SEX-DEPENDENT ENGAGEMENT OF ANTERIOR INSULAR CORTEX INPUTS TO THE DORSOLATERAL STRIATUM IN BINGE ALCOHOL DRINKING

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Abstract

How does alcohol consumption alter synaptic transmission across time, and do these alcohol-induced neuroadaptations occur similarly in both male and female mice? Previous work shows that anterior insular cortex (AIC) projections to the dorsolateral striatum (DLS) are uniquely sensitive to alcohol-induced neuroadaptations in male, but not female mice, and play a role in governing binge alcohol consumption in male mice. Here, by using high-resolution behavior data paired with in-vivo fiber photometry, we show how similar levels of alcohol intake are achieved by different behavioral strategies across sex, and how inter-drinking session thirst states predict future alcohol intakes in females, but not males. Further, we show how presynaptic calcium activity recorded from AIC synaptic inputs in the DLS across 3 weeks of water consumption followed by 3 weeks of binge alcohol consumption change across, fluid, time, sex, and brain circuit lateralization. By time-locking presynaptic calcium activity from AIC inputs to the DLS to peri-initiation of drinking bouts we show that AIC inputs into the left DLS robustly encode binge alcohol intake behaviors relative to water consumption and AIC inputs into the right DLS in males, but not females. Finally, we demonstrate how binge alcohol intake decreases AIC input engagement as alcohol intake progresses in both the left and right inputs into the DLS for males. These findings suggest a fluid-, sex- and lateralization-dependent role for the engagement of AIC inputs into the DLS that encode binge alcohol consumption behaviors, and contextualize alcohol-induced neuroadaptations at AIC inputs to the DLS.

Introduction

Binge alcohol consumption, defined as consuming 4 to 5 or more drinks in about two hours for females and males, respectively, represents a large proportion of the deaths associated with problematic alcohol use and is one of the strongest risk factors for developing alcohol use disorder (AUD) [1-3]. Binge drinking is particularly prevalent amongst young adults, especially among females who have been increasing their alcohol use rates year on year for the past half-decade [4,5].

Modeling binge drinking in rodent animal models is largely accomplished through the behavioral paradigm, Drinking in the Dark (DID), in which mice have *ad libitum* access to alcohol (20% v/v) in their home cage for two-hour sessions, 3 hours into their dark-light cycle for 3 to 4 days a week, and for one 4-hour “binge” session for a single day, followed by two or 3 days of abstinence from alcohol [6]. This procedure has great construct and predictive validity for human binge drinking. Mice display similar drinking patterns to humans like front-loading behavior wherein they consume larger amounts of alcohol earlier in the task compared to later, which can also be a measurement for intoxication rate [7]. DID also successfully induces pharmacologically relevant levels of blood alcohol content (BAC) in mice that is equivalent to the legal intoxication limit in humans [8,9].

It is also commonly accepted that female mice tend to drink more than males across a wide range of alcohol consumption models, including DID [10-13]. Possible reasons that females tend to drink more than males is due to an increased sensitivity to the rewarding and decreased sensitivity to the aversive properties of alcohol, different pharmacokinetic and pharmacodynamic properties of alcohol, and differences in sex hormone expression levels, such as estrogen which fluctuates across the menstrual cycle [13-19]. Yet, there are also reported instances in which male and female mice consume identical levels of alcohol in these drinking models, such as for mice genetically selected to consume high binge levels of alcohol [20]. And, while it is known that estrogen plays a role in increasing alcohol intakes in some models, there is evidence to suggest there is no effect of estrous cycle on alcohol intake specifically during DID [12].

Nonetheless, when female sex-dependent differences in alcohol intake are present during DID, they are often shown in comparison to males and/or other experimental controls (*e.g.*, ovariectomized mice). However, while we may observe that females drink more, and in some cases how their BAC differs, there remains very little knowledge on the behavioral strategies used to achieve those differential alcohol intakes compared to males.

Additionally, the neural circuitry that underlies binge drinking remains poorly understood, and descriptions of sex differences for neural circuits currently implicated in binge alcohol consumption remain even more elusive. Of the neural circuits implicated in binge drinking, only a few of these studies to date have included sex as a factor in their analyses [21-27]. Previous work from our laboratory that investigates such circuitry shows that alcohol uniquely alters anterior insular cortex (AIC) inputs into the dorsolateral striatum (DLS) via synaptic alterations in mu opioid and ionotropic glutamate receptors in male, but not female mice [27,28]. Optogenetically controlling these inputs during DID can govern how much alcohol male mice, binge consume.

To further detail the sex differences displayed between male and female mice in DID and build on our understanding of how AIC inputs into the DLS are altered by binge alcohol exposure across time and sex, we measured detailed drinking behaviors and presynaptic calcium activity at AIC inputs into the DLS for 3 weeks of water drinking followed by 3 weeks of binge alcohol drinking. Here, we show that male and females consume water identically. Next, we described how the drinking mechanics and patterns differ between females and males that binge similar levels of alcohol, such that female mice consume alcohol more efficiently. Also, by measuring inter-DID session water intakes, we show how female, but not male, thirst states predict future alcohol intake. Finally, using intersectional genetics, we expressed GCaMP, a genetically encoded calcium indicator, bilaterally only in AIC inputs to the DLS to measure how presynaptic AIC input activity changes across time, sex, fluid type, and brain circuit lateralization. By recording GCaMP activity from AIC inputs bilaterally in the DLS, we show that the AIC inputs into the left DLS strongly encode binge alcohol drinking in males. Further, increasing history of alcohol binge drinking decreases the calcium activity in both AIC inputs into the right and left DLS, suggesting decreased engagement as a function of

83 increased drinking history. Together, we establish detailed sex-differences in the
84 behavioral strategies females use to binge consume similar amounts of alcohol
85 compared to males. Further, we describe how AIC inputs to the left DLS encode binge
86 alcohol drinking and how the engagement of these inputs changes over the course of
87 binge drinking history across sex and circuit lateralization. Together these findings
88 represent targets for future pharmacotherapies and therapeutic modalities that leverage
89 the unique alcohol-induced changes to AIC inputs into the DLS to create more targeted,
90 efficacious treatment approaches for those experiencing AUD.

91 **Materials and Methods**

92 See supplementary information for detailed methods

93 ***Animals and Experimental Design***

94 Male and female C57BL/6J mice were ordered from Jackson Laboratory (Bar Harbor,
95 ME). Animals arrived at six weeks of age and were allowed to acclimate to the animal
96 facility for at least one week before any surgery was performed. All mice were injected
97 bilaterally with AAV9-syn-FLEX-jGCaMP7s-WPRE (Addgene, 104491-AAV9) in the AIC
98 and AAVrg-Ef1a-mCherry-IRES-Cre (Addgene, 55632-AAVrg) in the DLS to express
99 GCaMP in AIC inputs to the DLS. Additionally, 200 µm fiber optic cannula were inserted
100 bilaterally into the DLS. Example images of viral expression and viral schematics are
101 described in **Fig. 1a**. Animals were singly housed. Animal care and experimental
102 protocols for this study were approved by the Institutional Animal Care and Use
103 Committee at the Indiana University School of Medicine.

104 DID was conducted as described in **Fig. 1b** and as previously described in [27]. Mice
105 were subjected to 3 weeks of water DID followed by 3 weeks of alcohol DID using
106 lickometers to track microstructure of drinking behavior [29]. Mice had *ad libitum* access
107 to their homecage water bottles that were weighed once a day to calculate inter-DID
108 session water intakes. Repeating weekly DID cycles are referred to as DID Weeks.

109 ***In vivo Fiber Photometry***

110 Fiber photometry was performed with a Neurophotometrics fiber photometry system
111 (FP3002, Neurophotometrics LTD, San Diego, CA) and signal processing and data

analysis were performed with a custom python pipeline (see supplementary information for code). Briefly, animals were attached to cabling before a DID session in their own homecage and time locked signals from the fiber photometry acquisition system and lickometer were recorded. A 415 nm isosbestic channel and 470 nm GCaMP channel were recorded bilaterally for the duration of each DID session. A bi-exponential decay was fit to the 415 nm emission signal and was then robustly linearly scaled (RANSAC) to the 470 GCaMP emission signal. The 470 nm GCaMP signal was then divided by the scaled 415 nm isosbestic signal to compute a corrected observed fluorescence value respective to the signal recorded across the entirety of the DID session, defined as ΔF/F. To compare animals, fluid, sex, and lateralization effects, the data were globally z-scored, chopped, and aligned to the initiation of each drinking bout inclusive of the 5 seconds before and after each drinking event.

Modeling, Statistics, and Data Analysis

Drinking microstructure events were modeled using licks, lick durations, bouts, and the timestamps associated with these events to compute other detailed features such as inter-drink interval and latency to drink. Data preprocessing, transformations, and scaling were performed using SciPy and scikit-learn. Statistical modeling and data visualization was performed using pingouin, statsmodels, matplotlib, and seaborn.

Results

Sex-dependent differences in water and alcohol Drinking in the Dark

We isolated AIC→DLS circuitry by injecting an anterograde adeno-associated virus (AAV) into the AIC encoding a cre-dependent genetically encoded calcium indicator (GCaMP) and a retrograde AAV to express cre, a recombinase enzyme, in the DLS and implanted bilateral optic cannulas in the DLS to visualize and record calcium activity in synaptic inputs that arise from AIC projections to the DLS in both male and female mice **(Figure 1a)**. For all viral and fiber placements please see **Figure Supplement 1**. Animals then underwent 6 weeks of the Drinking in the Dark (DID) paradigm where they had 0, 2, or 4 hours of access to water each day for 3 weeks, and then were transitioned to alcohol access for an additional 3 weeks **(Figure 1b)**. For DID sessions in the first 3 weeks, animals achieved stable levels of water intake across 15 DID

142 sessions, and there were no sex differences between male and female water drinking
143 **(Figure 1c-d)**. By averaging water intakes by week, a clearer understanding of weekly
144 water intakes can be computed with no noted water intake differences across week or
145 by sex **(Figure 1e-f)**. During drinking weeks 4 to 6, when animals were transitioned from
146 water to alcohol intake, mice reliably consumed binge-like levels of alcohol during the 4-
147 hour sessions, which correlated with pharmacologically relevant levels of intake as
148 based on previous experiments in our laboratory using identical drinking measures
149 **(Figure 1g)** [27]. We failed to find differences in alcohol intake between males and
150 females across all DID sessions in this paradigm **(Figure 1h)**. Nonetheless, when
151 analyzing alcohol consumption by week, for both sexes, there was a greater amount of
152 alcohol drank during week 4 in comparison to week 5 **(Figure 1i)**. This could be due to
153 the transition from water to alcohol, in which the total fluid volume consumed during
154 water sessions is larger than alcohol sessions and may be supported by the decreasing
155 levels of alcohol intake seen in early alcohol DID sessions 16-19 as animals adapt to
156 consuming a new fluid type **(Figure 19h)**. By week, both male and female animals
157 experienced this decrease in alcohol intake in week 5 and 6 as compared to week 4 of
158 DID, but there were no sex differences or interactions between sex and drinking week
159 **(Figure 1j)**. Ultimately, both sexes consume binge-like levels of alcohol, and in relation
160 to the measured calcium activity at AIC inputs into the DLS presented below, one can
161 be confident that differences in these signals are not a function of the total alcohol
162 intake by sex.

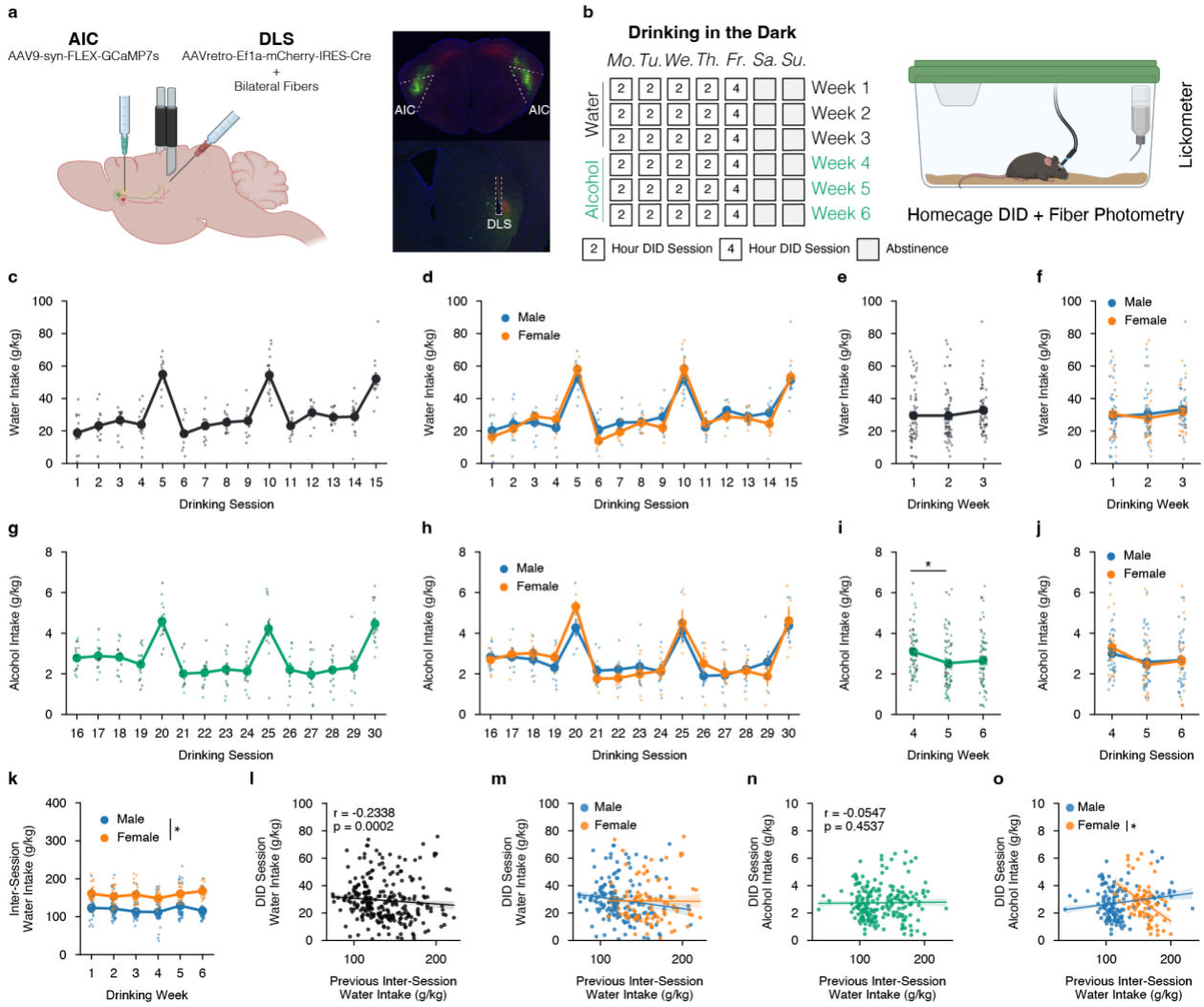

Figure 1: Sex-dependent differences in water and alcohol Drinking in the Dark (DID) (a) Representation of viral strategy and expression to record presynaptic calcium activity of AIC inputs within the DLS. **(b)** Schematic of Drinking in the Dark (DID) protocol. **(c)** Group and individual animals' water consumption by DID session (n = 14 animals). **(d)** Group and individual animals' water consumption by DID session and by sex (mixed ANOVA, Sex $F_{(1,12)} = 0.1032$, $p = 0.7535$; male n = 9, female n = 5). **(e)** Group and individual animals' water consumption by DID week (rmANOVA, Week $F_{(2,26)} = 2.4712$, $p = 0.1041$). **(f)** Group and individual animals' water consumption by DID week and by sex (mixed ANOVA, Sex $F_{(1,7)} = 0.1032$, $p = 0.7535$; male n = 9, female n = 5). **(g)** Group and individual animals' alcohol consumption by DID session (n = 14 animals). **(h)** Group and individual animals' alcohol consumption by DID session and by sex (mixed ANOVA, Sex $F_{(1,7)} = 0.4714$, $p = 0.5144$; male n = 9, female n = 5). **(i)** Group and individual animals' alcohol consumption by DID week (rmANOVA, Week $F_{(2,26)} = 7.8861$, $p = 0.0021$; Week 4 to Week 5, $p = 0.0012$; male n = 9, female n = 5). **(j)** Group and individual animals' alcohol consumption by DID week and by sex (mixed ANOVA, Sex $F_{(1,7)} = 0.4714$, $p = 0.5144$; male n = 9, female n = 5). **(k)** Group and individual animals' inter-session water consumption by DID week and by sex (mixed ANOVA, Sex $F_{(1,12)} = 13.5883$, $p = 0.0031$; male n = 9, female n = 5). **(l)** Previous inter-session water intakes negatively correlate with next DID session water intake (Shepherd's pi correlation, $r = -0.2383$, $p = 0.0002$). **(m)** Previous inter-session water intakes do not correlate with next DID session water intake by sex (ANCOVA, Sex $F(1,257) = 0.3048$, $p = 0.5814$; male n = 9, female n = 5). **(n)** Previous inter-session water intakes do not correlate with next

DID session alcohol intake (Shepherd's pi correlation, r = -0.0547, p = 0.4537). **(o)** Previous inter-session water intakes do not correlate with next DID session alcohol intake by sex (ANCOVA, Sex $F_{(1,315)}$ = 0.0101, p = 0.9201; male n = 9, female n = 5; males only r = 0.1554, p = 0.0916; females only r = -0.4441, p = 0.0002).

The amount of water intake between DID sessions was also measured daily using the home cage water bottle. Interestingly, females consumed more water between both water and alcohol DID sessions across all 6 weeks compared to males **(Figure 1k)**. Previous inter-session DID water intakes were also correlated to the next DID session intake to see if the amount of water consumed before any water DID session had any influence on the amount of water consumed during the upcoming DID session. We found a modest, yet statistically significant negative correlation **(Figure 1l)**. This suggests that increased water intake, a proxy for a quenched thirst state, being achieved before a water DID sessions can predict lower levels of water consumed during the DID session itself. We found no sex differences in this correlation between males and females **(Figure 1m)**. Similarly, we questioned if inter-session water intakes influenced alcohol DID intakes, which we did not find to be true **(Figure 1n)**. Yet, the patterning between males and females for thirst states looks strikingly different. While we did not find a statistically significant difference using sex as a grouping variable in a co-variate analysis of variance (ANCOVA) to determine if the slopes of the regression fits were statistically different, if one a priori separates sex and then runs a regression, the correlation for male intersession water intakes predicting alcohol DID intakes is not significant (p = 0.0916), whereas increasing female intersession water intake is negatively and significantly correlated (p = 0.0002, r = -0.4441) with lower alcohol DID intakes **(Figure 1o)**. Together, this may suggest that the elevated inter-session DID water intakes correlate with decreased levels of alcohol intake during DID sessions for females and may offer another explanation for why females and males binge drank similar levels of alcohol in this behavioral paradigm.

Sex-dependent differences in water and alcohol drinking mechanics

We also aimed to understand how well animals' intake correlated with both water and alcohol microstructure measurements directly from the lickometer in a sex-dependent manner. First, we plotted licks, lick duration, and number of drinks (i.e. a count of any drinking activity that occurred in a 3 second window) and correlated them to water

intake across all DID water sessions. For licks, lick duration, and number of drinks, all 3 measures were significantly correlated with intake and there were no differences in how male and female mice consumed water out of the lickometers across all DID water sessions **(Figure Supplement 2a-c)**. Similarly, licks, lick duration, and number of drinks were all highly correlated to alcohol intake **(Figure Supplement 2d-f)**. For lick and lick durations, there were no sex differences, yet for number of drinks, female mice showed an increased correlation with alcohol intake at lower number of drinks compared to males, suggesting they might be more efficient drinkers, such that efficiency measures the ability to consume more alcohol in a single bout **(Figure Supplement 2f)**.

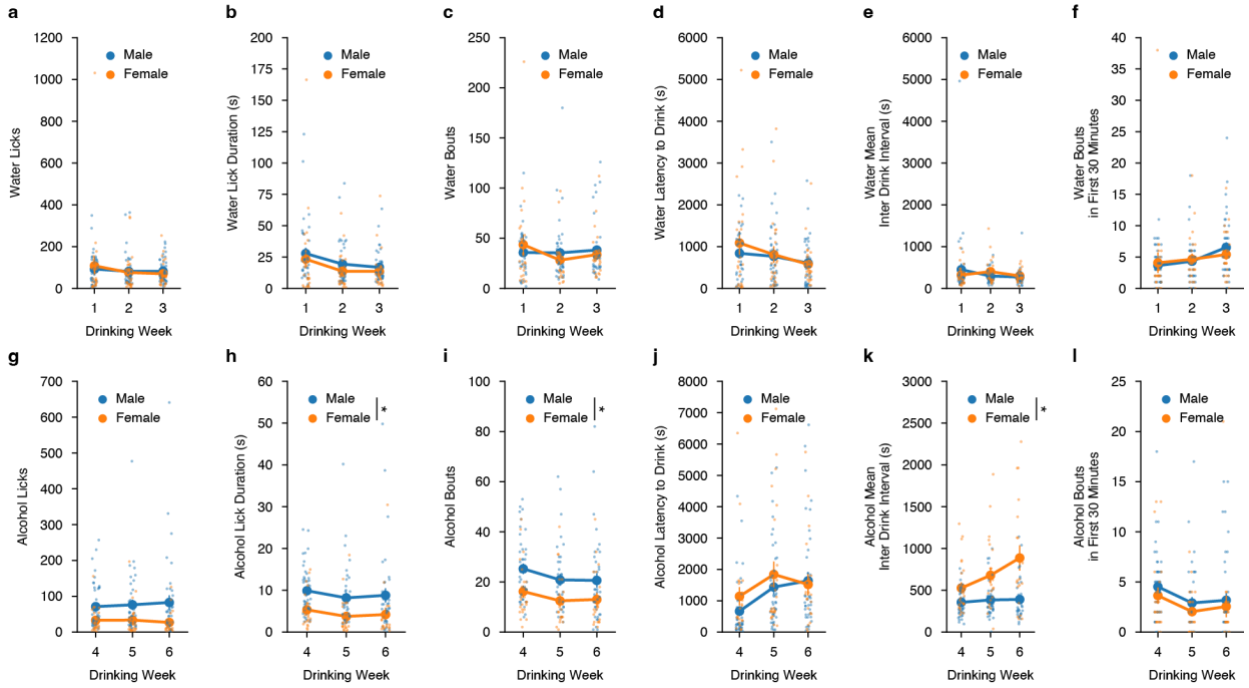

Figure 2: Alterations in drinking microstructure represent different strategies used to consume similar levels of alcohol, but not water intake in a sex-dependent manner. Water **(a)** licks (mixed ANOVA, Sex $F_{(1,11)} = 0.0141$, $p = 0.9077$; male n = 9, female n = 5), **(b)** lick duration (mixed ANOVA, Sex $F_{(1,11)} = 0.5306$, $p = 0.4816$; male n = 9, female n = 5), **(c)** bouts (mixed ANOVA, Sex $F_{(1,11)} = 0.0603$, $p = 0.8105$; male n = 9, female n = 5), **(d)** latency to drink (mixed ANOVA, Sex $F_{(1,11)} = 0.3347$, $p = 0.5746$; male n = 9, female n = 5) **(e)**, mean inter-drink interval (mixed ANOVA, Sex $F_{(1,11)} = 0.0189$, $p = 0.8930$; male n = 9, female n = 5), and **(f)** bouts in the first 30 minutes of a DID session (mixed ANOVA, Sex $F_{(1,11)} = 0.0646$, $p = 0.8040$; male n = 9, female n = 5) do not differ by sex across drinking week. Alcohol **(g)** licks do not differ by sex across week (mixed ANOVA, Sex $F_{(1,12)} = 3.1134$, $p = 0.1031$; male n = 9, female n = 5), but alcohol **(h)** lick duration (mixed ANOVA, Sex $F_{(1,12)} = 5.7522$, $p = 0.0336$; male n = 9, female n = 5), and **(i)** bouts are decreased in female compared to male binge alcohol drinkers (mixed ANOVA, Sex $F_{(1,12)} = 5.8813$, $p = 0.0320$; male n = 9, female n = 5). There were no sex differences in alcohol **(j)** latency to drink (mixed ANOVA, Sex $F_{(1,12)} = 0.9559$, $p = 0.3475$; male n = 9, female n = 5) **(k)**, but alcohol mean inter-drink interval is increased in females compared to males (mixed ANOVA, Sex $F_{(1,12)} = 17.0779$, $p = 0.0014$; male n =

9, female n = 5). There are no sex-dependent changes in **(l)** bouts in the first 30 minutes of an alcohol DID session by week (mixed ANOVA, Sex $F_{(1,12)} = 1.0208$, $p = 0.3323$; male n = 9, female n = 5).

We also wanted to see how these microstructure analyses were altered across time between sex. For water intake, across DID weeks 1-3 there were no sex differences in the number of licks, lick durations, water bouts, the latency to drink water, the mean inter-drink interval between water bouts, nor differences in front loading behavior for water intake **(Figure 2a-f)**. For alcohol intake, across DID weeks 4-6 there were no sex differences for the number of alcohol licks, but females did have a decreased time for alcohol lick durations and number of alcohol bouts compared to males **(Figure 2g-i)**. These findings across time further support that females may be more efficient in consuming alcohol, such that it requires less time and bottle interactions to reach similar levels of alcohol intake that males achieve. Females and males displayed the same latency to drink alcohol across time, but as DID weeks progressed, females displayed an increased inter-drink interval **(Figure 2j-k)**. Finally, both males and females displayed similar levels of front-loading behavior for alcohol **(Figure 2l)**. Together, these data suggest that when alcohol intake levels are similar between males and females, the way in which they achieve those intakes may be dissimilar in both the manner in how females consume alcohol and how those differences in consumption evolve across time.

Calcium dynamics of AIC inputs into the DLS during drinking bouts

To visualize changes in calcium dynamics at the AIC presynaptic inputs to both the left and right DLS, we aligned the changes in calcium fluorescence to the beginning of water and alcohol drinking bouts, creating a 10 second window with 5 seconds before the initiation of the drinking bout and 5 seconds after. All recordings from the left and right presynaptic terminals were z-scored across all DID sessions for comparison across time, fluid, brain lateralization, and sex.

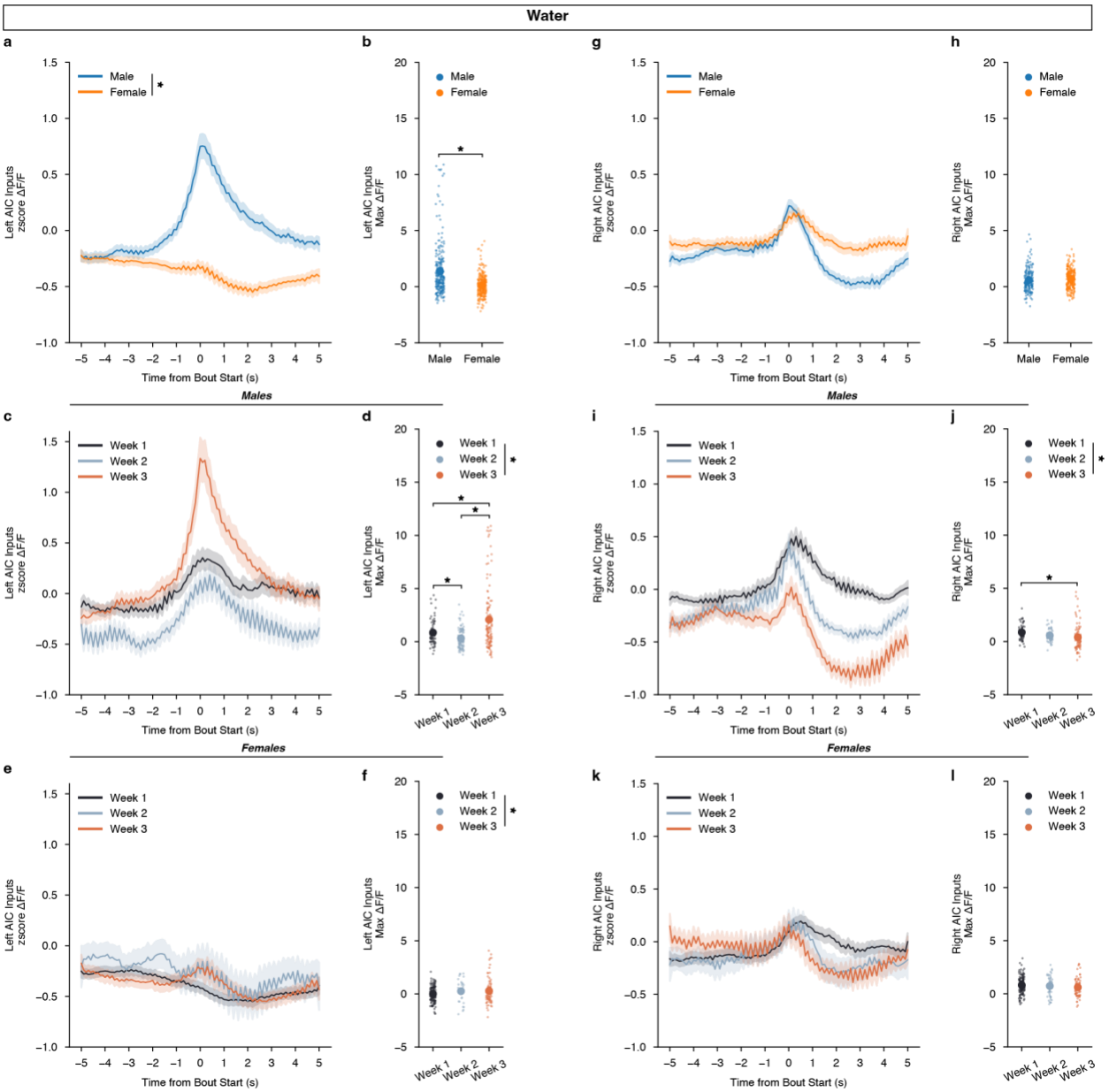

Figure 3: Calcium dynamics of AIC inputs into the left and right DLS during water drinking bouts. (a) Left AIC inputs to the DLS display a sex and time dependent change in ΔF/F (mixed ANOVA, Sex x Time $F_{(100,900)}$ = 1.3268, p = 0.0223) **(b)** and males show larger maximum ΔF/F values in the lick window (t-test, $t_{(348.0439)}$ = 8.3639, p = 1.4829e-15). **(c)** For males, by week, there was no time by week change in ΔF/F (rmANOVA, Week x Time $F_{(200,200)}$ = 0.6676, p = 0.9978) but, **(d)** there was a significant increase in maximum ΔF/F across weeks (ANOVA, Week $F_{(2,118.0693)}$ = 12.2468, p = 8e-6; Week 1 to Week 3 p = 1.40e-4; Week 2 to Week 3 p = 3e-6). **(e)** For females, by week, there was no time by week change in ΔF/F (repeated-measures ANOVA, Week x Time $F_{(200,200)}$ = 0.8667, p = 0.8438), but **(f)** there was a change in maximum ΔF/F by week, but with no post-hoc significance (ANOVA, Week $F_{(2,5.3748)}$ = 3.0771, p = 0.0477). **(g)** Right AIC inputs to the DLS do not display a sex and time dependent change in ΔF/F (mixed ANOVA, Sex x Time $F_{(100,800)}$ = 0.4966, p = 0.9998) **(h)** or change in maximum ΔF/F values in the lick window (t-test, $t_{(394.3894)}$ = -1.9659, p = 0.050006). **(i)** For males, by week, there was no time by week change in ΔF/F (rmANOVA, Week x Time $F_{(200,400)}$ = 1.2085, p = 0.05771) but, **(j)** there was a significant decrease in maximum ΔF/F across weeks (ANOVA, Week

$F_{(2,7.2055)}$ = 3.9152, p = 0.02152; Week 1 to Week 3 p = 0.0171). **(k)** For females, by week, there was no time by week change in ΔF/F (rmANOVA, Week x Time $F_{(200,200)}$ = 0.7551, p = 0.9761), nor **(l)** a change in maximum ΔF/F by week (ANOVA, Week $F_{(2,1.876)}$ = 1.2762, p = 0.2808). GCaMP activity is aligned to the initiation of the water drinking bout with 5 seconds before and 5 seconds after the bout plotted. Solid lines represent means and banded shading or error bars represent the standard error of the mean.

During water drinking sessions, calcium activity from inputs to the left DLS are observed near the initiation of the drinking bout in male, but not in female mice as measured by the interaction between sex and time for ΔF/F as well as the maximum ΔF/F value achieved during the recording window **(Figure 3a-b)**. By splitting the data by time, for males, the strength of the ΔF/F signal by week also increased in DID week 3 compared to weeks 1 and 2, suggesting that as time progresses for water drinking, AIC inputs to the left DLS become more engaged during water drinking behaviors **(Figure 3c-d)**. For females, there was no significant change in ΔF/F for week by time, but similarly to males, a slight increase in maximum ΔF/F was seen in week 3, but without post-hoc significance to differentiate these changes by week **(Figure 3e-f)**. Interestingly, for AIC inputs into the right DLS, there were no sex by time differences in ΔF/F as well as no differences in maximum ΔF/F measured in the lick window by sex **(Figure 3g-h)**. For males, by time, the opposite is observed for AIC inputs to the right DLS as compared to the AIC inputs to the left DLS, where a decrease in the maximum ΔF/F was observed from week 1 to week 3, suggesting a disengagement of these AIC inputs as male mice drink water **(Figure 3i-j)**. Finally, for females, there were no differences in AIC inputs to the right DLS across time or by maximum ΔF/F across water drinking weeks **(Figure 3k-l)**.

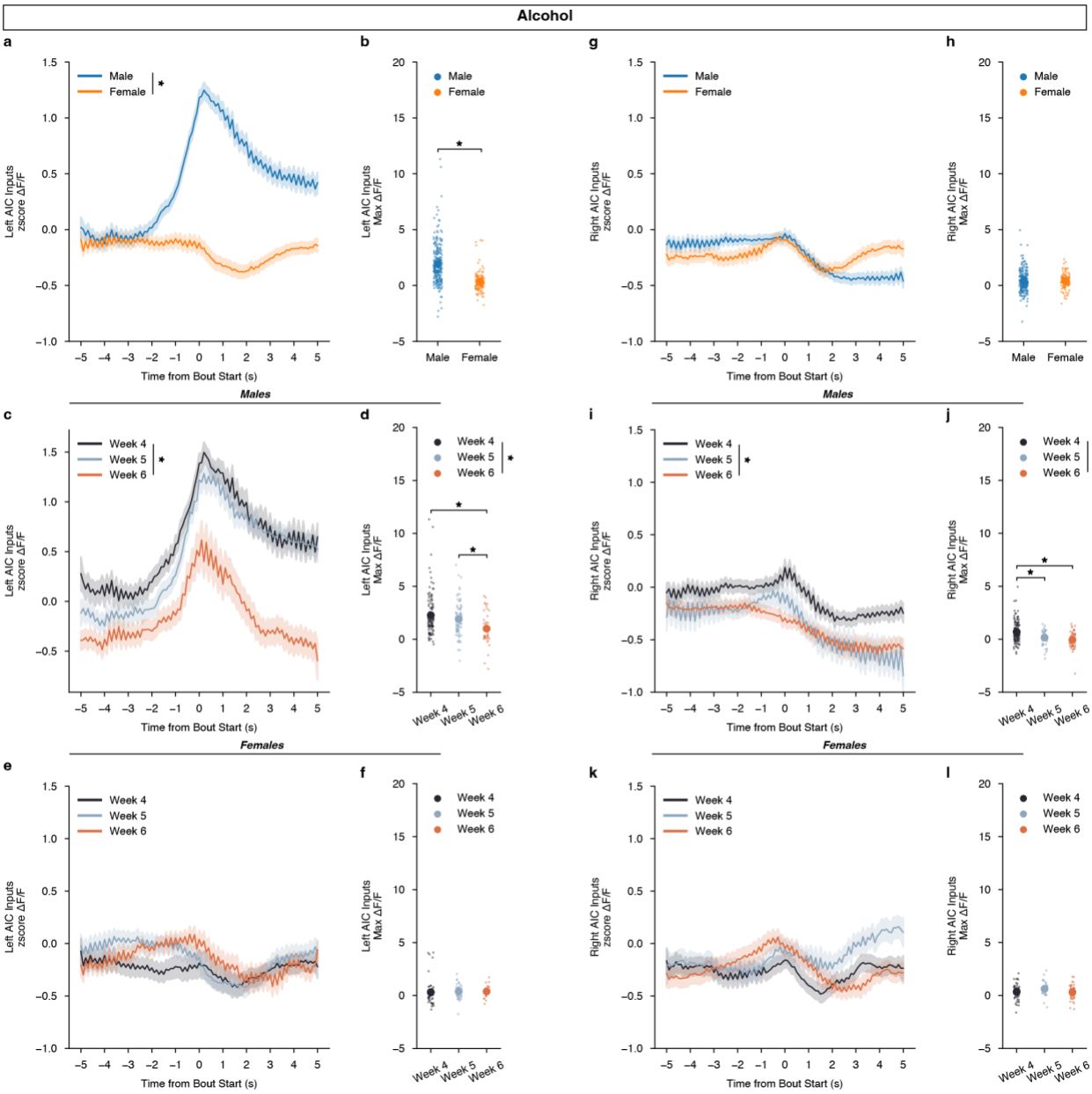

Figure 4: Calcium dynamics of AIC inputs into the left and right DLS during alcohol drinking bouts. (a) Left AIC inputs to the DLS display a sex and time dependent change in ΔF/F (mixed ANOVA, Sex x Time $F_{(100,1200)}$ = 3.2003, p = 4.0388e-21) **(b)** and males show larger maximum ΔF/F values in the lick window for alcohol (t-test, $t_{(381.7545)}$ = 11.8034, p = 1.2686e-27). **(c)** For males, by week, there was a significant decrease in the weekly ΔF/F (rmANOVA, Week x Time $F_{(200,200)}$ = 1.3395, p = 0.0197) and, **(d)** there was a significant decrease in maximum ΔF/F across weeks (ANOVA, Week $F_{(2,48.1234)}$ = 8.0179, p = 4.22e-4; Week 4 to Week 6 p = 2.67e-4; Week 5 to Week 6 p = 0.01003). **(e)** For females, by week, there was no time by week change in ΔF/F (rmANOVA, Week x Time $F_{(200,400)}$ = 1.1616, p = 0.1066), nor **(f)** was there a change in maximum ΔF/F by week (ANOVA, Week $F_{(2,0.1533)}$ = 0.1011, p = 0.9039). **(g)** Right AIC inputs to the DLS do not display a sex and time dependent change in ΔF/F (mixed ANOVA, Sex x Time $F_{(100,1200)}$ = 1.1124, p = 0.2188) **(h)** or change in maximum ΔF/F values in the lick window (t-test, $t_{(369.2551)}$ = -0.4997, p = 0.6175). **(i)** For males, by week, there was a decrease in time by week change in ΔF/F (rmANOVA, Week x Time $F_{(200,800)}$ = 2.09006, p = 7.553e-13) and **(j)** there was a significant decrease in maximum ΔF/F across weeks (ANOVA, Week $F_{(2,31.2873)}$ = 22.3352,

p = 1.1184e-9; Week 4 to Week 5 p = 2.6892e-4; Week 4 to Week 6 p = 3.2726e-10). **(k)** For females, by week, there was no time by week change in ΔF/F (rmANOVA, Week x Time $F_{(200,400)}$ = 0.9869, p = 0.5371), nor **(l)** a change in maximum ΔF/F by week (ANOVA, Week $F_{(2,2.3316)}$ = 2.6558, p = 0.0737). GCaMP activity is aligned to the initiation of the water drinking bout with 5 seconds before and 5 seconds after the bout plotted. Solid lines represent means and banded shading or error bars represent the standard error of the mean.

When transitioning from water DID to binge alcohol DID sessions in week 4, the AIC inputs to the left DLS continue to show large calcium transients in males compared to female mice as measured by the interaction between sex and time for ΔF/F as well as the maximum ΔF/F value achieved during the recording window **(Figure 4a-b)**. By splitting the data by sex, for males, the strength of the ΔF/F signal by week decreased in DID week 6 compared to weeks 4 and 5, suggesting that as time progresses for alcohol drinking, AIC inputs to the left DLS become disengaged during alcohol drinking behaviors **(Figure 4c-d)**. These data suggest that alcohol may disrupt the encoding of alcohol drinking behaviors across time from AIC inputs into the left DLS. For females, there was no significant change in ΔF/F for week by time, and no change to the maximum ΔF/F by week **(Figure 4e-f)**. Like water, for AIC inputs into the right DLS, there were no sex by time differences in ΔF/F as well as no differences in maximum ΔF/F measured in the lick window by sex **(Figure 4g-h)**. For males, by time, like the AIC inputs to the left DLS, a decrease in the maximum ΔF/F was observed from week 4 to week 6 for the AIC inputs to the right DLS, suggesting a global disengagement of AIC inputs as male mice drink alcohol across time **(Figure 4i-j)**. Finally, for females, there were no differences in AIC inputs to the right DLS across time or by maximum ΔF/F across alcohol drinking weeks **(Figure 4k-l)**. Together, across weeks for both left and right AIC inputs to the DLS in males, alcohol decreases calcium activity at both left and right AIC inputs to the DLS.

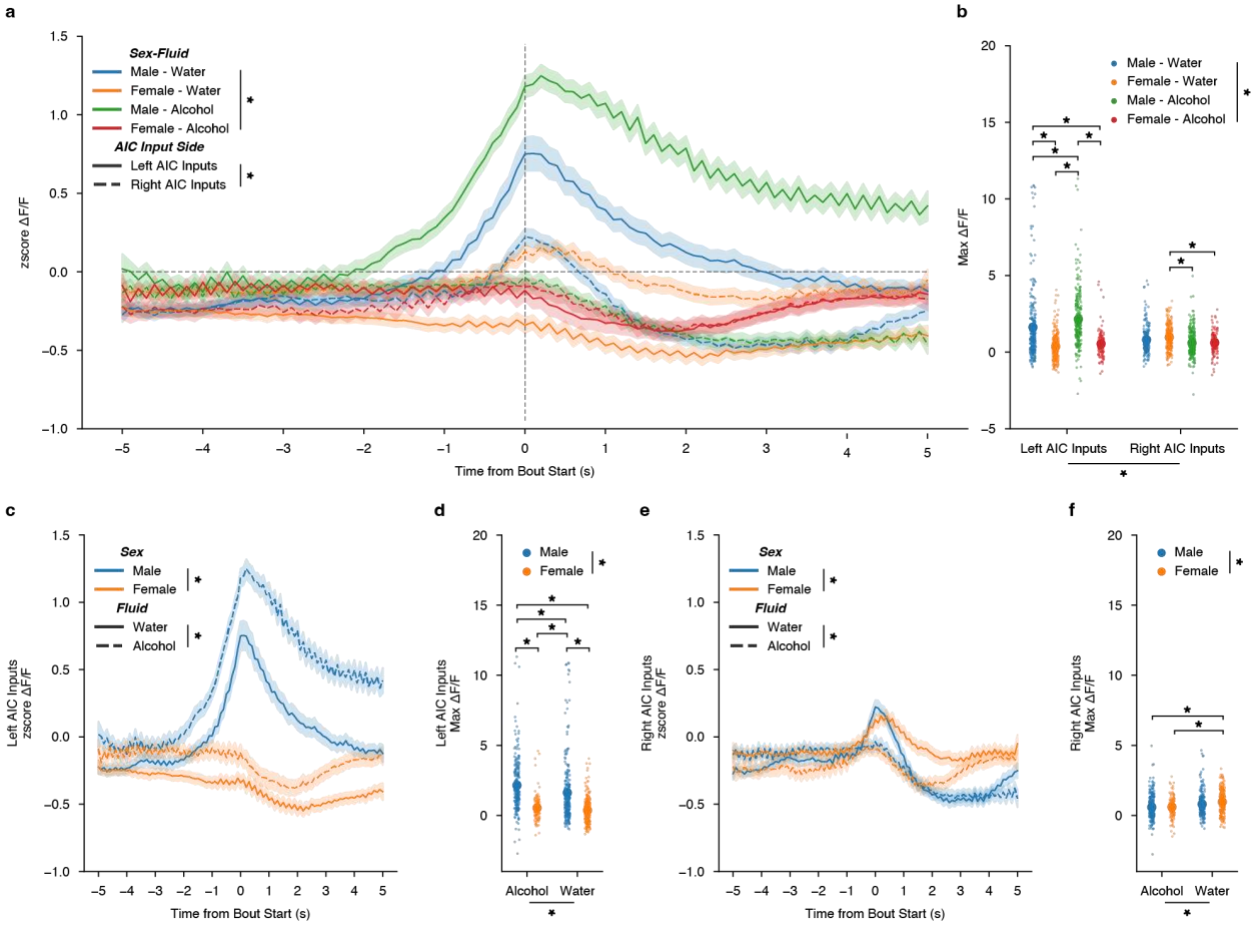

Figure 5: Sex-dependent calcium dynamics of AIC inputs into the left and right DLS during water and drinking bouts. (a) AIC inputs to the DLS are most strongly engaged by alcohol at AIC inputs into the left DLS (mixed ANOVA, Sex x Fluid x Time x Input Side $F_{(100,192.8931)}$ = 2.1336, p = 3.3646e-18) and **(b)** maximum ΔF/F changes are associated with changes in AIC inputs to the left DLS than with the right DLS (mixed ANOVA, Sex x Fluid x Input Side $F_{(3,336.8538)}$ = 64.2211, p = 2.0104e-39). **(c)** For left AIC inputs into the DLS, there were both sex and fluid interactions by time for changes in ΔF/F (mixed ANOVA, Sex x Fluid x Time $F_{(100,279.0092)}$ = 2.4303, p = 6.7612e-14) and **(d)** increases in maximum ΔF/F for alcohol compared to water were observed for males (mixed ANOVA, Sex x Fluid $F_{(3,173.1181)}$ = 62.9854, p = 4.9096e-37). **(e)** For right AIC inputs into the DLS, there were also both sex and fluid interactions by time for changes in ΔF/F (mixed ANOVA, Sex x Fluid x Time $F_{(100,86.0651)}$ = 1.3375, p = 0.01369) and **(f)** decreases in maximum ΔF/F for alcohol compared to water were observed for females (mixed ANOVA, Sex x Fluid $F_{(3,22.0906)}$ = 10.8346, p = 5.4322e-7). GCaMP activity is aligned to the initiation of the water drinking bout with 5 seconds before and 5 seconds after the bout plotted. Solid lines represent means and banded shading or error bars represent the standard error of the mean.

Finally, to summarize the differences between calcium activity at left and right inputs to the DLS across fluid, sex, and lateralization more generally, we collapsed all water and alcohol drinking sessions' calcium activity and compared them for sex, fluid, lateralization, and time differences. Altogether, it showed that drinking behaviors are largely encoded by AIC inputs into the left DLS, with alcohol displaying the largest

368 change in magnitude for ΔF/F, and water the second largest when time locked to
369 drinking bouts **(Figure 5a-b)**. Further, by looking at all AIC inputs into the left DLS by
370 sex and fluid, calcium activity is increased for alcohol compared to water, especially in
371 males **(Figure 5c-d)**. Whereas by looking at all AIC inputs into the right DLS by sex and
372 fluid, that finding is reversed, such that a decrease in calcium transients for alcohol
373 compared to water is present, especially in females **(Figure 5e-f)**. Thus, a net shift
374 towards increased engagement of AIC inputs into the left DLS in the transition from
375 water drinking to alcohol drinking is achieved via both increasing AIC engagement to the
376 left DLS and decreasing AIC engagement to the right DLS.

377 **Discussion**

378 In summary, here we show not only how male and female mice binge consume both
379 water and alcohol in similar amounts, but also the differences associated with the
380 behavioral strategies to accomplish such task. Based on these data, we show how
381 female mice may be more efficient drinkers, more susceptible to inter-session water
382 drinking that modulates future alcohol intake, and time-resolved differences in how their
383 binge drinking behaviors evolve compared to male mice. These robust measurements
384 and resultant sex differences of binge-associated behavioral strategies must continue to
385 be explored, but we hope these initial data serve as proof for the diversity between
386 sexes that exist in alcohol consumption models even when animals consume similar
387 amounts of alcohol.

388 The calcium imaging at pre-synaptic AIC inputs to the DLS data suggest: 1) there is a
389 time-dependent activation of AIC inputs into the DLS that occurs across drinking weeks
390 where calcium activity becomes increasingly time locked to drinking behaviors, and
391 depending on the sex and lateralization, becomes more or less engaged to alcohol
392 drinking and 2) lateralization affects insula projections into the DLS, such that insula
393 signals received in the right and left DLS are remarkably different.

394 First, we saw strong evidence that AIC inputs into the DLS become engaged around
395 water drinking behaviors, but ultimately most strongly engaged at AIC inputs into the left
396 DLS early in binge alcohol consumption in male mice. Interestingly, these robust
397 calcium signals encoding binge consumption decayed in amplitude near drinking bout

initiations later in binge alcohol exposure history, suggesting that insular signals into the DLS can dynamically respond to alcohol across time. Further, while we did not directly assess the kinetics of alcohol calcium activity, it appears that the AIC inputs to the left DLS also become left-shifted, such that the initiation of calcium activity begins around 2 seconds before the bottle contact, whereas in water this activity begins ~ 1 second before the bottle contact **(Figure 5a)**. Also, the off-kinetics of the calcium activity in alcohol AIC inputs to the left DLS compared to water show a longer sustained activity across time such that the calcium activity does not return to baseline within 5 seconds post-lick, whereas in water, the calcium activity returns to baseline ~ 3 seconds post bottle contact **(Figure 5a)**. Interestingly, when looking at the time course of the signal for alcohol AIC inputs to the left DLS by week in males, the left-shifted on-kinetics remain stable across alcohol history, but the amplitude and off-kinetics decrease and shorten, respectively comparing week 4 to week 6 of DID **(Figure 4c)**. Together, these data suggest that while AIC inputs into the left DLS are most strongly engaged in alcohol drinking, that the change in their kinetics and thus what they might be encoding in a behavioral and/or interoceptive context may be altered due to increasing alcohol history. While these generalized kinetic and amplitude changes in calcium activity are interesting observations, future studies employing faster and higher resolution imaging techniques will be needed to tease apart *in-vivo* presynaptic release changes due to alcohol in this circuitry, but nonetheless these data represent an intriguing initial finding.

Secondly, lateralization of insular activity is well documented in human and rat studies, yet few, if any, mouse studies show clear lateralization effects within the insular cortex [30]. To our knowledge this is the first demonstration, in a water or alcohol drinking paradigm, to show lateralized effects of AIC inputs in the mouse. Also, in our previous work we did not see lateralization effects when stimulating AIC inputs into the left DLS compared to right DLS, but it is important to note that the time-resolution in which we were photoactivating AIC inputs in the DLS was around 10 order of magnitudes longer than the time-resolution of the measured calcium activity, suggesting we could have been occluding lateralization effects by stimulating for too long, at the non-biologically relevant hertz, and/or with too much light power [27].

428 Together, these findings suggest there exist both sex-dependent, fluid-dependent, and
429 brain lateralization effects for AIC inputs into the DLS in how they encode water and
430 alcohol drinking activity. Further, these findings help contextualize our previous work on
431 AIC input control over binge alcohol drinking behaviors such that alcohol exposure in
432 males decreases robust calcium activity from AIC inputs that when restored with
433 photoactivation of AIC inputs into the DLS leads to decreases in binge alcohol
434 consumption **(Figure 4a-d)**. Yet, in females the lack of perturbed calcium dynamics may
435 correlate with the lack of alcohol-induced changes in synaptic plasticity that were
436 observed in slice electrophysiology at AIC inputs to the DLS after 3 weeks of binge
437 alcohol exposure [27]. In conclusion, leveraging these sex-dependent differences in AIC
438 circuitry as binge drinking behaviors develop may represent novel biological levers to
439 target in the quest for developing new pharmacotherapies and therapeutic modalities to
440 treat those experiencing AUD.

Author Contributions

DLH: Conceptualization, Resources, Data Curation, Software, Formal Analysis, Funding Acquisition, Investigation, Visualization, Methodology, Writing - Original Draft, Project Administration, Writing – Review and Editing

BKA: Conceptualization, Supervision, Funding Acquisition, Methodology, Project Administration, Writing – Review and Editing

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Competing Interests

No competing interests declared

References

1 Addolorato G, Vassallo GA, Antonelli G, Antonelli M, Tarli C, Mirijello A, et al. Binge Drinking among adolescents is related to the development of Alcohol Use Disorders: results from a Cross-Sectional Study. Sci Rep. 2018;8(1):12624.

2 Kanny D, Naimi TS, Liu Y, Lu H, Brewer RD. Annual Total Binge Drinks Consumed by U.S. Adults, 2015. Am J Prev Med. 2018;54(4):486-96.

3 Mokdad AH, Marks JS, Stroup DF, Gerberding JL. Actual causes of death in the United States, 2000. JAMA. 2004;291(10):1238-45.

4 Keyes KM, Jager J, Mal-Sarkar T, Patrick ME, Rutherford C, Hasin D. Is There a Recent Epidemic of Women's Drinking? A Critical Review of National Studies. Alcohol Clin Exp Res. 2019;43(7):1344-59.

5 White AM. Gender Differences in the Epidemiology of Alcohol Use and Related Harms in the United States. Alcohol Res. 2020;40(2):01.

6 Thiele TE, Navarro M. "Drinking in the dark" (DID) procedures: a model of binge-like ethanol drinking in non-dependent mice. Alcohol. 2014;48(3):235-41.

7 Ardinger CE, Lapish CC, Czachowski CL, Grahame NJ. A critical review of front-loading: A maladaptive drinking pattern driven by alcohol's rewarding effects. Alcohol: Clinical and Experimental Research. 2022;46(10):1772-82.

8 Barkley-Levenson AM, Crabbe JC. High drinking in the dark mice: a genetic model of drinking to intoxication. Alcohol. 2014;48(3):217-23.

9 Huynh N, Arabian NM, Asatryan L, Davies DL. Murine drinking models in the development of pharmacotherapies for alcoholism: drinking in the dark and two-bottle choice. JoVE (Journal of Visualized Experiments). 2019(143):e57027.

10 Blednov YA, Metten P, Finn DA, Rhodes JS, Bergeson SE, Harris RA, Crabbe JC. Hybrid C57BL/6J x FVB/NJ mice drink more alcohol than do C57BL/6J mice. Alcoholism, clinical and experimental research. 2005;29(11):1949-58.

11 Hwa LS, Chu A, Levinson SA, Kayyali TM, DeBold JF, Miczek KA. Persistent escalation of alcohol drinking in C57BL/6J mice with intermittent access to 20% ethanol. Alcohol Clin Exp Res. 2011;35(11):1938-47.

12 Satta R, Hilderbrand ER, Lasek AW. Ovarian hormones contribute to high levels of binge-like drinking by female mice. Alcoholism: Clinical and Experimental Research. 2018;42(2):286-94.

13 Sneddon EA, White RD, Radke AK. Sex Differences in Binge-Like and Aversion-Resistant Alcohol Drinking in C57BL/6J Mice. Alcohol Clin Exp Res. 2019;43(2):243-49.

14 Czerniak R. Gender-based differences in pharmacokinetics in laboratory animal models. Int J Toxicol. 2001;20(3):161-3.

15 Elvig SK, McGinn MA, Smith C, Arends MA, Koob GF, Vendruscolo LF. Tolerance to alcohol: A critical yet understudied factor in alcohol addiction. Pharmacol Biochem Behav. 2021;204:173155.

16 Finn DA, Helms ML, Nipper MA, Cohen A, Jensen JP, Devaud LL. Sex differences in the synergistic effect of prior binge drinking and traumatic stress on subsequent ethanol intake and neurochemical responses in adult C57BL/6J mice. Alcohol. 2018;71:33-45.

17 Overstreet DH, Knapp DJ, Breese GR. Modulation of multiple ethanol withdrawal-induced anxiety-like behavior by CRF and CRF1 receptors. Pharmacol Biochem Behav. 2004;77(2):405-13.
18 Schramm-Sapyta NL, Walker QD, Caster JM, Levin ED, Kuhn CM. Are adolescents more vulnerable to drug addiction than adults? Evidence from animal models. Psychopharmacology (Berl). 2009;206(1):1-21.
19 Vandegrift BJ, Hilderbrand ER, Satta R, Tai R, He D, You C, et al. Estrogen Receptor α Regulates Ethanol Excitation of Ventral Tegmental Area Neurons and Binge Drinking in Female Mice. The Journal of Neuroscience. 2020;40(27):5196-207.
20 Savarese AM, Ozburn AR, Barkley-Levenson AM, Metten P, Crabbe JC. The impact of Drinking in the Dark (DID) procedural manipulations on ethanol intake in High Drinking in the Dark (HDID) mice. Alcohol. 2021;93:45-56.
21 Dornellas APS, Burnham NW, Luhn KL, Petruzzi MV, Thiele TE, Navarro M. Activation of locus coeruleus to rostromedial tegmental nucleus (RMTg) noradrenergic pathway blunts binge-like ethanol drinking and induces aversive responses in mice. Neuropharmacology. 2021;199:108797.
22 Burnham NW, Chaimowitz CN, Vis CC, Segantine Dornellas AP, Navarro M, Thiele TE. Lateral hypothalamus-projecting noradrenergic locus coeruleus pathway modulates binge-like ethanol drinking in male and female TH-ires-cre mice. Neuropharmacology. 2021;196:108702.
23 Ferguson LB, Zhang L, Kircher D, Wang S, Mayfield RD, Crabbe JC, et al. Dissecting Brain Networks Underlying Alcohol Binge Drinking Using a Systems Genomics Approach. Mol Neurobiol. 2019;56(4):2791-810.
24 Levine OB, Skelly MJ, Miller JD, Rivera-Irizarry JK, Rowson SA, DiBerto JF, et al. The paraventricular thalamus provides a polysynaptic brake on limbic CRF neurons to sex-dependently blunt binge alcohol drinking and avoidance behavior in mice. Nat Commun. 2021;12(1):5080.
25 Rinker JA, Marshall SA, Mazzone CM, Lowery-Gionta EG, Gulati V, Pleil KE, et al. Extended Amygdala to Ventral Tegmental Area Corticotropin-Releasing Factor Circuit Controls Binge Ethanol Intake. Biol Psychiatry. 2017;81(11):930-40.
26 Siciliano CA, Noamany H, Chang CJ, Brown AR, Chen X, Leible D, et al. A cortical-brainstem circuit predicts and governs compulsive alcohol drinking. Science. 2019;366(6468):1008-12.
27 Haggerty DL, Munoz B, Pennington T, Viana Di Prisco G, Grecco GG, Atwood BK. The role of anterior insular cortex inputs to dorsolateral striatum in binge alcohol drinking. Elife. 2022;11:e77411.
28 Munoz B, Fritz BM, Yin F, Atwood BK. Alcohol exposure disrupts mu opioid receptor-mediated long-term depression at insular cortex inputs to dorsolateral striatum. Nat Commun. 2018;9(1):1318.
29 Godynyuk E, Bluitt MN, Tooley JR, Kravitz AV, Creed MC. An Open-Source, Automated Home-Cage Sipper Device for Monitoring Liquid Ingestive Behavior in Rodents. eNeuro. 2019;6(5):ENEURO.0292-19.2019.

30 Centanni SW, Janes AC, Haggerty DL, Atwood B, Hopf FW. Better living through understanding the insula: Why subregions can make all the difference. Neuropharmacology. 2021;198:108765.