Whole-brain activity changes in male and female C57BL/6J mice following binge-like ethanol drinking.

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Introduction

- Growing parity between men and women in alcohol use and alcohol use disorder diagnoses necessitates study of sex differences in alcohol related behaviors.[1,2]
- C57BL/6J (B6) mice exhibit sex differences related to binge-like ethanol drinking.
  - DREADD stimulation of the nucleus accumbens core (NAcc) reduces ethanol intake in female B6 mice, no change in males.[6,4]
  - DREADD inhibition of the NAcc reduces ethanol intake in males, but increases intake in females.[3,4,5]
  - Sex influences in NAcc transcriptional changes following binge-like drinking.[6]

Research Question:

- Are different brain regions engage in males and females following Drinking-In-the-Dark (DID) binge-like drinking?

Hypotheses:

- Higher c-Fos expression in ethanol than water drinking mice
- Males have greater engagement of excitatory input [e.g. cortical regions (infralimbic, prelimbic, insular) or ventral hippocampus] following DID.
- Females have greater engagement of inhibitory or peptidergic regions [e.g. central amygdala (CeA), pallidum, or hypothalamus].

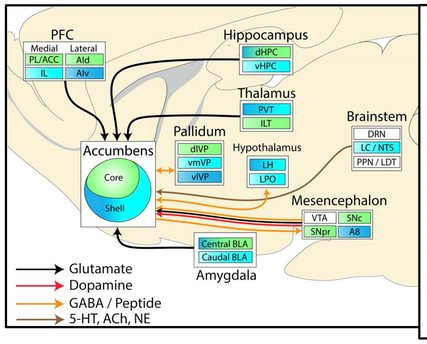

Figure 1. The rodent NAcc receives diverse anatomical and neuromodulatory inputs. Regions are color coded to reflect the accumbens subregion they primarily project to: NAcc (green), medial NAc shell (light blue), lateral NAc shell (dark blue), or both (white). Notable NAcc projections: ACC, anterior cingulate cortex; Ald, dorsal anterior insular; BLA, basolateral amygdala, dHPC, dorsal hippocampus; IL, infralimbic cortex; PL, prelimbic cortex; SNc, substantia nigra pars compacta; SNpr, substantia nigra pars reticulata (Scofield et al., 2016).

Methods

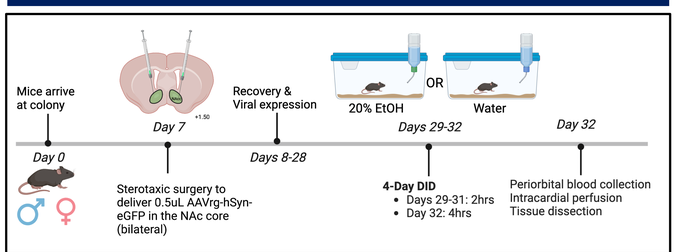

Figure 2. Experimental Timeline. (Created with BioRender.com)

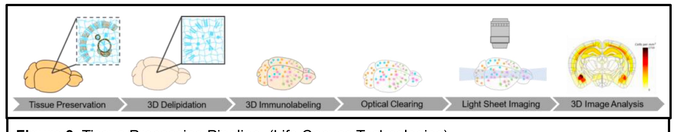

Figure 3. Tissue Processing Pipeline. (Life Canvas Technologies)

Adult (10 weeks old) male and female B6 mice (n=17-19/sex/fluid) were purchased from Jackson Labs and housed under a 12/hr reverse light-dark cycle. All mice underwent stereotaxic surgery to deliver AAVrg-hSyn-eGFP to the NAcc, then were allowed to recover for 3 weeks. Mice drank 20% ethanol or water using 4-day DID procedure[7], drinking session began 3 hrs into the animal's dark cycle. Immediately the finals DID, we collected periorbital blood for determination of blood ethanol concentration (BEC), perfused mice, and collected brain tissue. Whole-brain clearing, immunolabeling for c-Fos and GFP, and imaging was completed by Life Canvas Technologies, then image atlas registration and cell detection was conducted using SmartAnalytics software.

1. Male and Female B6 Mice Drink Ethanol to Intoxication

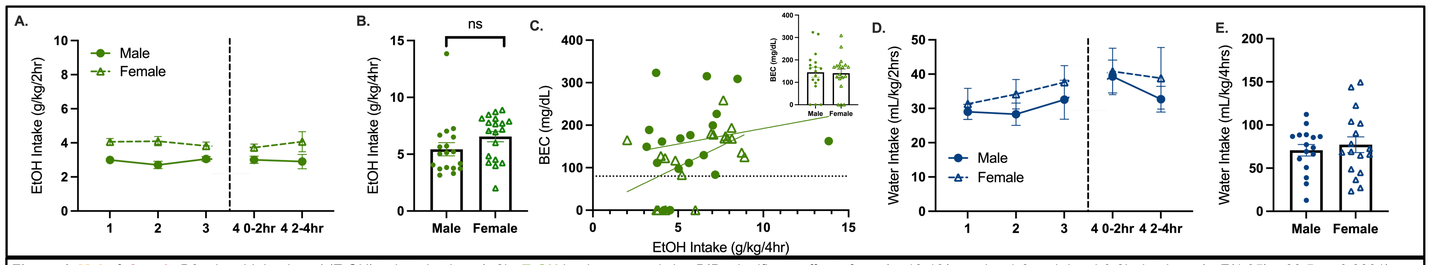

Figure 4. Male & female B6 mice drink ethanol (EtOH) to intoxication. *A.* 2hr EtOH intake across 4-day DID, significant effect of sex (n=18-19/sex; day 1-3 and day 4 0-2hr intake only, $F(1,35) = 33.5$, $p<0.0001$), no effect of day or interaction. *B.* 4hr EtOH intake on day 4, no effect of sex. *C.* Scatterplot showing 4hr EtOH intake vs BEC. Dotted line at 80 mg/dL indicates threshold for intoxication. (Pearson's correlation values: male R^2 =0.03980; female R^2 = 0.2845, no effect of sex on slope or intercept). *Inset:* No effect of sex on BECs. *D.* 2hr water intake across 4-day DID, no significant effects (n=17/sex). *E.* 4hr water intake on day 4, no effect of sex. Data reported as mean ± SEM. Closed circles denote males, open triangles denote females.

2. Binge-like Ethanol Drinking Changes c-Fos Expression

2.1 Representative c-Fos expression and cell density heatmaps

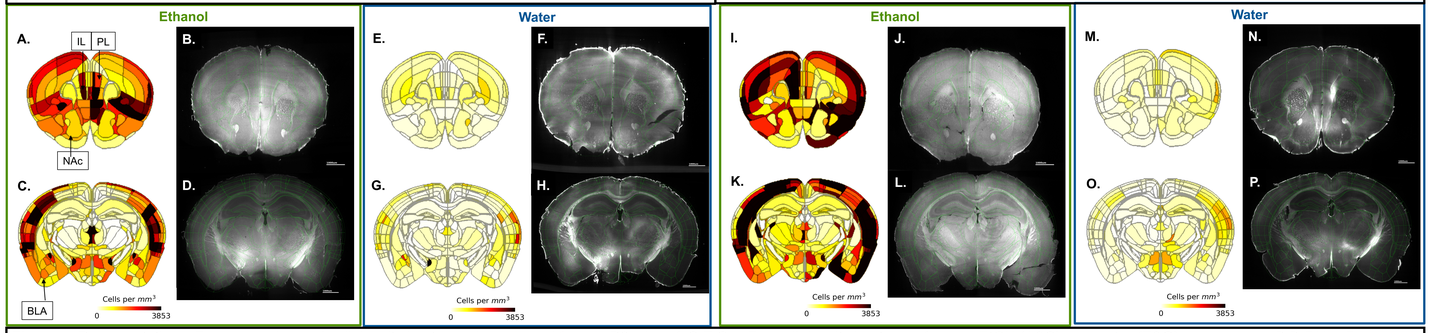

Figure 5. Representative c-Fos expression and cell density heatmaps in male and female B6 mice. *A,C,E,G.* c-Fos cell density heatmaps from an EtOH (A&C) or a water (E&G) male mouse. *B,D,F,H,* c-Fos expression with Allen Brain Atlas (ABA) overlay from an EtOH (B&D) or a water (F&H) male mouse. I,K,M,O. c-Fos cell density heatmaps from an EtOH (I&K) or a water (M&O) female mouse. J,L,N,P. c-Fos expression with ABA overlay from an EtOH (J&L) or a water (N&P) female mouse. A-D from one male EtOH drinking mouse; E-H from one male water drinking mouse; I-L from one female EtOH drinking mouse; M-P from one female water drinking mouse. Scale bar = 1000um. BLA, basolateral amygdala; IL, infralimbic cortex; NAc, nucleus accumbens; PL, prelimbic cortex.

2.2 Visualization of c-Fos cell Density Distribution

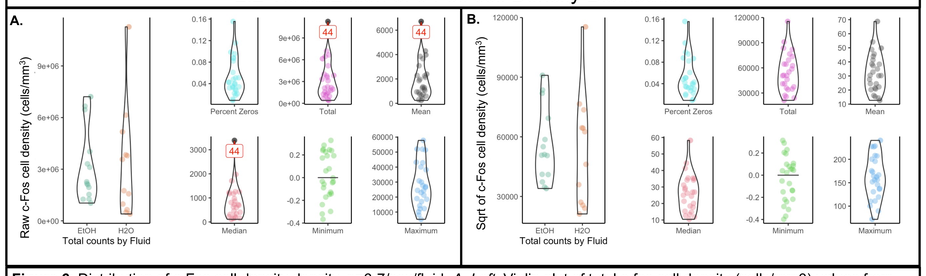

Figure 6: Distribution of c-Fos cell density density. n=6-7/sex/fluid. *A. Left:* Violin plot of total c-fos cell density (cells/mm3) values from mice that drank ethanol or water from the untransformed data set. *Right:* Violin plots of the percent zeros, total, mean, median, minimum and maximum c-fos cell density values in the untransformed data set. *B. Left:* Violin plot of total c-fos cell density (cells/mm3) values from mice that drank ethanol or water from square root (Sqrt) transformed data set. *Right:* Violin plots of the percent zeros, total, mean, median, minimum and maximum c-fos cell density values in the Sqrt transformed data set. c-Fos cell density values (cells/mm^3) include separate values for left and right hemisphere. Plots created with Rstudio packages ggpubr, ggstatsplot, and tidyverse (version 2022.07.2+576)

2.3 Principal Components Analysis

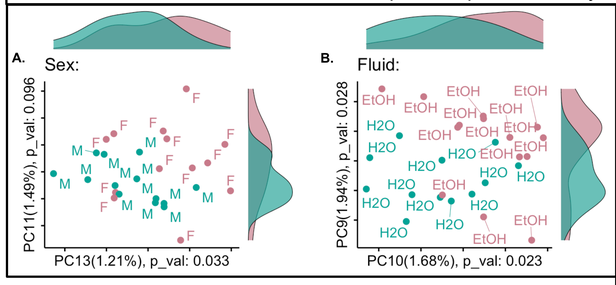

Figure 7: Principal Component Analysis (PCA) of c-Fos cell density. n=6-7/sex/fluid. *A.* Plot of top PCs associated with sex, PCs 13 and 11, with respective density plots. Sex is significantly associated with PC 13 (1.214% of variance, $p <0.05$. *B.* Plot of top PCs associated with fluid, PC 10 and 9, with respective density plots.. Fluid is significantly associated with PC 2, 9 and 10 (10.043% of variance, p-value <0.05). c-Fos cell density values (cells/mm^3) include separate values for left and right hemisphere, and values square-root normalized. Plots created with Rstudio packages ggpubr, ggstatsplot, and tidyverse (version 2022.07.2+576)

2.4 c-Fos Expression in Regions of Interest

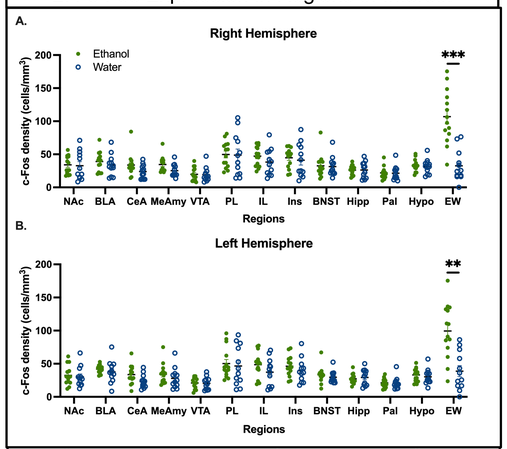

Figure 8: c-Fos expression in regions of interest. *A.* c-Fos cell density in right hemisphere for mice that drank ethanol or water. Effect of region ($F(2.898, 69.56)=26.49$; $p<0.01$), subject ($F(24,288)=11.61$; $p<0.001$, and region by fluid ($F(12,288)=15.05$; $p<0.001$]. Multiple comparisons test revealed effect of fluid ($p<0.0001$) in the Edinger Westphal nucleus (EW). *B.* c-Fos cell density in left hemisphere of mice that drank ethanol or water. Effect of region ($F(2.984, 71.61)=21.38$; $p<0.0001$], subject ($F(24,288)=7.104$; $p<0.0001$], and region by fluid $F(12, 288)=8.216$; $p<0.0001$]. Multiple comparisons test revealed an effect of fluid ($p< 0.01$) in the EW. n=6-7/sex/fluid. Data reported as mean ± SEM. No effect of sex or interactions in any tests, data collapsed by sex. c-Fos cell density values (cells/mm^3) were square-root normalized Closed circles denote mice that drank ethanol, open circles denote mice that drank water. MeAmy, medial amygdala; VTA, ventral tegmental area; Ins, insular cortex; BNST, bed nuclei of the stria terminalis; Hipp, hippocampal region; Pal, pallidum; hypo, hypothalamus.

Conclusions

- Male and female B6 mice drink ethanol to intoxication in a 4-day DID procedure.
- Mice that drank ethanol have greater c-Fos expression in the Edinger-Westphal nucleus than mice that drank water.
- PCA results indicate that fluid and sex explain a significant amount of variation in the data.

Future Directions

- Complete quantification of c-Fos expression on remaining samples.
- Determine if hemisphere explains a significant amount of variation in the data
  - Collapse data by hemisphere if not
- Perform hierarchical clustering and apply graph theory/network science to understand large scale differences in c-Fos expression between sex and fluid groups
- Quantify co-labeled c-Fos and GFP positive neurons to answer these questions:
  - Which anatomical NAcc inputs are active during binge-like ethanol intake?
  - Are the same inputs active in male and female B6 mice?

References

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- For questions please email Amy Chan at chanamy@ohsu.edu