

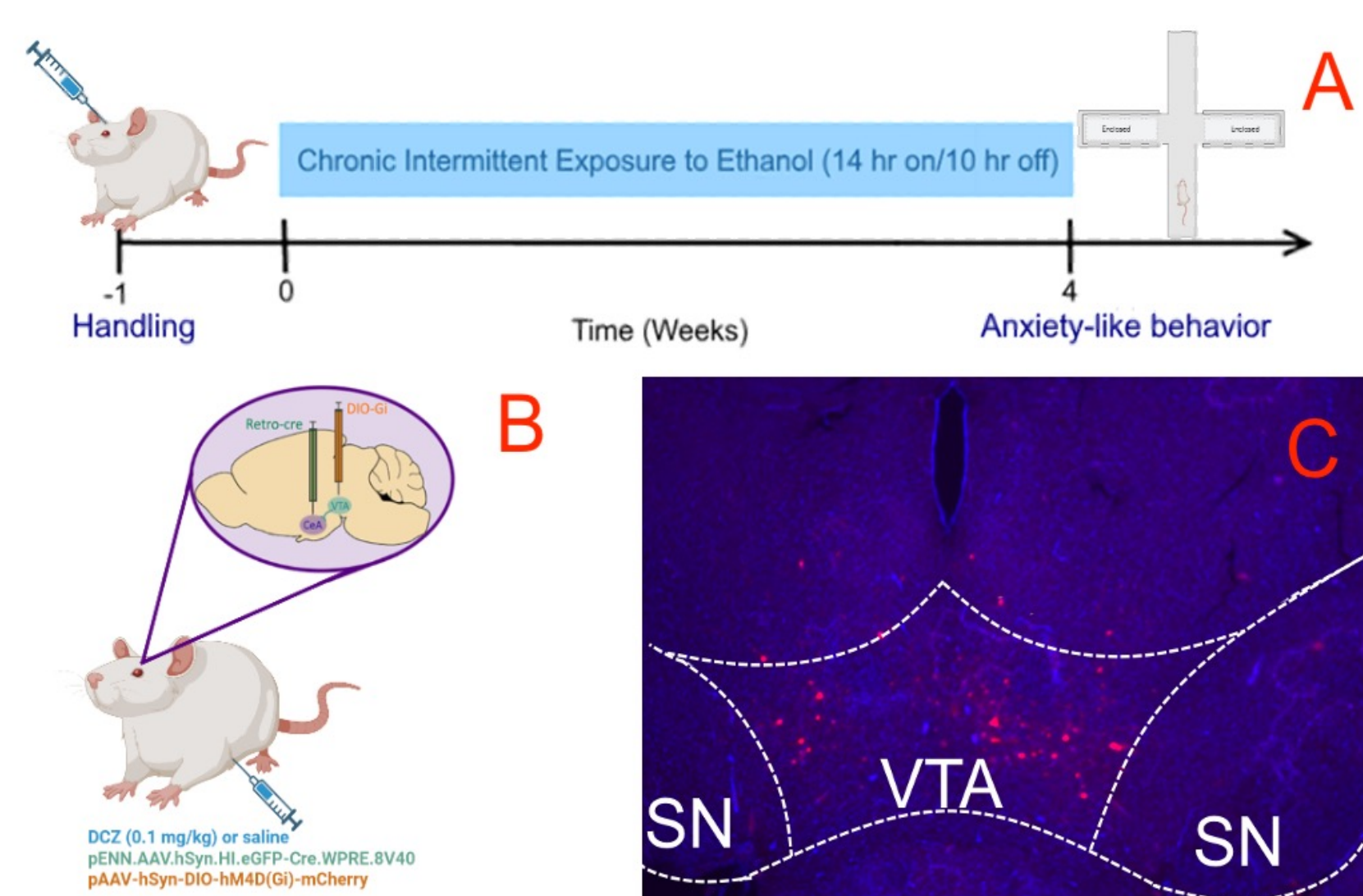
Sydney Long, Sydney Vita, PhD, Elizabeth Avegno, PhD  
Department of Physiology, Louisiana State University Health Sciences Center

## Introduction

- Humans with alcohol use disorder (AUD) often experience anxiety during withdrawal (WD), which is associated with greater risk of relapse
- The neurobiology underlying WD-associated anxiety may involve the ventral tegmental area (VTA) and the central amygdala (CeA)
- VTA: associated with reward
- CeA: associated with emotional response
- Previous work has demonstrated activation of CeA-projecting VTA neurons during alcohol WD in a rat model of alcohol dependence, raising the possibility that this circuit contributes to WD-associated behaviors
- We hypothesized that inhibition of the VTA-CeA circuit may rescue anxiety-like behavior of dependent rats during WD
- To test this hypothesis, we used a chemogenetic strategy to inhibit CeA-projecting VTA neurons and evaluating anxiety-like behavior during WD
- We used a designer receptor exclusively activated by designer drugs (DREADD) for our chemogenetic strategy
- Gi: inhibitory DREADD
- DCZ: designer drug that selectively activates Gi-expressing cells

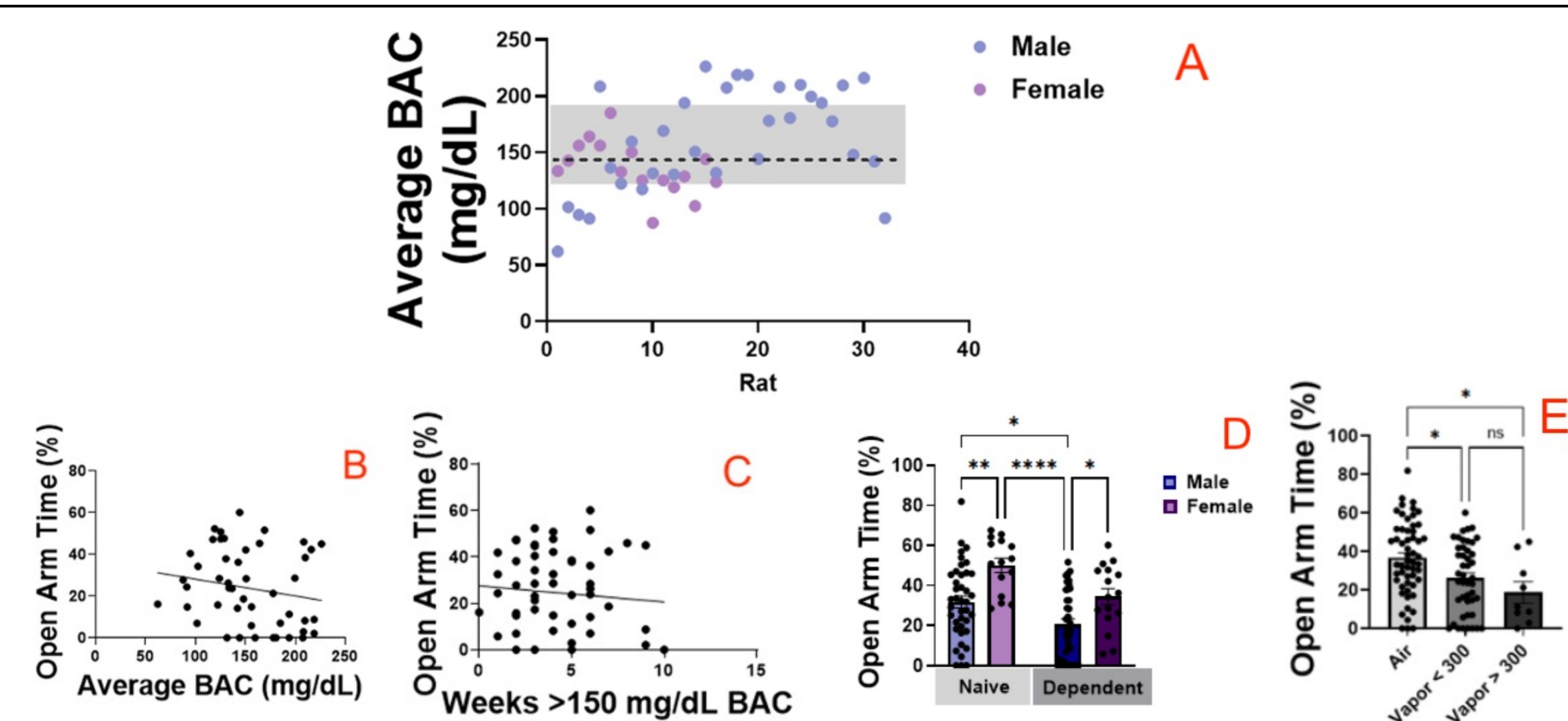
## Methods

- We first analyzed existing data to determine whether experimental parameters influence behavioral outcomes (Figure 2)
- We used a dual virus approach to selectively transfect CeA-projecting VTA neurons with an inhibitory DREADD. Retro-cre was bilaterally injected into the CeA, and a cre-dependent inhibitory (Gi) DREADD (or control inactive virus) was injected into the VTA of adult male Wistar rats. (see Figure 1)
- To model alcohol dependence, we used a chronic intermittent exposure (CIE) to ethanol vapor paradigm, where rats were exposed to vapor for 14 hours a day and pure air for 10 hours a day
- Blood alcohol concentrations (BAC) were monitored and targeted between 150-300 mg/dL
- Following 4 weeks of vapor exposure, rats were tested for anxiety-like behavior in an elevated plus maze (EPM) during withdrawal
- To inhibit the VTA-CeA circuit, DCZ (0.1 mg/kg, i.p.) was administered 30 minutes prior to behavioral testing
- Brains were sectioned to confirm virus placement and stained to confirm inhibition of cells using phosphorylation of pyruvate dehydrogenase (pPDH)



**Figure 1:** Depiction of methods involving alcohol exposure and virus and drug administration.  
**Figure 1a:** Timeline of chronic intermittent exposure to ethanol from handling to behavior testing.  
**Figure 1b:** Depiction of where retro-Cre and DIO-Gi (or mCherry) are injected as well as the concentration of DCZ (or saline) that is administered.  
**Figure 1c:** 2x representative injection site of mCherry in VTA. Blue dots are nuclei visualized with DAPI. Red dots are virus (mCherry) containing cells. Pink dots are pPDH-expressing cells, indicating cellular inhibition. Green is indicative of the presence of retro-Cre and shows the retrograde pathway of VTA neurons projecting to the CeA.

## Effects of Vapor Parameters on Behavioral Outcomes



**Figure 2:** Average BAC, total number of weeks with a BAC > 150 mg/dL, sex, and whether rats ever achieved a BAC > 300 mg/dL were plotted for multiple rat cohorts against behavioral outcomes

**Figure 2a:** Average BAC of rats plotted against individual rat IDs, with male rats in blue and female rats in purple. Dashed line indicates the average BAC across all rats. The shaded region indicates rats in the range between the lower 25<sup>th</sup> quartile and the upper 75<sup>th</sup> quartile.

**Figure 2b:** Percent of time spent in the open arm of the EPM plotted against average BAC. Linear regression analysis revealed no significant relationship between average BAC on time spent in the open arm of an EPM

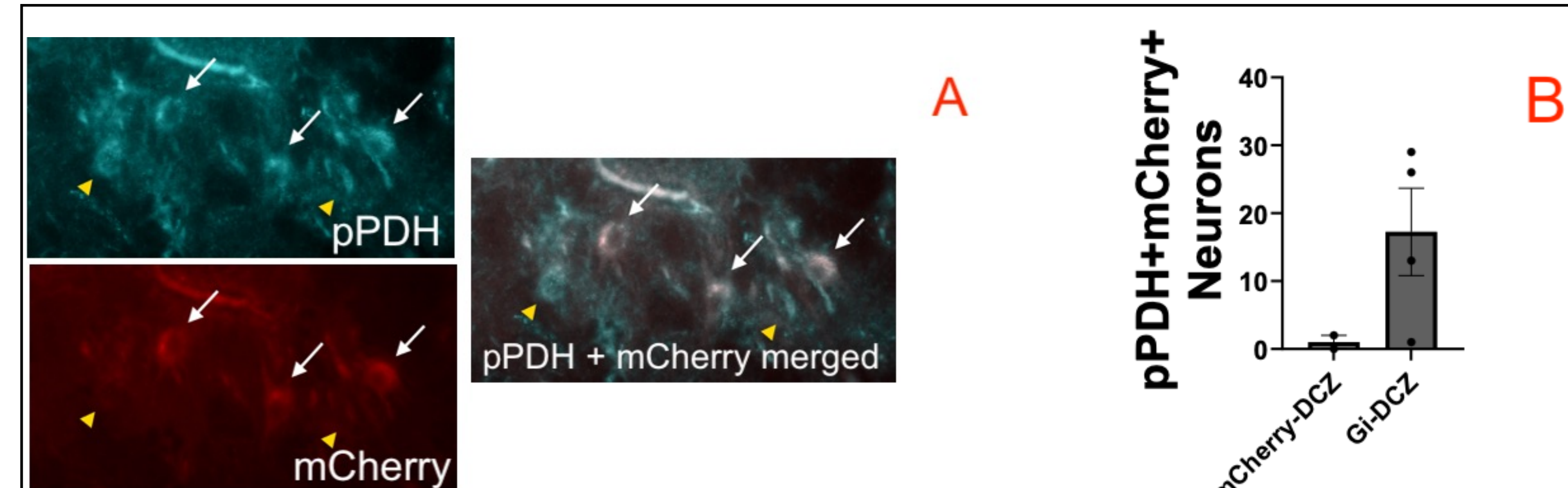
**Figure 2c:** Percent of time spent in the open arm of the EPM plotted against the number of weeks each rat had a BAC above 150 mg/dL. Linear regression analysis revealed no significant relationship between number of weeks of vapor exposure on time spent in the open arm of an EPM

**Figure 2d:** Percent of time spent in the open arm of the EPM plotted against naïve males (light blue), naïve females (light purple), dependent males (dark blue), and dependent females (dark purple)

**Figure 2e:** Percent of time spent in the open arm of the EPM plotted against rats exposed to air (light gray), rats who did not reach a BAC above 300 mg/dL (gray), and rats who reached a BAC above 300 mg/dL (dark gray)

- A 2-way ANOVA indicates main effects of sex and vapor on behavior (\* $p < 0.05$ ; \*\* $p < 0.005$ ; \*\*\*\* $p < 0.0001$ ; Tukey's multiple comparisons test)
- When comparing data from rats that did achieve BACs > 300 mg/dL to those that did not, and to data from naïve controls (right-most graphs), a one-way ANOVA indicated a significant relationship between intoxication levels and behavior (\* $p < 0.05$ , Tukey's multiple comparisons test)
- Average BACs across cohorts were aggregated and used to determine a threshold for inclusion in future studies, defined as the lower quartile ( $\leq 130$  mg/dL), which was then applied to our analysis of rats following chemogenetic inhibition of the VTA-CeA circuit during WD

## DCZ Administration Inhibits CeA-projecting VTA Neurons in Gi-Expressing Rats



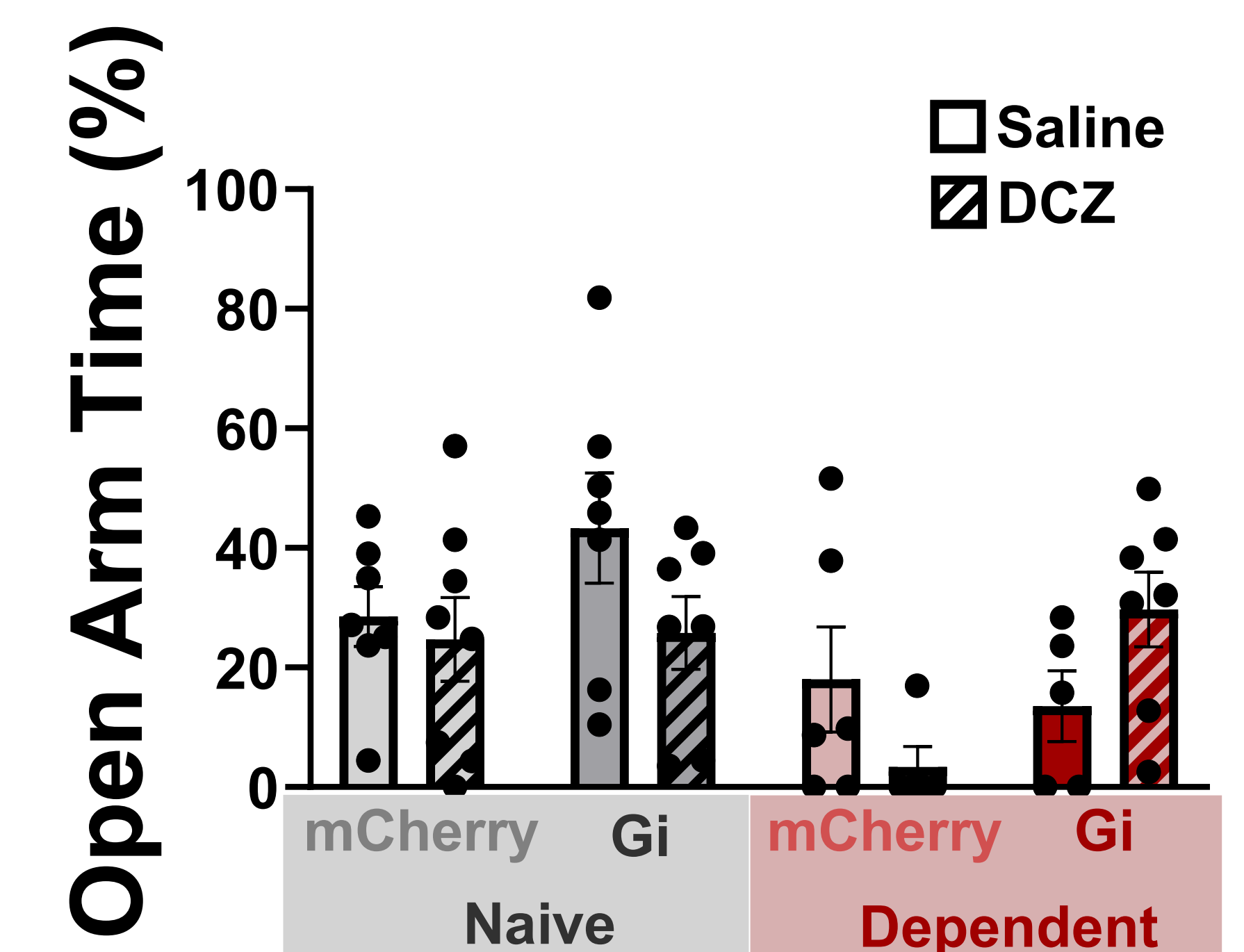
**Figure 3:** Image analysis of cells containing pPDH and mCherry in rats administered mCherry and DCZ and rats administered Gi and DCZ.

**Figure 3a:** Visualization of cellular inhibition using pPDH (blue) and visualization of virus-containing cells using mCherry (red) and their layered imaging to analyze coexpression. White arrows indicate pPDH+mCherry+ cells. Yellow arrowhead indicates pPDH+ mCherry- cells

**Figure 3b:** Number of pPDH and mCherry coexpressing cells in cells containing mCherry and DCZ (control) and cells containing Gi and DCZ (experimental).

- To confirm cell type-specific inhibition following DCZ administration, we stained VTA-containing sections
- Greater number of pPDH+, mCherry+ expressing VTA neurons of Gi-expressing rats compared to inactive virus controls ( $p > 0.05$  due to low n; two-tailed  $t$ -test)

## Administration of Gi and DCZ May Rescue Anxiety-Like Behavior



**Figure 4:** Percent of time spent in the open arm of the EPM plotted against experimental and control rats. Naïve rats shown in gray. Dependent rats shown in red. Rats who were administered saline shown as a solid bar. Rats who were administered DCZ shown as a striped bar. Rats who were administered mCherry are plotted on the left of each set of columns. Rats who were administered Gi plotted on the right of each set of columns.

- Less time in the open arm of the elevated plus maze (EPM) is recognized as a demonstration of anxiety-like behavior
- Preliminary data suggests that inhibition of the VTA-CeA circuit in alcohol dependent rats tested during WD may rescue increased anxiety-like behavior, although this did not achieve statistical significance
- Rats that received Gi and DCZ showed results most similar to naïve control rats
- Rats that were dependent on alcohol but received no inhibitory DREADD (received mCherry) overall spent the least amount of time in the open arm

## Conclusion

- Our data indicate that inhibiting the CeA-projecting VTA circuit may rescue increased anxiety-like behavior associated with alcohol withdrawal
- Further tests with a larger data set to perform a full statistical analysis is necessary to verify the current findings
- Additionally, future experimentation is necessary to see whether CeA-projecting VTA neurons have collateral projections which may influence behavior
- Ongoing work is utilizing a brain clearing and whole-brain imaging strategy to see if CeA-projecting VTA neurons project elsewhere in the brain
- If other regions are identified as being involved, these previous experiments can be repeated with site-specific drug and virus administration to test the role of these regions
- As a result of these experiments, we hope to better understand the neurobiology underlying alcohol withdrawal-associated increases in anxiety, potentially allowing for improved therapeutic options for individuals with AUD