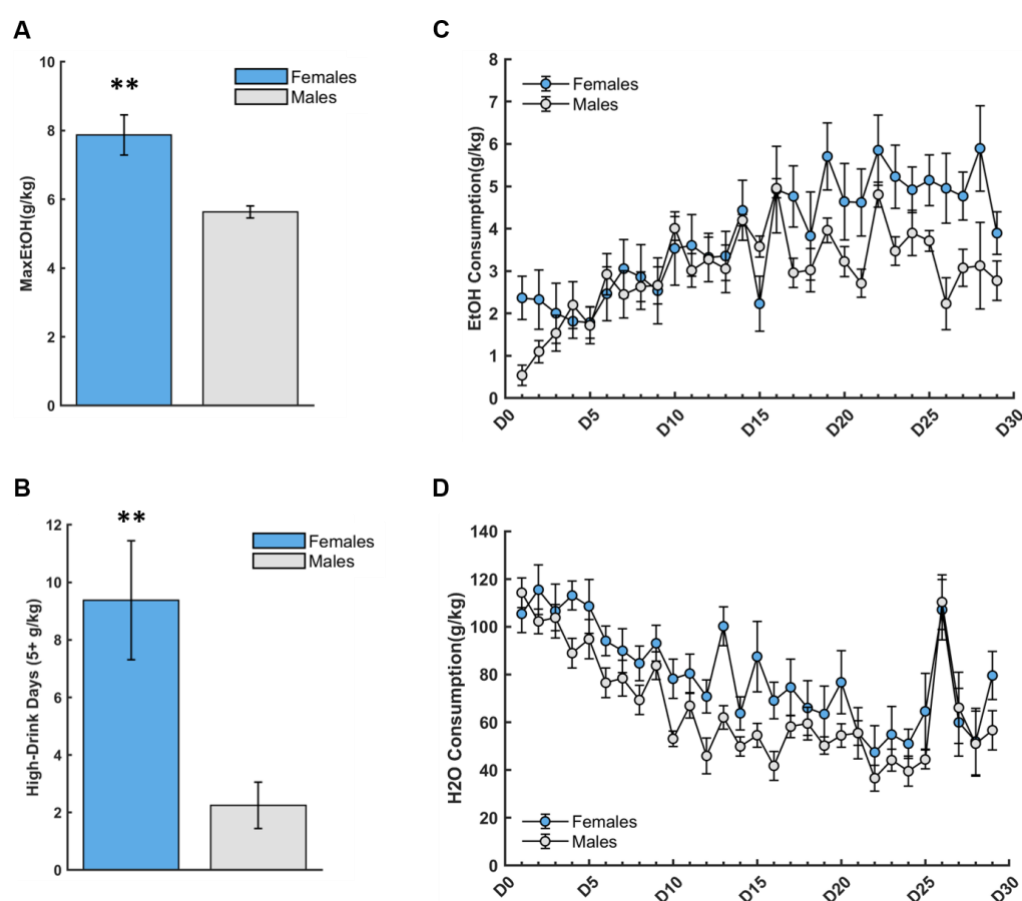


Fig 2. 20% Ethanol (EtOH) consumption (g/kg) across drinking days (D1-D29) is more pronounced in females. (A) Females reached a greater maximum EtOH level of consumption than males. (B) Females exhibited more high-drinking (5-10 g/kg/24 hr) days than males. (C) There was a within-subject effect of day, with both males and females escalating their EtOH consumption over time, a marginally significant sex*day interaction, and a trend for a main effect of sex. (D) There was a de-escalation of water consumption over the 29 days of alcohol access, but no main effect of sex or sex*day interaction. Bars indicate \pm S.E.M. $n=8$ males, $n=8$ females, ** $p<0.01$.

Full Model

Generalized linear models were first run to include all between- (drinking group, sex) and within-subject (learning phase, day) factors, to test for differences among individual factors and (2- and 3-way) interactions on our main behavioral outcomes (i.e. percent correct, denoted as choice of the better option, and rewards collected), omissions (initiation, choice), and latencies (initiation, correct vs. incorrect choice, reward collection), as well as differences in stimulus-based use of advantageous (i.e., win-stay, lose-shift) and less advantageous (i.e., win-lose, lose-stay) strategies.

We found a significant effect of day on choosing the better option (GLM: $\beta_{\text{day}} = 0.01$, $p = 0.001$), indicative of learning over time, as well as a significant group*day interaction (GLM: $\beta_{\text{group*day}} = 0.02$, $p = 0.01$), with the H2O-drinking animals exhibiting higher rates of learning across days. Additionally, there were 3 and 4-way interactions that were significant or marginally-significant, including group*sex*day (GLM: $\beta_{\text{group*sex*day}} = -0.03$, $p = 0.01$), group*phase*day (GLM: $\beta_{\text{group*phase*day}} = -0.02$, $p = 0.05$), and group*sex*phase*day (GLM: $\beta_{\text{group*sex*phase*day}} = 0.02$, $p = 0.03$). All animals also increased their number of rewards collected (GLM: $\beta_{\text{day}} = 1.76$, $p < .0001$), with notable group (GLM: $\beta_{\text{group}} = 19.70$, $p = 0.02$) and sex differences (GLM: $\beta_{\text{sex}} = 17.26$, $p = 0.04$), such that H2O-drinking animals and males collected a greater number of rewards overall compared to EtOH-drinking animals and females, respectively. Significant sex*day (GLM: $\beta_{\text{sex*day}} = -1.73$, $p < .0001$), and marginally-significant phase*day (GLM: $\beta_{\text{phase*day}} = -0.74$, $p = 0.05$) interactions indicated that the rate at which rewards were collected increased more rapidly in females and during the discrimination learning phase. We then tested the effect of all factors on initiation omissions, which, as expected, decreased across testing days (GLM: $\beta_{\text{day}} = -0.26$, $p = 0.04$), but were greater in number among EtOH-drinking animals (GLM: $\beta_{\text{group}} = -10.62$, $p = 0.001$), females (GLM: $\beta_{\text{sex}} = -6.03$, $p = 0.05$), and during the discrimination phase (GLM: $\beta_{\text{phase}} = -7.42$, $p = 0.02$).

Our analyses on omissions resulted in an increased number of initiation omissions in EtOH-drinking animals (GLM: $\beta_{\text{group}} = -304.12$, $p=0.01$), and a significant 3-way group*sex*phase interaction for choice omissions (GLM: $\beta_{\text{group*sex*phase}} = -3.81$, $p=0.02$). Conversely, H2O-drinking animals exhibited longer reward collection latencies (GLM: $\beta_{\text{group}} = 0.17$, $p=0.02$), and incorrect choice latencies (GLM: $\beta_{\text{group}} = -0.09$, $p=0.04$). Finally, we assessed if there were any differences in use of advantageous vs. less advantageous learning strategies and found a greater use of the advantageous strategies (i.e. win-stay, lose-shift) compared to less advantageous strategies (i.e. win-shift, lose-stay) during the discrimination learning phase (GLM: $\beta_{\text{phase}} = -0.13$, $p=0.004$). Akaike Information Criteria (AIC) and Bayesian Information Criteria (BIC) were computed for the full model ($\text{AIC}_{\text{percent-correct}} = -6375.40$; $\text{BIC}_{\text{percent-correct}} = -5446.70$), as well as for models excluding the phase factor by separately analyzing discrimination ($\text{AIC}_{\text{percent-correct}} = -1501.50$; $\text{BIC}_{\text{percent-correct}} = -1287.40$), and reversal learning phases ($\text{AIC}_{\text{percent-correct}} = -1441.2$; $\text{BIC}_{\text{percent-correct}} = -1234.30$), while still including all the other factors (i.e. group, sex, days). The latter models (for which phases were analyzed separately) yielded lower AIC and BIC values, indicative of a better fit. Thus, subsequent analyses were further subdivided into discrimination vs. reversal phases, and early vs. late discrimination and reversal learning based on prior studies indicating these may be particularly informative to contrast (39–44).

Pretraining (PT) performance

As pretraining was a unique phase with slightly different dependent measures (i.e. forced choice omissions and latencies), a separate GLM was used to assess the effect of group (EtOH, H2O) and sex (female, male), and group*sex interactions on the number of pretraining sessions required to reach criterion to advance to the discrimination learning phase of the PRL task. A significant effect

of group and sex emerged, with the EtOH-experienced animals requiring a greater number of pretraining sessions than the H2O-only group (GLM: $\beta_{\text{group}} = -14.00$, $p=0.04$; **Fig 3A**), and females requiring a greater number of pretraining sessions than males (GLM: $\beta_{\text{sex}} = -11.38$, $p=0.001$; **Fig 3A**). A group*sex interaction was found (GLM: $\beta_{\text{group*sex}} = 12.00$, $p=0.01$) with EtOH-drinking females requiring more pretraining sessions than EtOH-drinking males (GLM: $\beta_{\text{sex}} = -11.38$, $p=0.03$). Both EtOH-drinking females (GLM: $\beta_{\text{group}} = -14.00$, $p=0.01$) and EtOH-drinking males (GLM: $\beta_{\text{group}} = -2.00$, $p=0.03$) needed more sessions to reach criterion than their H2O-drinking counterparts (**Fig 3A**). There was an overall average of 10.19 ± 1.47 ($M \pm \text{SEM}$) sessions to successfully meet the pretraining criterion and advance to discrimination learning.

We analyzed group and sex differences, as well as the group by sex interaction on initiation omissions, defined as a failure to nosepoke the center white square stimulus within 40s, and initiation latencies, defined as the duration until nosepoke of the center white square stimulus. There was an effect of both group (GLM: $\beta_{\text{group}} = -554.12$, $p=0.0001$; **Figs 3B**), and sex (GLM: $\beta_{\text{sex}} = -517.87$, $p=0.0003$; **Figs 3B**) on initiation omissions, with EtOH-drinking animals and females displaying more initiation omissions compared to H2O-drinking animals and males, respectively. A significant drinking group*sex interaction (GLM: $\beta_{\text{group*sex}} = 467.00$, $p=0.01$; **Fig 3B**), indicated that both EtOH-drinking females (GLM: $\beta_{\text{group}} = -554.00$, $p=0.01$) and males (GLM: $\beta_{\text{group}} = -87.13$, $p=0.01$) exhibited more initiation omissions than their H2O-drinking counterparts, and EtOH-drinking females exhibited more initiation omissions than EtOH-drinking males (GLM: $\beta_{\text{sex}} = -517.88$, $p=0.01$). The EtOH group also displayed longer initiation latencies than the H2O group (GLM: $\beta_{\text{group}} = -3.48$, $p=0.001$; **Figs 3C and S3A**), but there was no effect of sex (GLM: $\beta_{\text{sex}} = -1.37$, $p=0.18$; **Figs 3C and S3D**), or group*sex interaction (GLM: $\beta_{\text{group*sex}} = -0.09$, $p=0.95$; **Fig 3C**).

Next we analyzed differences in forced-choice omissions, defined as failure to nosepoke the stimulus presented on either the left or right side of the touchscreen after initiation of the trial, and forced-choice latencies, defined as duration until nosepoke of the stimulus presented on the left or right side. There was a significant effect of sex (GLM: $\beta_{\text{sex}} = -6.13$, $p=0.03$; **Fig 3D**), with females displaying more forced-choice omissions, but no significant group (GLM: $\beta_{\text{group}} = -4.25$, $p=0.13$; **Fig 3D**), or group*sex interaction (GLM: $\beta_{\text{group*sex}} = 5.38$, $p=0.17$; **Fig 3D**). There was a significant effect of both group (GLM: $\beta_{\text{group}} = -2.76$, $p=0.01$; **Fig 3E and S3B**), and sex (GLM: $\beta_{\text{sex}} = -2.37$, $p=0.03$; **Fig 3E and S3E**), on forced-choice latency, with the EtOH-experienced animals and females taking longer to select the stimulus, but no significant group*sex interaction (GLM: $\beta_{\text{group*sex}} = -0.45$, $p=0.76$). Finally, we analyzed data for differences in reward latency, defined as the duration to collect the sucrose reward from the food magazine, and found no effect of group (GLM: $\beta_{\text{group}} = 0.44$, $p=0.70$; **Figs 3F and S3C**), sex (GLM: $\beta_{\text{sex}} = -0.01$, $p=0.92$; **Figs 3F and S3F**), or group*sex (GLM: $\beta_{\text{group*sex}} = 0.15$, $p=0.35$; **Fig 3F**) interaction.

Collectively, the omission and latency data for initiations and forced-choice trials suggest an attenuating effect of EtOH experience, and specifically in EtOH-experienced females, on quickly responding to stimuli, while the reward collection data point to preserved motor responding and motivation for reward in EtOH-experienced animals.

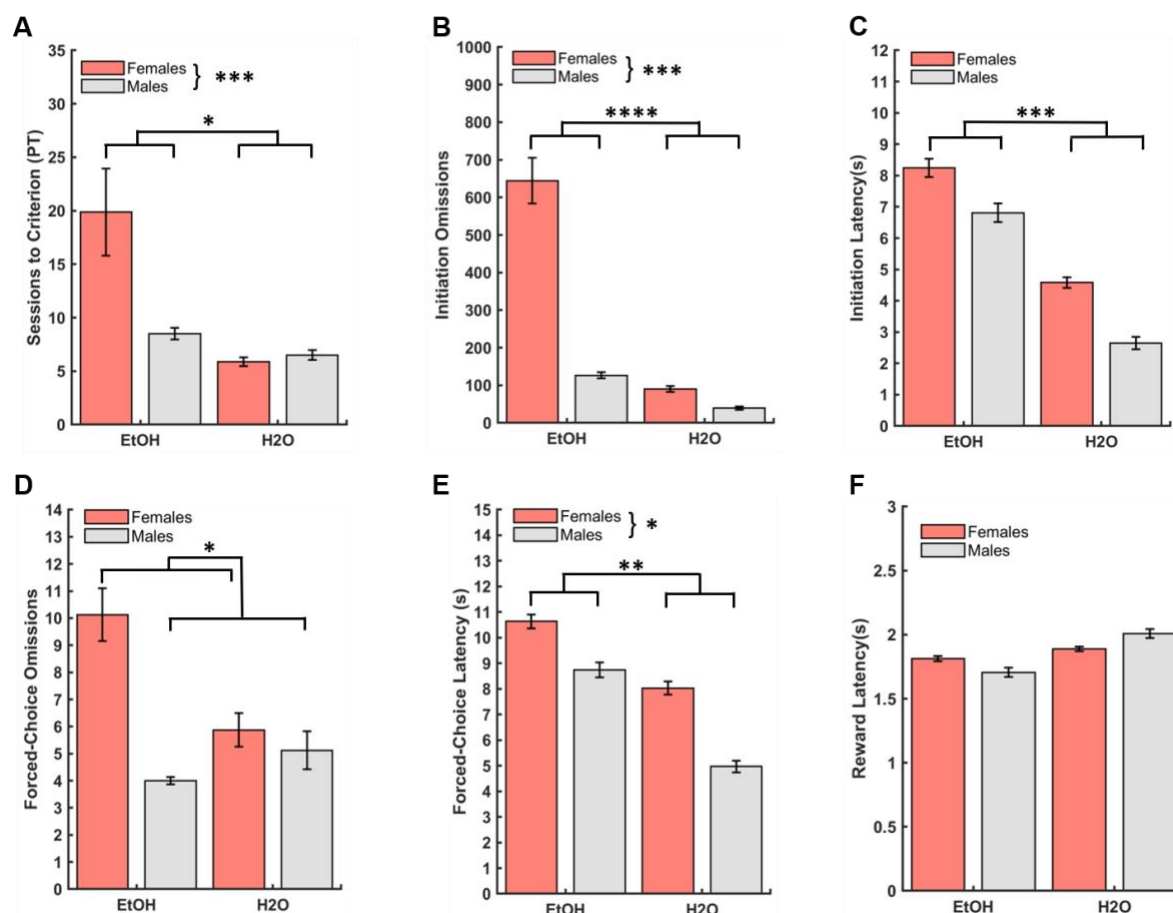


Fig 3. Drinking group, sex differences, and drinking group by sex interactions in operant pretraining.

(A) The number of pretraining sessions required to reach criterion to advance to the main PRL task was greater for EtOH-drinking (male and female) animals than their H2O-only drinking counterparts, and greater for EtOH-drinking females compared to EtOH-drinking males. Although a significant group*sex interaction was found for this measure, significant pairwise comparisons are not depicted for clarity. (B) EtOH group and females exhibited more initiation omissions than the H2O group and males, respectively. Although a significant group*sex interaction was also found for this measure, significant pairwise comparisons are not depicted for clarity. (C) EtOH group exhibited longer initiation latencies than the H2O group. No sex differences were found for initiation omissions. (D) Females displayed more forced-choice omissions. No group differences were found for forced-choice omissions. (E) EtOH group and females exhibited longer forced-choice latencies than the H2O group and males, respectively. (F) No group or sex differences were found for reward latencies. Latencies represent group medians. Bars indicate $\pm S.E.M.$ n=16 males, n=16 females, * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$

Probabilistic Discrimination (D) performance

There were no significant group (GLM: $\beta_{\text{group}} = -9.50$, $p=0.07$) or sex differences (GLM: $\beta_{\text{sex}} = 3.13$, $p=0.54$) on total number of sessions to reach criterion for the discrimination phase, but a marginally-significant group*sex interaction on this measure was observed (GLM: $\beta_{\text{group*sex}} = 14.88$, $p=0.047$; **Fig 4A**). Overall, all animals performed comparably regardless of group or sex, with an overall average of 27.41 ± 2.17 days required to successfully discriminate and advance to the reversal phase.

A GLM model was used to test the effect of drinking group (EtOH, H₂O), sex (female, male), days, and their 2-way and 3-way interactions on percent correct (i.e. choosing the better option), number of rewards (i.e. sucrose pellets), and initiation omissions across 20 testing days of discrimination learning (D1-D20). All animals demonstrated learning by showing an increase in choosing the better option across days, (GLM: $\beta_{\text{day}} = 0.01$, $p=0.001$; **Figs 4B**), regardless of group or sex. There was no effect of drinking group (GLM: $\beta_{\text{group}} = -0.01$, $p=0.79$), or sex (GLM: $\beta_{\text{sex}} = 0.07$, $p=0.17$), and no group*sex (GLM: $\beta_{\text{group*sex}} = -0.03$, $p=0.65$), or sex*day (GLM: $\beta_{\text{sex*day}} = 0.01$, $p=1.00$) interaction on percent correct. There was however a significant group*day interaction (GLM: $\beta_{\text{group*day}} = 0.02$, $p=0.02$), with the H₂O-drinking animals choosing the better option increasingly more across days than the EtOH-drinking animals. There was also a significant group*sex*day interaction (GLM: $\beta_{\text{group*sex*day}} = -0.02$, $p=0.01$). Post-hoc comparisons revealed males learned quicker than females (GLM: $\beta_{\text{sex}} = 0.01$, $p<0.001$), that H₂O-drinking females chose the better option increasingly more across days than their EtOH-drinking counterparts (GLM: $\beta_{\text{group}} = 0.01$, $p=0.002$); and that the same pattern was not observed for males.

All animals increased the number of rewards collected across days, (GLM: $\beta_{\text{day}} = 1.83$, $p<0.0001$; **Fig S1A**). Drinking group (GLM: $\beta_{\text{group}} = 22.59$, $p=0.01$), and sex differences (GLM: $\beta_{\text{sex}} = 17.60$, $p=0.04$) also emerged, with both H₂O-drinking animals and males collecting a greater

number of rewards (**Fig S1A**). No significant group*sex ($p=0.07$), group*day ($p=0.78$), or group*day*sex ($p=0.08$) interactions on number of rewards collected were found, only a significant sex*day interaction (GLM: $\beta_{\text{sex*day}}=-1.75$, $p<0.0001$), with females displaying a greater increase in rewards collected across days (GLM: $\beta_{\text{day}}=1.69$, $p<0.0001$), compared to males (**Fig S1A**).

Conversely, all animals decreased the number of initiation omissions across days, (GLM: $\beta_{\text{day}}=-0.29$, $p=0.02$; **Fig S1B**), regardless of group or sex. There were significant group (GLM: $\beta_{\text{group}}=-11.17$, $p=0.001$) and sex differences (GLM: $\beta_{\text{sex}}=-6.36$, $p=0.04$), with EtOH-drinking animals and females exhibiting more initiation omissions than H2O-drinking and males, respectively (**Fig S1B**). A significant group*sex interaction (GLM: $\beta_{\text{group*sex}}=14.81$, $p=0.01$), with EtOH-drinking females displaying more initiation omissions than H2O-drinking females (GLM: $\beta_{\text{group}}=-304.13$, $p=0.02$); the same pattern was not observed in males (**Fig S1B**). Similarly, a significant group*sex*day (GLM: $\beta_{\text{group*sex*day}}=-0.69$, $p=0.02$) revealed that EtOH-drinking females decreased their number of initiation omissions across discrimination learning at a slower rate than EtOH-drinking males ($p=0.03$) and H2O-drinking females ($p=0.03$). There were no differences between male and female H2O drinkers, or between EtOH- vs. H2O-drinking males. Finally, we conducted GLM analyses on total omissions and median latencies in discrimination learning. Our results indicated EtOH-drinking animals exhibited more initiation omissions than H2O-drinking animals (GLM: $\beta_{\text{group}}=-304.12$, $p=0.01$), whereas H2O-drinking animals had longer reward collection latencies than EtOH-drinking animals (GLM: $\beta_{\text{group}}=0.17$, $p=0.02$). All other types of omission and latency analyses yielded non-significant results.

First 500 trials. Because interesting latency differences were obtained for the pretraining phase and based on prior studies comparing early vs. late discrimination learning, we conducted

further latency analyses for other phases of learning to assess if these trends were maintained. An analysis of initiation latencies and omissions, choice (correct and incorrect) latencies and omissions, and reward latencies was conducted for the first 500 trials of discrimination learning to capture early learning in this phase, with animals averaging 89.19 ± 3.38 committed trials per day. There was a significant group difference on initiation omissions (GLM: $\beta_{\text{group}} = -158.75$, $p = 0.002$; **Fig 4C**), with EtOH-drinking animals exhibiting more initiation omissions than H2O-drinking animals, but no sex differences (GLM: $\beta_{\text{sex}} = -56.50$, $p = 0.36$; **Fig 4C**). A significant drinking group*sex interaction on initiation omissions emerged (GLM: $\beta_{\text{group*sex}} = 219.00$, $p = 0.02$; **Fig 4C**), with EtOH-drinking females exhibiting more initiation omissions than their H2O-drinking female counterparts ($p = 0.01$), and H2O-drinking males exhibiting marginally more initiation omissions than H2O-drinking females ($p = 0.05$), but EtOH-drinking males were no different than H2O-drinking males. There was no effect of drinking group (**Figs 4D and S4A**), sex (**Figs 4D and S4E**), or group*sex interaction (**Fig 4D**) on initiation latencies, choice omissions, correct or incorrect choice latencies, or reward collection latencies (**Figs S4B and S4F**), with the exception of a marginally significant group effect (GLM: $\beta_{\text{group}} = 0.88$, $p = 0.05$), with H2O-drinking animals exhibiting more choice omissions than EtOH-drinking animals. It should be noted that choice omissions represented a small number of occurrences (normally ranging from 0-2) and this effect was driven by a single outlier with 4 choice omissions, which upon removal yielded a non-significant group effect (GLM: $\beta_{\text{group}} = 0.48$, $p = 0.14$). In summary, the data for early discrimination learning suggest an enduring effect of EtOH experience on initiating trials, with females most affected.

Last 500 trials. An analysis of initiation latencies and omissions, choice (correct and incorrect) latencies and omissions, and reward latencies was also conducted for the last 500 trials

of discrimination learning to capture late phase learning, with animals averaging 118.98 ± 3.89 committed trials per day. There was a marginally-significant effect of group (GLM: $\beta_{\text{group}} = -49.13$, $p=0.05$; **Fig 4E**), with EtOH-drinking animals exhibiting more initiation omissions than H2O-drinking animals, but no sex differences (GLM: $\beta_{\text{sex}} = 92.88$, $p=0.21$; **Fig 4E**) or group*sex interaction (GLM: $\beta_{\text{group*sex}} = -67.50$, $p=0.38$; **Fig 4E**). There were no group (GLM: $\beta_{\text{group}} = -0.92$, $p=0.24$; **Figs 4F and S4C**), or sex differences (GLM: $\beta_{\text{sex}} = 1.26$, $p=0.20$; **Figs 4F and S4G**) on initiation latencies, as well as no group*sex interaction (GLM: $\beta_{\text{group*sex}} = -0.76$, $p=0.51$; **Fig 4F**). We did, however, find a significant effect of group on incorrect choice latencies (GLM: $\beta_{\text{group}} = 0.28$, $p=0.02$), with H2O-drinking animals displaying longer latencies than EtOH-drinking animals, but no significant effect of sex, or group*sex interaction was found for this measure. Similar to what was found during early discrimination, there was a significant group effect on choice omissions in late discrimination (GLM: $\beta_{\text{group}} = 1.13$, $p=0.01$), with the H2O-drinking animals exhibiting more choice omissions than the EtOH-drinking animals, but no effect of sex, or group*sex interaction was found for this measure. However, this effect was largely driven by the same animal as during early discrimination, which upon removal, yielded non-significant results (GLM: $\beta_{\text{group}} = 0.43$, $p=0.06$). Finally, our results indicated that H2O-drinking animals displayed longer reward collection latencies than EtOH-drinking animals (GLM: $\beta_{\text{group}} = 0.23$, $p=0.01$; **Fig S4D**), but no effect of sex (**Fig S4H**) or group*sex interaction emerged. Thus, the pattern of prior EtOH experience rendering animals more likely to fail to initiate trials was also observed through late discrimination learning, but rats did not take longer to initiate trials when they did so, as in pretraining and early discrimination phases. Although we did observe greater choice omissions by the H2O-drinking animals during both early and late discrimination, this was driven largely by one animal.

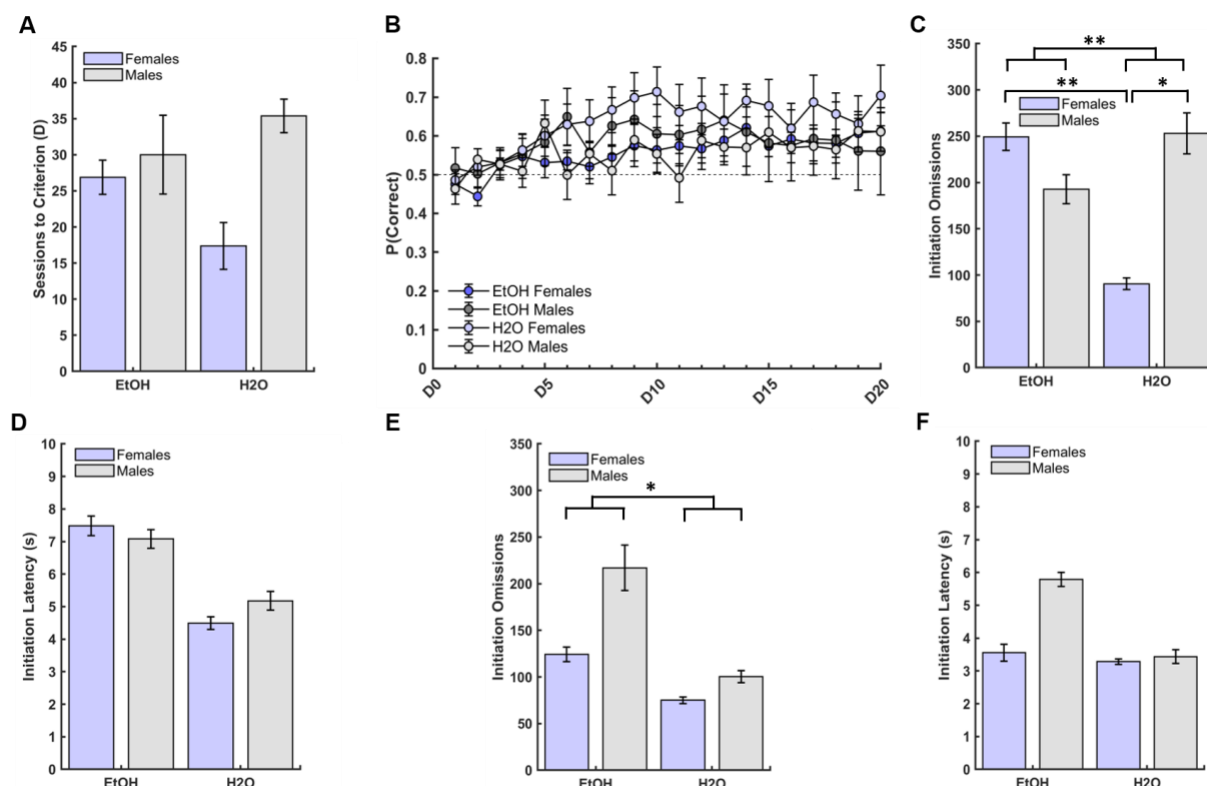


Fig 4. Drinking group differences in initiation omissions, but not latencies, during early and late probabilistic discrimination learning performance. (A) There were no group or sex differences in the number of sessions to reach criterion for the discrimination learning phase, only a marginally significant group*sex interaction. (B) There was no effect of group or sex on probability of choosing the better option, only an effect of day (D1-D20), with the probability of choosing the better option increasing across testing days irrespective of sex or group. (C) EtOH group exhibited more initiation omissions compared to the H2O group. EtOH-drinking females exhibited more initiation omissions than H2O-drinking females, and H2O-drinking males exhibited marginally more initiation omissions than H2O-drinking females in early discrimination learning. (D) No group or sex differences emerged for initiation latencies during early discrimination learning. (E) EtOH-drinking animals (male and female) exhibited marginally more initiation omissions than H2O-drinking animals, but no sex differences emerged for late discrimination learning. (F) No group or sex differences were found for initiation latencies during late discrimination learning. Latencies were medians. Bars indicate $\pm S.E.M.$ $n=16$ males, $n=16$ females, * $p \leq 0.05$, ** $p \leq 0.01$

PRL reversal (R) performance

There were no significant group differences (GLM: $\beta_{\text{group}} = 5.75$, $p=0.18$), sex differences (GLM: $\beta_{\text{sex}} = -5.25$, $p=0.22$), or group*sex interaction (GLM: $\beta_{\text{group*sex}} = -1.88$, $p=0.74$) on sessions to reach criterion for the reversal learning (Fig 5A). There was an overall average of 25.27 ± 1.62 days to successfully complete the PRL phase.

A GLM model was used to test the effect of drinking group (EtOH, H₂O), sex (female, male), days, and their 2-way and 3-way interactions on probability of choosing the better option, number of rewards, and initiation omissions across 15 days of reversal learning. Two females were excluded because they either failed to meet criterion for discrimination learning, and never advanced to reversal learning. All other animals demonstrated learning by exhibiting an increase in choosing the better options across days, (GLM: $\beta_{\text{day}} = 0.01$, $p = 0.01$; **Fig 5B**), irrespective of group or sex. There was no effect of group (GLM: $\beta_{\text{group}} = -0.07$, $p = 0.10$), or sex (GLM: $\beta_{\text{sex}} = -0.04$, $p = 0.51$), and no group*sex (GLM: $\beta_{\text{group*sex}} = 0.10$, $p = 0.17$), sex*day (GLM: $\beta_{\text{sex*day}} = 0.002$, $p = 0.60$), group*day (GLM: $\beta_{\text{group*day}} = 0.002$, $p = 0.47$), or group*sex*day (GLM: $\beta_{\text{group*sex*day}} = -0.004$, $p = 0.46$) interactions on the probability of choosing the better option.

All animals increased the number of rewards collected over days, (GLM: $\beta_{\text{day}} = 0.82$, $p = 0.01$; **Fig S1C**). There was no significant effect of group (GLM: $\beta_{\text{group}} = 3.69$, $p = 0.75$), or sex (GLM: $\beta_{\text{sex}} = 2.40$, $p = 0.85$), and no significant group*sex (GLM: $\beta_{\text{group*sex}} = 5.73$, $p = 0.74$), group*day (GLM: $\beta_{\text{group*day}} = -0.02$, $p = 0.95$), sex*day (GLM: $\beta_{\text{sex*day}} = 0.66$, $p = 0.08$), or group*sex*day (GLM: $\beta_{\text{group*sex*day}} = 0.48$, $p = 0.40$) interactions on number of rewards collected. All animals decreased the number of initiation omissions across days, (GLM: $\beta_{\text{day}} = 0.10$, $p = 0.55$; **Fig S1D**), regardless of group or sex. There was no significant effect of group (GLM: $\beta_{\text{group}} = -0.44$, $p = 0.91$), or sex (GLM: $\beta_{\text{sex}} = -1.81$, $p = 0.61$), and no significant group*sex (GLM: $\beta_{\text{group*sex}} = 0.67$, $p = 0.91$), group*day (GLM: $\beta_{\text{group*day}} = -0.06$, $p = 0.78$), sex*day (GLM: $\beta_{\text{sex*day}} = 0.09$, $p = 0.70$), or group*sex*day (GLM: $\beta_{\text{group*sex*day}} = -0.51$, $p = 0.09$) interactions on initiation omissions. Finally, we conducted GLM analyses on total omissions and median latencies in reversal learning. Similar to the above measure for across-day learning, we found no significant effect of group (GLM: $\beta_{\text{group}} = 7.88$, $p = 0.97$), sex (GLM: $\beta_{\text{sex}} = -234.25$, $p = 0.17$), or group*sex

(GLM: $\beta_{\text{group} \times \text{sex}} = -122.1$, $p = 0.59$) interaction for initiation omissions. However, though there was no effect of sex (GLM: $\beta_{\text{sex}} = 0.67$, $p = 0.49$), we found that H2O-drinking animals exhibited more choice omissions than EtOH-drinking animals (GLM: $\beta_{\text{group}} = 2.54$, $p = 0.03$), with a significant group*sex interaction revealing H2O-drinking females exhibited more choice omissions than EtOH-drinking females and H2O-drinking males (GLM: $\beta_{\text{group} \times \text{sex}} = -3.29$, $p = 0.03$). Additionally, there was a marginally-significant effect of group on incorrect choice latencies (GLM: $\beta_{\text{group}} = 0.15$, $p = 0.05$), with H2O-drinking animals exhibiting longer incorrect choice latencies than EtOH-drinking animals. Analyses on initiation, incorrect, and reward latencies yielded non-significant results. Unlike for the discrimination phases, upon removal of 2 outliers, the effect of drinking group remained significant, with H2O-drinking animals exhibiting more choice omissions than EtOH-drinking animals (GLM: $\beta_{\text{group}} = 1.59$, $p = 0.03$).

First 500 trials. An analysis of initiation latencies and omissions, choice (correct and incorrect) latencies and omissions, and reward latencies was conducted for the first 500 trials of reversal learning, to capture early reversal learning. Animals averaged 119.70 ± 8.32 committed trials per day. There were no significant group (GLM: $\beta_{\text{group}} = -38.04$, $p = 0.65$; **Fig 5C**), or sex differences (GLM: $\beta_{\text{sex}} = -60.29$, $p = 0.41$; **Fig 5C**) on initiation omissions, and no significant group*sex interaction (GLM: $\beta_{\text{group} \times \text{sex}} = 7.67$, $p = 0.94$; **Fig 5C**). Similarly, there was no effect of drinking group (**Figs 5D and S5A**), sex (**Figs 5D and S5E**), or group*sex interaction (**Fig 5D**) on initiation latencies, choice omissions, correct choice latencies, or reward collection latencies (**Figs S5B and S5F**), with the exception of a significant group difference on incorrect choice latencies (GLM: $\beta_{\text{group}} = 0.32$, $p = 0.004$), with H2O-drinking animals exhibiting longer latencies when choosing the incorrect stimulus compared to EtOH-drinking animals. There was also a significant group*sex interaction (GLM: $\beta_{\text{group} \times \text{sex}} = -0.31$, $p = 0.04$) on incorrect choice latency, with H2O-

drinking females displaying longer latencies than both H₂O-drinking males ($p=0.05$) and their EtOH-drinking counterparts ($p=0.02$). In summary, the data for early reversal learning suggests there was no longer any effect of prior EtOH experience on initiating trials, as had been previously observed during early discrimination learning, but it is important to note that EtOH-drinking animals were less tentative than the H₂O-drinking animals given their faster incorrect choice latencies.

Last 500 trials. An analysis of initiation latencies and omissions, choice (correct and incorrect) latencies and omissions, and reward latencies was also conducted for the last 500 trials of reversal learning, to capture late phase learning. Animals averaged 155.11 ± 4.06 committed trials per day. Our analyses on initiation omissions indicated females exhibited more initiation omissions (GLM: $\beta_{\text{sex}} = -67.96$, $p=0.02$; **Fig 5E**), but there was no effect of group (GLM: $\beta_{\text{group}} = -8.46$, $p=0.85$; **Fig 5E**), or group*sex interaction (GLM: $\beta_{\text{group*sex}} = -8.79$, $p=0.85$; **Fig 5E**). There was a marginally-significant effect of sex (GLM: $\beta_{\text{sex}} = -2.08$, $p=0.05$; **Figs 5F and S5G**), with females taking longer to initiate trials, but no significant group differences (GLM: $\beta_{\text{group}} = -0.78$, $p=0.47$; **Figs 5F and S5C**), or group*sex interaction (GLM: $\beta_{\text{group*sex}} = 0.01$, $p=0.99$; **Fig 5F**). Females also exhibited more choice omissions than males (GLM: $\beta_{\text{sex}} = -0.33$, $p=0.02$), but no group differences or group*sex interaction were found. Upon removal of a single outlier, the effect of sex remained (GLM: $\beta_{\text{sex}} = -0.33$, $p=0.01$). Males displayed longer correct choice latencies than females (GLM: $\beta_{\text{sex}} = 25.46$, $p=0.003$), whereas H₂O-drinking animals exhibited longer reward latencies than EtOH-drinking animals (GLM: $\beta_{\text{group}} = 0.27$, $p=0.01$; **Fig S5D**). We did not find any other significant effect of group, sex, or group*sex interaction.

Thus, the pattern of prior EtOH experience rendering animals more likely to fail to initiate trials and taking longer to do so in early discrimination learning, was not preserved through

reversal learning. In summary, the late reversal phase was characterized by predominantly female-specific attenuations in initiation of trials (both omissions and latencies), as well as correct choice latencies.

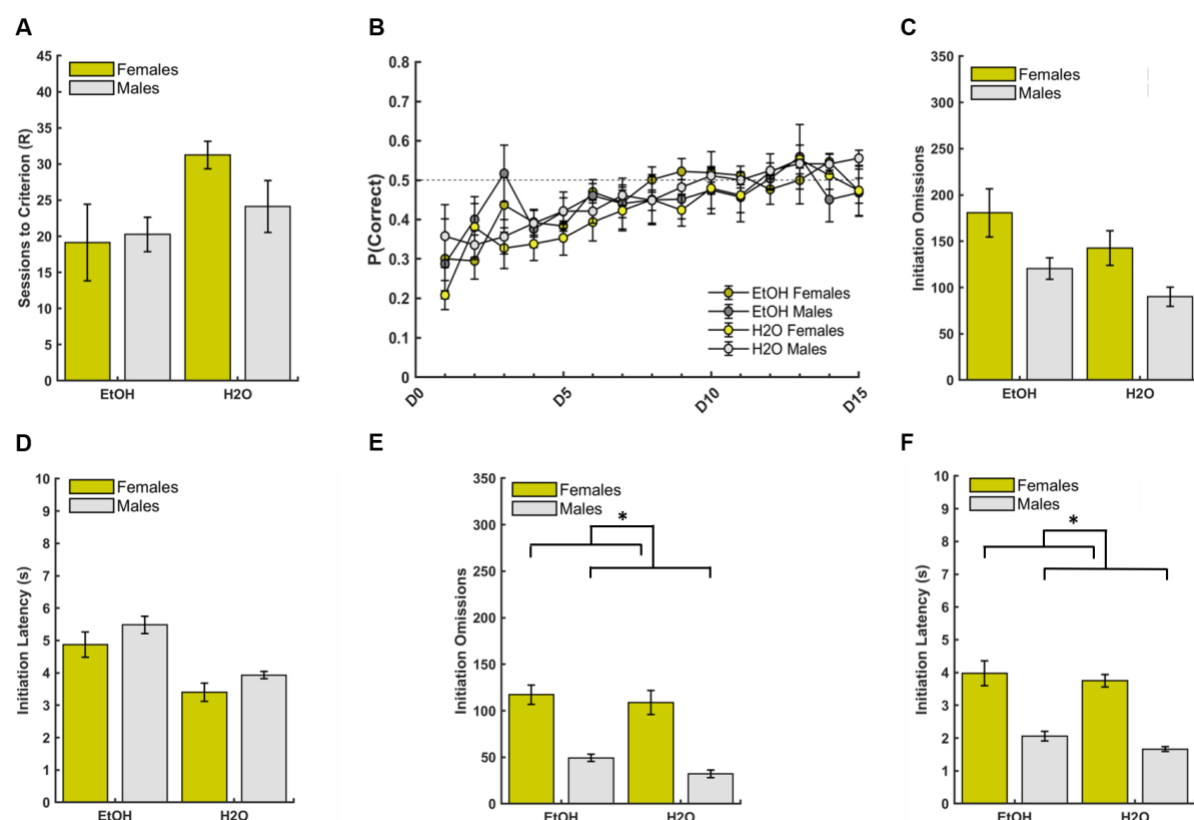


Fig 5. Sex differences, but no drinking group differences, in initiation omissions and latencies during late probabilistic reversal learning performance. (A) There were no group or sex differences in the number of sessions to reach criterion for the reversal learning phase. (B) There was no effect of group or sex on probability of choosing the better option, only an effect of day (D1-D25), with the probability of choosing the better option increasing across the fifteen testing days regardless of sex or group. (C) No group or sex differences were found for initiation omissions during early reversal learning. (D) No group or sex differences were found for initiation latencies during early reversal learning. (E) Females exhibited more initiation omissions than males during late reversal learning. (F) Females exhibited marginally longer initiation latencies than males during late reversal learning. Latencies represent group medians. Bars indicate $\pm S.E.M.$ $n=16$ males, $n=14$ females, $*p \leq 0.05$

Win-Stay/Lose-Shift (WSLS) Strategies

Potential differences in win-stay/lose-shift (WSLS) strategies on stimulus responses employed by each group (EtOH, H2O) and by sex (male, female) were tested for the first 500 committed trials

and last 500 committed trials of the discrimination phase and reversal phase by calculating the frequency of each strategy individually. We compared the frequency of using advantageous strategies (i.e., win-stay, lose-shift) vs. less advantageous strategies (i.e., win-shift, lose-stay) by generating an adaptive score: (win-stay + lose-shift) - (win-shift + lose-stay).

There were no significant effects of group, sex, or a group*sex interaction in stimulus-based strategies individually, or as an ‘adaptive score’ comparison during early or late discrimination learning (**Figs 6A and 6B**). However, during early reversal learning, we found a greater use of the lose-shift strategy in the EtOH-drinking rats than the H2O-drinking rats (GLM: $\beta_{\text{group}} = -0.10$, $p = 0.01$; **Fig S2B**), but a greater lose-stay strategy (GLM: $\beta_{\text{group}} = 0.10$, $p = 0.01$; **Fig S2D**), as well as greater use of the less adaptive strategies (GLM: $\beta_{\text{group}} = -0.20$, $p = 0.0002$; **Fig 6C**) among the H2O-drinking animals compared to the EtOH-drinking animals. For late reversal learning, we found group differences for all four stimulus-based strategies, with the H2O-drinking animals using the win-stay strategy (GLM: $\beta_{\text{group}} = 0.10$, $p = 0.03$; **Fig 7A**) and the lose-stay strategy (GLM: $\beta_{\text{group}} = 0.05$, $p = 0.04$; **Fig 7D**) more than the EtOH-drinking animals, suggesting overall more stimulus persistence. Conversely, the EtOH-drinking animals used the win-shift strategy (GLM: $\beta_{\text{group}} = -0.10$, $p = 0.03$; **Fig 7C**) and lose-shift strategies (GLM: $\beta_{\text{group}} = -0.05$, $p = 0.04$; **Fig 7B**) more than the H2O-drinking animals, indicating more of an exploration-based strategy. No significant effects were uncovered for adaptive score in the late reversal phase.

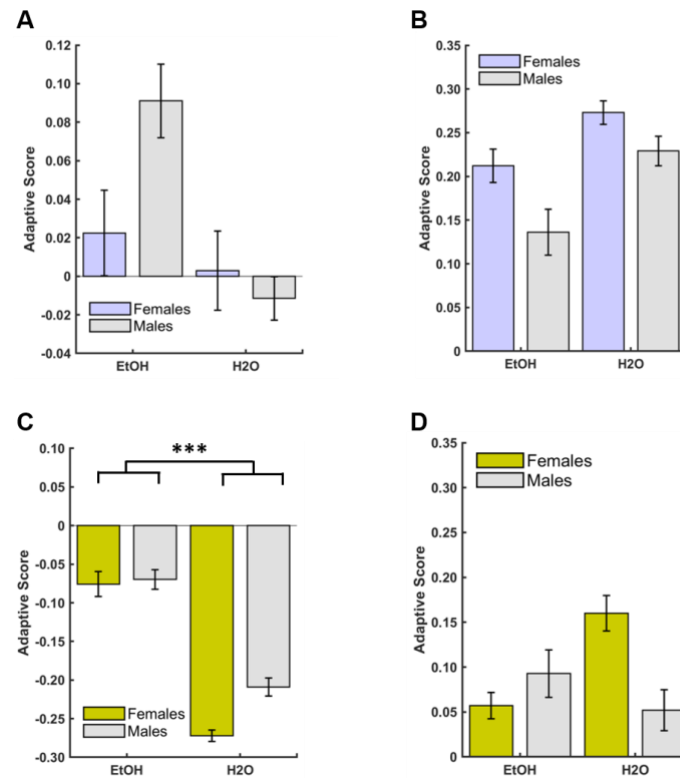


Fig 6. Drinking group differences in early reversal learning strategies. An adaptive score was calculated as the difference between advantageous strategies and less advantageous strategies: (win-stay + lose-shift) - (win-shift + lose-stay). (A) There were no group or sex differences in adaptive scores during early discrimination learning. (B) There were no group or sex differences in adaptive scores during late discrimination learning. (C) EtOH group exhibited higher adaptive scores than H2O group during early reversal learning, but there were no sex differences. (D) There were no group or sex differences in adaptive scores during late reversal learning. Bars indicate $\pm S. E. M.$ $n=16$ males, $n=16$ females, * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$

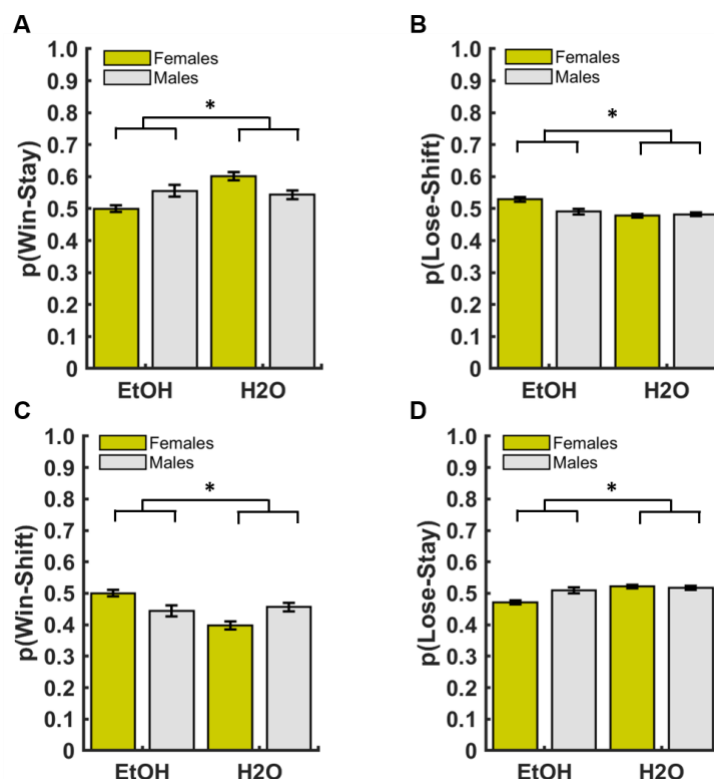


Fig 7. Drinking group differences in the use of Win-Stay/Lose-Shift strategies during late probabilistic reversal learning. (A) H2O-drinking animals used the win-stay strategy more than EtOH-drinking animals. (B) EtOH-drinking animals used the lose-shift strategy more than H2O-drinking animals. (C) EtOH-drinking animals used the win-shift strategy more than H2O-drinking animals. (D) H2O-drinking animals used the lose-stay strategy more than EtOH-drinking animals. Bars indicate $\pm S.E.M.$ * $p \leq 0.05$

Discussion

The present study used an intermittent access model to study the effect of voluntary EtOH consumption on cognitive flexibility using a probabilistic reversal learning paradigm. We included sex as an a-priori moderator. Although forced-exposure models such as intraperitoneal (i.p.) injections (45,46) and EtOH vapor inhalation (47–49) are well-established methods in rodents, they may not be as representative of human alcohol consumption. Therefore, we used a two-bottle choice procedure that allows for oral consumption of EtOH, resulting in increased ecological validity and variability in consumption patterns, which may be important in generating individual differences in alcohol consumption to study subsequent flexible reward learning. To our

knowledge, there had only been one study that previously used this voluntary consumption model to test the effects of EtOH on reversal learning, and found no effect (6). However, it is important to note that although the rats in that EtOH group had access to EtOH for 6 weeks, the rats in that study did not demonstrate escalation normally seen with intermittent voluntary EtOH consumption models, including our study. Though we also corroborate no pronounced effects of EtOH exposure on overall learning, a more fine-grained analysis of trial-by-trial and latency data revealed that EtOH-experienced animals were less likely to initiate trials and were slower to initiate trials throughout pretraining and discrimination learning. Since EtOH drinking rats were unaffected in their reward collection and stimulus response times, collectively the data support the interpretation that the most pronounced attentional decrements appearing closest in time to drinking, despite intact motivation for reward and motor responding throughout learning. We further elaborate on these attentional effects, as well as reversal-specific EtOH effects on WSLS strategies below.

Consumption patterns of EtOH

We observed an escalation of drinking over the course of the twenty-nine days of alcohol access, irrespective of sex, an expected pattern when using intermittent-access models compared to continuous access models. Several studies administering intermittent exposure have shown that alternating brief periods of alcohol access with brief periods of no access can actually escalate alcohol consumption to excessive levels (25,50–55) compared to continuous daily access (25,50,51) which typically exhibit more moderate, but stable levels of intake. However, despite the recent popularity of the intermittent-access model, the underlying psychological and neurobiological mechanisms that promote the escalation of alcohol consumption remain unclear and should be investigated in future studies.

We found that females reached a higher EtOH consumption level and exhibited greater high-drinking days than males. These findings are consistent with previous studies showing that female rodents drink more EtOH than males (22–25) and exhibit less aversion to EtOH, as demonstrated by conditioned taste aversion using EtOH-saccharin pairings (27–29), with males developing an aversion after only one pairing and females after the third pairing and only at higher doses of EtOH (28). Other groups have reported that the rewarding effects of EtOH are enhanced in females and therefore, may be hormone-dependent (56), which may explain the increased EtOH intake over time that may lead to increases in potential for over-consumption. However, the role of gonadal hormones on ethanol intake and preference remain unclear, as other studies have shown that the removal of testicular hormones in males decreases alcohol intake, and no differential consumption in ovariectomized vs. intact females (24). Although seemingly contradictory, these findings may provide further evidence of the dissociation between chromosomal and gonadal sex, given that studies have found alcohol reinforcement is mediated by chromosomal sex, independent of gonadal phenotype (57). Taken together, the present findings add to a growing body of evidence for sex differences in alcohol consumption patterns.

Attentional deficits following EtOH across learning stages

We observed the most pronounced impairment following EtOH on sessions to reach criterion during pretraining; a pattern that was not maintained through discrimination or reversal learning. Prior studies testing the relationship between alcohol exposure and performance on reversal learning tasks have largely been mixed, with some studies demonstrating alcohol produced impairments in both discrimination and reversal learning (4,58), other showing no impairments for either (5,6,45,59,60), and some only showing impairments on reversal, with the

discrimination learning phase largely intact (5,47,58,61,62). These conflicting findings may be due to variations in alcohol administration procedures, most of which have used forced-exposure models, variations in maximum blood ethanol concentration (BEC) levels, and/or types of reversal learning paradigms employed. Even similar methods of administrations produce variable results with studies showing vapor EtOH exposure impairs reversal learning (47), does not impair (60), or improves reversal learning (59), with the doses of i.p. EtOH administration determining whether an impairment is observed (45). Our study is consistent with the only other study to our knowledge that has used a 2-bottle choice procedure to assess effects on reversal learning, which similarly found no overt learning impairment (6). Although we did not measure BEC levels during the 2-bottle choice procedure, it is likely they never reached BECs known to impair reversal learning based on previous experiments using forced-exposure models (150-550 mg/dl). Task parameters (i.e. stimulus modalities, probability of reward) may also contribute to differential effects, with groups using lever or touchscreen-based responding reporting no pronounced impairments on discrimination or reversal learning (5,6,59), whereas groups employing Morris Water and Barnes Maze tasks reporting impairments in reversal learning. Discrimination learning seems to remain mostly intact across diverse paradigms (58,61–63). We maintain that many past studies of EtOH effects on reversal learning typically report omnibus measures of learning and do not probe more micro (trial-by-trial) analyses that may be more sensitive to EtOH effects, as reported here.

We found impairments associated with prior EtOH experience, such that animals previously exposed to EtOH required more sessions to reach criterion and exhibited longer initiation and choice latencies during pretraining. Similar deficits were also found during discrimination learning, with a greater number of initiation omissions and longer initiation latencies in the EtOH-experienced group. These effects were most pronounced in female animals

(discussed below). There have been studies of EtOH exposure on attention using the 5-choice serial reaction time task (64–72), considered to be the gold standard for measuring attention in rodents, revealing that EtOH-exposed animals exhibit attention deficits. An evaluation of attentional capacity using a 5-choice continuous performance task following EtOH exposure in rats found this group exhibited more omissions and longer choice latencies relative to control rats, while motivation remained intact. Indeed, there were no differences in accuracy or reward latencies (66), similar to our present findings. However, it is important to note these differences were observed only during acute, not prolonged, abstinence from EtOH exposure- the latter, as we report here. Other groups have previously reported an EtOH dose-dependent decrease in the ability to direct and sustain attention to brief stimuli, but not a complete disruption in overall performance (i.e. percentage correct), also suggestive of an impairment in attentional processing (70). Similarly, we found differences in measures of attention processing (i.e. initiation omissions and latencies), but observed no overall performance deficit in the probability of choosing the better option for both the discrimination and reversal phases of learning. Importantly, attentional deficits following EtOH experience have also been found in human binge-drinkers (i.e. more omitted trials, lower accuracy), particularly under task variants meant to increase attentional load in a human version of the 5-CSRTT (64). Indeed, the pattern we observed here- that EtOH-drinking animals exhibited more initiation omissions and longer initiation latencies (particularly in early phases of pretraining and discrimination)- stand in contrast to their quick reward collection times and intact accuracy measures, relative to H₂O-drinking animals in these same phases.

Sex differences in pretraining and reversal learning

Some interesting sex differences on reversal learning emerged in our experiment. The most pronounced impairments were observed during late reversal, with females exhibiting greater omissions (initiation and choice) and longer initiation latencies than males, irrespective of prior EtOH exposure. This is in agreement with the human literature, which has shown that males outperform females on reversal learning (73,74), and with observations in marmosets where females require more trials to learn reversals than males. Interestingly, though we find sex differences in reversal learning, there were no differences in the number of omitted trials or reaction times (i.e. latencies) (75). Relevant to this, Grissom et al. (2019) conducted an extensive review of sex differences in several aspects of executive function, including attention, and did not find evidence to support robust sex differences in this domain. Prior studies have reported that male rodents show higher levels of novelty-seeking (76), with higher novelty-seeking related to higher levels of impulsivity in males relative to females (77).

Sex-dependent EtOH effects in early learning and attentional measures

A sex-dependent drinking group difference was observed, with EtOH-exposed females more affected than males on measures of attention: they exhibited more initiation omissions than their H₂O-drinking counterparts during both pretraining and early discrimination learning, which is also reflected in a greater number of sessions required to reach criterion in early learning. Although prior research has not provided sufficient evidence supporting an attentional deficit specific to females (78), there is now substantial evidence to support a potential EtOH-specific effect on attentional processing (64,66,70). Therefore, it is plausible that sex effects we observe here are moderated by EtOH-experience, resulting in more pronounced deficits in attentional processing in EtOH-drinking females. It is worth noting that EtOH-exposed males also exhibited

some impairments (i.e. more sessions to reach criterion and initiation omissions), but this effect was only observed early in pretraining and did not extend to discrimination or reversal learning.

Sex-independent EtOH effects on WSLs in reversal learning

We observed sex-independent EtOH effects on WSLs strategies during reversal learning. Rats with prior EtOH experience were more likely to use a “shift” strategy whereas H₂O-drinking animals were more likely to “stay” with the previous stimulus choice in the reversal phase. Similarly, animals with prior EtOH experience were generally more flexible in early reversal learning (i.e. they exhibited a greater ‘adaptive’ score) than H₂O-drinking animals. This suggests that EtOH-experienced rats had a more tenuous representation of stimulus-outcome contingencies upon criterion-level performance than the H₂O-drinking control rats, and could consequently be more flexible. However, all rats generally increased their choice of the better option and rewards collected over time, while decreasing the number of initiation omissions for both the discrimination and reversal phase. The lack of pronounced EtOH impairments on overall discrimination and reversal phases of learning- as measured by global measures such as the probability choosing the better option over time- may be attributed to plasticity following protracted abstinence in rodents (79–81), and humans (82,83). Similarly, the probability of using WSLs strategies across time was ~0.5, suggesting these strategies were not used effectively for learning. Indeed, dissociations in learning and WSLs have been reported before (84). It will be important to investigate the extent to which the later pro-exploratory phenotype relies on an early attentional decrement, or if these are orthogonal effects of chronic EtOH experience.

Conclusions

In summary, we observed pronounced trial initiation omissions following EtOH experience in females during pretraining and discrimination learning. These phases are closest in time to the last EtOH experience and constitute the early abstinence period. Additionally, this attentional decrement, which was most pronounced in female animals, was partnered by an enhanced exploration strategy in all EtOH drinking animals, both males and females, later in reversal learning.

Alterations related to attention and processing speed in early EtOH abstinence (during pretraining) may have a domino effect on later learning, leading to the sex by drinking group interaction we observe in discrimination learning, and perhaps contribute to the enhanced exploration phenotype in reversal learning. A true test of this would require animals to undergo pretraining, discrimination learning, and drinking prior to any reversal learning. Ultimately, all rats exhibited intact motivation and motor timing, and were able to increase their probability of choosing the better option and number of rewards, while decreasing their initiation omissions. Although voluntary alcohol consumption models, such as the one employed here, do not model severe alcohol dependence like forced-exposure models, they do however reflect escalating, chronic intermittent drinking that corresponds to the early stages of problematic drinking, before individuals transition to alcohol dependence. Attenuated attentional mechanisms in early abstinence may not contribute to decrements in flexible learning per se, but may instead detract from executive functions important in limiting (over)consumption. Future studies should investigate the brain mechanisms and the role of gonadal hormones on alcohol consumption and attention, and systematically compare these measures as predictors of consumption (i.e. relapse) during acute vs. prolonged abstinence.

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Author contributions

813 **Conceptualization:** AI, CA, LR, **Data curation:** AS, CA, VM, **Formal analysis:** CA, AS, AI
814 **Funding acquisition:** AI, IS, **Investigation:** CA, KD, SK, AI, **Methodology:** AI, CA, **Project**
815 **administration:** CA, AI, **Resources:** AI, IS, **Software:** AS, CA, AI, **Supervision:** CA, AI
816 **Validation:** CA, AI, **Visualization:** CA, AI, **Writing – original draft:** CA, AI, **Writing –**
817 **review and editing:** CA, AI, VM, IS, LR

818

Supplemental Figures

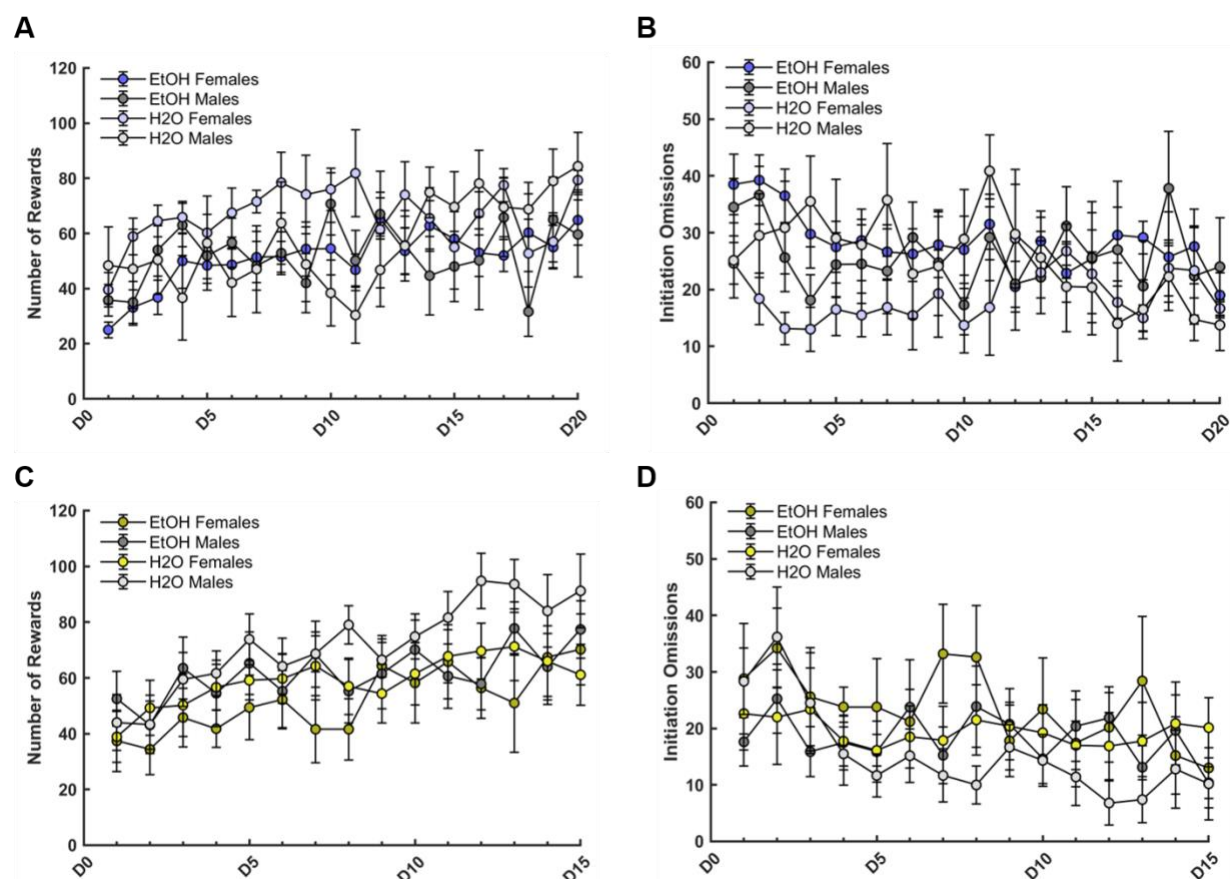


Fig S1. Drinking group and sex differences on number of rewards and initiation omissions during probabilistic discrimination and reversal learning. (A) All animals regardless of drinking group or sex increased their number of rewards collected over the twenty testing days of discrimination learning. H2O-drinking animals and males collected a greater number of rewards than EtOH-drinking animals and females, respectively. Females displayed a greater increase in rewards collected across days. (B) All animals regardless of drinking group or sex decreased their initiation omissions over the twenty testing days of discrimination learning. EtOH-drinking animals and females had more initiation omissions than H2O-drinking animals and males, respectively. EtOH-drinking females displayed more initiation omissions than H2O-drinking females. (C) All animals regardless of drinking group or sex increased their number of rewards collected over the fifteen testing days of reversal learning. There were no group or sex differences on number of rewards collected. (D) All animals regardless of drinking group or sex decreased the number of initiation omissions over the fifteen testing days of reversal learning. There were no group or sex differences on initiation omissions. Bars indicate $\pm S.E.M.$

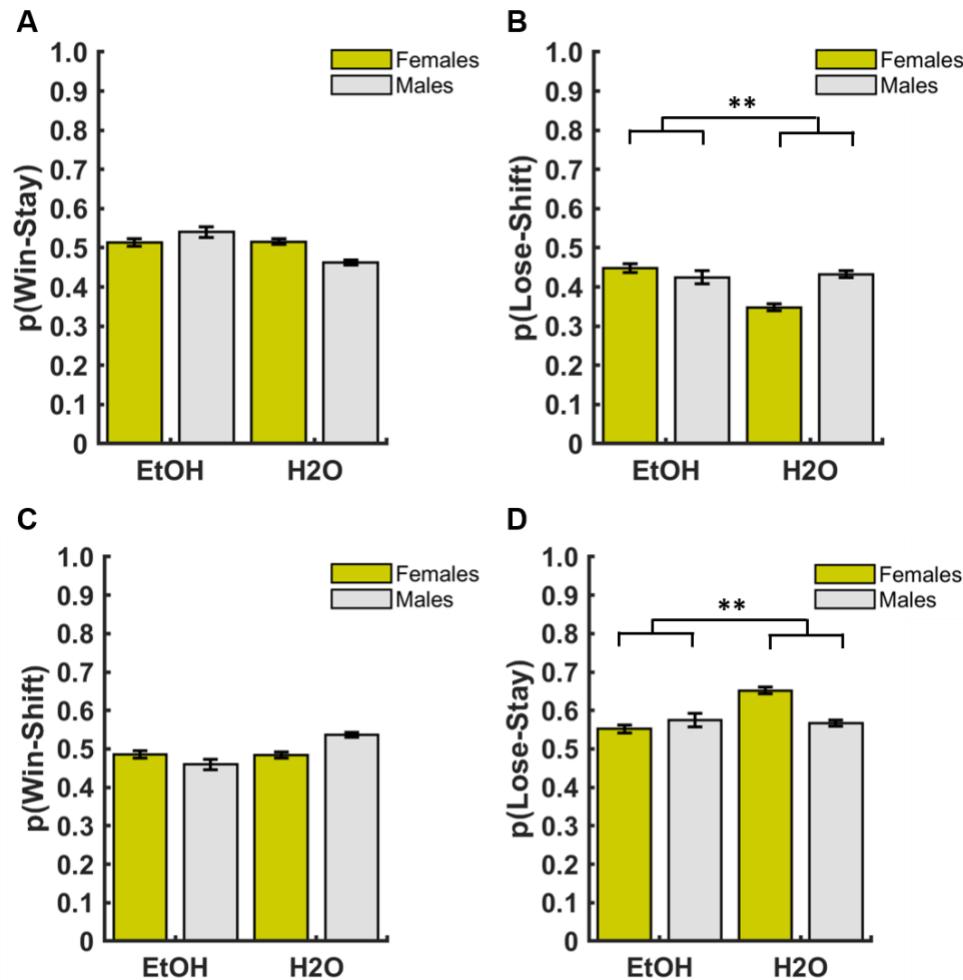


Fig S2. Drinking group differences in use of lose-shift and lose-stay strategies during early probabilistic reversal learning. (A) No group or sex differences in the use of the win-stay strategy. (B) EtOH-drinking animals used the lose-shift strategy more than H2O-drinking animals. (C) No group or sex differences in the use of the win-shift strategy. (D) H2O-drinking animals used the lose-stay strategy more than EtOH-drinking animals. Bars indicate $\pm S.E.M.$ * $p \leq 0.05$, ** $p \leq 0.01$

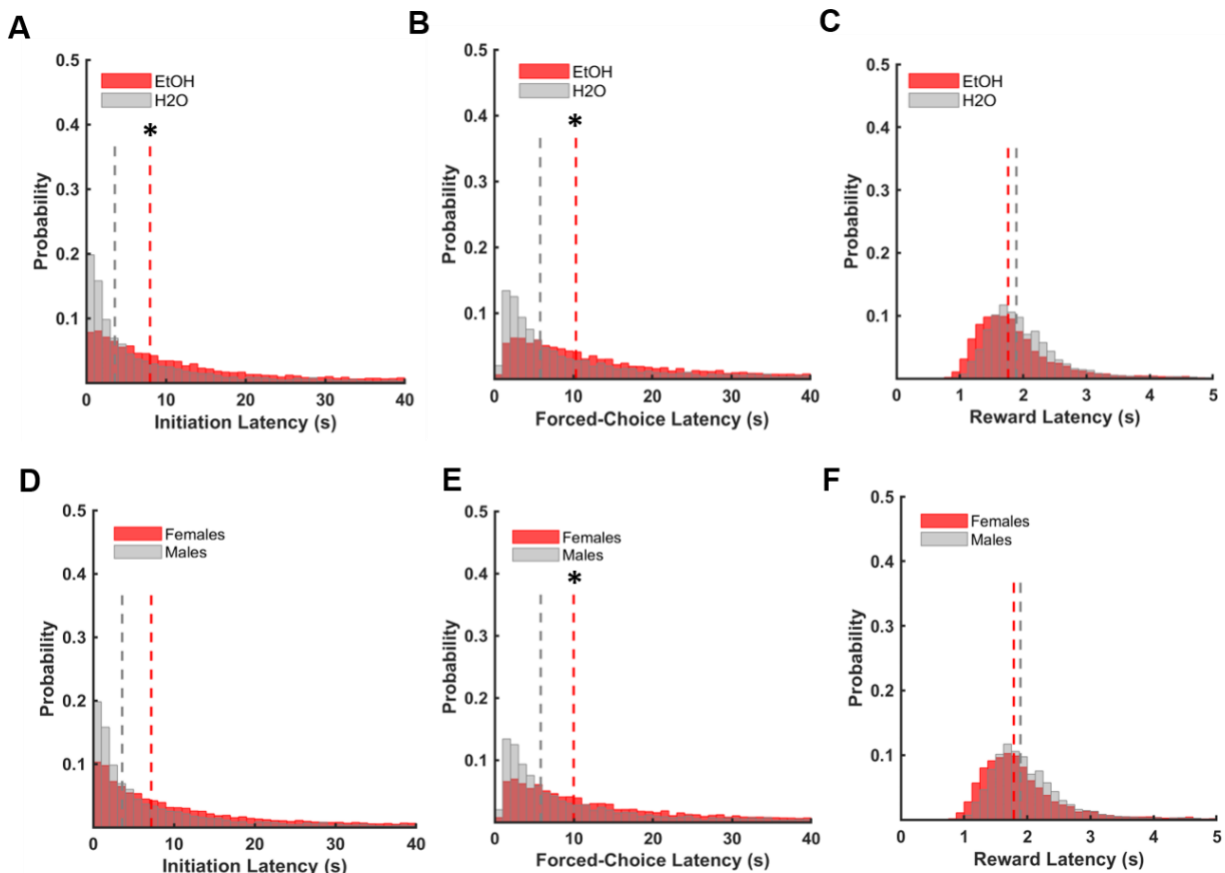


Fig S3. Drinking group and sex differences in latencies during operant pretraining. (A) EtOH group exhibited longer initiation latencies than the H₂O group. (B) EtOH group exhibited longer forced-choice latencies than the H₂O group. (C) No group differences were found for reward latencies. (D) No sex differences were found for initiation latencies. (E) Females exhibited longer forced-choice latencies than males. (F) No sex differences were found for reward latencies. Dashed lines in latency histograms represent group medians. Bars indicate $\pm S.E.M.$ n=16 males, n=16 females, *p<0.05

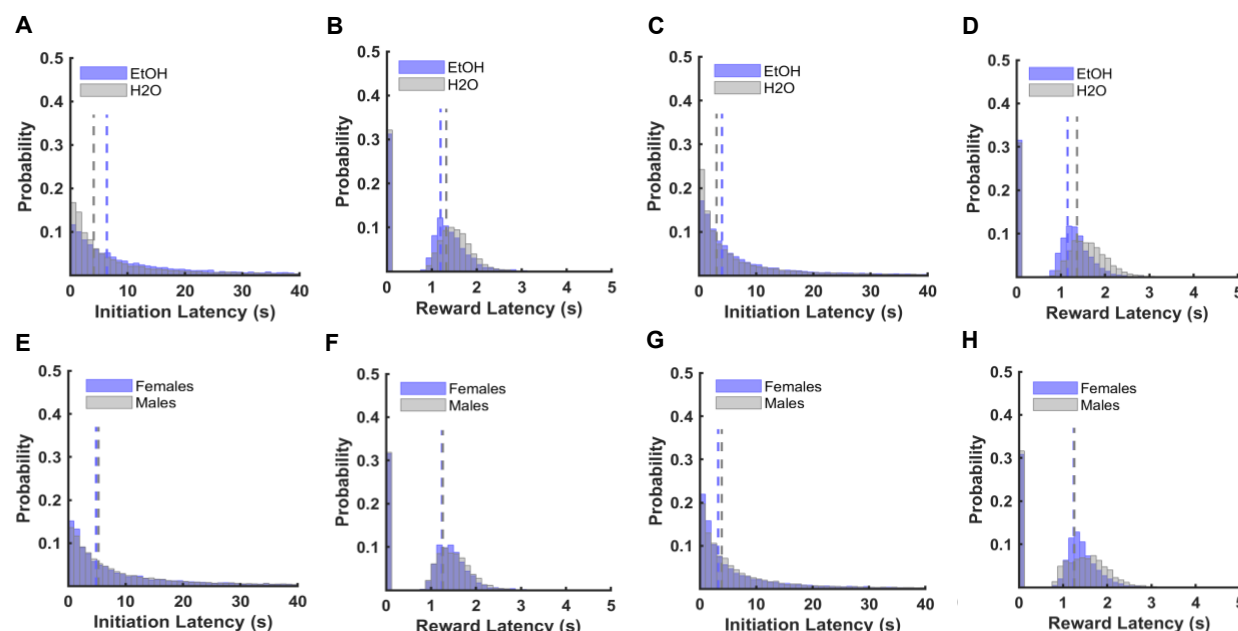


Fig S4. No drinking group or sex differences in latencies during early or late probabilistic discrimination learning. (A) No group differences were found for initiation latencies for the first 500 trials. (B) No group differences were found for reward latencies for the first 500 trials. (C) No group differences were found for initiation latencies for the last 500 trials. (D) No group differences were found for reward latencies for the last 500 trials. (E) No sex differences were found for initiation latencies for the first 500 trials. (F) No sex differences were found for reward latencies for the first 500 trials. (G) No sex differences were found for initiation latencies for the last 500 trials. (H) No sex differences were found for reward latencies for the last 500 trials. Dashed lines in histograms of latencies represent group medians. Bars indicate $\pm S.E.M.$ $n=16$ males, $n=16$ females.

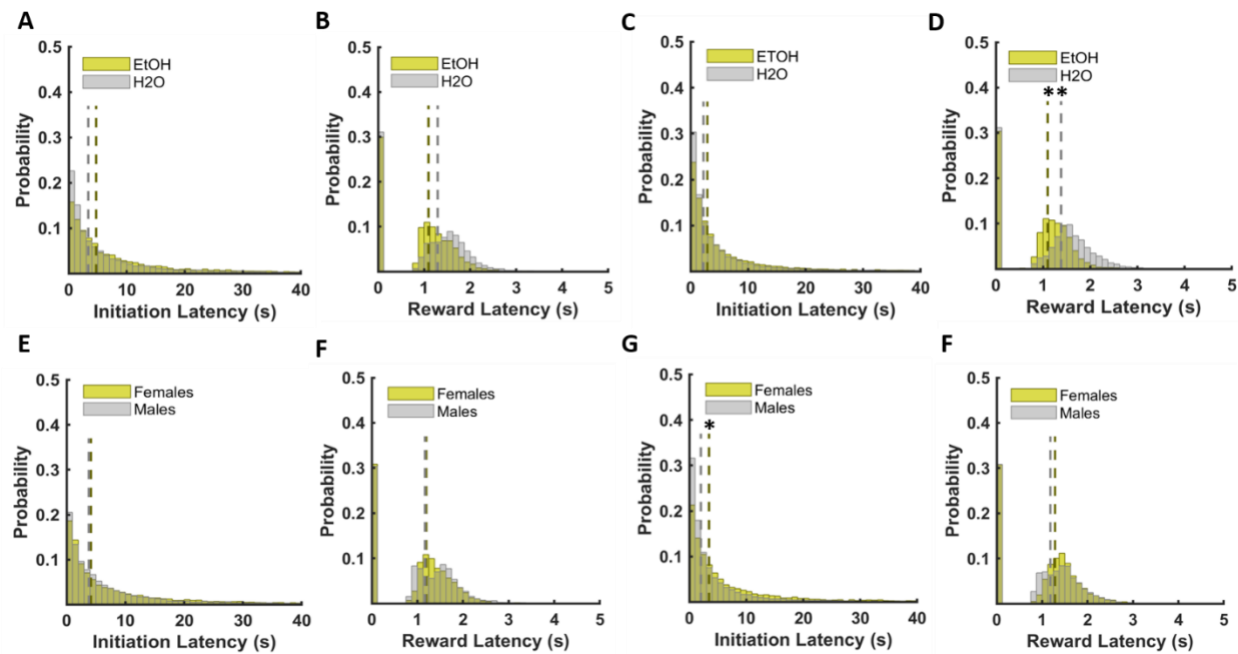


Fig S5. Drinking group and sex differences in latencies during early and late probabilistic reversal learning. (A) No group differences were found for initiation latencies for the first 500 trials. (B) No group differences were found for reward latencies for the first 500 trials. (C) No group differences were found for initiation latencies for the last 500 trials. (D) H2O group exhibited longer reward latencies compared to the EtOH group for the last 500 trials. (E) No sex differences were found for initiation latencies for the first 500 trials. (F) No sex differences were found for reward latencies for the first 500 trials. (G) Females exhibited longer initiation latencies compared to males for the last 500 trials. (H) No sex differences were found for reward latencies for the last 500 trials. Dashed lines in histograms of latencies represent group medians. Bars indicate $\pm S.E.M.$ $n=16$ males, $n=14$ females, $*p \leq 0.05$, $**p \leq 0.01$

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