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Research report

Under the influence: Effects of adolescent ethanol exposure and anxiety on motivation for uncertain gambling-like cues in male and female rats

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ABSTRACT

Gambling disorder (GD) frequently co-occurs with alcohol use and anxiety disorders, suggesting possible shared mechanisms. Recent research suggests reward uncertainty may powerfully enhance attraction towards reward cues. Here, we examined the effects of adolescent ethanol exposure, anxiety, and reward uncertainty on cue-triggered motivation. Male and female adolescent rats were given free access to ethanol or control jello for 20 days. Following withdrawal, rats underwent autoshaping on a certain (100%-1) or uncertain (50%-1-2-3) reward contingency, followed by single-session conditioned reinforcement and progressive ratio tasks, and 7 days of omission training, during which lever pressing resulted in omission of reward. Finally, anxiety levels were quantified on the elevated plus maze. Here, we found that uncertainty narrowed cue attraction by significantly increasing the ratio of sign-tracking to goal-tracking, particularly amongst control jello and high anxiety animals, but not in animals exposed to ethanol during adolescence. In addition, attentional bias towards the lever cue was more persistent under uncertain conditions following omission training. We also found that females consumed more ethanol, and that uncertainty mitigated the anxiolytic effects of ethanol exposure observed in high ethanol intake animals under certainty conditions. Our results further support that reward uncertainty biases attraction towards reward cues, suggesting also that heightened anxiety may enhance vulnerability to the effects of reward uncertainty. Chronic, elevated alcohol consumption may contribute to heightened anxiety levels, while high anxiety may promote the over-attribution of incentive value to reward cues, highlighting possible mechanisms that may drive concurrent anxiety, heavy drinking, and problematic gambling.

1. Introduction

Gambling disorder (GD) is characterized by a preoccupation with, intense desire to engage in, and loss of control over gambling behaviors [1]. Rates of problem gambling have been steadily increasing over the past few decades [2–4]. However, some studies report that although a majority of the population (up to 78.4%) has engaged in some form of gambling activity, only about 1–5% of individuals report gambling behaviors and symptoms that resemble addictive disorders [5–8]. This suggests that there are large individual differences in the susceptibility to problematic gambling behaviors. For example, the prevalence of GD appears to vary by age, with adolescents and young adults at a heightened risk [5,9,10].

GD also frequently co-occurs with other psychiatric disorders, particularly substance use (SUD) and anxiety disorders [7,11–13], which have been highlighted as conferring risk for the onset of GD, particularly in adolescence [14,15]. Alcohol for example, is consistently reported as one of the most commonly misused substances by individuals with GD, with rates of alcohol use disorder two to nine times greater amongst individuals with GD than in the general population [16,17]. Of note, anxiety disorders often precede the onset of both GD and SUDs [7,18]. Individuals with anxiety disorders often report gambling to cope with anxiety, with several studies supporting anxiety as a significant correlate or predictor of problematic gambling behavior [19,20], suggesting that individual differences in anxiety may predict gambling propensity. Gender also appears to play a major role in risk for compulsive gambling, with males consistently reported at higher risk for problem and pathological gambling [7,21–23]. Additionally, risk factors for GD vary by gender, with GD in females associated with emotional distress and anxiety disorders while substance use and impulsivity align more closely with clinical presentations in males [24–26].

Overall, evidence suggests that GD shares many characteristics with SUDs [8], particularly the ability of Pavlovian conditioned reward cues to become motivational magnets capable of eliciting powerful cue-triggered motivation [27–29]. The incentive sensitization theory

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proposes that the excessive attribution of incentive value to reward-related cues may be a core diathesis in addiction and addictive behaviors [30,31]. According to the theory, excessive attraction to rewards and their cues results from sensitization of mesolimbic dopaminergic pathways involved in objective craving or 'wanting', following repeated drug use, stress or addictive behaviors, such as gambling [28,32,33]. Individual differences in attentional bias or sensitivity to reward cues may thus underlie individual vulnerability to addictive spectrum disorders [34,35]. In at-risk individuals, neural reward circuits may become hypersensitized and promote intense craving or 'wanting' in response to reward-paired cues. Research in humans suggests that gambling cues, such as the lights and sounds characteristic of slot machines, can powerfully elicit craving, the urge to gamble, and narrowed attention towards gambling-related cues [36–40].

Reward uncertainty is believed to contribute to the attractiveness and motivational capacity of gambling [41]. Utilizing Pavlovian Conditioned Approach, or autoshaping, we have shown in rodent models that reward uncertainty, particularly in the probability and magnitude of the reward, may enhance attentional bias and the incentive value attributed to reward cues, measured as sign-tracking, over that of the impending reward, known as goal-tracking [42–45]. This enhancement of incentive salience and attraction to reward cues occurs despite the reduced predictive value of the Pavlovian cue under conditions of reward uncertainty [28,46,47]. Many others have shown in both humans and rodents that sensitivity to the behavioral influence of reward-paired cues may mediate the transition from recreational gambling to problem gambling [48–50]. For example, Winstanley and colleagues demonstrated that the addition of win-related cues to the rodent gambling task (rGT) exacerbates risky decision-making [51]. Similarly, studies in humans have shown that exposure to gambling-related stimuli causes gamblers to overestimate their wins, promotes further gambling, and triggers craving, suggesting the ability of such cues to induce relapse [38–40]. We have also shown that once established, this enhanced cue attraction generated by reward uncertainty persists even when animals are transitioned from conditions of reward uncertainty to certainty, in which the predictive value of the cue is increased [44]. More recently, Chang and Smith demonstrated that under conditions of reward certainty, despite changes in the topography of the sign-tracking response, the attraction and incentive value afforded to predictive cues is resistant to changes in contingencies, such as exposure to an omission schedule during which lever deflections cancel reward delivery [52]. This suggests that cue attraction remains strong even when it is paired with negative consequences, such as omission of reward. Uncertain gambling-like cues may possess even greater resistance to changes in reward contingencies, retaining their attractive qualities even when reward contingencies are degraded.

Alcohol and stress, similar to reward uncertainty, have also been associated with excessive attribution of incentive value to reward-associated cues [45,53–55]. Sensitivity to stress and alcohol-related cues is strongly associated with a high risk for relapse, implying that stress and drug-related cues may similarly promote and maintain addictive behaviors [56], while in some cases simultaneously increasing anxiety [57]. In recent studies, alcohol exposure has also been shown to increase risky choices in the rat gambling task [58]. Adolescent alcohol exposure appears to potentiate dopamine release, increasing attraction towards Pavlovian reward cues as expressed by decreased goal-tracking and increased sign-tracking behavior [54]. These results are observed to an even greater extent for individuals engaging in high alcohol consumption [59]. Together, these findings underscore the potential for mechanisms and interactions between reward uncertainty, anxiety, and alcohol use that may sensitize attraction towards reward cues in gambling, subsequently conferring risk for and maintaining problematic gambling behavior.

Here, we aimed to study how individual differences in adolescent alcohol use and anxiety may contribute to problematic gambling behavior by enhancing the incentive salience of reward-related cues under conditions of reward uncertainty. In line with the literature to date on uncertainty, anxiety, alcohol, and reward cues, we hypothesized that (1) reward uncertainty would enhance attraction to associated cues, (2) adolescent intake of alcohol would further this attraction towards reward cues, particularly for those with high voluntary intake, and (3) heightened anxiety would be associated with both increased ethanol intake and increased fixation on uncertain reward cues.

2. Materials and methods

2.1. Animals and housing conditions

Male and female Sprague-Dawley rats (N = 65; female = 28, male = 37) were bred in-house and weaned at 21-days of age (PND 21). Animals were housed in groups of 2–3 animals in a temperature regulated room (68–72 °F) under a 12:12 h reverse light/dark cycle, and handled for a minimum of three days. Animals were singly housed from PND 28–48 for the 20-day ethanol exposure. After ethanol exposure, animals were rehoused in groups of 2–3 for the remainder of the experiment. Rats were initially given ad lib. access to water and chow (Teklad), and were food-restricted to approximately 90% body weight prior to the first autoshaping session (PND 60). Animals were weighed daily during ethanol exposure and weekly for the remainder of the experiment. Animals were tested in red light conditions for all tasks. All procedures were approved by the Wesleyan University Institutional Animal Care and Use Committee.

2.2. Groups and conditions

Rats were first randomly assigned to control and ethanol groups at 28 days of age, controlling for litter and sex. After the ethanol exposure period, control and ethanol groups were randomly assigned to certain and uncertain reward contingencies (counterbalanced) for the autoshaping task (see Fig. 1A for timeline of experimental procedures). In the certain reward condition (100%-1), lever presentations (CS) were always immediately followed by the delivery of 1 sucrose pellet (UCS) to the magazine dish on every trial. In the uncertain reward condition (50%-1-2-3), 1, 2, or 3 sucrose pellets were delivered following 50% of CS presentations to the magazine entry dish. On the other 50% of trials, no sucrose reward was delivered. Reward was therefore uncertain for these animals in both probability and magnitude, yet the number of CS presentations and reward deliveries was identical across all groups. This yielded four conditions:

- Control Certain: control jello + certain reward (n = 16; female = 7, male = 9).
- Control Uncertain: control jello + uncertain reward (n = 16; female = 7, male = 9).
- Ethanol Certain: ethanol jello + certain reward (n = 16; female = 7, male = 9).
- Ethanol Uncertain: ethanol jello + uncertain reward (n = 17; female = 7, male = 10).

2.3. Ethanol exposure

2.3.1. Apparatus

A glass jar of gelatin was suspended from the cage top in each animal's home cage (34.5 × 43.7 × 18.5 cm) by a metal clamp. Each gelatin jar contained approximately 50 g of control or ethanol jello, depending on the condition (see Fig. 1B).

2.3.2. Ethanol exposure procedure

Individual differences in ethanol intake were assessed using voluntary intake of ethanol gelatin (jello), rather than forced methods of intake, such as injection procedures [60,61]. Male and female adolescent rats were given free access to ethanol or control jello over a

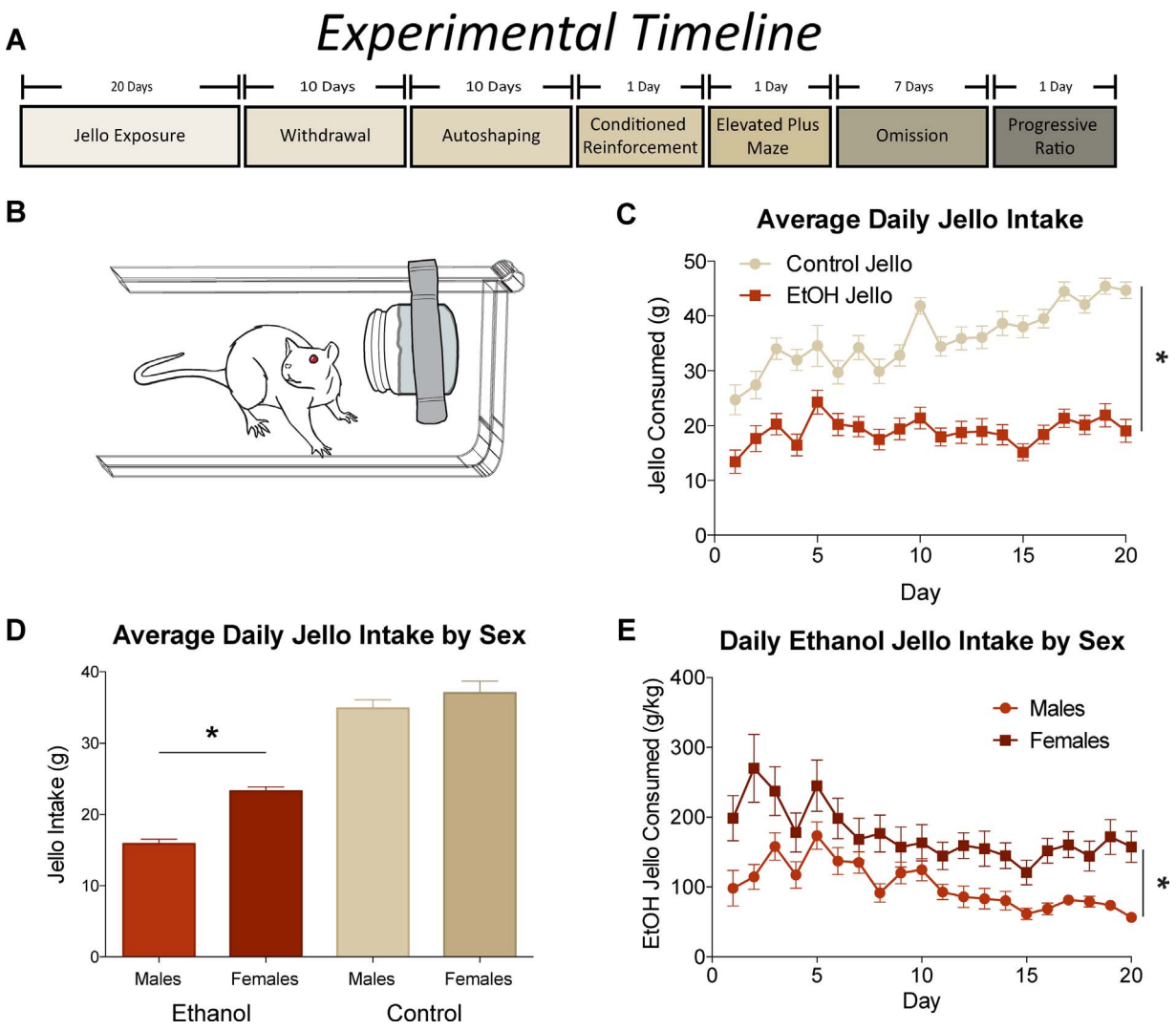

Fig. 1. Experimental design and the consumption of control or 10% ethanol jello during adolescence. (A) Overview of the experimental timeline of procedures and tasks throughout the experiment along with (B) a depiction of the home cage apparatus used during ethanol exposure. (C) Animals given control jello consumed significantly more jello than those given ethanol jello, although all animals increased their jello consumption over the 20-day exposure period (PND 28–48). (D) Females on average consumed significantly greater amounts of ethanol jello than males, however there was no such sex difference in the amount of control jello consumed. (E) Females consumed a significantly greater amount of ethanol jello (g/kg) each day than males, when adjusted for body weight (grams of ethanol jello consumed/kg of bodyweight), across the exposure period. Data presented are Mean +/− SEM. *$p < 0.05$.

prolonged 20-day exposure period, followed by a 10-day withdrawal period before behavioral training.

At prenatal day 28, rats were single-housed in new cages with access to 50 g of a control or 10% ethanol gelatin. The glass jars of gelatin, or "jello shots," were made in-house weekly using a standardized recipe of deoinzed water, gelatin, maltodextrin, and ethanol [60,62]. Jello shots were weighed and replaced each day to record daily intake over the 20-day exposure period. Rats were weighed daily to calculate ethanol consumption with respect to weight. After ethanol exposure, animals were re-housed socially for a 10-day withdrawal period prior to behavioral testing. The quantity of ethanol jello consumed by each animal was calculated in respect to body weight based (ethanol jello intake = grams jello consumed/kilograms body weight).

2.4. Autoshaping/conditioned reinforcement/omission/progressive ratio

2.4.1. Apparatus

All procedures were conducted in standard Med-Associates chambers (25.8 × 32.2 × 33.2 cm; St. Albans, VT, USA) with metal bar floors and plexiglas walls as previously described [63]. Briefly, each chamber was equipped with two retractable levers on the front wall of the chamber on either side of a recessed food magazine, which delivered 45 mg sucrose pellets (TestDiet, St. Louis, MO, USA). A speaker at the top of the chamber delivered a 2.9 kHz tone. For the conditioned reinforcement session, the back wall was outfitted with two nosepoke holes (one active, one inactive, location counterbalanced), located on either side of a retractable lever. During this time, the food magazine on the front wall was covered with a custom metal plate. Med-PC® software (Med-Associates) automatically collected lever responses (LP), nosepokes (NP), and magazine entries (ME) for all sessions. Chambers were placed in sound attenuating cabinets to reduce ambient light and noise. Red LED lights were mounted on the wall inside the cabinet and were turned on during all sessions.

2.4.2. Autoshaping procedure

To examine the degree of attraction for reward-related cues, animals underwent 10 days of Pavlovian autoshaping. Prior to testing rats were exposed to sucrose pellets in their home cages to reduce neophobia. This was followed by two magazine training sessions which consisted of delivery of 30 sucrose pellets (UCS; VI-45) to the food magazine. To

ensure that prior ethanol exposure did not devalue the sucrose pellets or produce any conditioned taste aversion, sucrose pellet consumption was recorded during magazine training. Here, animals previously exposed to ethanol consumed 100% of the sucrose pellets delivered, while a few control animals demonstrated more hesitance to approach the novel reward on the initial day. However, by the second day of magazine training, all animals had successfully acquired magazine training and readily consumed all of their sucrose pellets. Subsequent Pavlovian autoshaping training was conducted as previously described [44,45]. Briefly, each autoshaping session consisted of 36 CS presentations (VI-45) and delivery of 36 UCS sucrose pellets, regardless of condition. During each CS trial an illuminated lever was presented with an auditory stimulus for 8-s. Retraction of the lever CS was immediately followed by delivery to the food magazine of either 1 pellet (certain reward condition) or 1, 2, or 3 sucrose pellets (with equal probability) 50% of the time, or no pellet for the remaining 50% of trials (uncertain reward condition). A control lever was extended into the chamber for the duration of the session. Responses on the CS or control lever, or entries into the food magazine were recorded but had no programmed consequence. Cue-induced attraction was quantified across sessions by the ratio of interaction with the lever (lever presses) over the sucrose delivery dish (magazine entries). Rats were tested in the same operant box for each session.

2.4.3. Sign-tracking and goal-tracking

Although reward delivery is non-contingent on the animal's behavior, animals typically interact (e.g. sniffing, nibbling, biting, pressing) with the predictive lever (sign-tracking) or the location of food delivery (goal-tracking), which may be quantified as a measure of the incentive salience attributed to that cue and reveal individual differences in cue attraction [34]. An animal was classified as a sign-tracker if it performed 4 times more lever press responses than magazine entries (lever presses ≥ 75% of lever presses + magazine entries) and a goal-tracker if it performed 4 times more magazine entries than lever responses during the CS presentations of the last day (Day 10) of Pavlovian autoshaping. An animal's response bias towards either cue (LP = lever press; ME = magazine entry) was determined using the following equation (LP-ME)/(LP + ME) based on the PCA index [64], with scores ranging from 1 to −1. Animals with a strong preference for the lever (sign-trackers) had a response bias between 1 and 0.5, whereas goal-trackers had a response bias between −0.5 and −1. An individual was classified as an intermediate if it directed between 25% and 75% of its responses to either the lever or the food magazine, giving it a response bias between 0.5 and −0.5. All animals developed a conditioned response after initial training.

2.4.4. Conditioned reinforcement procedure

Following autoshaping, rats completed a single conditioned reinforcement session (30 min) to assess the incentive value of the CS (lever + tone), and to measure to what extent it could act as a reinforcer in the absence of reward. Rats were given the opportunity to work to gain access to a lever + auditory cue CS by poking in the active nosepoke hole. A nosepoke into the active hole resulted in a 3-s presentation of the reward-related cue (lever + tone), during which time additional pokes were recorded but had no programmed outcome. In contrast, a nosepoke in the inactive hole had no programmed consequence at any time. Med-PC recorded active and inactive nosepokes and the number of presses on the lever during the session.

2.4.5. Omission procedure

Rats were subsequently trained for 7 consecutive days on an omission schedule, in which cue attraction in the form of lever presses cancelled reward delivery. This behavioral assay captured the ability to suppress CS lever pressing acquired in autoshaping, sensitivity to changes in instrumental contingency, and persistence of incentive salience when CS lever pressing resulted in a negative consequence, such as the omission of reward. Like the autoshaping sessions, the omission sessions consisted of 36 total CS trials (VI-45) under the same certain or uncertain reward contingencies. However, in omission sessions, contact with the CS lever resulted in the omission of reward delivery for that trial. The total number of omitted trials, presses on the CS lever, and entries into the food magazine were recorded during each omission session by Med-PC.

2.4.6. Progressive ratio procedure

A progressive ratio paradigm was then utilized to assess motivation for the sucrose reward and the possible modulation of incentive value attributed to the reward under conditions of reward uncertainty. During a 30-min session, rats were initially trained to press a lever for sucrose pellets on fixed ratio (FR1) reward contingency, where each lever press resulted in the delivery of 1 pellet. The next day, rats were tested using a progressive ratio (PR) schedule where the number of presses required for the delivery of a sucrose pellet increased after each reward, according to an exponential progression (PR schedule = 1, 2, 4, 6, 9, 12, 15, 20, 25, 32, 40, 50, 62, 77, 95, …) based on the formula PR = $[5e^{(rewardnumber \times 0.2)}] - 5$ and rounded to the nearest integer [65–67]. Med-PC recorded the number of rewards earned and lever presses an animal completed during the task. The highest number of lever presses an animal was willing to perform for one sucrose pellet was used as a measure of breakpoint.

2.5. Elevated plus maze

2.5.1. Apparatus

The plus-shaped maze was elevated off the floor by 97 cm and consisted of four arms measuring 40 cm in length and 15 cm in width. Two arms were "closed", restricted by walls 40.5 cm in height on all three sides, and two arms were "open" with no walls. Each arm was located directly across from its matching arm with a 15 × 15 cm square intersection in the middle, joining each arm of the maze. An overhead infrared camera was positioned to capture behavior on the maze for the duration of each session.

2.5.2. Elevated plus maze procedure

After autoshaping training and the conditioned reinforcement session, all animals completed a one-day session on the elevated plus maze, as a measure of anxiety levels [68]. Exploratory behavior on the elevated plus maze was recorded during a 5-min session using an infrared camera suspended above the maze. All rats were placed in the center of the maze at the beginning of the session, facing the same open arm.

2.5.3. Video scoring

Videos were recorded using Monitor Station (Video Insight, Houston, TX, USA) and scored manually by an investigator blind to the experimental conditions. Time spent and entries into the two closed and two open arms were recorded for each animal's first five minutes on the maze. An arm entry was recorded when all four paws of the animal were located in one arm. The entry ended when all four paws of the animal were no longer in the arm. The duration of all arm entries was summed to determine the total time spent on each of the closed and open arms. The amount of time spent on the two open arms was summed together and used as a measure of anxiety. Open arm time inversely correlates with anxiety; thus, the most anxious rats will spend the least amount of time in the open arms [68,69].

2.6. Statistical analysis

Data from all tasks were analyzed using univariate/repeated measures ANOVAs or paired/unpaired *t*-tests (SPSS 21 and Graphpad PRISM 6), where appropriate. As stated in our hypotheses (Introduction), our primary intention in this study was to examine the

relative contribution of reward contingency (certain vs uncertain) and ethanol exposure (control vs ethanol) on conditioned approach behavior. As such, after performing factorial analyses across all factors, data were also analyzed separately across these two factors using repeated measures ANOVAs. Sex was included as a factor in initial factorial ANOVAs, and in the absence of any effects of sex, data was collapsed across this factor and re-analyzed. Correlations were used to assess group effects across tasks. K-means clustering based on anxiety and jello intake data was used to separate animals into high and low intake/anxiety groups. All analyses were two-tailed and performed at a level of significance of $p < 0.05$.

3. Results

3.1. Ethanol intake

All animals in the control and ethanol conditions readily consumed jello, and consumption steadily increased over the 20 days (PND 28-48) of free access to jello (Main effect of Day: $F_{(19,1159)} = 11.914$, $p = 0.000$). However, the ethanol group consumed substantially less jello on average, presumably because of the 10% ethanol content (Main effect of Jello type: $F_{(1,61)} = 84.411$, $p = 0.000$), and ethanol jello consumption across the 20 days of exposure increased to a lesser degree (Day × Jello type interaction: $F_{(19,1159)} = 6.546$, $p = 0.000$; Fig. 1C). Specifically, consumption of control jello nearly doubled between the first and last day of the exposure period (24.7 ± 2.8 g vs 44.7 ± 1.5 g; Main effect of Day for Control: $F_{(19,570)} = 15.908$, $p = 0.000$), whereas the amount of ethanol jello consumed increased by only 140% (13.4 ± 2.1 g vs 19.1 ± 2.1 g; Main effect of Day for Ethanol: $F_{(19,589)} = 2.508$, $p = 0.000$).

3.1.1. Sex differences in ethanol intake – female rats consume more ethanol

In addition to differences in jello intake based on the presence or absence of ethanol, we also found sex differences in jello consumption (Main effect of Sex: $F_{(1,61)} = 7.079$, $p = 0.010$; Day × Sex interaction: $F_{(19,1159)} = 2.078$, $p = 0.004$). Specifically, females consumed more grams of ethanol jello than males (Ethanol Male vs. Female: $F_{(1,31)} = 9.762$, $p = 0.004$), whereas no sex difference was noted in the consumption of control jello (Control Male vs. Female: $F_{(1,30)} = 0.634$, $p = 0.432$; Fig. 1D). This suggests that the increased ethanol jello consumption observed in females was likely due to the effects of ethanol rather than an enhanced palatability of the jello vehicle for female rats. Furthermore, in order to account for sex differences in body weight, since females are typically lighter than males, ethanol jello consumption was also calculated in grams per kilogram body weight (g/kg). This resulted in an even greater difference between female and male ethanol intake, with females consuming on average > 170% (73.4 ± 2.7 g/kg) more ethanol jello per day (Main effect of Sex: $F_{(1,31)} = 16.089$, $p = 0.000$; Sex × Day interaction: $F_{(19,589)} = 1.396$, $p = 0.122$; Fig. 1E). Nonetheless, overall ethanol consumption (g/kg) appeared to decrease for both groups across days (Main effect of Day: $F_{(19,589)} = 7.611$, $p = 0.000$; Fig. 1E), which is in apparent contrast with the significant increase in consumption of ethanol jello noted above. However, this can likely be explained by a greater increase in body weight (> 220%) during adolescence than the increase in jello intake (> 140%), rather than reflective of an actual quantitative decrease in jello consumption (ethanol jello intake = grams jello consumed/kilograms body weight).

3.2. Autoshaping – uncertainty enhances incentive salience and attention focused on the CS lever

During the autoshaping task, animals were trained through CS-UCS pairings to associate the delivery of sucrose pellets with a lever + auditory cue following a certain or uncertain reward contingency (certain, 100%-1 pellet; uncertain 50%-1-2-3 pellets). Overall, rats demonstrated acquisition of the task across training sessions in the form of increased lever pressing (Main effect of Day for Lever Presses: $F_{(9,576)} = 78.148$, $p = 0.000$) and decreased magazine entries (Main effect of Day for Magazine Entries: $F_{(9,576)} = 33.650$, $p = 0.000$; Fig. 2A), with a large majority of animals (62 out of 65) displaying a sign-tracker phenotype.

In order to determine whether uncertainty significantly narrowed attraction towards the lever cue and away from the magazine dish, we calculated the ratio of lever presses to magazine entries within each session (Ratio = LP/ME). We found that across groups, the ratio of lever presses to magazine entries steadily increased (Main effect of Day: $F_{(9,513)} = 45.196$, $p = 0.000$), and that uncertainty significantly increased the ratio of lever presses to magazine entries across days relative to certain reward conditions (Main effect of Uncertainty: $F_{(1,57)} = 5.252$, $p = 0.026$; Uncertainty × Day interaction: $F_{(9,513)} = 1.877$, $p = 0.053$; Fig. 2B). In addition, we also found that males displayed a greater ratio of lever presses to magazine entries than females (Main effect of Sex: $F_{(1,57)} = 8.649$, $p = 0.005$; Fig. 2C), and although there was no clear effect of ethanol exposure (Main effect of Jello: $F_{(1,57)} = 0.857$, $p = 0.358$; Fig. 2D), there was a strong trend towards an interaction between uncertainty, ethanol consumption and sex (Uncertainty × Ethanol × Sex interaction: $F_{(1,57)} = 3.548$, $p = 0.065$). To further understand these effects, we began by examining the impact of uncertainty and sex in animals exposed to either ethanol or control jello. As shown in Fig. 3A, reward uncertainty significantly elevated the ratio of lever presses to magazine entries in control animals (Main effect of Uncertainty: $F_{(1,28)} = 4.859$, $p = 0.036$; Uncertainty × Day interaction: $F_{(9,252)} = 2.086$, $p = 0.031$), although there was no effect of sex (Main effect of Sex: $F_{(1,28)} = 2.093$, $p = 0.159$; Sex × Day interaction: $F_{(9,252)} = 0.778$, $p = 0.637$; Fig. 3B). The effect of uncertainty on cue attraction was however particularly prominent in males, but not in females (Main effect of Uncertainty in Males: $F_{(1,16)} = 6.212$, $p = 0.024$; Females: $F_{(1,12)} = 0.553$, $p = 0.471$; Fig. 3C–D). Here the effect of uncertainty on cue attraction appeared to be largely driven by a rapid decline in magazine entries in control animals (Main effect of Uncertainty on Magazine Entries: $F_{(1,30)} = 6.714$, $p = 0.015$; Males: $F_{(1,16)} = 6.081$, $p = 0.025$; Females: $F_{(1,12)} = 1.696$, $p = 0.217$), rather than any significant change in lever pressing (Main effect of Uncertainty on Lever Presses: $F_{(1,30)} = 1.661$, $p = 0.207$; Males: $F_{(1,16)} = 2.579$, $p = 0.128$; Females: $F_{(1,12)} = 0.049$, $p = 0.828$).

In contrast, chronic exposure to ethanol appeared to abolish the enhanced focus on the lever cue (ratio of LP/ME) due to reward uncertainty (Main effect of Uncertainty: $F_{(1,29)} = 0.905$, $p = 0.349$; Uncertainty × Day interaction: $F_{(9,261)} = 0.543$, $p = 0.842$; Fig. 3E). Yet, ethanol exposure appeared to enhance cue attraction in males over females (Main effect of Sex: $F_{(1,29)} = 7.896$, $p = 0.009$; Sex × Day interaction: $F_{(9,261)} = 2.017$, $p = 0.038$; Fig. 3F), possibly due to the high consumption of ethanol in females. There was as a result, no effect of uncertainty in either males or females exposed to ethanol during adolescence (Main effect of Uncertainty in Males: $F_{(1,17)} = 0.245$, $p = 0.627$; Females: $F_{(1,12)} = 3.148$, $p = 0.101$; Fig. 3G–H). In addition, and in contrast to previous findings [70], it should be noted that there was no effect of ethanol exposure under conditions of reward certainty (Main effect of Ethanol under Certain Reward Conditions: $F_{(1,30)} = 0.088$, $p = 0.769$).

The effect of uncertainty can be further seen when examining its impact on response bias (LP-ME)/(LP + ME) [64], which determines the degree to which an animal develops a strong sign-tracking (ST) or goal-tracking (GT) phenotype. Here, rats developed a strong bias towards sign-tracking across the 10 days of training (Main effect of Day: $F_{(9,513)} = 128.922$, $p = 0.000$), and reward uncertainty produced even stronger sign-tracking behavior (Main effect of Uncertainty: $F_{(1,57)} = 4.637$, $p = 0.036$; Main effect of Sex: $F_{(1,57)} = 0.881$, $p = 0.352$). In particular, rats given control jello and exposed to uncertain conditions developed a stronger sign-tracking phenotype than rats exposed to certain conditions across training (Main effect of

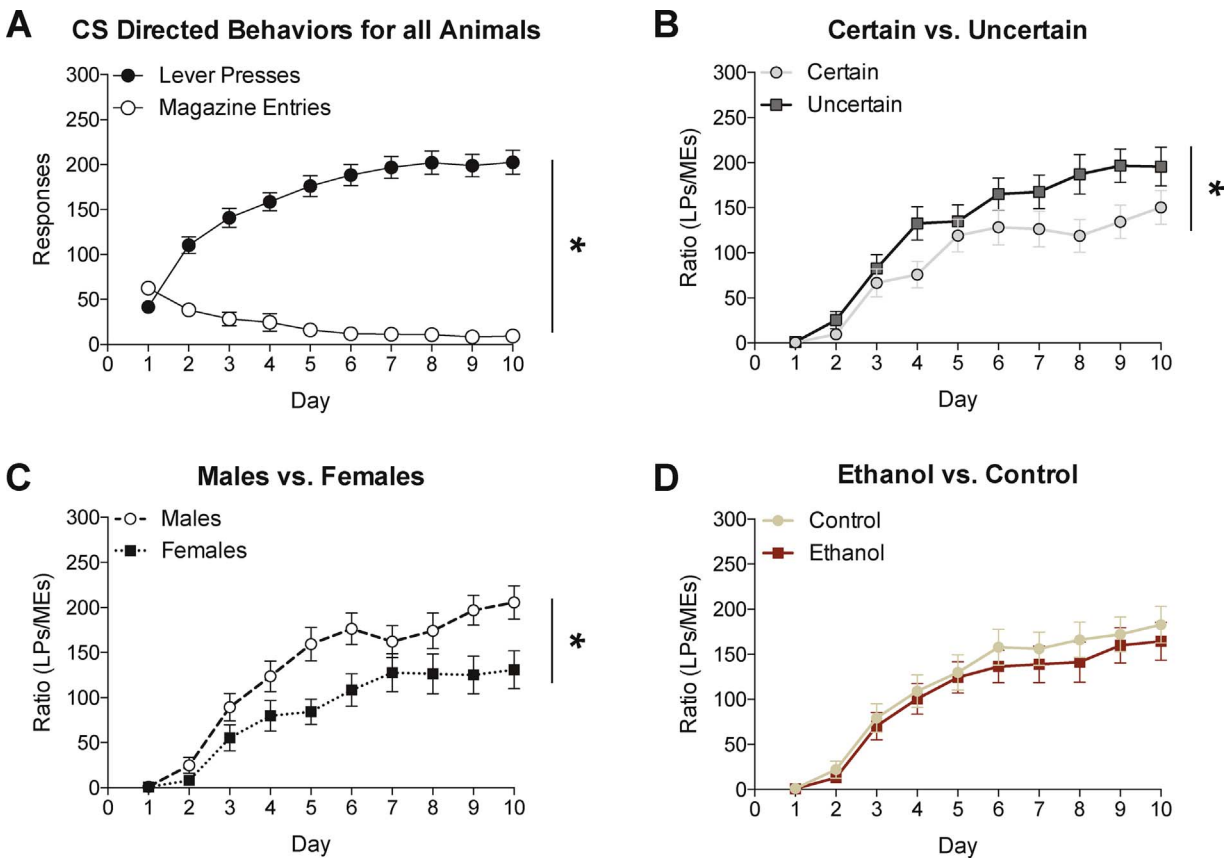

Fig. 2. Acquisition of autoshaping, and the impact of reward uncertainty, sex, and ethanol on cue attraction (ratio). (A) The majority of rats (62 out of 65) developed a strong sign-tracking response across the 10 days of autoshaping, consisting of a rapid increase in lever directed responses and decrease in magazine entries during the CS. (B) Uncertainty in reward probability and magnitude (50%-1-2-3) resulted in greater cue attraction measured as the ratio of lever presses (LP) to magazine entries (ME) when compared to certain reward conditions (100%-1). (C) Males displayed more cue attraction than females, measured by ratio (LP/ME) of lever presses to magazine entries. (D) There was no impact of ethanol jello exposure on cue attraction. Data presented are Mean +/− SEM. *$p < 0.05$.

Uncertainty for Controls: $F_{(1,30)} = 4.506$, $p = 0.042$, Fig. 3I). Again the effect of uncertainty appeared negated by ethanol jello exposure (Main effect of Uncertainty for Ethanol: $F_{(1,30)} = 0.593$, $p = 0.447$; Fig. 3J).

3.3. Conditioned reinforcement – CS lever becomes a conditioned reinforcer across conditions

In order to assess whether the lever cue (CS) could act as a conditioned reinforcer, rats were tested in a one-day conditioned reinforcement task after the completion of autoshaping training, during which animals were able to work to gain brief access to the CS by nosepoking in the active hole. Overall, rats displayed strong conditioned reinforcement, preferentially working for the presentation of the CS (Main effect of Nosepoke: $F_{(1,57)} = 108.533$, $p = 0.000$). However, as shown in Fig. 4A, there were no effect of any other factors (Main effect of Uncertainty: $F_{(1,57)} = 0.060$, $p = 0.807$; Main effect of Jello: $F_{(1,57)} = 1.449$, $p = 0.234$; Main effect of Sex: $F_{(1,57)} = 1.122$, $p = 0.294$). Specifically, animals exposed to certain reward conditions preferentially responded in the active vs. the inactive nosepoke hole, suggesting that the CS had acquired strong reinforcing properties (Control Certain: $t_{(15)} = 5.188$, $p = 0.000$; Ethanol Certain: $t_{(15)} = 5.491$, $p = 0.000$). The same was also true for animals under uncertain reward conditions despite the reduced predictive value of the CS (Control Uncertain: $t_{(15)} = 4.662$, $p = 0.000$; Ethanol Uncertain: $t_{(16)} = 7.587$, $p = 0.000$; Fig. 4A). This was also the case for both males and females (Males: t's > 3.088, p's < 0.016; Females: t's > 3.170, p's < 0.020), with the exception of the female control certain group which trended towards significance ($t_{(6)} = 2.203$, $p = 0.07$).

3.4. Omission – enhanced motivation for uncertain reward cues persists despite negative consequences

Animals were next trained on an omission contingency task on the same certain or uncertain reward contingencies. In the omission task, lever pressing during the CS presentation resulted in the omission of reward for that trial. Overall, animals learned the task effectively over the seven sessions and progressively extinguished lever pressing behavior across all groups (Lever Presses: Main effect of Day: $F_{(6,378)} = 110.669$, $p = 0.000$). However, under conditions of reward uncertainty, animals appeared more rigid in their behavior, continuing to lever press at significantly higher levels (Lever Presses: Main effect of Uncertainty: $F_{(1,63)} = 6.527$, $p = 0.013$) and decreasing lever pressing at a slower rate (Day × Uncertainty interaction: $F_{(6,378)} = 5.405$, $p = 0.000$; Fig. 4B). In contrast, rats under certain reward conditions more rapidly adapted their behavior than their uncertain counterparts, and increasingly redirected their attention towards the magazine, measured by growing magazine entries, across omission sessions (Magazine Entries: Main effect of Uncertainty: $F_{(1,63)} = 9.238$, $p = 0.003$; Main effect of Day: $F_{(6,378)} = 17.107$, $p = 0.000$; Day × Uncertainty interaction: $F_{(6,378)} = 3.217$, $p = 0.004$; Fig. 4B). The ratio of lever presses to magazine entries therefore persisted at significantly higher levels in conditions of reward uncertainty (Main effect of Uncertainty: $F_{(1,63)} = 12.85$, $p = 0.001$; Day × Uncertainty interaction: $F_{(6,378)} = 10.794$, $p = 0.000$), and as shown in Fig. 4C–D, this was the case for both control and ethanol animals (Main effect of Uncertainty for Control: $F_{(1,30)} = 6.955$, $p = 0.013$; Ethanol: $F_{(1,31)} = 6.340$, $p = 0.017$; Fig. 4C–D). Animals in uncertain reward conditions also extinguished their ratio of lever presses to magazine entries at a

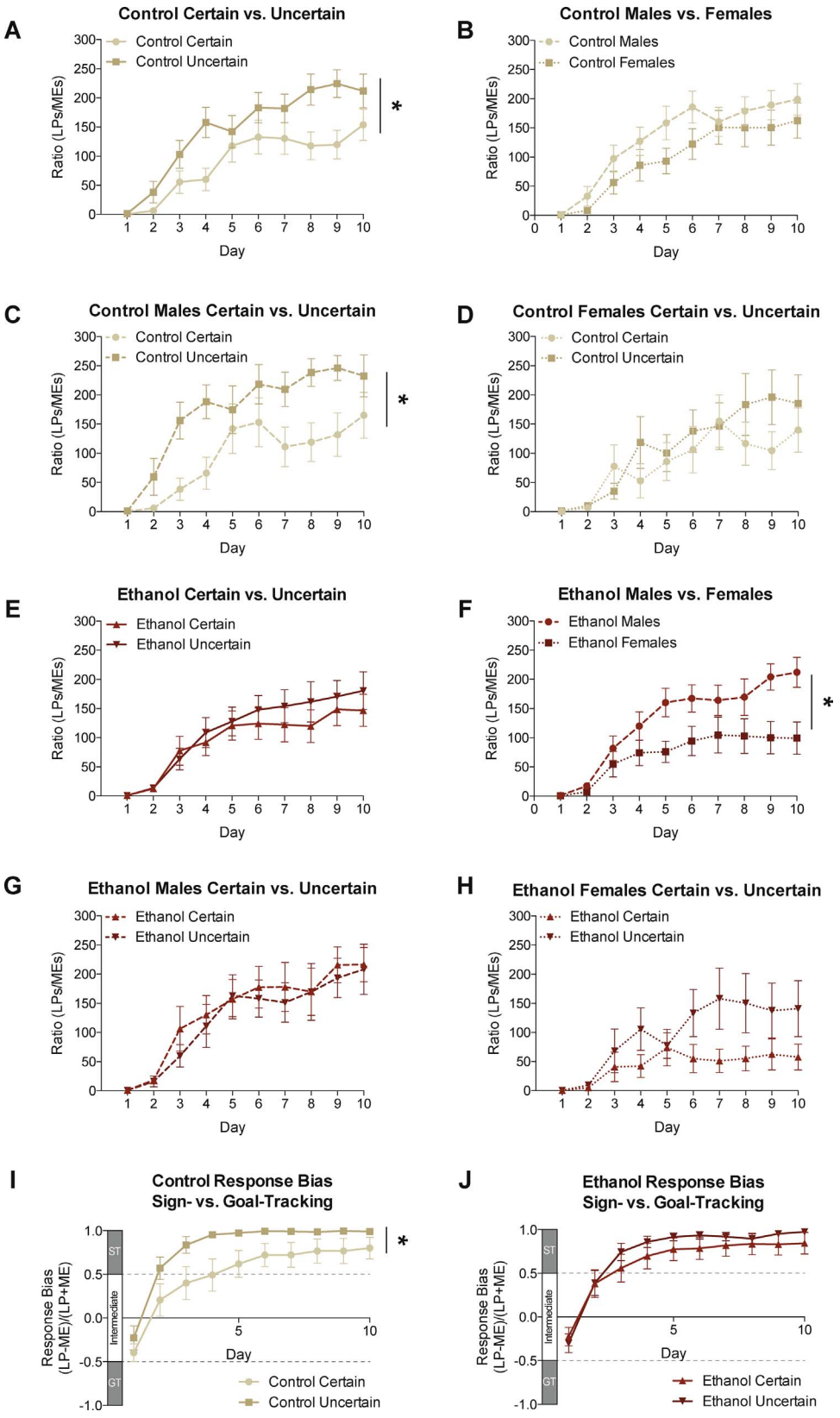

Fig. 3. The impact of reward uncertainty, sex, and ethanol on cue attraction (ratio of lever presses (LPs) divided by magazine entries (MEs)) and response bias during autoshaping. (A) Uncertainty increased cue attraction in control animals. (B) There was no sex difference in the ratio of lever presses to magazine entries in animals given control jello. (C) Reward uncertainty significantly increased cue attraction in control males, (D) but not in control females. (E) Ethanol exposure during adolescence appeared to blunt the impact of uncertainty on cue attraction. (F) Males exposed to ethanol displayed greater cue attraction than their female counterparts. (G) There was no significant impact of reward uncertainty on the ratio of lever presses to magazine entries in either males, (H) or females. (I) Animals exposed to control jello more rapidly developed a stronger sign-tracking phenotype under reward uncertainty than their certain counterparts, as measured by their response bias (LP-ME)/(LP + ME). (J) Exposure to ethanol during adolescence muted these observed effects of uncertainty on response bias. Data presented are Mean +/− SEM. *$p < 0.05$.

significantly slower rate than animals in certain reward conditions (Day × Uncertainty interaction for Control: $F_{(6,180)} = 4.839$, $p = 0.000$; Ethanol: $F_{(6,186)} = 6.410$, $p = 0.000$). The narrowed focus on the CS seen under conditions of reward uncertainty in the autoshaping task persisted in omission, despite the negative consequence of reward loss. Further, animals exposed to uncertain reward conditions demonstrated reduced behavioral flexibility, as demonstrated by persistent lever pressing, and were unable to reshape CS-induced motivation as rapidly

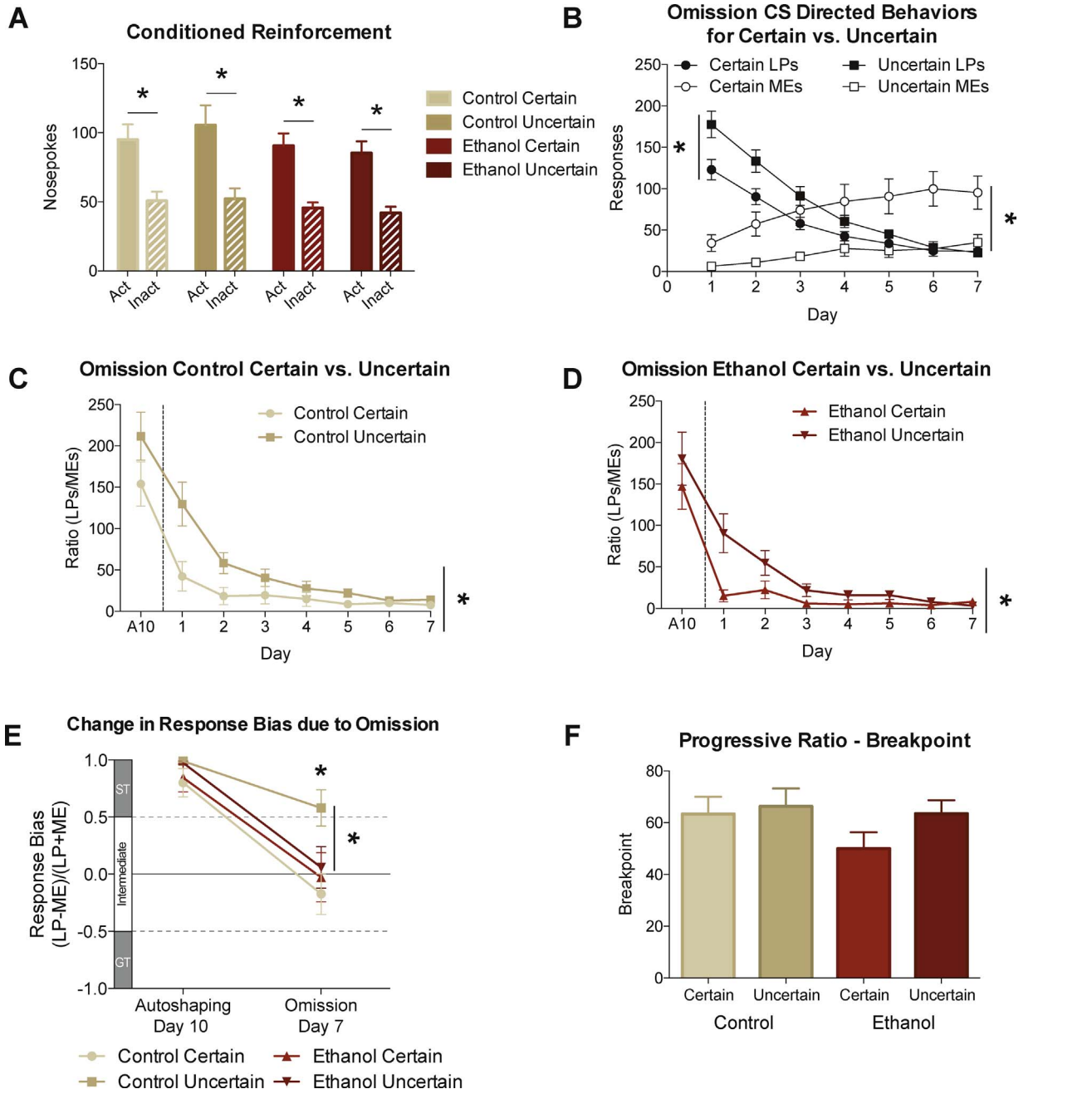

Fig. 4. The effect of uncertainty and ethanol on cue-related behavior in conditioned reinforcement, omission, and progressive ratio tasks. (A) The lever + tone CS acquired reinforcing properties similarly across all groups, shown by preferential responding in the active nosepoke hole, despite the reduced predictive value of the CS under uncertain reward conditions. (B) Exposure to 7 days of omission training, where responses on the CS lever omitted reward delivery, decreased lever responses and increased magazine entries under both certain and uncertain reward conditions. However, under conditions of reward uncertainty, animals appeared more rigid in their behavior, by continuing to lever press at significantly higher levels for longer and by more slowly increasing their magazine entries. (C) Despite no initial difference in the ratio of lever presses to magazine entries on the last day of autoshaping (A10), uncertain control rats maintained a more persistent attraction to the lever CS under omission conditions, when responses on the lever CS omitted reward delivery. (D) Similarly, rats in uncertain reward conditions exposed to ethanol during adolescence maintained a higher ratio of lever presses to magazine entries when placed under omission contingencies. (E) By the last day of autoshaping, all groups displayed a significant response bias in favor of a sign-tracking phenotype. However, over the course of 7 days of exposure to omission conditions, the response bias for almost all groups rapidly moved away from sign-tracking and towards an intermediate phenotype. This was not the case for control uncertain rats who maintained a response bias favoring sign-tracking behavior. (F) There was no difference between groups in the breakpoint, a quantification of the maximal effort an animal will make to gain access to the UCS sucrose reward, during a progressive ratio task, suggesting that uncertainty and ethanol exposure had little impact on the motivation for the sucrose reward. Data presented are Mean +/− SEM. *$p < 0.05$.

as animals under certain reward conditions. Specifically, rats in the uncertain control group displayed a muted shift away from sign-tracking between the last day of autoshaping (Day 10) and the last day of omission training (Day 7) (Main effect of Group: $F_{(1,61)} = 3.538$, $p = 0.020$; Fig. 4E). The Control Uncertain group's persistent attraction to the lever CS can best be seen by the maintained response bias towards the lever CS (sign-tracking) even after 7 days of omission training (Control Uncertain vs. other groups: t's > 2.144, p's < 0.041; Fig. 4E). In fact, the control uncertain group was the only group that remained primarily composed of sign-trackers (Day 7 omission: 10/16 were STs) and retained an average response bias within the sign-tracker range (all other groups were intermediates on average).

3.5. Progressive ratio – uncertainty does not increase motivation for the reward itself

So far, our findings suggest that uncertainty can both influence the expression of CS attraction and the persistence of CS attraction in the face of negative consequences. However, it is unclear whether the effects of uncertainty on cue attraction are driven by an enhanced incentive value of the cue, the reward, or both. In order to assess the motivational value of the reward itself, all animals completed a one-day progressive ratio task. In the task, animals were able to work for sucrose pellets by pressing a lever. The maximum amount of lever presses an animal performed for one pellet, or "breakpoint," was used as a measure of motivation for the sucrose pellet reward. No difference was noted across conditions overall or within sex in breakpoint (F's $\leq$ 1.338, p's $\geq$ 0.270; see Fig. 4F). On average, animals across groups were willing to perform 60.846 $\pm$ 25.447 lever presses for 1 sugar pellet. Uncertainty therefore does not appear to enhance the incentive value of reward-cues by increasing the value of the reward itself.

3.6. Elevated plus maze – females exposed to ethanol in adolescence show higher anxiety levels

Anxiety levels were quantified for all animals using the elevated plus maze, where anxiety is inversely correlated with the amount of time spent on the open arms of the maze. We found overall sex differences with female rats displaying lower anxiety than males (Main effect of Sex: $F_{(1,57)} = 7.964$, $p = 0.007$; Fig. 5A). Additionally, adolescent ethanol exposure was associated with increased anxiety in adulthood (Main effect of Ethanol: $F_{(1,57)} = 4.189$, $p = 0.045$; Fig. 5B). Specifically, females exposed to ethanol spent significantly less time on the open arms of the maze, exhibiting greater anxiety than controls (Females: Ethanol vs Control: $t_{(26)} = 2.399$, $p = 0.024$; Fig. 5C), which was not the case in males (Males: Ethanol vs Control: $t_{(35)} = 0.687$, $p = 0.497$). There was no apparent overall association between reward uncertainty and anxiety (Main effect of Uncertainty: $F_{(1,57)} = 0.039$, $p = 0.845$). However, there was a trending effect of heightened anxiety following ethanol exposure in animals of both sexes under certain compared to uncertain reward conditions (Certain: $t_{(30)} = 1.932$, $p = 0.063$; Uncertain: $t_{(31)} = 0.787$, $p = 0.437$; Fig. 5D).

3.7. Anxiety clusters – looking at effects by high and low anxiety

3.7.1. High/low clustering

In order to investigate the effects of individual differences in anxiety levels on sensitivity to reward-related cues, animals were separated into high and low anxiety groups. K-means clustering was performed based on the amount of time spent in the open arms of the elevated plus maze. All animals were clustered in one analysis to create an overall high and low anxiety group, regardless of sex or condition. As shown in Fig. 5E, clustering effectively produced high and low anxiety groups with significantly different levels of anxiety (Main effect of High/Low Anxiety: $F_{(1,63)} = 155.396$, $p = 0.000$; High vs. Low by Group: t's > 5.058, p's < 0.001).

When examining the distribution of high and low anxiety individuals in each of the four groups, it became apparent that ethanol exposure during adolescence appeared to shift the distribution of high and low animals across clusters. In particular, there were more high anxiety animals in both ethanol conditions than in the control conditions and respectively, more low anxiety animals in the control conditions compared to ethanol conditions (see Fig. 5F). This was supported by a significant interaction between the number of animals in the high and low anxiety clusters and whether they were exposed to control or ethanol jello (High/Low Anxiety $\times$ Jello interaction: $F_{(1,2)} = 33.8$, $p = 0.028$; Main effect of Jello: $F_{(1,2)} = 1.0$, $p = 0.423$; Main effect of High/Low Anxiety: $F_{(1,2)} = 0.2$, $p = 0.698$; Fig. 5F). Nonetheless, it is important to take caution in weighing these results, considering the small n for comparison with only two ethanol and two control conditions. However, this finding further supports that adolescent ethanol exposure may have enduring effects on increased anxiety in adulthood.

3.7.2. Anxiety and autoshaping – high anxiety increases cue attraction under uncertain reward conditions

The relationship between anxiety and cue attraction was examined in high and low anxiety clusters across factors of interest, specifically for its potential interaction with the effects of uncertainty and jello type observed in Section 3.2 (Autoshaping). Notably, high anxiety animals displayed a greater ratio of lever presses to magazine entries than their low anxiety counterparts during autoshaping (Main effect of High/Low Anxiety: $F_{(1,61)} = 4.441$, $p = 0.039$; Fig. 6A). Further, for animals exposed to control jello during adolescence, there was both a significant effect of uncertainty and anxiety (Main effect of High/Low Anxiety: $F_{(1,28)} = 4.817$, $p = 0.037$; Main effect of Uncertainty: $F_{(1,28)} = 7.287$, $p = 0.012$) and a trending interaction between the two (High/Low Anxiety $\times$ Uncertainty interaction: $F_{(1,28)} = 3.361$, $p = 0.077$). Further examination showed that reward uncertainty elevated the ratio of lever presses to magazine entries in the high anxiety group to a greater extent than the low anxiety group (Main effect of High/Low Anxiety: $F_{(1,14)} = 10.118$, $p = 0.007$; Fig. 6B), suggesting that highly anxious individuals may be more vulnerable to developing excessive cue attraction in conditions of reward uncertainty. This was further supported by a significant correlation between greater anxiety and higher levels of lever pressing and cue attraction (ratio) on certain autoshaping days for control animals trained under uncertainty (Control Uncertain Ratio Days 6–7: $r_{(14)}$s < -0.522, p's < 0.038; Fig. 6C). In contrast, there was no difference in the ratio of lever presses to magazine entries between high and low anxiety animals under conditions of reward certainty (Main effect of Anxiety Cluster: $F_{(1,14)} = 0.054$, $p = 0.82$; Fig. 6D).

In order to assess the impact of anxiety on reward uncertainty and exposure to control or ethanol jello, cue attraction (ratio LP/ME) was examined separately in high and low anxiety animals. Within the high anxiety animals, reward uncertainty significantly increased the ratio of lever presses to magazine entries (Main effect of Uncertainty: $F_{(1,25)} = 6.421$, $p = 0.018$), as seen in Section 3.2 (Autoshaping), and adolescent exposure to ethanol additionally modulated this effect on cue attraction (Uncertainty $\times$ Jello interaction: $F_{(1,25)} = 4.365$, $p = 0.047$). However, the impact of uncertainty was not replicated within low anxiety animals (Main effect of Uncertainty: $F_{(1,24)} = 1.907$, $p = 0.180$; Uncertainty $\times$ Jello interaction: $F_{(1,24)} = 0.018$, $p = 0.895$). Behavior was then further examined based on whether animals were exposed to control or ethanol jello. This revealed that for high anxiety animals exposed to control jello, reward uncertainty significantly increased the ratio of lever presses to magazine entries, when compared to certain conditions (Main effect of High/Low Anxiety: $F_{(1,11)} = 6.547$, $p = 0.027$; Fig. 6E), but had no effect in low anxiety animals ($F_{(1,17)} = 0.583$, $p = 0.456$; see Fig. 6F Control Certain vs. Control Uncertain).

In the case of animals exposed to ethanol during adolescence, and similar to results reported for overall autoshaping in Section 3.2, ethanol exposure disrupted the effects of uncertainty and anxiety noted above in high anxiety control animals (Main effect of High/Low Anxiety: $F_{(1,29)} = 1.214$, $p = 0.280$; Main effect of Uncertainty: $F_{(1,29)} = 0.68$, $p = 0.416$; High/Low Anxiety $\times$ Uncertainty: $F_{(1,29)} = 0.250$, $p = 0.621$). There was therefore no difference in cue attraction (ratio) during autoshaping between high and low anxiety animals exposed to ethanol and reward uncertainty (Main effect of High/Low Anxiety: $F_{(1,15)} = 0.198$, $p = 0.663$; Fig. 6G). Indeed, under reward uncertainty, high anxiety animals exposed to ethanol during adolescence displayed a significantly lower cue attraction (ratio) than their high anxiety counterparts given control jello (Main effect of Jello: $F_{(1,15)} = 5.836$, $p = 0.029$; Fig. 6H), a difference which was not seen in

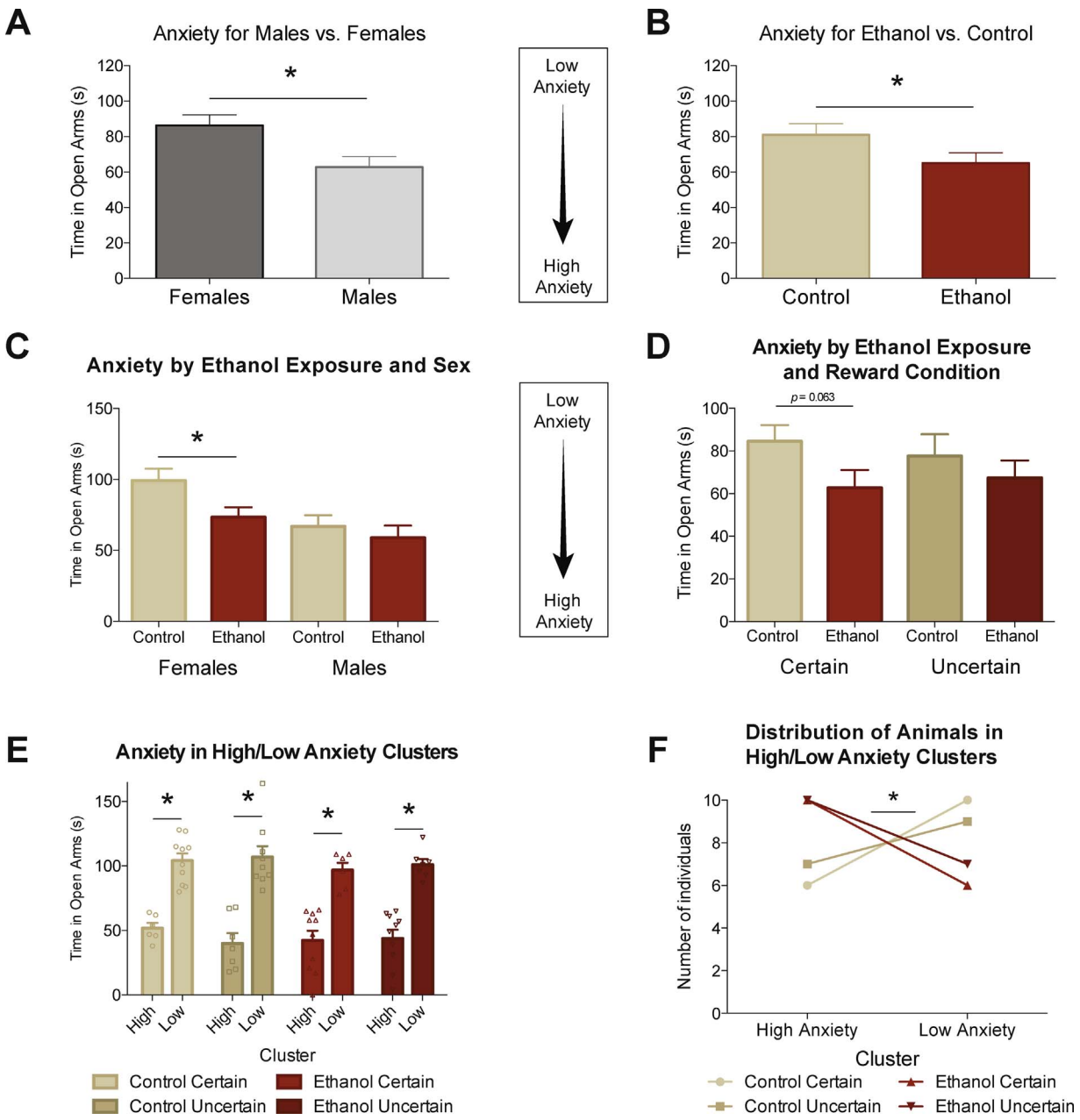

Fig. 5. The impact of sex, ethanol, and reward uncertainty on anxiety, and characterization of high/low anxiety clusters. (A) Females spent significantly more time in the open arms of the elevated plus maze, suggesting that males were more anxious. (B) Adolescent exposure to ethanol resulted in higher levels of anxiety in adulthood. (C) Adolescent exposure to ethanol increased anxiety and decreased the time spent in the open arms of the elevated plus maze in female rats, while no significant sex difference following ethanol exposure was present in males. (D) There was a trend towards greater anxiety in rats exposed to ethanol and certain reward conditions, but not in uncertain conditions. (E) Animals across each group were clustered into high and low anxiety groups using K-means clustering, creating high and low anxiety subgroups that significantly differed in the amount of time spent in the open arms of the elevated plus maze. (F) There was a significantly greater number of rats distributed into the high anxiety subgroup, than to the low anxiety subgroup, for the animals exposed to ethanol rather than control jello during adolescence. Data presented are Mean +/− SEM. *$p < 0.05$.

low anxiety animals (Main effect of Jello: $F_{(1,14)} = 0.003$, $p = 0.956$; see Fig. 6F Control Uncertain vs. Ethanol Uncertain). In fact, animals displaying low levels of anxiety all showed similar levels of lever press to magazine entry ratio, irrespective of condition (F's < 0.769, p's > 0.4; Fig. 6F). The elevated focus on the CS lever cue as a result of uncertainty therefore appeared to be driven primarily by high anxiety individuals, and largely restricted to animals in the control jello condition. Together these results suggest that high anxiety control individuals might be more susceptible to enhanced cue attraction under conditions of reward uncertainty.

3.8. Jello intake clusters – looking at effects in high and low intake animals

3.8.1. High/low clusters

Rats were also clustered into high and low jello intake groups using K-means clustering. Due to the significant differences in intake by jello type and sex, animals were clustered in four separate analyses by jello condition and sex (see Fig. 7A–B). This clustering method was effective in producing high and low intake groups with significantly different average intake across the 20-day exposure period (Main effect of High/Low Intake: $F_{(1,49)} = 164.342$, $p = 0.000$), with intake varying significantly across clusters by sex and type of jello consumed (High/Low

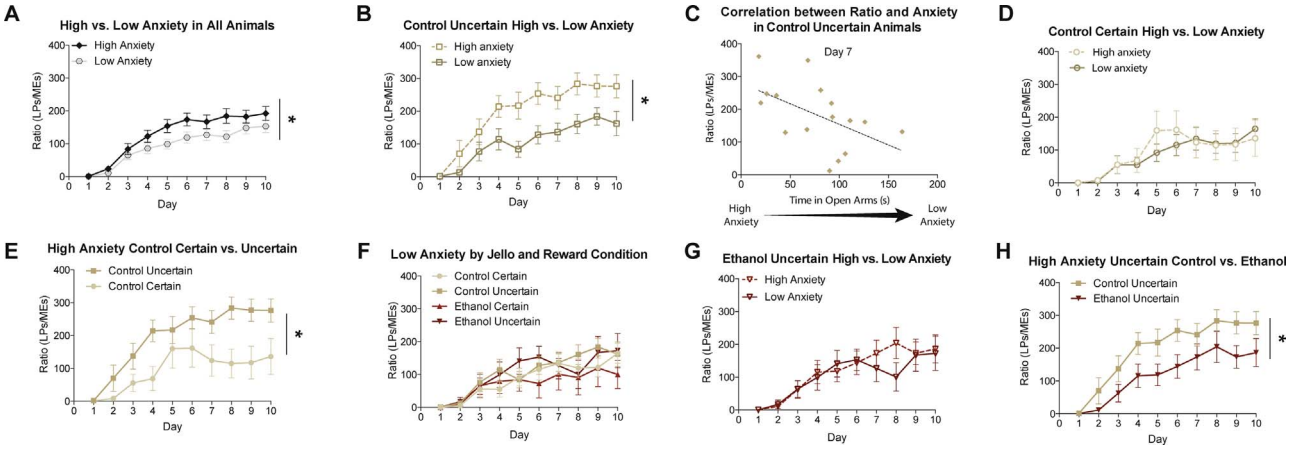

Fig. 6. Individual differences in high and low anxiety and the interaction of high anxiety with the effects of reward uncertainty and ethanol on cue attraction. (A) High anxiety animals overall showed greater cue attraction (ratio of lever presses (LPs) to magazine entries (MEs)) than low anxiety animals. (B) Among control uncertain rats, high anxiety in the elevated plus maze was associated with a significantly greater ratio of lever presses to magazine entries when compared to low anxiety rats. (C) This finding was further demonstrated in control rats exposed to uncertain reward conditions by a significant correlation between high anxiety, as measured by reduced open arm time, and greater cue-triggered focus on the lever CS, as exampled in day 7 of autoshaping. (D) Under conditions of reward certainty in control animals, there was no effect of anxiety on cue attraction. (E) Reward uncertainty also increased cue attraction in high anxiety animals exposed to control jello. (F) In contrast, there was no difference in cue attraction amongst low anxiety animals, irrespective of reward conditions or jello type. (G) Unlike the effect observed in figure (B), there was no effect of anxiety on cue attraction in ethanol animals exposed to uncertain reward conditions. (H) Specifically, ethanol exposure significantly attenuated the enhanced cue attraction noted in high anxiety control animals under conditions of reward uncertainty, demonstrated in figure (B). Data presented are Mean +/− SEM. *$p < 0.05$.

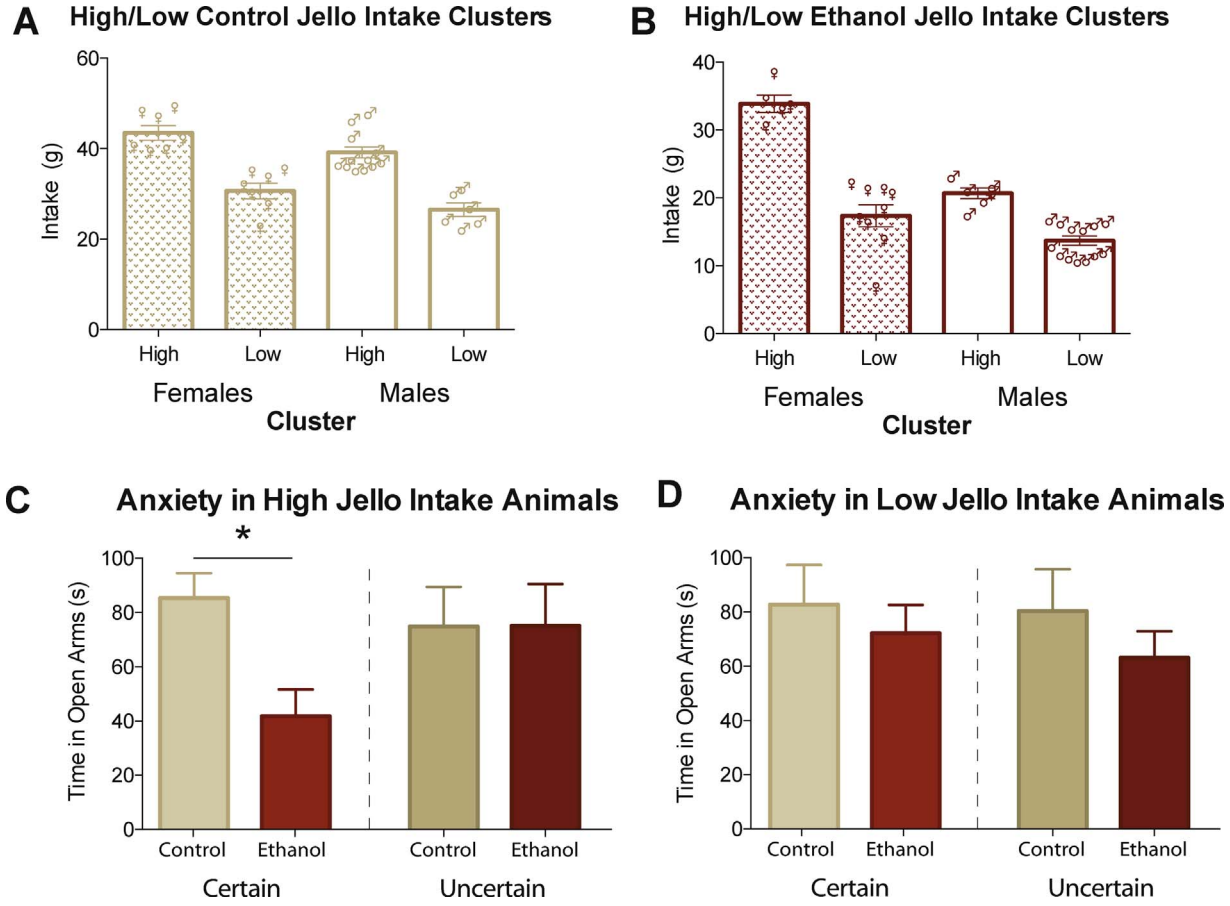

Fig. 7. High and low jello intake clusters for animals exposed to ethanol and control jello, and their impact on anxiety. (A-B) Animals exposed to either control (A) or ethanol (B) jello during adolescence were clustered into high and low jello consumption groups, with high and low intake females consuming significantly more ethanol than their male counterparts, respectively. (C) Across high jello intake animals, rats exposed to ethanol during adolescence showed greater anxiety than their control jello counterparts, particularly if trained under certain but not uncertain reward conditions. (D) No effect of jello or reward uncertainty was seen in low jello intake animals. Data presented are Mean +/− SEM. *$p < 0.05$.

Intake × Sex interaction: $F_{(1,49)} = 4.86$, $p = 0.032$; High/Low Intake × Sex × Jello interaction: $F_{(1,49)} = 7.165$, $p = 0.010$; High vs. Low × Group: F's > 29.864, p's < 0.001).

3.8.2. Sex differences in intake replicated in high/low ethanol intake animals

The overall finding of greater ethanol intake in females was replicated in the high and low ethanol groups. As shown in Fig. 7B, females in the high and low intake groups consumed more grams of ethanol jello on average than their male counterparts in the high and low intake groups, respectively (Main effect of Sex in Ethanol High Intake: $F_{(1,9)} = 83.803$, $p = 0.000$; Ethanol Low Intake: $F_{(1,20)} = 5.506$, $p = 0.029$). In addition, the difference in ethanol intake between high and low groups was significantly greater in females compared to the males (High/Low Intake by Sex interaction: $F_{(1,29)} = 17.375$, $p = 0.000$). High intake animals also consumed consistent amounts of ethanol jello across the exposure period, with no significant change across exposure days ($F_{(19,171)} = 0.873$, $p = 0.616$). In contrast, low intake ethanol animals showed an overall decrease in ethanol jello intake across days of ethanol exposure ($F_{(19,399)} = 2.5$, $p = 0.001$).

3.9. Ethanol intake and anxiety

As shown above, when animals are not segregated by high and low ethanol intake, a history of chronic ethanol intake produced increased anxiety, and a trend towards increased anxiety for ethanol exposed animals under certain reward conditions (see Section 3.6 and Fig. 5B & D). This effect was therefore further examined separately for high and low jello intake animals. Notably, in the high jello intake animals, adolescent ethanol exposure resulted in significantly more anxiety for animals compared to their control counterparts under certain, but not uncertain reward conditions (Main effect of Jello: Certain: $F_{(1,15)} = 8.103$, $p = 0.013$; Uncertain: $F_{(1,13)} = 0.000$, $p = 0.989$; Fig. 7C). However, this was not the case for low ethanol intake animals under either certain or uncertain reward conditions (Main effect of Jello: Certain: $F_{(1,15)} = 0.333$, $p = 0.573$; Uncertain: $F_{(1,18)} = 0.987$, $p = 0.334$; Fig. 7D). This suggests that in individuals with a high ethanol intake, uncertainty may have mitigated some of the additional anxiety created by adolescent exposure to ethanol.

4. Discussion

4.1. Reward uncertainty

The results of the present study demonstrate the complex interactions that occur between anxiety, adolescent alcohol consumption, and reward uncertainty on the attraction to Pavlovian reward-related cues. Our results are in line with previous reports showing that uncertainty in the magnitude and probability of reward increases response bias and attraction towards the CS during a Pavlovian Autoshaping Task, further demonstrating that reward uncertainty characteristic of gambling may cultivate a biased attention and attraction towards associated reward cues [43–45]. Sign-tracking behavior has previously been associated with behavioral and neurobiological vulnerability factors implicated in addiction [71,72]. The propensity to approach and fixate on the reward signal (sign-track) rather than the location of food delivery (goal-track) may represent a "pathological attentional bias" that mirrors susceptibility to developing an addictive spectrum disorder [73]. Thus, individual differences in the sensitivity to reward cues may interact with the motivational capacity of reward uncertainty evidenced in our findings, predisposing individuals to compulsive gambling behavior [28,34]. Individuals with this predisposition for gambling disorder may be similarly reinforced and triggered to relapse by the environmental cues associated with uncertain reward in gambling.

In this study, nearly all animals were sign-trackers, predominantly approaching and engaging the lever cue rather than the goal associated with reward delivery. Repeated CS-UCS pairings during autoshaping increased sign-tracking behavior across days, with a steady increase in lever pressing and consequent decrease in magazine entries. Animals exposed to reward uncertainty demonstrated a more rapid and elevated shift in the ratio of sign-tracking to goal-tracking behaviors, displaying a stronger response bias towards sign-tracking [64]. These findings were specific to animals given control jello during adolescence and were most prominent in males. Ethanol exposure in adolescence appeared to mitigate these differences in response bias under conditions of reward uncertainty, with no significant differences in the ratio of sign-tracking to goal-tracking behaviors emerging during autoshaping between certain and uncertain reward conditions following ethanol exposure.

We also report that the ability of the CS to act as a conditioned reinforcer was not diminished under conditions of reward uncertainty, despite its reduced predictive value. Here, the CS acted as an equally effective conditioned reinforcer across all conditions, and animals were similarly motivated to acquire and perform a novel nosepoking behavior in order to gain access to the CS. This highlights the dissociable nature of the three fundamental characteristics that apply to cues or conditioned stimuli imbued with incentive salience; notably, (1) that cues can become 'motivational magnets' capable of attracting motivated and, in extreme cases, irrational behavior (autoshaping), (2) that cues can elicit cue-triggered 'wanting' (Pavlovian-to-Instrumental-Transfer), and finally (3) that cues can reinforce the acquisition of a new instrumental response (conditioned reinforcement) [31,74]. Our findings show that uncertainty specifically enhances the motivational magnet properties of a cue while leaving its ability to act as a conditioned reinforcer unchanged. However, it remains possible that uncertainty diminishes the cue's predictive value while enhancing its incentive value, sufficient to compensate for the loss in predictive reinforcing properties [46]. This enhanced incentive value under conditions of reward uncertainty may contribute to the intense cue-induced craving seen for reward cues in GD [75–77]. Further, the observed results support that reward uncertainty may enhance the incentive value of the reward cue rather than for the reward itself. Specifically, there was no significant difference in effort price (breakpoint) that animals were willing to work for the sucrose reward, demonstrating that the enhanced attribution of incentive salience to the cue was not due to uncertainty increasing the value of the reward. These results are in line with theories and neuropsychological findings that GD may arise from both a blunted sensitivity to reward and an increased vulnerability to reward-related cues [78]. Individuals at risk for gambling disorder may be less sensitive to reward and therefore gamble more to achieve the same positive effects, further increasing exposure to reward-related cues in this vulnerable population.

4.2. Persistent cue attraction under reward uncertainty despite loss of reward

Our results also demonstrate that reward uncertainty during autoshaping training persistently elevates response bias in favor of the reward cue, reducing behavioral flexibility and the ability to adapt to a new reward contingency when contact with the reward cue became actively disadvantageous in an omission task. Animals exposed to reward uncertainty, regardless of ethanol condition, struggled to suppress attraction and redirect attention away from the cue previously associated with uncertain reward even when engagement of the lever resulted in the omission of reward. These effects of uncertainty persisted even longer for animals given control jello, with most animals continuing to display a sign-tracker phenotype even after 7 days of omission contingencies. Our results support that uncertainty, characteristic of gambling, may promote a narrowed and extinction-resistant attraction to reward cues. A recent study by Chang and Smith [52] showed under conditions of reward certainty that although animals successfully learn to extinguish lever pressing during omission procedures,

attraction to the lever is not necessarily extinguished. Across sessions, they demonstrated that the extinction of lever pressing was complemented by a reciprocal, progressive increase in sniffs and orientations to the lever. Cue attraction persisted robustly as animals learned to reshape cue-directed behaviors to avoid reward omission. In the present study, although lever pressing was largely diminished across most conditions, animals trained under uncertain reward conditions remained fixated on the lever cue almost exclusively, while those trained in certain reward conditions more readily shifted focus from the lever to the magazine. Reward uncertainty may thus contribute to rigid, narrowed motivated behaviors that persist even when disadvantageous consequences are presented in gambling. Our findings thus suggest even greater persistence and attribution of incentive salience to reward cues in conditions of uncertainty, than has previously been reported under conditions of reward certainty [52].

It is important to acknowledge that this finding may in part be influenced by the similarity of the uncertain reward condition (50%-1-2-3) and a partial reinforcement schedule. In partial reinforcement, animals are also rewarded on an uncertain reward probability, although there is typically no uncertainty in the reward magnitude. There is robust support that partial reinforcement during acquisition of instrumental tasks makes animals more resistant to extinction [79]. Partial reinforcement may have therefore contributed to the persistent value of uncertain reward cues seen during omission. However, the partial reinforcement effect in instrumental learning is often associated with a significant decrease in the speed of acquisition of behavior and learning [80]. Animals exposed to reward uncertainty in the present study actually demonstrated a quicker and more intense acquisition of lever presses in respect to magazine entries across autoshaping sessions, suggesting other motivational factors likely influenced cue attraction and behavior beyond those attributable to partial reinforcement. However, partial reinforcement under Pavlovian conditioning has also recently been shown to be more resistant to extinction [81,82], which is in line with our current findings. Further research is needed to examine whether the quantity and type of CS-directed behaviors that persist during omission contingencies under conditions of reward uncertainty are similar to those seen with reward certainty [52]. The elevated, extinction-resistant incentive salience of uncertain reward cues observed here may similarly motivate continued gambling behavior in the face of negative consequences, such as financial losses. Gambling-cues may hold persistent value despite serious costs, contributing to the maintenance of gambling behavior and significant vulnerability for relapse.

4.3. Ethanol and incentive attribution

Interestingly, we found that uncertainty heightened cue attraction only in control animals. The heightened cue attraction resulting from uncertainty was not present in animals exposed to alcohol during adolescence, nor was there a heightened cue attraction effect of ethanol in animals under more typical certain reward conditions. This is surprising given that previous research has suggested that adolescent ethanol exposure increases dopaminergic activity and results in increased attribution of value to reward cues [54]. One possible explanation for these results is that the effects of adolescent ethanol exposure masked the effect of uncertainty. Animals in both uncertain reward conditions engaged in 1–2 lever presses per second on each 8-s lever presentation throughout the 36 trial sessions. Based on the high level of conditioned responding present in both uncertainty groups, animals may have lever pressed at maximal levels producing a ceiling effect that concealed any interaction effect between ethanol and reward uncertainty. Additionally, ethanol exposure may have increased animals' attraction to reward cues in general [54], elevating cue attraction for both certain and uncertain animals, masking the difference observed under conditions of uncertainty for control animals. One recent study reported that intermittent exposure to ethanol during adolescence increased sign-tracking behavior under conditions of reward certainty, with females across the sample performing stronger sign-tracking than goal-tracking [70]. Our results in the certain reward groups also fail to replicate these reported sex differences or enhanced sign-tracking following adolescent ethanol exposure. It is unclear why these effects were not present in our sample, however it is possible, at least in the case of the article by Madayag and colleagues, that the at-will consumption procedures utilized in the present study for prolonged ethanol exposure contributed to discrepancies with previous findings and hypotheses, which relied upon an intermittent and potentially mildly aversive intragastric administration method. Our findings suggest that ethanol may enhance attraction to reward cues, sufficiently to mask the effect of uncertainty, but further research is needed to elucidate this interaction.

4.4. Anxiety effects on cue attraction in uncertain reward conditions

Anxiety has been shown to play an important role in gambling behavior [7,11–13]. Our results demonstrate that the ability of uncertainty to enhance the incentive salience of the reward cue was not uniform across individuals. In particular, we found individual differences in anxiety, with high anxiety quantified as less time spent on the open arms of an elevated plus maze. Our results showed that heightened anxiety enhanced sensitivity to the effects of reward uncertainty, as demonstrated by elevated approach and response bias towards the lever cue. This influential role of anxiety was absent, however, following ethanol exposure in adolescence and for animals exposed to reward certainty. In fact, the heightened attraction for the lever cue in high anxiety individuals seemed to require both conditions of reward uncertainty and no adolescent exposure to ethanol. High anxiety control animals exposed to reward uncertainty displayed greater cue attraction than both high anxiety animals exposed to certainty and high anxiety animals exposed to reward uncertainty and ethanol during adolescence. To our knowledge no previous study has demonstrated an effect of anxiety on autoshaping, particularly for reward uncertainty.

As previously discussed, the effects of adolescent ethanol exposure may be confounding the effect of uncertainty, thus masking an interaction between uncertainty and anxiety as well. However, our findings do still suggest high anxiety may elevate sensitivity to the motivational effects of reward cues in gambling-like conditions of reward uncertainty. There is in fact some evidence in the literature to support this finding and a potential modulating role of anxiety in incentive salience attribution. Previous animal studies have associated higher anxiety with higher levels of endogenous corticotropin-releasing factor (CRF) [83], and microinjections of CRF to the nucleus accumbens in rats results in increased sensitivity to Pavlovian paired reward cues [53]. The high anxiety rats in this study may have had naturally higher levels of endogenous CRF and therefore, been more vulnerable to over-attribute value to cues associated with uncertain reward. This finding provides support for one direct mechanism by which high anxiety levels, characteristic of anxiety disorders, may confer risk for the onset of GD and subsequently increase gambling severity. Of note, the directionality of the relationship between anxiety and sign-tracking is unclear as the behavioral assay for anxiety in the present design was performed after autoshaping. Therefore, another possible interpretation is that high levels of sign-tracking increased anxiety. Heightened sign-tracking acquisition and performance has been associated with greater session-induced corticosterone release, a stress hormone in rodents comparable to cortisol in humans [72,84]. Those results do not provide sufficient support to conclude that high anxiety levels were the result of high lever pressing in this study, however, as findings in previous reports required anxiety to be measured immediately post-session. Rather, it seems that sign-tracking is closely associated with elevated corticosterone release, with literature supporting higher corticosterone levels as both a biomarker for increased vulnerability to fixate on reward-paired cues and a result of sign-tracking in autoshaping procedures [72]. Based on current findings and literature, it is more compelling to conclude

that high anxiety may interact with reward uncertainty and enhance attraction to uncertain reward cues.

In the context of gambling, anxiety may powerfully influence the attribution of value to reward cues. Individuals with anxiety disorders may be more susceptible to over-attribute value to gambling cues, conferring risk for the onset of GD. This interpretation aligns with the temporal relationship observed in the comorbidity between anxiety disorders and GD, with anxiety often preceding the onset of addictive spectrum disorders [7,18]. The elevated symptom severity seen in GD presentations with comorbid anxiety disorders may in part be the result of this enhanced vulnerability to attribute value to and fixate on uncertain reward cues in gambling [85]. Individuals with anxiety disorders may experience intensified craving and difficulty suppressing gambling urges, as a result of this increased vulnerability to uncertain reward cues.

Anxiety is also suggested to contribute to the onset of GD through negative reinforcement in individuals that use gambling to escape from or cope with negative affect [14,86–88]. Poor coping styles in high anxiety individuals may contribute to initial gambling involvement and provide cognitive motivation for gambling. Based on our present findings, these same high anxiety individuals may also be more at risk to excessively value gambling cues and experience powerful, extinction-resistant reinforcement by sensory cues such as the lights and sounds in a casino. This motivational mechanism may work concurrently with coping strategies, confounding the risk for GD and further contributing to symptom severity on both a cognitive and neurobiological level for high anxiety individuals [89].

4.5. Females intake significantly more ethanol in social isolation

Our results also show that females are far more susceptible than males to consume very high amounts of ethanol in social isolation. When animals were clustered into high and low intake groups, it was evident that a handful of female rats exhibited much higher ethanol intake than all other ethanol-exposed animals. Previous research has reported mixed findings on sex differences in adolescent ethanol consumption. Contrary to the results of this study, some studies have reported no sex difference in ethanol intake, while others have reported opposite findings [90,91]. One study reported that social setting influences the relationship between sex and ethanol intake [92,93]. Similar to the results of the present study, females consumed more ethanol than males when alone. Males, on the other hand, were more likely to consume more ethanol in social settings. The disparity in findings on sex difference in ethanol intake is likely influenced by the inconsistency in ethanol procedures used. Across the studies reviewed ethanol concentrations ranged from 5%-20%, housing conditions varied, exposure duration ranged from a few days to weeks, and the schedules of access varied from limited to free access. The results of the present study most closely align with previous findings that females will consume more ethanol under extended exposure in social isolation [93]. The increased amount of ethanol intake seen in females may therefore not generalize to ethanol consumption overall, but may instead be more specific to ethanol intake as a result of social isolation or stressful environments during adolescence.

4.6. Ethanol intake is associated with heightened anxiety in females and following reward certainty

Adolescent females were not only heavier ethanol drinkers in social isolation, but they were also more anxious in adulthood after ethanol exposure. It is known that social isolation during adolescence can elevate anxiety in rats, particularly in females [94]. Rats show elevated corticosterone levels and higher ethanol intake after social isolation [95,96]. However, it is unlikely that heightened anxiety levels in ethanol females were the result of social isolation alone in this study. During the jello exposure procedure, control and ethanol animals were both housed in social isolation. Yet, female animals given ethanol jello were significantly more anxious than those given control jello. This finding suggests that repeated ethanol use may have independent effects on anxiety and interact with social isolation for adolescent females. There is evidence in mice to suggest that a similar length of ethanol exposure leads to increased corticosterone levels [97]. This increase in anxiety for ethanol-exposed females may therefore have been the result of the anxiety-promoting effects of alcohol use. Furthermore, the anxiety caused by social isolation [94] may have also contributed to escalated ethanol intake in females [98], further compounding the anxiogenic effects of alcohol use. Female problem gamblers often report using substances and gambling to cope with and escape negative emotions, including anxiety [19,99]. Though alcohol may initially be used to cope with anxiety, the results of this study suggest repeated use may interact with stressful conditions and ultimately increase anxiety levels. Therefore, it is possible individuals with GD, particularly females, may initially use alcohol and gambling to escape anxiety, and progressively become more dependent on gambling as anxiety increases due to chronic alcohol use.

An alternative explanation for the greater alcohol intake seen in females comes from a recent study showing that females may experience more of the rewarding and reinforcing effects of alcohol than males [100]. In this study, Torres and colleagues demonstrated that females preferred contexts paired with moderate doses of ethanol significantly more than males. Removal of the ovaries was sufficient to eliminate the observed sex difference in preference for ethanol-paired contexts. These findings suggest female hormones may enhance the rewarding effects of ethanol. It is possible, therefore, that females may also be more susceptible to heavy drinking due to this enhanced sensitivity to positive reinforcement by ethanol. Over time, however, females in particular may become more anxious as a result of chronic use. Further study to understand how the rewarding and anxiogenic effects of alcohol may confer risk for problematic heavy drinking and in turn, problematic gambling in females, may elucidate a significant role for anxiety in the etiology and maintenance of GD.

High ethanol intake animals in this study were also significantly more anxious than control intake animals, but these findings were specific to animals exposed to reward certainty. Prior research has supported a positive correlation between anxiety and heightened intake levels [93,95], where rats that consume the highest amounts of ethanol show the highest levels of anxiety in a behavioral assay for social anxiety. It is unclear why the relationship between ethanol and anxiety was not present in high intake animals exposed to reward uncertainty, however it is possible that the rewarding effects of uncertainty may have masked or mitigated the anxiogenic effects of ethanol.

Stressful environmental conditions in adolescence, similar to the stress of social isolation in this study, may interact with increased anxiety and promote problem drinking [101]. Based on our results, female adolescents may be particularly susceptible to experiencing increased anxiety as a result of chronic heavy drinking. Given that women are more likely to experience anxiety disorders and report using alcohol as a coping mechanism, this finding is particularly concerning [16,102,103]. Women may be both more susceptible to heavy ethanol intake to cope with anxiety and more at risk to experience increased anxiety as a result of chronic alcohol use. This may contribute to a powerful, mutually reinforcing cycle of increased alcohol use to cope with increased anxiety, that may confer elevated risk for dependence on coping behaviors like gambling.

5. Conclusion

The goal of the present study was to investigate how adolescent alcohol use and anxiety may interact and contribute to severe GD symptoms. Specifically, the study investigated how anxiety and alcohol may enhance the motivational power of gambling-like cues. Prior research has suggested that stress, alcohol, and uncertainty in gambling

may all similarly sensitize neural reward pathways and contribute to the impairing craving and relapse seen in addiction. Our findings support that reward uncertainty, similar to drugs of abuse, enhances sensitivity to discrete cues associated with uncertain reward. Further, the results suggest that high anxiety levels may make individuals more vulnerable to over-attribute value to gambling-like reward cues, increasing vulnerability to addiction and inducing a powerful cue-induced craving to gamble. Contrary to expectations, ethanol exposure did not observably sensitize animals to uncertain reward. Ethanol, however, was associated with significantly increased anxiety in females and in high intake animals in certain reward conditions. Based on the results of the present study, adolescents that gamble to cope with anxiety may also experience elevated craving and vulnerability to GD as a result of enhanced sensitivity to gambling cues. Additionally, the use of alcohol as a coping mechanism may elevate anxiety, particularly for females, increasing the motivation to gamble and severity of gambling symptoms. This might constitute a serious risk factor for problematic gambling behaviors and GD in women displaying levels of high anxiety and who engage in cue-reinforced gambling, such as slot machines where the light and sound cues are truly uncertain.

Author contributions

Experiments were designed by SNH, JDL, MJFR, data was collected by SNH, JDL, analyzed, interpreted, and written by SNH, MJFR, and edited by SNH, JDL, MJFR.

Conflicts of interest

None.

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