Negative Affect and BNST Cellular Activation during Withdrawal from Adolescent Alcohol Exposure

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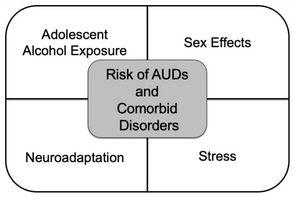
Background

- Adolescence is a time of brain maturation, lending vulnerability to the effects of alcohol.
- Most alcohol use is initiated during adolescence, typically in a binge-like manner.



- Adolescent alcohol use is the highest risk factor for developing an alcohol use disorder (AUD) later in life.
- Current data shows that adolescent alcohol use in females is outpacing males.
- Females are more motivated to consume alcohol to relieve negative affect, while males drink for the positive rewarding effects of alcohol.
- Negative affect is produced during alcohol withdrawal, and this negative affect is a driver for continued alcohol use and later stress-induced relapse.
- The bed nucleus of the stria terminalis (BNST) is a highly sexually dimorphic brain region that is known to be critical in alcohol-mediated negative affect and subsequent relapse.
- BNST CRF-CRFR1 signaling is known to be involved in alcohol withdrawal, negative affect, and stress.

The current work will test the hypotheses that withdrawal from adolescent alcohol exposure produces sex differences in:

- **Negative affect phenotypes**
- **BNST cellular activation, specifically in CRF and CRFR1 containing cells**

Methods

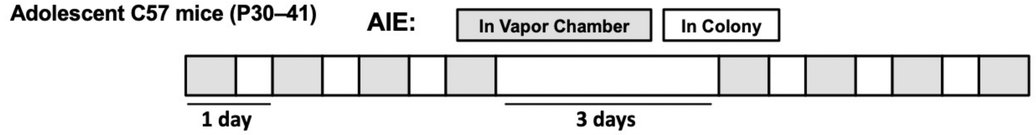
Adolescent intermittent ethanol (AIE) vapor exposure: Adolescent (PND30–41) C57Bl/6J female and male mice were given a daily injection of either pyrazole (air control, 1 mmol/kg) or pyrazole + ethanol (ethanol group, 1 mmol/kg + 0.8 g/kg, respectively) to impair the metabolism of ethanol. Thirty minutes after the injection, mice were placed in their home cages, which were then placed into a chamber filled with volatilized ethanol (20.3 ± 0.2 mg/L) or volatilized water (air group). Airflow through the chambers was maintained at 5.5 L/min, and volatilization was maintained at 1.5 L/min. After 16 hours of exposure, mice were removed from the chambers and returned to standard animal housing. Ethanol chamber exposure occurred from 1600–0800 the following day. Using these parameters, we were able to reliably obtain blood ethanol concentrations in the range of 150–185 mg/dL in adolescent mice. This protocol was run for two, four-day cycles of 16 hours in vapor chambers and eight hours out of vapor chambers. Five hours after the final alcohol exposure, behavioral tasks were performed, or brains were collected for RNAscope.

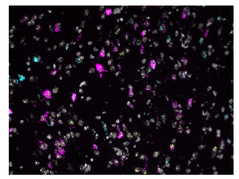
Elevated plus maze: Mice were placed on a plus sign-shaped maze with two wall-less "open" arms and two walled "closed" arms. This test examined the drive to explore an open arm versus remaining "safe" in a closed arm.
- Two or three paws on open arm = "partial entry" (sheltered exploratory behavior)
- All four paws on open arm = "full entry" (unsheltered exploratory behavior)

Marble-burying task: A simple measure of OCD/anxiety-attributed burying or digging behaviors which can be attenuated by anxiolytic drugs. Mice were placed in a cage with evenly-spaced black glass marbles for 20 minutes, and the number of marbles buried (2/3 covered) was recorded.

RNAscope: Brains were collected and flash frozen using isopentane. Brains were stored at -80ºC, and twenty-four hours before slicing, brains were moved to -20ºC. Brains were sliced on a Cryostat (CryoStar NX50) at 10µm and adhered to warm Fisher plus slides and immediately refrozen. RNAscope was performed on slides containing the BNST following the steps of the ACD Fluorescent Multiplex Kit. Tissues were fixed using cold 4% paraformaldehyde, and the following three probes were used for RNAscope: C1 for cfos mRNA, C2 for CRF mRNA, and C3 for CRFR1 mRNA. Twenty-four hours after completing RNAscope, slides were imaged using an Epifluorescent Slide Scanning Microscope using corresponding FITC, TX Red, and CY5 filter cubes. Images were captured using Metamorph at 20X magnification and overlayed onto each other with color using ImageJ. ImageJ was also used to count the number of cells containing specific fluorescence in order to quantify the activation of different cell populations in the BNST. Negative control probe quantification was used to detect background levels of non-specific fluorescence.

Results

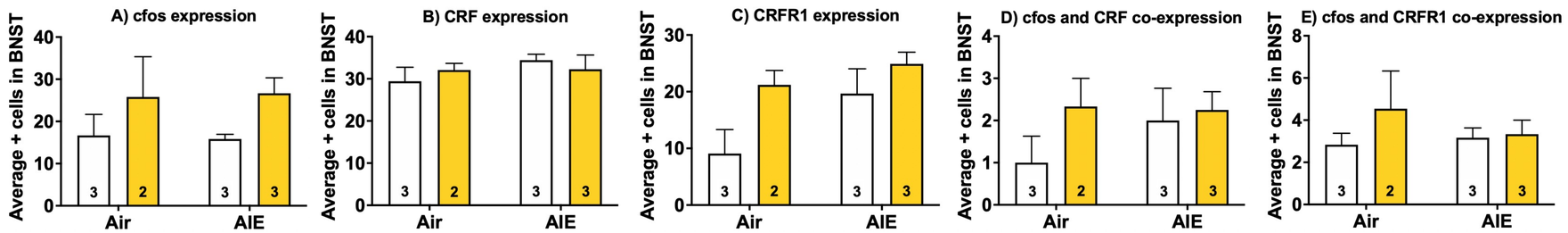




Blue = cfos
Pink = CRF
Green = CRFR1
White = DAPI

RNAscope:

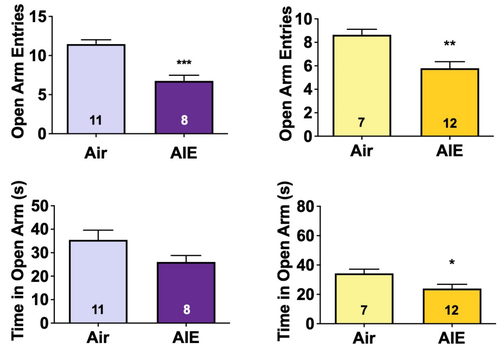
Male Female



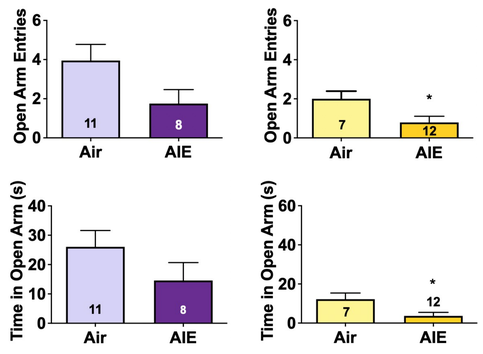
- Overall, RNAscope data reveals several non-significant but trending results. Female mice have greater cfos, CRFR1, and cfos/CRF co-expression compared to males but were not impacted by AIE. AIE appeared to increase CRFR1 and cfos/CRF co-expression in male mice.

Behavioral Tasks:

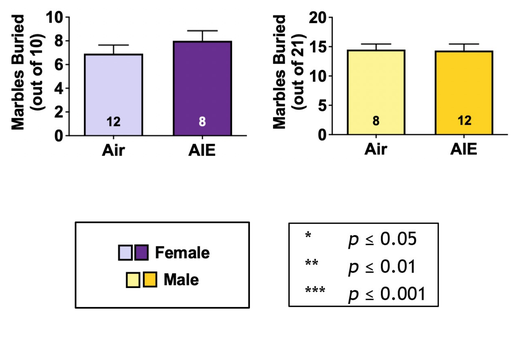
F) Acute AIE withdrawal reduced partial open-arm entries on EPM in both males and females.



G) Acute AIE withdrawal altered full open-arm entries on EPM only in males.



H) Acute AIE withdrawal did not affect marble burying.



Discussion:

This work demonstrated numerous sex differences during withdrawal from AIE.
- Anxiety-like behavior measured by the EPM was more robust in male mice.
- In the BNST, female mice have higher overall cellular activation, CRFR1, and cfos/CRF co-expression compared to males, but these levels are not impacted by AIE treatment.
- In male mice, AIE enhances CRFR1 expression and cfos/CRF co-expression, demonstrating a greater sensitivity of BNST CRF-CRFR1 signaling from alcohol withdrawal in male mice.

Future Directions:

- Cfos activation was expected to be higher overall in the AIE treatment group, therefore future work will explore other withdrawal timepoints and interactions with stress.
- Increase the number of mice in RNAscope experiments.
- Additional behavioral tests could be used to study if sex differences occur in other behavioral phenotypes.

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