Sex Differences in Home-Cage Ethanol Drinking and Operant Self-Administration in C57BL/6J Mice with Equivalent Regulation by Glutamate AMPAR Activity

Sara Faccidomo[1,2], Vallari R Eastman[1], Taruni S Santanam[1], Katarina S Swaim[1], Seth M Taylor and Clyde W Hodge[1,2]

[1] Bowles Center for Alcohol Studies, University of North Carolina at Chapel Hill, Chapel Hill, North Carolina 27599, USA

[2] Department of Psychiatry, University of North Carolina at Chapel Hill, Chapel Hill, North Carolina 27599, USA

*** Correspondence:**
Clyde W Hodge
chodge@med.unc.edu

Keywords: Sex as a Biological Variable (SABV); Ethanol; Alcohol Drinking; Glutamate; AMPA Receptors; Operant Self-Administration

Abstract

Introduction: Considering sex as a biological variable (SABV) in preclinical research can enhance understanding of the neurobiology of alcohol use disorder (AUD). However, the behavioral and neural mechanisms underlying sex-specific differences remain unclear. This study aims to elucidate SABV in ethanol (EtOH) consumption by evaluating its reinforcing effects and regulation by glutamate AMPA receptor activity in male and female mice.

Methods: C57BL/6J mice (male and female) were assessed for EtOH intake under continuous and limited access conditions in the home cage. Acute sensitivity to EtOH sedation and blood clearance were evaluated as potential modifying factors. Motivation to consume EtOH was measured using operant self-administration procedures. Sex-specific differences in neural regulation of EtOH reinforcement were examined by testing the effects of a glutamate AMPA receptor antagonist on operant EtOH self-administration.

Results: Female C57BL/6J mice exhibited a time-dependent escalation in EtOH intake under both continuous and limited access conditions. They were less sensitive to EtOH sedation and had lower blood levels post-EtOH administration (4 g/kg) despite similar clearance rates. Females also showed increased operant EtOH self-administration and progressive ratio performance over a 30-day baseline period compared to males. The AMPAR antagonist GYKI 52466 (0–10 mg/kg, IP) dose-dependently reduced EtOH-reinforced lever pressing in both sexes, with no differences in potency or efficacy.

Discussion: These findings confirm that female C57BL/6J mice consume more EtOH than males in home-cage conditions and exhibit reduced acute sedation, potentially contributing to higher EtOH intake. Females demonstrated increased operant EtOH self-administration and motivation, indicating higher reinforcing efficacy. The lack of sex differences in the relative effects of GYKI 52466 suggests that AMPAR activity is equally required for EtOH reinforcement in both sexes.

1. Introduction

The consideration of sex as a biological variable (SABV) in alcohol research is of paramount importance. Despite the increasing recognition of sex differences in alcohol consumption and susceptibility to alcohol use disorder (AUD), preclinical studies have predominantly used male subjects. This male bias in alcohol research has limited our understanding of potential sex-specific mechanisms of alcohol action.

Preclinical findings suggest that female rodents may be more vulnerable to the reinforcing effects of alcohol (Sneddon et al., 2020), which could contribute to escalated alcohol consumption, diminished withdrawal symptoms, and increased stress-induced reinstatement (Becker and Koob, 2016). For example, female mice consistently consume more alcohol per body weight than males under standard limited- and continuous-access procedures (Jury et al., 2017; Radke et al., 2021; Perry et al., 2023; Rivera-Irizarry et al., 2023; Szumlinski et al., 2023; Magee et al., 2024) but this is not found in all studies (reviewed by (Radke et al., 2021)). This sex difference in alcohol drinking is influenced by environmental conditions and may be enhanced by alcohol concentration, stress, and duration of exposure. However, despite these and other findings, the mechanisms underlying sex differences in alcohol consumption remain to be fully characterized. Therefore, there is a pressing need for research that explicitly addresses SABV in the motivation to consume alcohol. Such research could provide valuable insights into the sex-specific neurobiology of AUD and inform the development of treatments (Becker et al., 2017).

Recent research has focused on α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors (AMPAR), a subtype of ionotropic glutamate receptors that have been identified as key players in the reinforcing properties of alcohol (Sanchis-Segura et al., 2006; Cannady et al., 2013; Faccidomo et al., 2020; Faccidomo et al., 2021; Hoffman et al., 2021; Hoffman et al., 2022; Hoffman et al., 2023). Ethanol consumption modulates the function and expression of AMPA receptors in various brain regions associated with reward and addiction, including the nucleus accumbens and the amygdala (Marty and Spigelman, 2012; Wang et al., 2012; Salling et al., 2016; Faccidomo et al., 2021). Specifically, the positive reinforcing effects of alcohol require the synaptic insertion of GluA1-containing AMPA receptors (Faccidomo et al., 2021). Importantly, systemic pharmacological manipulation of AMPA receptors, such as the use of AMPA receptor antagonists, has been shown to reduce alcohol consumption and relapse in animal models of AUD (Backstrom & Hyytia, 2004). Thus, AMPA receptors represent a critical mechanism underlying the reinforcing properties of alcohol and a promising target for the treatment of AUD. More research is needed regarding the role of AMPARs as a potential regulator of the sex-specific reinforcing effects of alcohol.

The present preclinical study had three primary goals that address the role of SABV in the motivation to consume alcohol. First, we sought to clarify divergent findings in the literature regarding differential EtOH drinking by male and female mice. To address this objective, we evaluated EtOH intake by male and female C57BL/6J mice using standard home-cage continuous access (Hodge et al., 1999; Hodge et al., 2004) and limited access (Thiele and Navarro, 2014; Agoglia et al., 2015a; Agoglia et al., 2015b) methods. Second, to address the role of SABV in the motivation to self-administer alcohol, we utilized a well-characterized method of operant alcohol self-administration to evaluate the positive reinforcing aspects of alcohol under free access and progressive ratio testing (Besheer et al., 2008b; Faccidomo et al., 2009). Third, we evaluated the potential sex-specific role of AMPAR activity as a neurobiological mechanism of alcohol's positive reinforcing effects. Incorporating SABV into research that addresses the motivation to seek and consume alcohol has potential to improve the translational relevance of preclinical findings to both sexes.

2. Materials and methods

2.1 Subjects

Ten-week-old male and female C57BL/6J mice were obtained from Jackson Laboratory (Bar Harbor, ME) and were either group housed (operant self-administration experiment)- or single-housed (home-cage drinking experiments) upon arrival in clear Techniplast cages (28 x 17 x 14 cm). Group-housed ages were lined with corn cob bedding and a triangular red hut and cotton nestlet was provided for environmental enrichment. Enrichment was removed from cages for home-cage drinking studies because the presence of the nestlets and huts interferes with the accuracy of home cage drinking measurements (ie: clogged and climbing on sipper tubes). For all mice, standard rodent chow (Prolab® IsoPro® RMH 3000) and water were available ad libitum. The vivarium was maintained on a reverse light/dark cycle (12:12; lights off at 0700) and temperature and humidity were maintained at 21 + 1°C and 40 + 2%, respectively. All procedures were conducted in accordance with the Institutional Care and Use Committee at the University of North Carolina-Chapel Hill and the Guide for the Care and Use of Laboratory Animals (Committee for the Update of the Guide for the Care and Use of Laboratory Animals, 2011).

2.2 Continuous access two-bottle choice ethanol drinking

Male and female mice (n=12, n=6/group) were singly housed upon arrival for 2 weeks prior to initiation of home-cage alcohol drinking studies. These voluntary drinking studies were conducted as previously reported in (Hodge et al. 1999; Holstein et al. 2011, Stevenson et al. 2009, Salling et al. 2016, Faccidomo et al. 2018; Hoffman et al. 2019). Mice were given 24-h access to two drinking tubes (10 mL, 0.1 graduation intervals) containing ethanol (6-20% w/v) and water, respectively. The drinking tubes were constructed using a 10mL stripette, stainless steel ball-bearing sipper tube and rubber stopper such that small changes in volume could be recorded without removal from the cage during time course studies. The concentration of ethanol gradually increased, in 2-day increments, from 6-10-20%, and was maintained at 20% from day 5 to the end of the experiment. Fluid consumption was measured every 24-h and the position of the tubes was alternated daily to control for potential side preference. The timecourse of ethanol drinking was measured at 6 timepoints (7:00 AM – 7:00 PM) during the 12 h dark cycle on days 16 and 27 of the continuous access procedure to assess potential time-dependent changes in the diurnal pattern of intake. Dependent measures included volume of fluid consumed, ethanol dose (g/kg/day) and ethanol preference.

2.3 Limited access binge-like ethanol drinking

Following the 24-hr self-administration studies, mice were given 3 weeks of abstinence prior to initiation of a limited access self-administration study. For this protocol, mice were given one 10mL tube of either water or 20% (w/v) alcohol for 4 hrs (1000-1400) on Monday, Wednesday and Friday to model intermittent binge access of alcohol. Water was freely available to all mice on non-binge drinking days and times. After 6 weeks of intermittent binge access to alcohol (17 sessions), blood samples were collected immediately after the 17th session via submandibular bleed to measure blood ethanol concentration (BEC) as described in Section 2.4.

2.4 Blood ethanol measurement

Whole blood samples were collected as described in experiments, placed on ice, and centrifuged for phase separation of plasma and blood cells. Plasma aliquots (5 µl) were then injected into an Analox AM1 Alcohol Analyzer (Analox Technologies, Atlanta, USA) for quantification of BEC.

2.5 Acute ethanol effects

2.5.1 EtOH-induced loss of righting reflex

Loss of righting reflex (LORR), an indicator of the sedative/hypnotic properties of drugs, is defined as a mouse's inability to right itself (flip from supine to standing on all four paws) within 30 seconds. Male (n=12) and female (n=12) mice received an intraperitoneal sedative dose of ethanol (4 g/kg, IP). After injection, mice were placed on their backs in a V-shaped trough until LORR was observed as previously described (Hodge et al., 1999; Sharko and Hodge, 2008). The onset of LORR was recorded, and continuous monitoring was conducted until the righting reflex was regained, defined as the mouse righting itself three times within one minute. Two female mice were excluded from the analysis due to failure to exhibit LORR.

2.5.2 Blood ethanol clearance

To assess ethanol clearance, male and female mice (n=24, n=6/condition) were administered acute EtOH (4 g/kg, IP). Tail blood was collected from each animal using heparinized capillary tubes at the following minute timepoints following injection: 10, 30, 60, 120, 180, and 240. BEC was measured through analysis of 5uL of plasma with an Analox apparatus as described above (Section 2.4).

2.6 Operant Ethanol Self-Administration

2.6.1 Apparatus

Operant self-administration sessions were conducted in Plexiglas mouse operant-conditioning chambers (Med Associates, Fairfax, VT) as previously described (Hodge et al., 2006; Faccidomo et al., 2009). The left and right wall of each operant chamber contained one ultrasensitive stainless steel response lever and a syringe pump liquid delivery system. Ethanol solutions were maintained in 60-ml syringes mounted on a programmable pump (PHM-100, Med Associates), which delivered 0.014 ml per activation into a recessed stainless-steel receptacle located to the left of the associated response lever. A photobeam detection system spanned each receptacle area to record head entries into the drinking cup upon beam interruption. Each chamber also contained a house-light and a stimulus light located above each lever (which was activated each time the lever was pressed). The operant-conditioning chambers were interfaced (Med Associates) to a Windows-compatible PC, which was programmed to record all lever presses and liquid deliveries.

2.6.2 Operant Training and Acquisition

One week after arrival, experimentally naïve group-housed male and female C57BL/6J mice (n=16, n=8/condition) were given 24-h access to two bottles in the home cage, with one containing sweetened ethanol (EtOH 9% v/v + sucrose 2% w/v) and the other containing water, for two weeks before operant conditioning training.

After two weeks of home-cage exposure, mice were water-restricted for 23 h to promote auto-shaping of lever pressing during four overnight training sessions (16 h each, 1730-0930). During these sessions, lever presses on the active lever activated the syringe pump, delivering ethanol solution into the liquid receptacle. Presses on the inactive lever were recorded but had no programmed consequences. The location of the active and inactive levers was randomly assigned and counterbalanced within each group. Responses during reinforcer delivery were recorded but did not count towards the response requirement (time-out period). Reinforcement delivery was accompanied by brief illumination of the cue light (800 ms) and pump sound. Drinking receptacles were checked

171 at the end of each session to verify consumption. The chambers and pumps were connected to an
172 interface and computer that recorded the behavioral data (MED-PC for Windows v.4.1).

173 Within training sessions, the reinforcement schedule increased incrementally from fixed-ratio 1
174 (FR1) to FR2 and finally to FR4 after 25 ratios at each level. Once each mouse completed the initial
175 training sequence, responding was maintained on an FR4 schedule for the remainder of the
176 experiment. Following the four overnight training sessions, ethanol self-administration was measured
177 during 30 daily baseline sessions. Each session lasted 1 h in duration and was conducted during the
178 dark phase of the 12-h light-dark cycle, 5 days per week.

179 **2.6.3 Progressive Ratio Testing**

180 Following the 30 d baseline phase, mice were tested for performance on a progressive ratio (PR)
181 schedule of reinforcement as an index of motivation or reinforcer strength (Hodos, 1961; Markou et
182 al., 1993; Richardson and Roberts, 1996; Stafford et al., 1998) as previously described by our group
183 (Besheer et al., 2008b; Faccidomo et al., 2009; Hoffman et al., 2019). Briefly, the response
184 requirement during a PR test session began at FR 4 then increased arithmetically by +3 as the mouse
185 earned a reinforcer at each response requirement (i.e.; response requirement of FR 4 for 1st
186 reinforcer, FR 7 for 2nd reinforcer, FR 10 for the 3rd reinforcer, etc). The session ended when a 15-
187 min period occurred without reinforcement. PR "breakpoint" was defined as the time at which the
188 last response occurred. Session duration (min) and number of headpokes into the fluid receptacle
189 were also measured as indices of motivation under the PR schedule (Ihara et al., 2023).

190 **2.6.4 Effects of GYKI 52466 on operant alcohol self-administration**

191 Male and female mice with a history of alcohol self-administration (n=16; n=8/condition) were
192 habituated to injections of 0.9% saline. Injections were given 30 minutes prior to the start of the
193 operant session with a minimum of 3 operant sessions between injections. Initially, habituation
194 injections were administered until self-administration following injection stabilized (4-6 injections).
195 Once responding was stable, vehicle and 4 doses of GYKI 52466 dihydrochloride (0.3, 1.0, 5.6, and
196 10.0 mg/kg, i.p.; Tocris Bioscience) were administered using a counterbalanced design. A maximum
197 of two injections per week per animal were given so that responding returned to baseline following
198 drug administration.

199 **2.7 Locomotor Testing + GYKI-52466 Dose Effect Curve**

200 After operant self-administration, mice were tested for non-specific locomotor effects of GYKI-
201 52466 using computer-controlled open field chambers (27 cm x 27 cm x 20 cm; ENV-510, Med
202 Associates) as previously reported (Riday et al., 2012; Agoglia et al., 2015b; Faccidomo et al., 2015;
203 Stevenson et al., 2019; Faccidomo et al., 2020). X-Y ambulatory movements were recorded with two
204 sets of 16 pulse-modulated infrared photobeams, assessing the mouse's position every 60 s to
205 quantify distance traveled (cm). Male and female mice with a history of ethanol self-administration
206 were habituated to the open field apparatus for 2 h. One week later, mice were administered the drug
207 and returned to their home cage for 30 minutes before being placed in the open field apparatus for 1
208 h. Vehicle and two doses of GYKI-52466 (5.6 and 10.0 mg/kg, IP) were administered in a counter-
209 balanced design, with at least one week between tests. Due to technical issues with two of the open
210 field boxes, 2 male mice were excluded from the final analysis. For this reason, a secondary analysis
211 of GYKI-52466, with male and female mice combined, was added to the results.

212

2.8 Drugs

Ethanol solutions for self-administration were prepared by mixing ethanol (95% v/v) with sucrose (w/v) to create a combined solution. For injections, ethanol (95% v/v) was mixed with 0.9% NaCl to create a 20% ethanol solution (w/v). The noncompetitive AMPAR antagonist GYKI 52466 [4-(8-Methyl-9H-1,3-dioxolo[4,5-h][2,3]benzodiazepin-5-yl)-benzenamine dihydrochloride] was purchased from Tocris (R&D Systems, Minneapolis, MN) and dissolved in a vehicle of 0.9% NaCl for intraperitoneal (IP) administration in mice at doses of (0.0, 0.3, 1.0, 5.6, and 10 mg/kg).

2.9 Data analysis

All data were analyzed and plotted using GraphPad Prism version 10.3.1 (GraphPad Software, Boston, MA). Data were expressed as group means ± standard error of the mean (SEM). Statistical analyses were performed using unpaired t-test, analysis of variance (ANOVA), or repeated-measures ANOVA (RM-ANOVA) where appropriate. In situations where individual data points were omitted (e.g., spillage from drinking tubes), results were analyzed by fitting a mixed-effects model using Restricted Maximum Likelihood (REML). Post-hoc comparisons to evaluate specific differences were conducted using Šídák's multiple comparisons test to control familywise error rates, or Fisher's LSD test when conducting 4-family pairwise comparisons as implemented in GraphPad Prism. Simple linear regression with two-tailed comparison of slopes and intercepts was used to evaluate sex differences in blood ethanol clearance and change in baseline operant ethanol self-administration over time. Sex was represented as an independent variable in all experiments. Parameters analyzed and specific statistical tests employed are detailed within each respective results section.

3. Results

3.1 Home-cage ethanol intake

3.1.1 Continuous access two-bottle choice ethanol drinking

As an initial strategy for exploring potential sex differences in the motivation to consume ethanol, we first evaluated voluntary ethanol drinking under continuous access, two-bottle choice conditions. Mice were first exposed to two bottles, one containing water and the other containing an ascending range of ethanol (6 – 20% v/v) concentrations for two days at each value (**Fig 1a**). Then, ethanol (20% v/v) vs water intake was measured during a chronic access phase from days 7 – 50 (**Fig 1a**). Male and female mice showed continued growth during the 50-day experimental procedure (**Fig 1b**).

Exposure to ascending concentrations of ethanol produced a concentration-dependent increase in ethanol intake ($F (1.719, 17.19) = 55.89F$, $P<0.0001$) in the absence of a main effect of sex or a sex x concentration interaction (**Fig 1c, left**). Ethanol preference exhibited an inverted-u shaped function (**Fig 1c, middle**) that also showed a significant effect of ethanol concentration ($F (1.802, 18.02) = 8.559$, $P=0.003$) but no effect of sex and no interaction. Water intake varied with corresponding changes in available ethanol concentration ($F (1.985, 19.85) = 16.37$, $P<0.0001$) according to a u-shaped function (**Fig 1c, right**), also in the absence of sex effects. Overall, there were no sex-specific differences in ethanol or water intake during initial exposure to ascending ethanol concentrations.

By contrast, during the chronic access phase, when ethanol(20% v/v) vs water was available, ethanol intake (g/kg/24-h) diverged in a sex-specific manner and resulted in a significant main effect for time $F (36, 358) = 4.440$, $P<0.0001$) and sex ($F (1, 10) = 29.88$, $P=0.0003$), and a time x sex interaction $F (36, 358) = 2.318$, $P<0.0001$) with female mice showing significantly greater intake than males on most days of the 50-day procedure (**Fig 1d**). Ethanol preference also showed a sex-specific

divergence during the chronic access period with main effects for time ($F(36, 354) = 3.681$, $P<0.0001$) and sex ($F(1, 10) = 8.847$, $P=0.014$) and a significant time x sex interaction ($F(36, 354) = 1.666$, $P=0.01$). Overall, male mice did not prefer ethanol over water, with relative intake (preference) remaining below 50%. Ethanol preference emerged (relative intake >50%) in female mice during the final week (**Fig 1e**).

To further investigate the time-dependent emergence of sex differences, we measured ethanol intake at 6 timepoints during the 12-hr reverse light cycle (lights off: 7:00 AM – 7:00 PM) on days 16 and 27 of exposure (**Fig 1f and 1g**). Three-way RM-ANOVA identified significant main effects for time of day ($F(5, 60) = 14.49$, $P<0.0001$), treatment day ($F(1, 57) = 71.14$, $P<0.0001$), and sex ($F(1, 60) = 40.28$, $P<0.0001$), and a treatment day by sex interaction ($F(1, 57) = 11.89$, $P<0.001$).

Separate two-way RM-ANOVA analyses were then conducted on Day 16 and Day 27 to evaluate potential experience-dependent changes in the timecourse of ethanol intake. On day 16, there was a significant effect of time of day ($F(5, 47) = 36.4$, $P<0.0001$) and an interaction between time of day and sex ($F(5, 47) = 2.9$, $P=0.02$); however, there was no significant effect of sex alone. By contrast, on day 27, there were significant effects of both time of day ($F(5, 50) = 85.82$) and sex ($F(1, 10) = 10.75$) as well as an interaction ($F(5, 50) = 12.81$, $P<0.0001$). Follow-up multiple comparison analyses of the interactions showed that female mice exhibited greater intake that males during the early portion of the dark cycle (7:00 AM – 1:00 PM) to a greater extent on day 27 than on day 16 (**Fig 1f and 1g)**, consistent with greater motivation to seek ethanol during the most active phase of the light-dark cycle.

3.1.2 Limited access ethanol drinking

To examine potential sex differences in EtOH drinking more comprehensively, we measured voluntary drinking under limited access, two-bottle choice conditions. Mice were given access to two bottles, one containing water and the other containing ethanol (20% v/v) for 4-h per day, three days per week over a 6-week period (**Fig 2a**). Blood ethanol concentration (BEC) was measured following the final session (**Fig 2a**).

There was no initial sex-dependent difference in ethanol (g/kg) intake; however, consumption patterns diverged, and female mice consumed significantly more ethanol (g/kg) that male mice during the 6-week exposure period. A Two-way (sex x exposure day) RM-ANOVA conducted on ethanol intake (g/kg/4-h) identified a significant main effect of sex ($F(1, 10) = 33.4$, $P=0.0002$) but no effect of day and no interaction (**Fig 2b**). Planned multiple comparisons between male and female mice showed that there were no sex-dependent differences on exposure days 1 – 3 but female mice consumed significantly more ethanol during 8 of the remaining 14 days (**Fig 2b**), suggesting an experience-dependent escalation of limited access ethanol intake by female mice.

To evaluate sex-dependent differences in the rate of ethanol intake (g/kg) over time, daily ethanol intake (g/kg) was plotted as a cumulative function of exposure days (**Fig 2c**). RM-ANOVA identified significant main effects of sex ($F(1, 10) = 37.5$, $P<0.0001$) and time ($F(16, 160) = 413.8$, $P<0.0001$) as well as a sex x time interaction ($F(16, 160) = 27.9$, $P<0.0001$). Multiple comparison analysis showed that the interaction was driven by an increase in cumulative ethanol intake by female mice that emerged on exposure day 6 (**Fig 2c**). Water intake (mls) did not differ between male and female mice when evaluated on an individual daily (**Fig 2d**) or cumulative (**Fig 2e**) basis. Thus, female mice showed elevated ethanol preference (EtOH mls / total fluid) as compared to male ($F(1, 10) = 5.519$, $P=0.0407$) over the course of the 17 exposure days, but neither sex preferred ethanol over water as evidenced by preference ratios remaining mostly below 50% (**Fig 2f**).

299 Finally, blood ethanol concentration (BEC) was measured immediately after the final exposure
300 session. Female mice consumed significantly more ethanol (g/kg/4-h) than male mice
301 **(**t(9)=3.16,P=0.006) and showed a corresponding elevation in BEC (t(9)=4.5,P=0.0007) as compared
302 to males (**Fig 2g**).

303 **3.2 Acute sensitivity to ethanol sedation**

304 Altered risk of developing AUD in humans and differential ethanol intake in rodent models is
305 associated with altered sensitivity to acute effects of ethanol (Schuckit, 1994; Hodge et al., 1999;
306 Ueno et al., 2001). When tested for sedative response to acute ethanol (4 g/kg, IP), female mice
307 showed an equivalent onset of the LORR (**Fig 3a**) but demonstrated a significant decrease in the
308 duration of the LORR (t=1.767, df=20, P=0.046) as compared to males (**Fig 3b**).

309 **3.3 Blood ethanol clearance**

310 Because differential absorption, distribution or clearance of ethanol may contribute to reduced acute
311 sensitivity observed in female C57BL/6J mice, blood ethanol concentrations were measured 10 – 240
312 min following ethanol (4 g/kg, IP) administration. RM-ANOVA identified significant main effects of
313 time (F (5, 103) = 14.19, P<0.0001) and sex (F (1, 22) = 5.422, P=0.03) but no interaction between
314 sex and time (F (5, 103) = 0.8931, P=0.48).

315 Visual inspection of the blood ethanol curve showed that male and female levels were identical at 10-
316 min and peaked at 30-min post injection (**Fig 3c**). However, the two curves diverged from 30 – 240
317 min with females exhibiting a consistently lower trend (**Fig 3c**). Linear regression of the descending
318 limb of the BEC curves (30 – 240 min) found a significant difference between the male (Y = -
319 0.7445*X + 415.0) and female (Y = -0.8021*X + 374.6) Y-intercepts (F (1,110) = 10.75, P =0.0014)
320 consistent with reduced blood ethanol absorption in females. However, there was no significant
321 difference in the slopes (F(1,109) = 0.09, P=0.76) suggesting equivalent rates of blood ethanol
322 clearance in male and female mice.

323 **3.4 Positive reinforcing effects of ethanol**

324 To evaluate the positive reinforcing effects of ethanol, male and female C57BL/6J mice were trained
325 to lever press for a sweetened ethanol solution in operant conditioning chambers during 4 initial
326 overnight (16 h) Acquisition sessions. Following training, behavior was recorded during a 30-d
327 period followed by progressive ratio (PR3) testing (**Fig 4a**). Results showed that male and female
328 mice exhibited sex-specific differential patterns of ethanol reinforced responding during each phase
329 of testing.

330 During acquisition, two-way RM-ANOVA found no main effect of sex or time but identified a
331 significant time x sex interaction (F (3, 42) = 7.965, P=0.00030). Follow-up comparisons within sex
332 showed that the male mice exhibited a significant increase in ethanol reinforced lever pressing as a
333 function of time with female mice showing no time-dependent increase (**Fig 4b**). RM-ANOVA
334 across the 30-day baseline showed no main effects of sex or time, and no interaction. However, linear
335 regression revealed a significant positive slope for ethanol-reinforced responding in female mice (Y
336 = 1.172X + 65.80, F(1,238) = 15.24, P < 0.0001), indicating an increasing trend. In contrast, male
337 mice had a flat function (Y = 0.2185X + 57.59) with a non-significant slope. The slopes were
338 significantly different (F(1,476) = 5.7, P = 0.017), indicating a time-dependent increase in responding
339 only in female mice (**Fig 4c**).

340 Visual inspection of ethanol-reinforced responding during acquisition and baseline suggested
341 differential levels of variability between male and female mice. The coefficient of variation (CV),
342 calculated from the mean responses over the 4 days of acquisition (**Fig. 4b**, n=32 values per group),
343 indicated that total variability during acquisition was nearly two-fold greater in the female group
344 compared to the male group (CV male = 41.52%; CV female = 79.9%). During baseline (**Fig. 4c**,
345 n=240 values per group) this difference in variability decreased (CV male = 57.78%; CV female =
346 49.35%). Furthermore, when variability was evaluated within each individual baseline day (**Fig. 4c**,
347 n=30 values per group), the CV values were nearly identical between groups (CV male = 16.32%;
348 CV female = 16.71%), suggesting that between-subject differences within group diminished in an
349 experience-dependent manner.

350 Further, RM-ANOVA comparing the mean of week 1 baseline to week 4 showed a significant effect
351 of time (F(1, 14) = 5.289, P=0.037)) and a significant time x sex interaction (F (1, 14) = 7.458,
352 P=0.016) due to a significant differences between weeks 1 and 4 in female mice and a significant
353 difference between male and female mice during week 4 (**Fig 4d**). This summary analysis confirms
354 the female-specific change in operant ethanol self-administration that occurred over time and the
355 scatter plots further illustrate the overlapping but diverging range of individual behavior between
356 male and female groups (**Fig 4d**).

357 Ethanol intake (g/kg) showed no sex-specific differences during acquisition due to variability in
358 female responding and the programmed increase (FR1 – FR4) in lever-press requirement during the
359 4-d period (**Fig 4e**). However, RM-ANOVA of intake over time showed a significant main effect of
360 sex (F(1, 14) = 9.926, P=0.0071)) indicating an overall increase in ethanol intake by female mice
361 during the 30-d baseline. Similar to ethanol reinforced responding, linear regression analysis also
362 showed that ethanol intake (g/kg) by female mice increased over time (Y = 0.01099X + 0.8100) with
363 a slope significantly different from zero (F(1,238)=9.5,P=. 0023)) and that the slopes of the male and
364 female regression lines were significantly different from each other (F(1,476)=5.4,P=0.02)) (**Fig 4f**).
365 Comparison of week 1 and week 4 of baseline showed that ethanol intake tracked responding and
366 exhibited a significant effect of sex (F(1, 14) = 9.159, P=0.0091)) and a significant time x sex
367 interaction (F(1, 14) = 4.769, P=0.0465)) with post hoc comparisons confirming the time-dependent
368 increase in females and the sex specific difference in ethanol intake (g/kg) during week 4 (**Fig 4g**).
369 These summary data further illustrate the female-specific experience-dependent increase in ethanol
370 intake (g/kg) as well the shifting range of individual intake values shown in scatter plots (**Fig 4g**).

371 **3.5 Motivation to self-administer ethanol**

372 Motivation to self-administer the ethanol solution was assessed directly under a progressive ratio – 3
373 (PR3) schedule of reinforcement. Results showed that female mice exhibited a strong trend for an
374 increase in PR breakpoint (t(14)=1.63,P=0.0627)) (**Fig 4h**) as well as corresponding significant
375 increases in PR session duration (t(14)=2.1,P=0.027) and number of headpokes per session
376 (t(12)=2.02, P=0.033) (**Fig 4i – j**). Individual data points for PR responding variables showed a few
377 female mice having higher breakpoint, duration and headpokes during the session. We have kept
378 these mice in the data set because they were not statistical outliers but irrespective of their higher
379 values, it is apparent when you look at the range of values for all dependent variables, female mice
380 show greater motivation to self-administer ethanol than male mice. Furthermore, the calculated effect
381 size for these data sets exceeds 0.7 so we are confident in our interpretation of the results.

382

383 **3.6 AMPAR regulation of ethanol reinforcement**

384 To investigate potential sex-specific regulation of the positive reinforcing effects of ethanol by
385 AMPAR activity, mice (M&F, n=8/sex) were trained to self-administer sweetened ethanol in operant
386 chambers. After establishing stable baseline performance, mice were administered the
387 noncompetitive AMPAR antagonist GYKI 52466 (0 – 10 mg/kg, IP) prior to self-administration
388 sessions (**Fig 5a**). Dose order was counterbalanced according to a Latin-Square regimen. Three male
389 mice were excluded from this phase of the experiment because they did not demonstrate stable levels
390 of responding after vehicle injection (>25% variability between repeated vehicle sessions).
391 Following EtOH self-administration testing, the highest effective doses were tested for potential
392 nonspecific effects on locomotor activity in an open field apparatus (**Fig 5a**).

393 Two-way RM-ANOVA comparing sex and dose showed that GYKI 52466 produced a significant
394 dose-dependent reduction in ethanol reinforced responding ($F(4, 44) = 12$, $P<0.0001$) but there was
395 no effect of sex and no interaction. Planned multiple comparisons showed that GYKI 52466 (5.6 and
396 10 mg/kg) reduced the number of ethanol reinforced responses as compared to vehicle in both male
397 and female mice (**Fig 5b)**. Analysis of the number of reinforcer earned also showed a significant
398 dose-dependent effect of GYKI 52466 ($F(4, 44) = 13$, $P<0.0001$) that was supported by individual
399 planned comparisons within sex (**Fig 5c**). Interestingly, RM-ANOVA conducted on ethanol intake
400 (g/kg) found a significant effect of sex ($F(1, 11) = 13$, $P=0.0041$) and GYKI 52466 ($F(4, 44) = 9.5$,
401 $P<0.0001$). Planned comparisons within sex showed that only GYKI 52466 (10 mg/kg) significantly
402 decreased ethanol intake in both sexes (**Fig 5d**).

403 Due to the different baseline levels of self-administration between male and female mice, parameters
404 of ethanol reinforced responding were transformed to percent control to evaluate the relative effects
405 of GYKI 52466 (**Fig 5e-g**). Visual inspection of the graphs showed a relatively identical degree of
406 change in ethanol self-administration between male and female mice. RM-ANOVA identified
407 significant main effects of GYKI 52466 on ethanol reinforced responses ($F(4, 44) = 12.26$,
408 $P<0.0001$)), number of ethanol reinforcers ($F(4, 44) = 5.5$, $P=0.0011$)), and ethanol intake ($F(4, 44) =$
409 4.7, $P=0.0030$)) with dose-dependent reductions in total responses and reinforcers, but not intake (**Fig**
410 **5e-g**).

411 Analysis of the temporal pattern of ethanol reinforced responding following the highest dose of
412 GYKI 52466 was conducted using two approaches. First, a two-way RM-ANOVA was performed on
413 raw response totals collected in 5-minute bins, with both Time (0 – 60 min) and GYKI 52466 (0 or
414 10 mg/kg) as within-subject factors. For males, there was a significant main effect of GYKI 52466 (F
415 $(1, 4) = 9.124$, $P=0.039$) but no effect of Time and no interaction, indicating that response totals were
416 reduced consistently throughout the session. For females, there was a significant effect of GYKI
417 52466 ($F (1, 7) = 39.78$, $P=0.0004$) and Time ($F (11, 77) = 2.876$, $P=0.0033$) but no interaction,
418 indicating a consistent reduction in responding across time. Second, ethanol-reinforced response
419 totals were converted to a cumulative distribution to assess the impact of GYKI 52466 on the
420 ongoing rate of operant behavior, which is critical to assess altered reinforcing function of ethanol.
421 Since each cumulative response value is influenced by previous values (Fitts, 2006), two-way RM-
422 ANOVAs were performed with Geisser-Greenhouse correction for violations of sphericity in
423 cumulative data (Time variable). For males, there were significant main effects of GYKI 52466 (F
424 $(1.000, 4.000) = 9.098$, $P=0.0393$, Greenhouse-Geisser $\varepsilon = 1.0$) and Time ($F (1.208, 4.832) = 13.10$,
425 $P=0.0146$, Greenhouse-Geisser $\varepsilon = 0.11$) on response rate, and a strong trend for an interaction (F
426 $(1.463, 5.852) = 5.534$, $P=0.0506$, Greenhouse-Geisser $\varepsilon = 0.133$). This is consistent with a drug-
427 induced reduction in ethanol-reinforced responding that continued at a steady rate over the session
428 (**Fig 5h**). For females, there were significant main effects of GYKI 52466 ($F (1.000, 7.000) = 27.65$,

429 P=0.0012, Greenhouse-Geisser ε = 1.0) and Time (F (1.376, 9.631) = 75.47, P<0.0001, Greenhouse-
430 Geisser ε = 0.125) and a significant GYKI x Time interaction (F (1.526, 10.68) = 5.945, P=0.0237,
431 Greenhouse-Geisser ε = 0.139). This analysis is consistent with a time-dependent reduction of
432 ethanol-reinforced response rate in females (**Fig 5h**).

433 Finally, to investigate potential nonspecific motor effects of AMPAR inhibition, open-field
434 locomotor activity was measured following GYKI 52466 (0, 5.6, and 10 mg/kg, IP) during separate
435 behavioral tests. A two-way RM ANOVA found no effect on spontaneous locomotor activity during
436 1 h sessions (**Fig 5i**). Because the n was low in the male group, a secondary analysis was conducted
437 in which we collapsed sex as a factor and ran a RM one-way ANOVA on the effects of GYKI 52466
438 on open field locomotor activity. Similarly, we found no effect on 1 hr locomotor activity in the open
439 field.

440 **4. Discussion**

441 **4.1 Home-cage ethanol intake**

442 Growing evidence indicates that female rodents consume more ethanol under home-cage drinking
443 conditions than males; however, this is not found in all studies (Radke et al., 2021). Thus, in the
444 present study, we first sought to compare ethanol intake by male and female C57BL/6J mice under
445 multiple exposure conditions to add further information to this growing area of research, and to gain
446 internal data to complement potential sex-specific differences in the positive reinforcing and
447 motivational properties of ethanol.

448 Results showed that female C57BL/6J mice consumed more ethanol than males during chronic
449 exposure conditions in both the continuous and limited access experiments. However, it is important
450 to note that there was no sex-specific difference in ethanol intake or preference during initial
451 exposure periods, including during an ethanol concentration manipulation in the continuous access
452 study. In both experiments, female mice exhibited a time-dependent increase in ethanol (20% v/v)
453 intake (g/kg) that emerged after 3 days of exposure, resulting in significantly higher blood ethanol
454 concentration as compared to males.

455 These results replicate prior studies and are consistent with evidence showing overall greater
456 voluntary home-cage drinking by female C57BL/6J mice (Jury et al., 2017) and with the absence of a
457 sex-specific difference during initial exposure followed by an escalation in female C57BL/6J mice
458 over time (Sneddon et al., 2019; Magee et al., 2024). Our analysis of the temporal pattern of ethanol
459 intake showed that the female escalation emerged during continuous access primarily in the early
460 portion of the 12-h dark cycle, which is a period of peak motivation in rodents. This finding is
461 particularly intriguing and suggests that sex-specific mechanisms may interact with motivational
462 factors that drive experience-dependent escalations in drinking.

463 **4.2 Acute sensitivity to ethanol sedation and blood ethanol clearance**

464 Results of the present study also highlight significant sex differences in acute response to ethanol
465 sedation and blood ethanol levels following acute ethanol (4 g/kg, IP) administration in C57BL/6J
466 mice. We found that female mice exhibited a shorter duration of the ethanol-induced LORR
467 compared to males, despite an equivalent onset. Reduced sensitivity to ethanol sedation was
468 associated with a significant main effect of sex on blood ethanol (4 g/kg, IP) levels, with females
469 displaying consistently lower ethanol levels from 30 to 240 minutes post-injection. Regression
470 analysis further supported this finding, showing a significant difference in the intercepts of the BEC

curves between males and females, suggesting a lower peak BEC in females. Despite these differences, the rate of ethanol clearance, as measured by comparing slopes of the regression lines, was not different between sexes. Overall, this pattern of results suggests that the observed sex-specific differences in acute ethanol sensitivity may be influenced by differential EtOH absorption following IP injection.

By contrast, blood samples taken immediately after the last limited access session (Day 17) showed an approximate 4-fold increase in female BEC as compared to male mice. This occurred despite the overall ethanol intake (g/kg) by female mice being only 3-fold higher than males. The reason for this relative difference in BEC is unclear but may reflect sex-specific differences in first-pass ethanol metabolism. Evidence indicates that women exhibit elevated BECs as compared to men following oral consumption of alcohol due to a 77 percent reduction in first-pass metabolism and a 59 percent decrease in gastric alcohol dehydrogenase activity (Frezza et al., 1990; Baraona et al., 2001). Similarly, female C57BL/6J mice exhibit higher BECs than males following gastric intubation due to reduced first-pass metabolism (Desroches et al., 1995). Importantly, this sex-specific increase observed in female mice was inverted following IP injection of ethanol, with females mice showing reduced BEC levels at 30-min post injection (Desroches et al., 1995). This is consistent with results of the present study and supports the conclusion that the sex-specific differences in BEC observed in the present study following IP injection and oral consumption may reflect differential first-pass ethanol metabolism.

It is also plausible that sex-specific difference in BEC measured in the present study after limited access ethanol drinking may have been influenced by differences in the temporal pattern of ethanol intake by male and female mice. That is, male mice might have consumed most of their ethanol during the early period of the 4-hr home-cage access, which could result in the recorded range of BECs found in males (mean = 26.5 mg/dL) after hours of clearance. Alternatively, female mice may have consumed significant amounts of ethanol near the end of the access period achieving the recorded BEC (mean = 100.2 mg/dL). Thus, the observed difference in BEC in our study may have been influenced by the timing of ethanol intake or by reduced first-pass metabolism in female as compared to male mice.

Overall, these preclinical findings enhance our understanding of sex differences in alcohol drinking behaviors because the reduced sensitivity to ethanol's sedative effects in females may contribute to higher alcohol consumption to achieve pharmacological effects (Hodge et al., 1999; Ueno et al., 2001), potentially increasing the risk of developing AUD (Schuckit, 1988; 1994). Additionally, the difference in ethanol metabolism could contribute to escalated intake, influence drinking patterns and tolerance development. Understanding these physiological differences is crucial for developing targeted prevention and treatment strategies for AUD that consider sex-specific vulnerabilities and responses to alcohol

4.3 Positive reinforcing effects of ethanol

The findings from this study provide significant insights into the sex-specific reinforcing effects of ethanol in C57BL/6J mice, highlighting important differences in ethanol consumption and motivation between male and female mice. The observed increase in ethanol-reinforced responding and intake over time in female mice, contrasted with the stable patterns in male mice, underscores the potential influence of sex on the time-dependent escalation of ethanol reinforcement mechanisms that occurs during the development of AUD.

514 In addition to the sex-specific differential escalation of operant ethanol reinforced responding, results
515 of this study also identified an experience-dependent reduction in between-subject differences within
516 each sex, with initial variability stabilizing over time. During acquisition, female mice displayed
517 nearly twice the variability in ethanol reinforced responding as compared to males, as indicated by
518 the CV, potentially reflecting sex-specific differences in the emergence or development of ethanol's
519 reinforcing properties. One possible contributor to the increased variability in females is the estrous
520 cycle, which has been shown to influence reinforcement sensitivity and motivated behaviors,
521 including cocaine and ethanol reinforced responding (Roberts et al., 1989; Roberts et al., 1998).
522 However, results from the present study also showed that variability decreased in both sexes after
523 acquisition and was equivalent between male and female groups during the 30-day baseline phase.
524 This finding is consistent with evidence from an array of studies across multiple drugs of abuse
525 showing that hormonal influences on drug-taking behavior in females are more pronounced during
526 initial acquisition and tend to diminish with repeated use (reviewed by (Becker and Koob, 2016)).
527 Moreover, the longitudinal reduction and convergence of variability between male and female mice
528 in ethanol reinforced responding may reflect the general conclusion from a meta-analysis of 293
529 studies showing that females are not more variable than males across a wide range of endpoints, and
530 aligns with the notion that individual variability may sometimes reflect other factors, such as housing
531 conditions, rather than intrinsic sex-based differences (Prendergast et al., 2014). Overall, these
532 findings underscore the importance of considering both sex and experience when interpreting
533 variability in reinforcement-related behaviors. Future studies could clarify these interactions by
534 testing how estrous phases may contribute to early-stage variability in reinforcement responses
535 among females, especially during acquisition, to better understand the dynamics of sex and
536 experience on ethanol reinforced behavior.

537 The progressive ratio (PR) findings, which revealed a trend toward increased PR breakpoints and
538 significant increases in both PR session duration and headpokes per session in female mice, suggest
539 heightened motivation to seek ethanol in females. PR schedules, introduced by Hodos in 1961, are
540 foundational in preclinical drug abuse research as they measure "reward strength" and "motivation"
541 by gradually increasing the response requirement for each subsequent reinforcer, ultimately
542 determining the point at which an animal stops responding (Hodos, 1961). This cessation point, or
543 breakpoint, serves as a quantitative measure of an animal's motivation to obtain a reward, offering
544 insight into the reinforcing efficacy of the drug. Consequently, PR schedules are invaluable for
545 assessing the abuse potential of various substances.

546 Recent studies emphasize that additional PR performance metrics, such as session duration and
547 exploratory behaviors like headpokes into reinforcement delivery areas, offer further insights into the
548 reinforcing properties of drugs (Ihara et al., 2023). Our findings align with this perspective, showing
549 that female mice demonstrate greater persistence and exploratory behavior during ethanol self-
550 administration under PR schedules, which may indicate enhanced reinforcing efficacy and motivation
551 for ethanol. These results contribute to the understanding of how sex differences influence motivation
552 and reinforcement in drug-seeking behavior. They also provide a basis for exploring mechanisms that
553 may drive these differences, such as the estrous cycle, which has been associated with increased PR
554 breakpoints for cocaine in both rats (Roberts et al., 1989) and non-human primates (Mello et al.,
555 2007).

556 However, it is important to note that sex differences in ethanol PR performance appear to vary by
557 strain: while Wistar rats show higher PR breakpoints in males (Toivainen et al., 2024), studies in
558 Long Evans (Randall et al., 2017) and selectively bred alcohol-preferring (P) rats (Moore and Lynch,

559 2015) have not found sex-specific differences. These strain-dependent findings highlight the
560 complexity of sex effects on ethanol reinforcement and underscore the need for further research to
561 clarify the biological and behavioral underpinnings of these differences across different genetic
562 backgrounds.

563 Translationally, these findings have significant implications for understanding AUD in humans. The
564 sex-specific differences observed in operant self-administration complement our home-cage drinking
565 data discussed above and provide further evidence that parallels clinical observations where women
566 often exhibit a faster progression to AUD (Brady and Randall, 1999; Hernandez-Avila et al., 2004;
567 Mann et al., 2005). These results suggest that women may exhibit greater sensitivity to alcohol's
568 reinforcing effects compared to men. The enhanced motivation to seek ethanol, as indicated by the
569 PR findings, further supports the notion that females may have a higher propensity for alcohol-
570 seeking behavior. By elucidating the behavioral patterns and motivational aspects of ethanol
571 consumption in a controlled animal model, this research offers valuable insights that could inform the
572 development of sex-specific prevention and treatment strategies for AUD. Understanding the
573 biological and behavioral underpinnings of these differences is crucial for creating more effective
574 interventions and improving outcomes for individuals struggling with AUD.

575 **4.4 AMPAR regulation of ethanol reinforcement**

576 The results of this study demonstrate that the noncompetitive AMPAR antagonist GYKI 52466
577 significantly reduced ethanol-reinforced responding in a dose-dependent manner in both male and
578 female mice. This finding suggests that the excitatory actions of glutamate at ionotropic AMPARs
579 plays a crucial role in the positive reinforcing effects of ethanol. The lack of sex differences in the
580 overall reduction of ethanol-reinforced responses indicates that AMPAR antagonism is equally
581 effective in both sexes, despite the significant difference in baseline levels of responding under
582 vehicle conditions. The temporal analysis of ethanol-reinforced responding revealed intriguing sex-
583 specific patterns. While the highest dose of GYKI 52466 produced a consistent reduction in response
584 rate throughout the session in male mice, female mice exhibited a delayed reduction, becoming
585 significant only after 30 minutes. This difference in temporal response suggests that female mice may
586 have a different sensitivity or adaptive response to AMPAR antagonism over time or following
587 consumption of pharmacologically detectable levels of ethanol. Understanding these temporal
588 dynamics is essential for developing targeted interventions that consider sex-specific responses to
589 pharmacological treatments for alcohol use disorders. Further research is needed to explore these
590 temporal patterns and their underlying mechanisms in greater detail.

591 These data are in agreement with prior results from our group showing that AMPAR activity is
592 required for the reinforcing effects of ethanol in both mice and rats (Cannady et al., 2013; Agoglia et
593 al., 2015a; Cannady et al., 2016; Salling et al., 2016; Faccidomo et al., 2021; Hoffman et al., 2021;
594 Hoffman et al., 2023), and complement evidence showing that GYKI 52466 has effects on ethanol-
595 seeking and relapse-like behavior in rats. In two behavioral models of ethanol craving and relapse, it
596 was observed that GYKI 52466 (0 – 10 mg/kg) dose-dependently reduced cue-induced reinstatement
597 of ethanol-seeking behavior and the ethanol deprivation effect in male Wistar rats (Sanchis-Segura et
598 al., 2006). We also found previously that cue-induced reinstatement of ethanol-seeking behavior was
599 associated with increased activation (phosphorylation) of CaMKII (e.g., p-CaMKIIα-T286) in
600 specific reward-related brain regions, which promotes activation and membrane insertion of
601 AMPARs (Salling et al., 2017). Thus, activation of the CaMKII-AMPAR molecular signaling
602 pathway may underlie abstinence and cue-induced relapse to ethanol-seeking behavior.

603 However, other data indicate that a similar dose range of GYKI 52466 had no effect on operant
604 ethanol self-administration by male Lister rats (Stephens and Brown, 1999). Although the specific
605 reason(s) for this discrepancy is unknown, it could be due to differences in experimental design, or
606 the specific animal models used. For example, that study utilized a liquid dipper system where
607 ethanol was available for consumption for only 5-seconds during each delivery. Our apparatus
608 infused ethanol into a receptacle where it remained until consumed. Thus, the effects of GYKI 52466
609 effects on operant ethanol self-administration may be context- or procedure-dependent, and further
610 research is needed to explore this possibility.

611 It is also important to consider that GYKI 52466 produced a dose-dependent but partial reduction in
612 ethanol-reinforced responses and the number of reinforcers earned, resulting in approximately a 30–
613 40% decrease in ethanol intake as compared to the vehicle control. This partial reduction suggests
614 that while AMPAR activity is necessary for the full expression of ethanol's reinforcing properties,
615 complete suppression may require a higher dose or a competitive antagonist rather than GYKI 52466,
616 which is a noncompetitive AMPAR antagonist. Additionally, this partial effect indicates that other
617 neurotransmitter systems are likely involved in ethanol reinforcement, including dopaminergic
618 (Samson et al., 1992; Hodge et al., 1997), GABAergic (Hodge et al., 1996; Hodge et al., 1999; Ueno
619 et al., 2001), and metabotropic glutamatergic (Schroeder et al., 2005; Besheer et al., 2006; Besheer et
620 al., 2008a; Salling et al., 2008) pathways. Moreover, although we did not test the effects of GYKI
621 52466 on home cage drinking, future studies could explore whether this AMPAR inhibitor might
622 similarly reduce ethanol consumption in continuous access or binge-like drinking models. Such
623 research would offer insights into the efficacy of GYKI 5246 across varied drinking contexts and
624 further elucidate the role of AMPARs role in modulating ethanol intake patterns in males and
625 females.

626 Finally, AMPAR activity is known to influence a wide range of neural functions and behaviors
627 beyond ethanol reinforcement, including learning, memory, and emotional processing (Kessels and
628 Malinow, 2009). AMPARs play a crucial role in synaptic plasticity, particularly in processes like
629 long-term potentiation (LTP), which underlie learning and memory formation (Malenka and Nicoll,
630 1999; Diering and Huganir, 2018). Given the critical role of AMPAR activity in these fundamental
631 brain and behavioral functions, the impact of AMPAR antagonism by GYKI 52466 may not be
632 confined to ethanol reinforcement. Emerging research suggests that alterations in AMPAR activity
633 can influence reinforcement from a variety of substances, including other drugs of abuse and non-
634 drug reinforcers, such as food or social interactions (Choi et al., 2011; Pitchers et al., 2012; Fetterly
635 et al., 2024; Xu et al., 2024). To more precisely understand the role of AMPAR activity in ethanol-
636 related reinforcement, a valuable follow-up study could examine effects of GYKI 52466 on sucrose
637 reinforced behavior or to examine sex-specific behavioral outcomes associated with alcohol use, such
638 as aggression (Miczek et al., 2004; Faccidomo et al., 2008) or altered emotional state (Stevenson et
639 al., 2009). This would help determine whether changes in AMPAR activity selectively affect ethanol
640 reinforcement or reflect broader alterations in motivation, reward or emotional processing.

641 **4.5 Conclusions and Implications for sex-specific AUD treatment**

642 This research underscores the critical role of AMPAR activity in the positive reinforcing effects of
643 ethanol, as evidenced by the dose-dependent reduction in ethanol-reinforced responding following
644 systemic administration of the noncompetitive AMPAR antagonist GYKI 52466. Importantly, the
645 findings highlight that AMPAR inhibition is equally effective in both male and female mice, despite
646 the nearly two-fold higher baseline levels of responding in female mice, demonstrating a consistent
647 reduction in ethanol reinforcement across sexes. under vehicle conditions. This uniform response

suggests that AMPAR antagonism could be a broadly applicable strategy for reducing ethanol reinforcement, regardless of sex or baseline level of intake.

The significance of these results also lies in their contribution to the growing body of evidence supporting AMPAR antagonists as promising therapeutic agents for AUD. By demonstrating that GYKI 52466 can reduce ethanol-seeking behavior without affecting motor function, this study provides a strong foundation for future research aimed at optimizing AMPAR-targeted treatments. The consistency of these findings with previous studies on AMPAR activity and ethanol reinforcement further validates the potential of this pharmacological strategy. It will be important to evaluate competitive AMPAR antagonists, other AMPAR modulatory mechanisms, and direct neural circuits in future studies.

In conclusion, this study advances our understanding of the neurobiological mechanisms underlying ethanol reinforcement and highlights the translational value of AMPAR inhibition in developing pharmacological treatments for AUD. The equal efficacy of AMPAR antagonism in both male and female mice emphasizes the broad applicability of this approach. Future research should focus on elucidating the specific mechanisms driving the observed sex differences and confirming the efficacy of AMPAR inhibitors in diverse experimental contexts. Such efforts will be crucial for translating these findings into clinical applications, ultimately improving outcomes for individuals struggling with AUD.

5. Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

6. Ethics statement

The animal study was approved by the University of North Carolina at Chapel Hill IACUC. The study was conducted in accordance with the local legislation and institutional requirements.

7. Author Contributions

SF: Conceptualization, Data curation, Formal analysis, Methodology, Investigation, Project administration, Software, Supervision, Validation, Visualization, Writing – original draft. Writing – review & editing. **VRE**: Investigation, Data Curation. **TSS**: Investigation, Data curation. **KSS**: Investigation, Data curation. **SMT**: Investigation, Writing – original draft. **CWH**: Funding acquisition, Conceptualization, Methodology, Formal analysis, Project administration, Resources, Software, Supervision, Validation, Writing – original draft, Writing – review & editing.

8. Funding

Funding for this research was provided by grants R01 AA028782 and P60 AA011605 to CWH and by support from the Bowles Center for Alcohol Studies at UNC Chapel Hill.

9. Acknowledgement

Panel “a” in each figure was created in BioRender. Hodge, CW. (2024) BioRender.com/a66s622

10. Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

688 **11. References**

689 Agoglia, A.E., Holstein, S.E., Reid, G., and Hodge, C.W. (2015a). CaMKIIalpha-GluA1 Activity
690 Underlies Vulnerability to Adolescent Binge Alcohol Drinking. *Alcoholism, clinical and*
691 *experimental research* 39(9)**,** 1680-1690. doi: 10.1111/acer.12819.

692 Agoglia, A.E., Sharko, A.C., Psilos, K.E., Holstein, S.E., Reid, G.T., and Hodge, C.W. (2015b).
693 Alcohol alters the activation of ERK1/2, a functional regulator of binge alcohol drinking in
694 adult C57BL/6J mice. *Alcoholism, clinical and experimental research* 39(3)**,** 463-475. doi:
695 10.1111/acer.12645.

696 Baraona, E., Abittan, C.S., Dohmen, K., Moretti, M., Pozzato, G., Chayes, Z.W., et al. (2001).
697 Gender differences in pharmacokinetics of alcohol. *Alcohol Clin Exp Res* 25(4)**,** 502-507.

698 Becker, J.B., and Koob, G.F. (2016). Sex Differences in Animal Models: Focus on Addiction.
699 *Pharmacological reviews* 68(2)**,** 242-263. doi: 10.1124/pr.115.011163.

700 Besheer, J., Faccidomo, S., Grondin, J.J., and Hodge, C.W. (2008a). Effects of mGlu1-receptor
701 blockade on ethanol self-administration in inbred alcohol-preferring rats. *Alcohol* 42(1)**,** 13-
702 20. doi: S0741-8329(07)00220-0 [pii] 10.1016/j.alcohol.2007.11.001.

703 Besheer, J., Faccidomo, S., Grondin, J.J., and Hodge, C.W. (2008b). Regulation of motivation to self-
704 administer ethanol by mGluR5 in alcohol-preferring (P) rats. *Alcohol Clin Exp Res* 32(2)**,**
705 209-221. doi: 10.1111/j.1530-0277.2007.00570.x.

706 Besheer, J., Stevenson, R.A., and Hodge, C.W. (2006). mGlu5 receptors are involved in the
707 discriminative stimulus effects of self-administered ethanol in rats. *Eur J Pharmacol* 551(1-
708 3)**,** 71-75. doi: 10.1016/j.ejphar.2006.08.071.

709 Brady, K.T., and Randall, C.L. (1999). Gender differences in substance use disorders. *Psychiatr Clin*
710 *North Am* 22(2)**,** 241-252. doi: 10.1016/s0193-953x(05)70074-5.

711 Cannady, R., Fisher, K.R., Durant, B., Besheer, J., and Hodge, C.W. (2013). Enhanced AMPA
712 receptor activity increases operant alcohol self-administration and cue-induced reinstatement.
713 *Addiction biology* 18(1)**,** 54-65. doi: 10.1111/adb.12000.

714 Cannady, R., Fisher, K.R., Graham, C., Crayle, J., Besheer, J., and Hodge, C.W. (2016). Potentiation
715 of amygdala AMPA receptor activity selectively promotes escalated alcohol self-
716 administration in a CaMKII-dependent manner. *Addiction biology* 22(3)**,** 652-664. doi:
717 10.1111/adb.12357.

718 Choi, K.H., Edwards, S., Graham, D.L., Larson, E.B., Whisler, K.N., Simmons, D., et al. (2011).
719 Reinforcement-related regulation of AMPA glutamate receptor subunits in the ventral
720 tegmental area enhances motivation for cocaine. *J Neurosci* 31(21)**,** 7927-7937. doi:
721 10.1523/JNEUROSCI.6014-10.2011.

722 Desroches, D., Orevillo, C., and Verina, D. (1995). Sex- and strain-related differences in first-pass
723 alcohol metabolism in mice. *Alcohol* 12(3)**,** 221-226. doi: 10.1016/0741-8329(94)00098-x.

724 Diering, G.H., and Huganir, R.L. (2018). The AMPA Receptor Code of Synaptic Plasticity. *Neuron*
725 100(2)**,** 314-329. doi: 10.1016/j.neuron.2018.10.018.

726 Faccidomo, S., Bannai, M., and Miczek, K.A. (2008). Escalated aggression after alcohol drinking in
727 male mice: dorsal raphe and prefrontal cortex serotonin and 5-HT(1B) receptors.
728 *Neuropsychopharmacology* 33(12)**,** 2888-2899. doi: 10.1038/npp.2008.7.

729 Faccidomo, S., Besheer, J., Stanford, P.C., and Hodge, C.W. (2009). Increased operant responding
730 for ethanol in male C57BL/6J mice: specific regulation by the ERK1/2, but not JNK, MAP
731 kinase pathway. *Psychopharmacology (Berl)* 204(1)**,** 135-147. doi: 10.1007/s00213-008-
732 1444-9.

733 Faccidomo, S., Cogan, E.S., Hon, O.J., Hoffman, J.L., Saunders, B.L., Eastman, V.R., et al. (2021).
734 Calcium-permeable AMPA receptor activity and GluA1 trafficking in the basolateral
735 amygdala regulate operant alcohol self-administration. *Addict Biol* 26(5)**,** e13049. doi:
736 10.1111/adb.13049.

737 Faccidomo, S., Holstein, S.E., Santanam, T.S., Saunders, B.L., Swaim, K.S., Reid, G.T., et al.
738 (2020). Pharmacological inhibition of glycogen synthase kinase 3 increases operant alcohol
739 self-administration in a manner associated with altered pGSK-3beta, protein interacting with
740 C kinase and GluA2 protein expression in the reward pathway of male C57BL/6J mice.
741 *Behav Pharmacol* 31(1)**,** 15-26. doi: 10.1097/FBP.0000000000000501.

742 Faccidomo, S., Salling, M.C., Galunas, C., and Hodge, C.W. (2015). Operant ethanol self-
743 administration increases extracellular-signal regulated protein kinase (ERK) phosphorylation
744 in reward-related brain regions: selective regulation of positive reinforcement in the prefrontal
745 cortex of C57BL/6J mice. *Psychopharmacology* 232(18)**,** 3417-3430. doi: 10.1007/s00213-
746 015-3993-z.

747 Fetterly, T.L., Catalfio, A.M., and Ferrario, C.R. (2024). Effects of junk-food on food-motivated
748 behavior and nucleus accumbens glutamate plasticity; insights into the mechanism of
749 calcium-permeable AMPA receptor recruitment. *Neuropharmacology* 242**,** 109772. doi:
750 10.1016/j.neuropharm.2023.109772.

751 Fitts, D.A. (2006). Misuse of ANOVA with cumulative intakes. *Appetite* 46(1)**,** 100-102. doi:
752 10.1016/j.appet.2005.09.005.

753 Frezza, M., di Padova, C., Pozzato, G., Terpin, M., Baraona, E., and Lieber, C.S. (1990). High blood
754 alcohol levels in women. The role of decreased gastric alcohol dehydrogenase activity and
755 first-pass metabolism. *N Engl J Med* 322(2)**,** 95-99. doi: 10.1056/NEJM199001113220205.

756 Hernandez-Avila, C.A., Rounsaville, B.J., and Kranzler, H.R. (2004). Opioid-, cannabis- and
757 alcohol-dependent women show more rapid progression to substance abuse treatment. *Drug*
758 *Alcohol Depend* 74(3)**,** 265-272. doi: 10.1016/j.drugalcdep.2004.02.001.

759 Hodge, C.W., Haraguchi, M., Chappelle, A.M., and Samson, H.H. (1996). Effects of ventral
760 tegmental microinjections of the GABAA agonist muscimol on self-administration of ethanol
761 and sucrose. *Pharmacol Biochem Behav* 53(4)**,** 971-977. doi: 10.1016/0091-3057(95)02146-
762 9.

763 Hodge, C.W., Kelley, S.P., Bratt, A.M., Iller, K., Schroeder, J.P., and Besheer, J. (2004). 5-HT(3A)
764 receptor subunit is required for 5-HT3 antagonist-induced reductions in alcohol drinking.
765 *Neuropsychopharmacology* 29(10)**,** 1807-1813. doi: 10.1038/sj.npp.1300498.

766 Hodge, C.W., Mehmert, K.K., Kelley, S.P., McMahon, T., Haywood, A., Olive, M.F., et al. (1999).
767 Supersensitivity to allosteric GABA(A) receptor modulators and alcohol in mice lacking
768 PKCepsilon. *Nat Neurosci* 2(11)**,** 997-1002. doi: 10.1038/14795.

769 Hodge, C.W., Miles, M.F., Sharko, A.C., Stevenson, R.A., Hillmann, J.R., Lepoutre, V., et al.
770 (2006). The mGluR5 antagonist MPEP selectively inhibits the onset and maintenance of
771 ethanol self-administration in C57BL/6J mice. *Psychopharmacology (Berl)* 183(4)**,** 429-438.
772 doi: 10.1007/s00213-005-0217-y.

773 Hodge, C.W., Samson, H.H., and Chappelle, A.M. (1997). Alcohol self-administration: further
774 examination of the role of dopamine receptors in the nucleus accumbens. *Alcohol Clin Exp*
775 *Res* 21(6)**,** 1083-1091. doi: 00000374-199709000-00022 [pii].

776 Hodos, W. (1961). Progressive ratio as a measure of reward strength. *Science* 134(3483)**,** 943-944.
777 doi: 10.1126/science.134.3483.943.

778 Hoffman, J.L., Faccidomo, S., Kim, M., Taylor, S.M., Agoglia, A.E., May, A.M., et al. (2019).
779 Alcohol drinking exacerbates neural and behavioral pathology in the 3xTg-AD mouse model
780 of Alzheimer's disease. *Int Rev Neurobiol* 148**,** 169-230. doi: 10.1016/bs.irn.2019.10.017.

781 Hoffman, J.L., Faccidomo, S., Saunders, B.L., Taylor, S.M., Kim, M., and Hodge, C.W. (2021).
782 Inhibition of AMPA receptors (AMPARs) containing transmembrane AMPAR regulatory
783 protein gamma-8 with JNJ-55511118 shows preclinical efficacy in reducing chronic repetitive
784 alcohol self-administration. *Alcohol Clin Exp Res* 45(7)**,** 1424-1435. doi: 10.1111/acer.14639.

785 Hoffman, J.L., Faccidomo, S.P., Taylor, S.M., DeMiceli, K.G., May, A.M., Smith, E.N., et al.
786 (2022). Inhibition of AMPA receptors bound to transmembrane AMPA receptor regulatory
787 protein γ-8 (TARP γ-8) blunts the positive reinforcing properties of alcohol and sucrose in a
788 brain region-dependent manner. *bioRxiv***,** 2022.2012.2014.520457. doi:
789 10.1101/2022.12.14.520457.

790 Hoffman, J.L., Faccidomo, S.P., Taylor, S.M., DeMiceli, K.G., May, A.M., Smith, E.N., et al.
791 (2023). Negative modulation of AMPA receptors bound to transmembrane AMPA receptor
792 regulatory protein gamma-8 blunts the positive reinforcing properties of alcohol and sucrose
793 in a brain region-dependent manner in male mice. *Psychopharmacology (Berl)* 240(6)**,** 1261-
794 1273. doi: 10.1007/s00213-023-06365-z.

795 Ihara, K., Shikano, Y., Kato, S., Yagishita, S., Tanaka, K.F., and Takata, N. (2023). A reinforcement
796 learning model with choice traces for a progressive ratio schedule. *Front Behav Neurosci* 17**,**
797 1302842. doi: 10.3389/fnbeh.2023.1302842.

798 Jury, N.J., DiBerto, J.F., Kash, T.L., and Holmes, A. (2017). Sex differences in the behavioral
799 sequelae of chronic ethanol exposure. *Alcohol* 58**,** 53-60. doi: 10.1016/j.alcohol.2016.07.007.

800 Kessels, H.W., and Malinow, R. (2009). Synaptic AMPA receptor plasticity and behavior. *Neuron*
801 61(3)**,** 340-350. doi: 10.1016/j.neuron.2009.01.015.

802 Magee, S.N., Sereno, A.C., and Herman, M.A. (2024). Sex differences in basal motivated behavior,
803 chronic ethanol drinking, and amygdala activity in female and male mice. *Alcohol* 120**,** 85-97.
804 doi: 10.1016/j.alcohol.2024.06.004.

805 Malenka, R.C., and Nicoll, R.A. (1999). Long-term potentiation--a decade of progress? *Science*
806 285(5435)**,** 1870-1874. doi: 7846 [pii].

807 Mann, K., Ackermann, K., Croissant, B., Mundle, G., Nakovics, H., and Diehl, A. (2005).
808 Neuroimaging of gender differences in alcohol dependence: are women more vulnerable?
809 *Alcohol Clin Exp Res* 29(5)**,** 896-901. doi: 10.1097/01.alc.0000164376.69978.6b.

810 Markou, A., Weiss, F., Gold, L.H., Caine, S.B., Schulteis, G., and Koob, G.F. (1993). Animal models
811 of drug craving. *Psychopharmacology (Berl)* 112(2-3)**,** 163-182. doi: 10.1007/BF02244907.

812 Marty, V.N., and Spigelman, I. (2012). Long-lasting alterations in membrane properties, k(+)
813 currents, and glutamatergic synaptic currents of nucleus accumbens medium spiny neurons in
814 a rat model of alcohol dependence. *Frontiers in neuroscience* 6**,** 86. doi:
815 10.3389/fnins.2012.00086.

816 Mello, N.K., Knudson, I.M., and Mendelson, J.H. (2007). Sex and menstrual cycle effects on
817 progressive ratio measures of cocaine self-administration in cynomolgus monkeys.
818 *Neuropsychopharmacology* 32(9)**,** 1956-1966. doi: 10.1038/sj.npp.1301314.

819 Miczek, K.A., Fish, E.W., De Almeida, R.M., Faccidomo, S., and Debold, J.F. (2004). Role of
820 alcohol consumption in escalation to violence. *Ann N Y Acad Sci* 1036**,** 278-289. doi:
821 10.1196/annals.1330.018.

822 Moore, C.F., and Lynch, W.J. (2015). Alcohol preferring (P) rats as a model for examining sex
823 differences in alcohol use disorder and its treatment. *Pharmacol Biochem Behav* 132**,** 1-9.
824 doi: 10.1016/j.pbb.2015.02.014.

825 Perry, T.W., Sneddon, E.A., Reichert, A.N., Schuh, K.M., Shand, N.A., Quinn, J.J., et al. (2023).
826 Sex, but not early life stress, effects on two-bottle choice alcohol drinking behaviors in mice.
827 *bioRxiv*. doi: 10.1101/2023.01.21.524642.

828 Pitchers, K.K., Schmid, S., Di Sebastiano, A.R., Wang, X., Laviolette, S.R., Lehman, M.N., et al.
829 (2012). Natural reward experience alters AMPA and NMDA receptor distribution and
830 function in the nucleus accumbens. *PLoS One* 7(4)**,** e34700. doi:
831 10.1371/journal.pone.0034700.

832 Prendergast, B.J., Onishi, K.G., and Zucker, I. (2014). Female mice liberated for inclusion in
833 neuroscience and biomedical research. *Neurosci Biobehav Rev* 40**,** 1-5. doi:
834 10.1016/j.neubiorev.2014.01.001.

835 Radke, A.K., Sneddon, E.A., Frasier, R.M., and Hopf, F.W. (2021). Recent Perspectives on Sex
836 Differences in Compulsion-Like and Binge Alcohol Drinking. *Int J Mol Sci* 22(7). doi:
837 10.3390/ijms22073788.

838 Randall, P.A., Stewart, R.T., and Besheer, J. (2017). Sex differences in alcohol self-administration
839 and relapse-like behavior in Long-Evans rats. *Pharmacol Biochem Behav* 156**,** 1-9. doi:
840 10.1016/j.pbb.2017.03.005.

841 Richardson, N.R., and Roberts, D.C. (1996). Progressive ratio schedules in drug self-administration
842 studies in rats: a method to evaluate reinforcing efficacy. *J Neurosci Methods* 66(1)**,** 1-11.
843 doi: 10.1016/0165-0270(95)00153-0.

844 Riday, T.T., Dankoski, E.C., Krouse, M.C., Fish, E.W., Walsh, P.L., Han, J.E., et al. (2012).
845 Pathway-specific dopaminergic deficits in a mouse model of Angelman syndrome. *J Clin*
846 *Invest* 122(12)**,** 4544-4554. doi: 10.1172/JCI61888.

847 Rivera-Irizarry, J.K., Zallar, L.J., Levine, O.B., Skelly, M.J., Boyce, J.E., Barney, T., et al. (2023).
848 Sex differences in binge alcohol drinking and the behavioral consequences of protracted
849 abstinence in C57BL/6J mice. *Biol Sex Differ* 14(1)**,** 83. doi: 10.1186/s13293-023-00565-0.

850 Roberts, A.J., Smith, A.D., Weiss, F., Rivier, C., and Koob, G.F. (1998). Estrous cycle effects on
851 operant responding for ethanol in female rats. *Alcohol Clin Exp Res* 22(7)**,** 1564-1569.

852 Roberts, D.C., Bennett, S.A., and Vickers, G.J. (1989). The estrous cycle affects cocaine self-
853 administration on a progressive ratio schedule in rats. *Psychopharmacology (Berl)* 98(3)**,** 408-
854 411. doi: 10.1007/BF00451696.

855 Salling, M.C., Faccidomo, S., and Hodge, C.W. (2008). Nonselective suppression of operant ethanol
856 and sucrose self-administration by the mGluR7 positive allosteric modulator AMN082.
857 *Pharmacol Biochem Behav* 91(1)**,** 14-20. doi: S0091-3057(08)00222-0
858 [pii]10.1016/j.pbb.2008.06.006.

859 Salling, M.C., Faccidomo, S.P., Li, C., Psilos, K., Galunas, C., Spanos, M., et al. (2016). Moderate
860 Alcohol Drinking and the Amygdala Proteome: Identification and Validation of
861 Calcium/Calmodulin Dependent Kinase II and AMPA Receptor Activity as Novel Molecular
862 Mechanisms of the Positive Reinforcing Effects of Alcohol. *Biological psychiatry* 79(6)**,** 430-
863 442.

864 Salling, M.C., Hodge, C.J., Psilos, K.E., Eastman, V.R., Faccidomo, S.P., and Hodge, C.W. (2017).
865 Cue-induced reinstatement of alcohol-seeking behavior is associated with increased CaMKII
866 T286 phosphorylation in the reward pathway of mice. *Pharmacology, biochemistry, and*
867 *behavior* 163**,** 20-29. doi: 10.1016/j.pbb.2017.10.011.

868 Samson, H.H., Tolliver, G.A., Haraguchi, M., and Hodge, C.W. (1992). Alcohol self-administration:
869 role of mesolimbic dopamine. *Ann N Y Acad Sci* 654**,** 242-253.

870 Sanchis-Segura, C., Borchardt, T., Vengeliene, V., Zghoul, T., Bachteler, D., Gass, P., et al. (2006).
871 Involvement of the AMPA receptor GluR-C subunit in alcohol-seeking behavior and relapse.
872 *J Neurosci* 26(4)**,** 1231-1238. doi: 10.1523/JNEUROSCI.4237-05.2006.

873 Schroeder, J.P., Overstreet, D.H., and Hodge, C.W. (2005). The mGluR5 antagonist MPEP decreases
874 operant ethanol self-administration during maintenance and after repeated alcohol
875 deprivations in alcohol-preferring (P) rats. *Psychopharmacology (Berl)* 179(1)**,** 262-270. doi:
876 10.1007/s00213-005-2175-9.

877 Schuckit, M.A. (1988). Physiological and psychological factors as predictors of alcoholism risk.
878 *Recent Developments in Alcoholism* 6**,** 311-322.

879 Schuckit, M.A. (1994). Low level of response to alcohol as a predictor of future alcoholism. *Am J*
880 *Psychiatry* 151(2)**,** 184-189. doi: 10.1176/ajp.151.2.184.

881 Sharko, A.C., and Hodge, C.W. (2008). Differential modulation of ethanol-induced sedation and
882 hypnosis by metabotropic glutamate receptor antagonists in C57BL/6J mice. *Alcohol Clin*
883 *Exp Res* 32(1)**,** 67-76. doi: ACER554 [pii] 10.1111/j.1530-0277.2007.00554.x.

884 Sneddon, E.A., Ramsey, O.R., Thomas, A., and Radke, A.K. (2020). Increased Responding for
885 Alcohol and Resistance to Aversion in Female Mice. *Alcohol Clin Exp Res* 44(7)**,** 1400-1409.
886 doi: 10.1111/acer.14384.

887 Sneddon, E.A., White, R.D., and Radke, A.K. (2019). Sex Differences in Binge-Like and Aversion-
888 Resistant Alcohol Drinking in C57BL/6J Mice. *Alcohol Clin Exp Res* 43(2)**,** 243-249. doi:
889 10.1111/acer.13923.

890 Stafford, D., LeSage, M.G., and Glowa, J.R. (1998). Progressive-ratio schedules of drug delivery in
891 the analysis of drug self-administration: a review. *Psychopharmacology (Berl)* 139(3)**,** 169-
892 184. doi: 10.1007/s002130050702.

893 Stephens, D.N., and Brown, G. (1999). Disruption of operant oral self-administration of ethanol,
894 sucrose, and saccharin by the AMPA/kainate antagonist, NBQX, but not the AMPA
895 antagonist, GYKI 52466. *Alcohol Clin Exp Res* 23(12)**,** 1914-1920. doi: 00000374-
896 199912000-00009 [pii].

897 Stevenson, J.R., Schroeder, J.P., Nixon, K., Besheer, J., Crews, F.T., and Hodge, C.W. (2009).
898 Abstinence following alcohol drinking produces depression-like behavior and reduced
899 hippocampal neurogenesis in mice. *Neuropsychopharmacology* 34(5)**,** 1209-1222. doi:
900 npp200890 [pii]10.1038/npp.2008.90.

901 Stevenson, R.A., Hoffman, J.L., Maldonado-Devincci, A.M., Faccidomo, S., and Hodge, C.W.
902 (2019). MGluR5 activity is required for the induction of ethanol behavioral sensitization and
903 associated changes in ERK MAP kinase phosphorylation in the nucleus accumbens shell and
904 lateral habenula. *Behav Brain Res* 367**,** 19-27. doi: 10.1016/j.bbr.2019.03.038.

905 Szumlinski, K.K., Herbert, J.N., Mejia Espinoza, B., Madory, L.E., and Scudder, S.L. (2023).
906 Alcohol-drinking during later life by C57BL/6J mice induces sex- and age-dependent changes
907 in hippocampal and prefrontal cortex expression of glutamate receptors and neuropathology
908 markers. *Addict Neurosci* 7. doi: 10.1016/j.addicn.2023.100099.

909 Thiele, T.E., and Navarro, M. (2014). "Drinking in the dark" (DID) procedures: a model of binge-like
910 ethanol drinking in non-dependent mice. *Alcohol* 48(3)**,** 235-241. doi:
911 10.1016/j.alcohol.2013.08.005.

912 Toivainen, S., Xu, L., Gobbo, F., Della Valle, A., Coppola, A., Heilig, M., et al. (2024). Different
913 mechanisms underlie compulsive alcohol self-administration in male and female rats. *Biol Sex*
914 *Differ* 15(1)**,** 17. doi: 10.1186/s13293-024-00592-5.

915 Ueno, S., Harris, R.A., Messing, R.O., Sanchez-Perez, A.M., Hodge, C.W., McMahon, T., et al.
916 (2001). Alcohol actions on GABA(A) receptors: from protein structure to mouse behavior.
917 *Alcohol Clin Exp Res* 25(5 Suppl ISBRA)**,** 76S-81S. doi: 10.1097/00000374-200105051-
918 00014.

919 Wang, J., Ben Hamida, S., Darcq, E., Zhu, W., Gibb, S.L., Lanfranco, M.F., et al. (2012). Ethanol-
920 mediated facilitation of AMPA receptor function in the dorsomedial striatum: implications for
921 alcohol drinking behavior. *The Journal of neuroscience : the official journal of the Society for*
922 *Neuroscience* 32(43)**,** 15124-15132. doi: 10.1523/JNEUROSCI.2783-12.2012.

923 Xu, Q.W., Larosa, A., and Wong, T.P. (2024). Roles of AMPA receptors in social behaviors. *Front*
924 *Synaptic Neurosci* 16**,** 1405510. doi: 10.3389/fnsyn.2024.1405510.

925

12. Figure Captions

<u>Figure 1</u>. Continuous access home-cage two-bottle choice ethanol vs H_2O drinking by male and female mice. (**a**) Timeline of experimental procedure showing ethanol concentrations presented in daily 24 h access periods. (**b**) Body weight (g) of male and female mice on Day 1 and 50 showing normal growth and development throughout the experiment. **** - significant difference between Day 1 and Day 50, P<0.0001. (**c**) Ethanol intake (g/kg), ethanol preference (EtOH / H_2O %), and H2O intake (mls) plotted as a function of EtOH concentration during acquisition. (**d - e**) Ethanol intake and preference plotted as a function of chronic access day. Horizontal dashed line indicates 50% preference that would be expected by chance. * - significantly different from male at corresponding day, P<0.05. (**f – g**) Cumulative ethanol intake (g/kg) plotted as a function of time of day, showing rate of ethanol consumption on Day 16 (**f**) and Day 27 (**g**). * - significantly different from male at corresponding time, P<0.05. All data are plotted as MEAN ± SEM. Symbols and P values indicate results of RM-ANOVA followed by Šídák's multiple comparisons test.

<u>Figure 2</u>. Limited access home-cage two-bottle choice ethanol vs H2O drinking by male and female mice. (**a**) Timeline of experimental procedure showing ethanol concentration presented in 3 4-h sessions per week. (**b**) Ethanol intake (g/kg) plotted as a function of exposure day. * - significantly different from male at corresponding day, P<0.05. (**c**) Cumulative ethanol intake (g/kg) showing the rate of total intake as a function of exposure day. Symbols denote significantly different from male at corresponding day, ** - P<0.01; † - P<0.001; ‡ - P<0.0001. (**d – e**) H_2O intake (mls) and cumulative H_2O intake (mls) plotted as a function of exposure day. (**f**) Ethanol preference (EtOH mls / total fluid, %) plotted as a function of exposure day. Horizontal dashed line indicates 50% preference that would be expected by chance. (**g**) Mean blood ethanol concentration (BEC) plotted as a function of mean ethanol intake (g/kg) from individual mice (scatter plots) and shown as MEAN ± SEM (symbol with two-way error bars). All other data are expressed as MEAN ± SEM. Symbols and P values indicate results of RM-ANOVA followed by Šídák's multiple comparisons test.

<u>Figure 3</u>. Ethanol-induced loss of righting reflex and blood ethanol clearance in male and female mice. (**a**) Schematic of procedure showing that separate groups of male and female mice were tested for LORR and BEC after acute EtOH (4 g/kg, IP) administration. (**b**) Onset (**left**) and Duration (**right**) of EtOH-induced LORR plotted as a function of sex. * - significantly different from male, t-test P<0.05. showing no difference between male and female mice. (**c**) Blood ethanol level after acute administration of EtOH (4.0 g/kg) plotted as a function of time. **†** - indicates significant difference between the Y-intercepts of the male and female regression lines, ANOVA, P=0.001 as described in text. All data are plotted as MEAN ± SEM.

<u>Figure 4</u>. Operant EtOH self-administration and progressive ratio testing in male and female mice. (**a**) Timeline EtOH self-administration procedure showing 3 experimental phases of acquisition, baseline, and progressive ratio testing as a function of week. (**b, e**) Total number of EtOH reinforced responses (**b**) and EtOH intake (**e**) by each mouse during four daily 16 h acquisition sessions, separated by sex. * - indicates significant mean difference between day 4 and day 1, Šídák's multiple comparisons test, P=0.007. (**c, f**) EtOH reinforced responses plotted as a function of baseline day. Black lines are linear regression lines derived from regression analysis showing that the female slopes were significantly different from zero and that the two lines were different from each other for both responses (**c**) and intake (**f**) (see text). (**d, g**) Average EtOH reinforced responses (**d**) and EtOH intake (**g**) from week 1 and 4 showing an escalating pattern in female mice and difference between sexes during week 4. * - P=0.05; ** - P<0.01, Fisher's LSD multiple comparisons test. Progressive ratio breakpoint (**h**), duration of progressive ratio session (**i**), and headpokes occurring during each

971 session (**j**) plotted as a function of sex. Data were analyzed via unpaired t-test, * - indicates P<0.05.
972 All data are plotted as MEAN ± SEM.

973 **<u>Figure 5</u>. Operant EtOH self-administration and progressive ratio testing in male and female**
974 **mice**. (**a**) Timeline showing establishment of operant EtOH self-administration baseline followed by
975 pre-session systemic administration of the noncompetitive AMPAR antagonist GYKI 52466. (**b – c**)
976 Total EtOH reinforced responses (**b**), number of EtOH reinforcers earned (**c**), and EtOH intake (**d**)
977 plotted as a function of GYKI 52466 dosage. GYKI 52466 administration produced a dose-dependent
978 reduction in all measures despite a significant sex difference in total self-administration (see Results).
979 (**e – f**) Self-administration data were expressed as a percentage of vehicle control within each sex to
980 facilitate normalized comparisons of the effects of the AMPAR antagonist. Results showed that
981 GYKI 52466 produced overlapping dose-response curves in male and female mice with relatively
982 equivalent reductions in EtOH reinforced responses (**e**) and number of reinforcers (**f**) with a similar
983 but not significant reduction in EtOH intake (**g**). (**h**) EtOH reinforced response rate (resp / 5-min)
984 plotted as a function of time during 1 h sessions following administration of GYKI 52466 (0 and 10
985 mg/kg) in male (**left**) and female (**right**) mice. Symbols indicate significant difference as compared
986 to vehicle within sex, RM-ANOVA followed by Šídák's multiple comparisons test, * - P<0.05, ** -
987 P<0.01. All data are plotted as MEAN ± SEM.

988

Figure 1

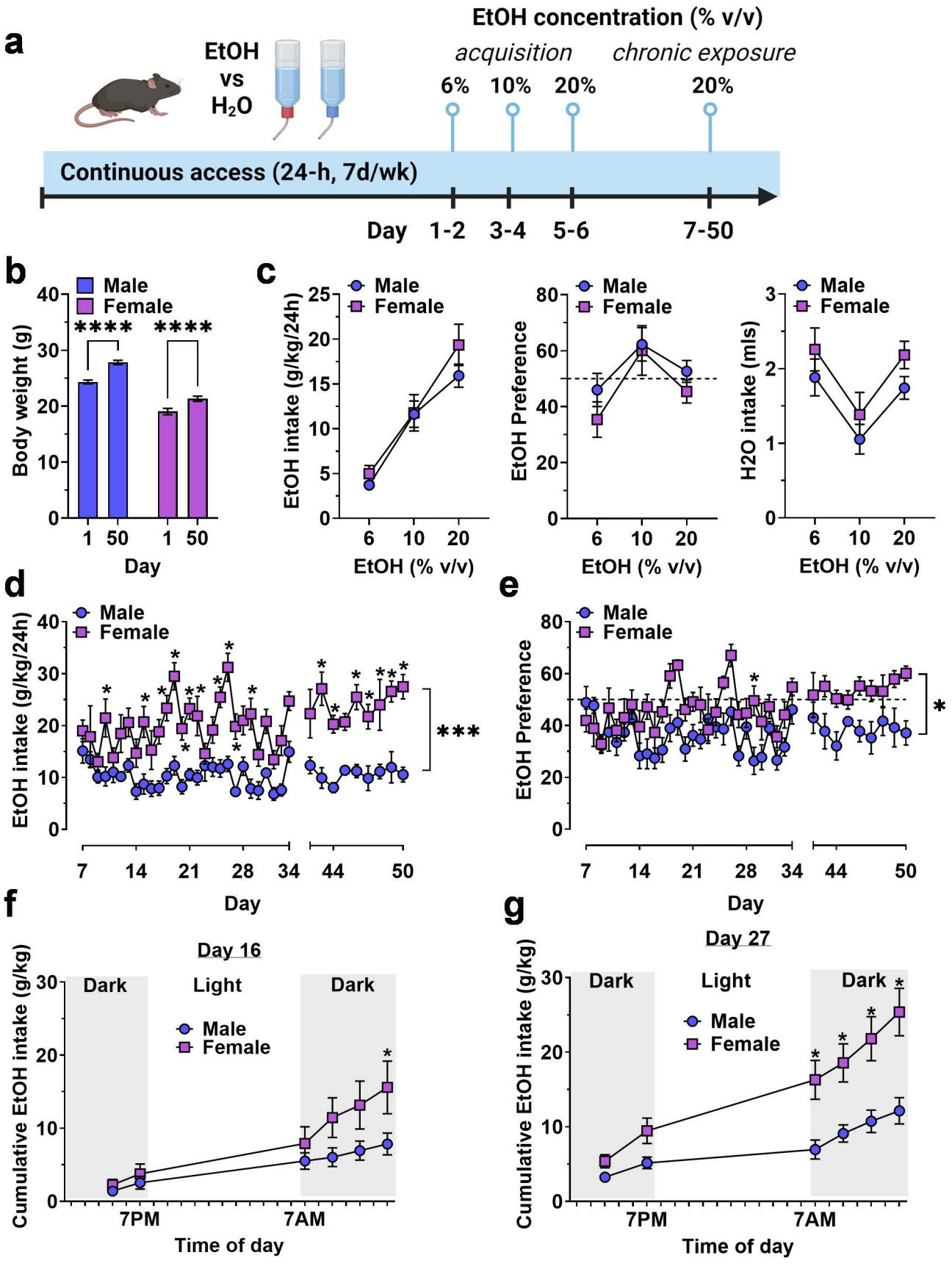

a
EtOH vs H2O
EtOH concentration (% v/v)
acquisition
chronic exposure
6%
10%
20%
20%
Continuous access (24-h, 7d/wk)
Day
1-2
3-4
5-6
7-50
b
Male
Female
Body weight (g)
Day
c
EtOH intake (g/kg/24h)
EtOH Preference
H2O intake (mls)
EtOH (% v/v)
d
EtOH intake (g/kg/24h)
Day
e
EtOH Preference
Day
f
Day 16
Dark
Light
Dark
Cumulative EtOH intake (g/kg)
7PM
7AM
Time of day
g
Day 27
Dark
Light
Dark
Cumulative EtOH intake (g/kg)
7PM
7AM
Time of day

Figure 2

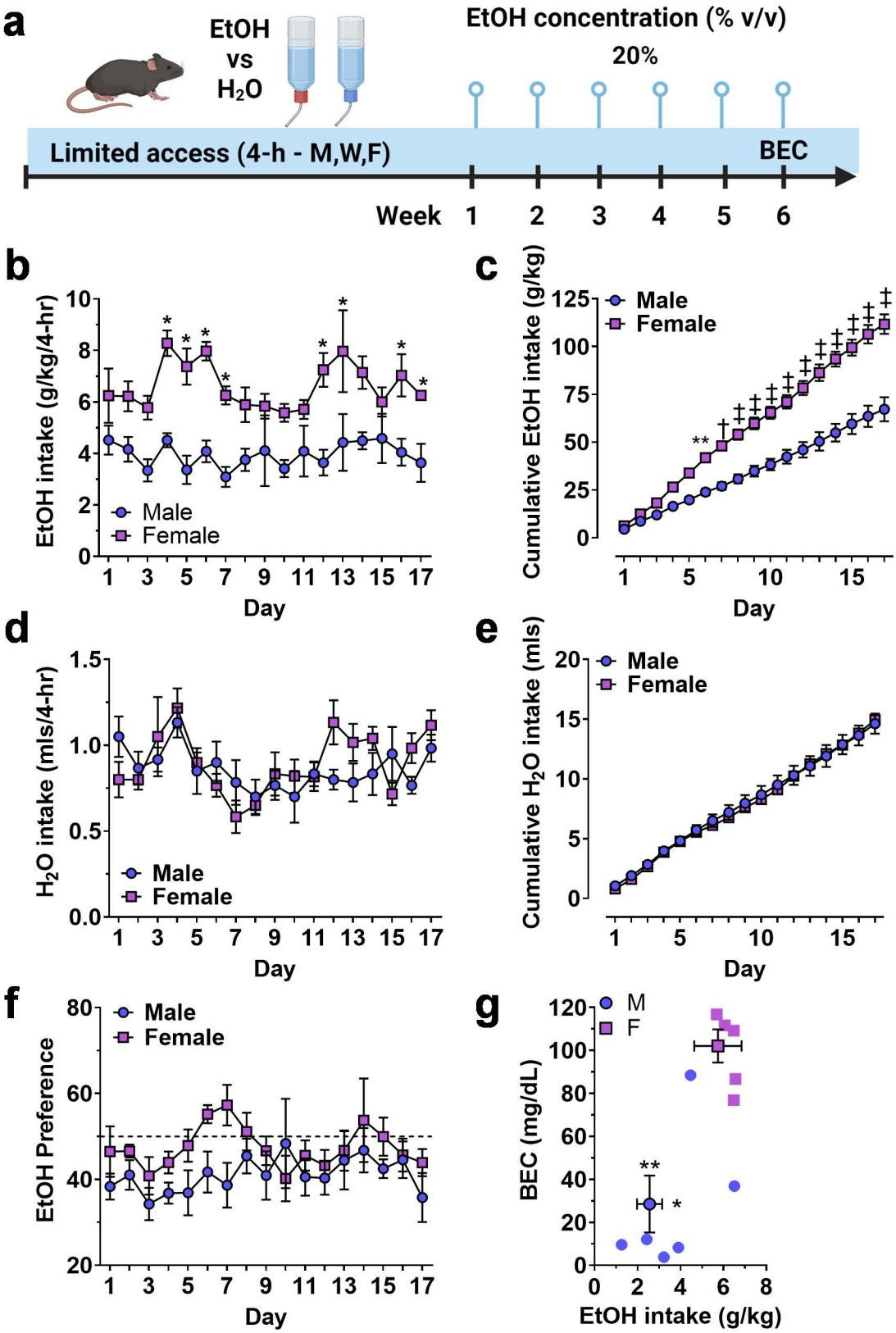

a
EtOH
vs
H2O
EtOH concentration (% v/v)
20%
Limited access (4-h - M,W,F)
BEC
Week 1 2 3 4 5 6
b
EtOH intake (g/kg/4-hr)
Male
Female
Day
c
Cumulative EtOH intake (g/kg)
Male
Female
Day
d
H2O intake (mls/4-hr)
Male
Female
Day
e
Cumulative H2O intake (mls)
Male
Female
Day
f
EtOH Preference
Male
Female
Day
g
BEC (mg/dL)
M
F
EtOH intake (g/kg)

Figure 3

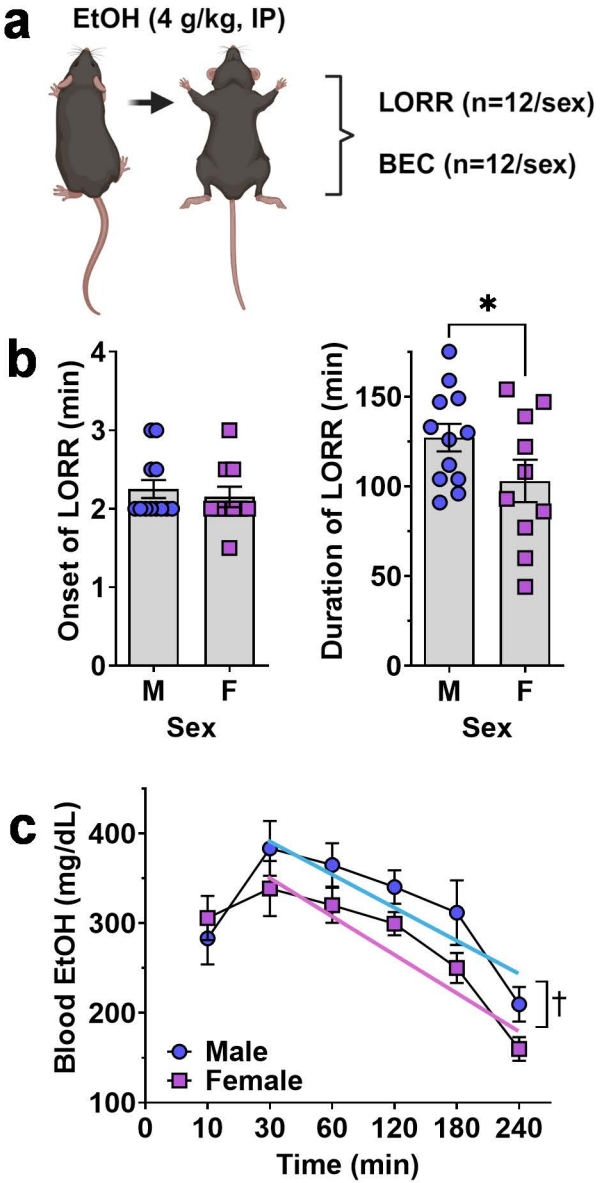

a
EtOH (4 g/kg, IP)
LORR (n=12/sex)
BEC (n=12/sex)
b
Onset of LORR (min)
0
1
2
3
4
M
F
Sex
Duration of LORR (min)
0
50
100
150
*
M
F
Sex
c
Blood EtOH (mg/dL)
100
200
300
400
0
10
30
60
120
180
240
Time (min)
Male
Female
†

Figure 4

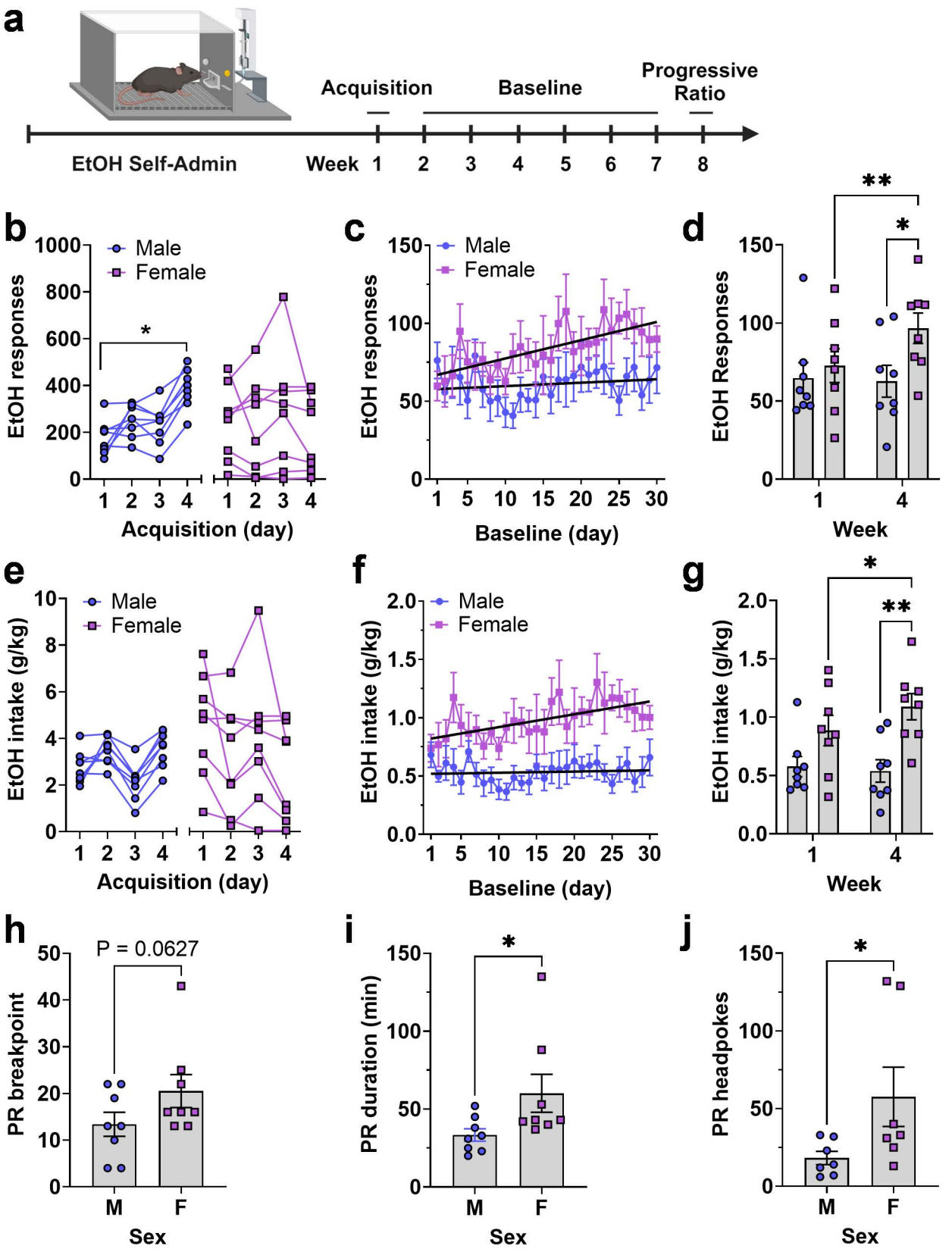
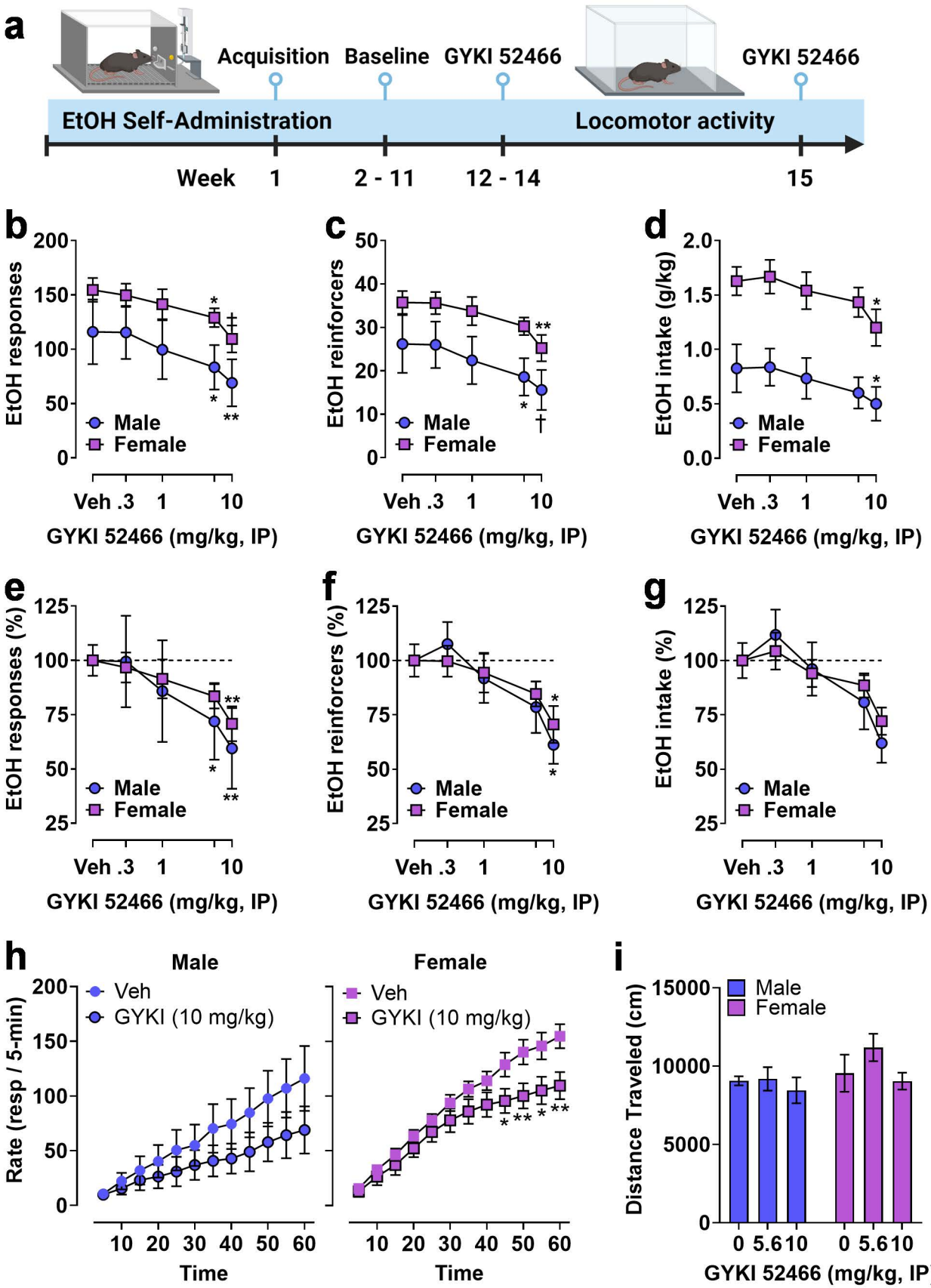

a
Acquisition
Baseline
Progressive Ratio
EtOH Self-Admin
Week 1 2 3 4 5 6 7 8
b
Male
Female
EtOH responses
Acquisition (day)
c
Male
Female
EtOH responses
Baseline (day)
d
EtOH Responses
Week
e
Male
Female
EtOH intake (g/kg)
Acquisition (day)
f
Male
Female
EtOH intake (g/kg)
Baseline (day)
g
EtOH intake (g/kg)
Week
h
P = 0.0627
PR breakpoint
M
F
Sex
i
PR duration (min)
M
F
Sex
j
PR headpokes
M
F
Sex

Figure 5


a
Acquisition
Baseline
GYKI 52466
GYKI 52466
EtOH Self-Administration
Locomotor activity
Week
1
2 - 11
12 - 14
15
b
EtOH responses
Male
Female
Veh .3 1 10
GYKI 52466 (mg/kg, IP)
c
EtOH reinforcers
Male
Female
Veh .3 1 10
GYKI 52466 (mg/kg, IP)
d
EtOH intake (g/kg)
Male
Female
Veh .3 1 10
GYKI 52466 (mg/kg, IP)
e
EtOH responses (%)
Male
Female
Veh .3 1 10
GYKI 52466 (mg/kg, IP)
f
EtOH reinforcers (%)
Male
Female
Veh .3 1 10
GYKI 52466 (mg/kg, IP)
g
EtOH intake (%)
Male
Female
Veh .3 1 10
GYKI 52466 (mg/kg, IP)
h
Male
Female
Veh
GYKI (10 mg/kg)
Rate (resp / 5-min)
Time
i
Male
Female
Distance Traveled (cm)
0 5.6 10 0 5.6 10
GYKI 52466 (mg/kg, IP)