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Adolescent alcohol disrupts development of noradrenergic neurons in the nucleus of the tractus solitarius and enhances stress behaviors in adulthood in mice in a sex specific manner

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Abstract

Alcohol use disorders (AUDs) are common mental health issues worldwide and can lead to other chronic diseases. Stress is a major factor in the development and continuation of AUDs, and adolescent alcohol exposure can lead to enhanced stress-responsivity and increased risk for AUD development in adulthood. The exact mechanisms behind the interaction between adolescence, stress, and alcohol are not fully understood and require further research. In this regard, the nucleus of the tractus solitarius (NTS) provides dense norepinephrine projections to the extended amygdala, providing a key pathway for stress-related alcohol behaviors. While NTS norepinephrine neurons are known to be alcohol sensitive, whether adolescent alcohol disrupts NTS-norepinephrine neuron development and if this is related to altered stress-sensitivity and alcohol preference in adulthood has not previously been examined. Here, we exposed male and female C57Bl/6J mice to the commonly used adolescent intermittent ethanol (AIE) vapor model during postnatal day 28-42 and examined AIE effects on: 1) tyrosine hydroxylase (TH) mRNA expression in the NTS across various ages (postnatal day 21-90), 2) behavioral responses

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Supplementary materials

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to acute stress in the light/dark box test in adulthood, 3) NTS TH neuron responses to acute stress and ethanol challenges in adulthood, and 4) ethanol conditioned place preference behavior in adulthood. Overall the findings indicate that AIE alters NTS TH mRNA expression and increases anxiety-like behaviors following acute stress exposure in a sex-dependent manner. These mRNA expression and behavioral changes occur in the absence of AIE-induced changes in NTS TH neuron sensitivity to either acute stress or acute alcohol exposure or changes to ethanol conditioned place preference.

Keywords

Adolescent alcohol; Nucleus of the tractus solitarius; Stress; Alcohol use disorder; Development

Introduction

Alcohol use disorders (AUDs) are one of the most pervasive mental health disorders worldwide and are an important risk factor for other chronic diseases [33]. AUDs are difficult to treat, in part, because they are not homogenous in nature. Indeed, AUD treatments must account for the complex interaction of underlying causes of the disorder as well as current mental health status of treatment-seeking individuals [20].

Numerous studies indicate that stress is a critical factor in both the development and maintenance of AUD. Early life exposure to alcohol or stress can also significantly influence an individual's subsequent risk for AUD. In the clinical population, adolescent alcohol drinking increases risks for AUDs in adulthood [11] and is associated with enhanced negative emotional reactivity and altered distress tolerance during sustained abstinence [49]. Animal models also indicate that adolescent ethanol (EtOH) exposure is associated with enhanced EtOH drinking and anxiety-like behaviors in adulthood [28]. Similarly, adolescent stress increases the propensity for AUDs in adulthood in both humans [9] and animal models [40]. These findings suggest that brain regions involved in stress-related behaviors that undergo plastic changes in adolescence might also be targeted by EtOH to promote increased risk for AUDs. The mechanisms involved in this interaction, however, are not fully understood.

In animal models, stress-induced reinstatement to drug seeking behaviors requires norepinephrine signaling from neurons in the A2 region of the nucleus of the tractus solitarius (NTS), which in turn increases neuronal activity in the bed nucleus of the stria terminalis (BNST) [41], particularly in Corticotropin Release Factor (CRF) neurons, to drive reinstatement behaviors [22,47]. Interestingly, the level of tyrosine hydroxylase (TH)-expression in the NTS, a key enzyme in the production of norepinephrine, surges in adolescence in rats during the normal course of development and tapers off in adulthood [24]. This change in expression suggests that adolescence is a critical window for the proper development of A2 neurons. Additionally, genetic modifications that knock out other NTS peptides can also alter the length of time NTS TH mRNA expression is enhanced during the adolescent-early adulthood period [24], resulting in modified TH fiber density in ascendant brain regions [25], increased stress responsivity [23], and increased alcohol

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drinking [42] in adulthood. Given these findings, we propose that developing NTS TH neurons are a critical target of adolescent EtOH exposure and hypothesize that adolescent intermittent EtOH (AIE) exposure will alter NTS TH neuron development to increase stress sensitivity and alter alcohol-related behaviors in adulthood. To test this hypothesis, we exposed male and female C57Bl/6J mice to the commonly used AIE vapor chamber model and examined NTS TH mRNA expression across adolescent and adult development. We further assessed how AIE might impact the sensitivity of NTS TH neurons to acute stress in adulthood. Finally, we assessed how AIE might impact EtOH sensitivity and EtOH conditioned place preference in adulthood. Overall the findings indicate that AIE alters NTS TH mRNA expression and increases anxiety-like behaviors following acute stress exposure in a sex-dependent manner, while maintaining NTS TH neuron sensitivity to both acute stress and acute EtOH exposure as well as maintaining EtOH conditioned place preference behavior.

Methods

Animals

Male and female C57Bl/6J mice were obtained from the Jackson Laboratory and used as breeders. Male and female offspring mice were generated at the Penn State College of Medicine Animal Research Facility to ensure mice underwent EtOH exposure at the appropriate age and had littermate controls. Litter sizes were 5-8 pups on average. To avoid potential litter-specific genetic effects, littermate mice were placed in separate groups such that no more than two mice from any litter were used in the same group. Mice were maintained on a 12-hour light/dark cycle with *ad libitum* water and food for the duration of the studies. All experimental protocols were approved by the Penn State College of Medicine Institutional Animal Care and Use Committee.

Adolescent intermittent EtOH (AIE) exposure

On postnatal day (PND) 28-42, mice were injected with 1mM/kg pyrazole to block EtOH metabolism 30 min prior to being placed in EtOH vapor chambers (La Jolla Alcohol Research, Inc) for 16hr/day for 2 consecutive days, followed by a 1 day off-period [38]. 95% EtOH was volatilized at 20mg/L with airflow through the chambers maintained at 5L/min with volatilization at 1.5L/min. Target blood EtOH concentrations (BECs) were 200-250 mg/dL and confirmed by use of age-adjusted sentinel mice within the vapor chamber during exposure periods. Control mice received the same handling/context exposure paradigm, including pyrazole injections, but volatilized EtOH was not introduced into their chambers.

Quantitative real-time PCR

mRNA expression of TH in the NTS from C57Bl6/J mice with and without AIE was examined at different time-points during AIE (PND29, PND36, PND43), one week after AIE (PND49) and in adulthood (PND70, PND90). Air exposed mice at these timepoints were used as controls. A pre-adolescence and pre-AIE exposure time point (PND21) was chosen as a comparator for all groups. To obtain samples at these time points, the NTS region was isolated via microdissection from coronal sections (500μm) and immediately frozen at −80°C until further use. Frozen tissue was homogenized in QIAzol Lysis Reagent

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using a TissueLyser II, with total RNA extracted using RNAeasy Lipid Tissue Mini kits and QIACube automated processing (Qiagen; Germantown, MD, United States). RNA concentration was measured with a NanoDrop spectrophotometer (ND-1000, Thermo Fisher Scientific; Waltham, MA, United States). cDNA was synthesized from total RNA using a high-capacity cDNA reverse transcription kit (ThermoFisher Scientific; Waltham, MA, United States). Quantitative real-time polymerase chain reaction (qRT-PCR) was performed on a QuantStudio 12K Flex system (Applied Biosystems; Foster City, CA, United States) using mouse specific Taqman gene primers for TH (Mm00447557_m1 ThermoFisher Scientific; Waltham, MA, United States). Each sample was measured in triplicate with cycle threshold (CT) values normalized to 18S ribosomal RNA (Rn18s, Mm03928990_g1). Relative gene expression was determined using the $2^{-\Delta\Delta CT}$ method.

Immunohistochemistry (IHC)

Mice were anesthetized with isoflurane and perfused through the left ventricle of the heart with phospho-buffered saline (PBS, 15 mL, Sigma-Aldrich P3619) followed by 4% paraformaldehyde in PBS (15 mL). Brains were removed, post-fixed for 24 h at 4°C in the same fixative, and then cryoprotected in PBS with 30% sucrose for an additional 48 h or until ready for sectioning. Forty micrometer semi-horizontal sections of the brainstem containing the NTS ([1], Doyle et al., 2004) were cut on a cryostat (Microm HM 550, Thermo Scientific) and stored in cryoprotectant before fluorescent immunohistochemical staining. Free-floating brain slices containing the NTS were washed in PBS (4 × 10 min), permeabilized with 0.5% Triton X-100 in PBS (30 min), and then blocked with 10% Normal Donkey Serum (Jackson Immuno Research) in PBS containing 0.1% Triton X-100 (1 h); all steps were at room temperature. Sections were then incubated with a rabbit anti-c-FOS antibody (1:200, sc-52, lot K0615, Santa Cruz Biotechnology Inc.) and a goat anti-TH antibody (ab76442, 1:250, lot GR21411-15, Abcam) in blocking solution for 72 h at 4°C. Following the incubation period, the slices underwent PBS washes (4 × 15 min) and were incubated with donkey anti-rabbit Alexa Fluor 488 (1:750, lot#129585, Jackson Immuno Research) and donkey anti-goat Alexa Fluor 594 (1:250, lot#122478, Jackson Immuno Research) fluorescent dye-conjugated secondary antibodies for 24 h at 4°C in PBS with 0.1% Triton X-100 and 10% Normal Donkey Serum. Finally, sections were washed (4 × 10 min), mounted on slides with Prolong Gold Mounting media (Lot#1837730, Invitrogen), and left overnight to dry. Stained NTS sections were z-stack imaged with a Keyence BZX-710 microscope at 4x and 40x magnification. 40x images (2–4 per animal) were analyzed with ImageJ software (NIH) by two researchers blinded to treatment condition independently. Since NTS area changes size by dorsal/ventral level in the horizontal orientation, final cell counts for each image were normalized to section size, averaged per mouse, and used for data analysis. ImageJ was used to autocorrect image brightness and create image overlays when necessary.

For effects of acute EtOH injection on TH/cfos immunohistochemistry, mice were injected with EtOH (0.5, 2, or 4 g/kg) or isovolumic saline (total injection volumes between 250 and 350 μL depending on body mass) and were individually housed for one hour until perfusion.

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To assess effects of stress on TH/cfos immunohistochemistry, mice were exposed to 10 min of forced swim stress as described below and perfused 30 min after exposure.

Forced swim and light/dark box

Mice were brought to the testing room and habituated for a minimum of 30 min. For the forced swim test, mice were placed individually in a 2-liter beaker of 21°C water for 10 min. Mice were then removed, towel dried, and placed in individual clean cages placed on top of a 32°C water circulating heating pad. After 20 min of recovery, mice were then placed in the Light chamber (400 lux) of the Light/Dark Box (Any-Maze) and allowed to move freely for 5 min while being video recorded (GoPro Hero 8). Latency time to exit the light side following initial placement in the chamber was examined as a separate measure from total time in the light side. The Light/Dark Box was cleaned with 70% EtOH solution between each test. Videos were analyzed via Any-Maze software automated analysis and confirmed by a researcher blinded to treatment conditions.

Conditioned place preference

Conditioned place preference (CPP) chambers (Any-Maze) consisted of three distinct compartments separated by manual dividers. Each compartment had distinct contextual characteristics: the middle (neutral) compartment was smaller (7.2 cm × 12.7 cm × 12.7 cm) with black walls, while the choice compartments were larger (16.8 cm × 12.7 cm × 12.7 cm, each) and had either black walls with white circles or black walls. All compartments were illuminated with dim light during use. Immediately following use, the entire preference chamber was cleaned thoroughly with a 70% EtOH solution. Mouse locations, activity counts, and time spent in each compartment were collected via automated video data-collection software (Any-Maze) and via infrared photo beam strips lining each compartment.

Habituation

Mice were placed in the center compartment with free access to all three compartments for 20 min once a day for 2 days. Time spent (seconds) in each compartment was recorded.

Conditioning

Twenty-four hours after habituation, mice received 5 days of conditioning training. EtOH-paired compartments were assigned based on the least preferred side (biased approach;) calculated by averaging time spent in each compartment over the two habituation days, similar to previous studies [14,46]. During conditioning, mice received an injection of saline and were placed into the most preferred compartment for 15 min in the mornings (08:00-10:00). Six hours later (14:00-16:00), mice received an injection of EtOH (2g/kg, I.P.)- a dose previously shown to induce reliable CPP in male and female C57Bl/6J mice [14,50]- and were placed into their least preferred compartment for 15 min.

Post-conditioning

24 hours after the last conditioning day, mice were placed in the 3-compartment chamber and allowed to move freely for 20 min. Our post-conditioning took place at the time point corresponding to time of EtOH conditioning (14:00-16:00).

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Statistical analysis

Data were analyzed and graphed using GraphPad Prism (GraphPad Software, versions 8-10; San Diego, CA, United States) and Microsoft Excel 365 (Microsoft Corporation; Redmond, WA, United States). Statistical tests used for analysis of data sets are described within the results section. Data are represented as mean ± standard error of the mean (SEM). Significance was determined at the $p < 0.05$ level for all analyses. Post hoc power analysis (G*power3.1) indicates >85% achieved power for all statistical tests.

Results

AIE induces early development of NTS TH mRNA expression in a sex specific manner

Previous research indicates that NTS TH neurons undergo age-dependent developmental changes during adolescence and that NTS TH neurons are EtOH-sensitive in adulthood. Here, we sought to test the hypothesis that adolescent EtOH exposure (AIE) via vapor chamber delivery would modify typical NTS TH developmental profiles in male and female C57Bl/6J mice. PND21 was used as a pre-adolescent and pre-EtOH vapor comparator group. Three-way ANOVA was used to examine NTS TH mRNA expression incorporating the variables of treatment (AIE vs Air), sex (male vs female), and age. Significant main effects of age ($F_{(6, 162)}= 9.258$, $p <0.0001$), sex ($F_{(1, 162)} = 80.76$, $p<0.0001$) and age-by-AIE interactions effects ($F_{(6, 162)} = 2.585$, $p=0.0203$) were found (Fig 1). Tukey's post hoc comparisons indicated males and females had significantly different TH mRNA expression at multiple timepoints as indicated in Supplement Table 1.

AIE induces sex-specific alterations to behavioral response to acute stress in adulthood

Since adolescence appears to be a natural epoch for NTS TH development that we demonstrate is vulnerable to modification by AIE, and since NTS TH neurons are critical to many behavioral and physiologic responses to stress, we next sought to determine if AIE might lead to long-term alterations in stress-related behaviors in adult mice. To that end, we serially combined two standard mouse models to induce and examine anxiety-like behavior: forced swim stress and the light dark box. Mice were initially exposed to forced swim for 10 min, towel dried and returned to their home cage for 20 min. After the home cage period, mice were then placed in the light-dark box for 5 min with activity video recorded.

While time in the light side is the typical readout used in the light-dark test, prior work indicates that male and female rodents display differing behavioral repertoires in various stress-related paradigms [10]. Therefore, we used multiple linear regression analysis to account for potential interaction of additional variables within the experimental design [51]. Specifically, light time was used as the dependent variable, with latency to first enter the dark side, dark side time, transitions, AIE, stress, and sex examined for main, two-way, and three-way interaction effects. This analysis indicated significant differences in light time ($F_{(41, 19)} = 376.1$ $p<0.0001$, adjusted $r^2=0.9961$, Fig 2) with "transitions" being the only non-significant variable for time in the light side. By incorporating all variables within this model, a significant AIE by stress by sex interaction was found ($p<0.0001$). This model did not fit a statistically significant regression when latency, dark time, or transitions were used as the dependent variables.

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Since the multiple regression analysis showed significant AIE, stress, and sex interactions, we next sought to confirm these findings in males and females separately using two-way ANOVA with preplanned Tukey's post-hoc analysis. In males, two-way ANOVA indicated significant main effects of AIE ($F_{(1, 20)} = 4.412$, p=0.0486) and stress ($F_{(1, 20)} = 19.73$, p=0.0003) on light time with no significant interaction effect (Fig 2A). Post-hoc analysis indicated significant differences between Air-No Stress vs AIE-Stress and Air-No Stress vs Air-Stress groups ($p<0.05$). In females, two-way ANOVA indicated significant main effects of AIE ($F_{(1, 31)} = 14.44$, p=0.0006) and stress ($F_{(1, 31)} = 7.350$, p=0.0108) with no significant interaction effect (Fig 2E). Post-hoc analysis indicated significant differences between Air-No Stress vs AIE-Stress and Air-Stress vs AIE-Stress groups ($p<0.05$). We then separately examined latency, dark time, and transitions as independent variables within each sex using two-way ANOVA with preplanned Tukey's posthoc comparison test to confirm and extend findings in the multiple linear regression model.

For dark time in male mice, two-way ANOVA indicated a significant effect of stress ($F_{(1, 20)} = 7.124$, p=0.0147) with no significant effect of AIE and no significant AIE x stress interaction ($p>0.05$). Posthoc analysis found no significant differences between any group (Fig 2B). For dark time in female mice, two-way ANOVA indicated a significant effect of AIE ($F_{(1, 31)} = 11.34$, p=0.0020) with no significant effect of stress and no significant AIE x stress interaction effect ($p>0.05$, Fig 2E).

For transitions in male mice, two-way ANOVA indicated significant stress ($F_{(1, 20)} = 8.997$, p=0.0071) and stress by AIE interaction ($F_{(1, 20)} = 4.548$, p=0.0455) effects. Post-hoc analysis indicated all groups were significantly different from the Air-No Stress group ($p<0.05$) while these groups were not statistically different from each other (Fig 2C). For transitions in female mice, two-way ANOVA indicated significant effects of stress ($F_{(1, 31)} = 6.900$, p=0.0133) and AIE ($F_{(1, 31)} = 13.54$, p=0.0009) but no significant stress by AIE interaction effect. Post-hoc analysis indicated all groups were significantly different from the Air-No Stress group ($p<0.05$) while these groups were not statistically different from each other (Fig 2G).

For latency in male mice, two-way ANOVA indicated a significant effect of stress ($F_{(1, 21)} = 15.59$, p=0.0007), with no significant AIE or stress x AIE interaction effects ($p>0.05$, Fig 2D). Posthoc analysis indicated significant differences between Air-No Stress and Air-Stress groups as well between AIE-No Stress and AIE-Stress groups ($p<0.05$). For latency in female mice, two-way ANOVA indicated no significant AIE, stress, or AIE x stress interaction effects ($p>0.05$, Fig 2H).

Overall, these data indicate that sex is a critical variable by which AIE alters NTS NE development and suggest that adolescent EtOH exposure results in sex-specific alterations in acute stress sensitivity of male and female mice. Such differences may be reflected as subtle changes within any one variable but significant interaction effects when all variables are accounted for in one statistical model.

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Stress increases NTS cfos expression in a sex-specific manner

The previous experiment indicates significant effects of AIE on NTS TH development as well as on adult stress-related behaviors. We therefore sought to test the hypothesis that AIE may alter how NTS TH neurons respond to stress, potentially in a sex-specific manner. We first sought to examine if AIE induced sex-specific changes in cfos colocalization with TH following forced swim stress (Fig 3). Three-way ANOVA indicated a significant main effect of stress ($F_{(1, 26)} = 6.880$, $p=0.0144$) and significant AIE-sex-stress interaction ($F_{(1, 26)} = 5.116$, $p=0.0323$) on overall cfos positive cells, with no other significant main or interaction effects ($p>0.05$). Post hoc analysis indicated a significant difference between male air-no stress and female AIE-stress mice ($p<0.05$). We further examined cfos co-localization with TH in these same brain sections. Importantly, there were no significant effects of AIE, stress, or sex on overall number of TH expressing neurons (Supplemental Figure 1). Since there was a significant AIE by stress by sex interaction with overall cfos expression as well as sex differences found in NTS TH neuron mRNA expression, we examined cfos/TH colocalization in both sexes separately. In males, two-way ANOVA indicated no significant effects of AIE, stress, or AIE by stress interaction effects ($p>0.05$). In females, two-way ANOVA indicated a significant main effect of stress ($F_{(1, 15)} = 9.394$, $p=0.0079$), with no significant effect of AIE or stress by AIE interactions. We further examined cfos expression in non-TH cells. Three-way ANOVA indicated a significant AIE by Sex by Stress interaction effect ($F_{(1, 27)} = 7.469$, $p>0.05$) with no other significant main or interaction effects detected ($p>0.05$). These data indicate that NTS TH neurons respond to forced swim stress in a sex-specific manner and that other NTS neurons may also become differentially regulated by AIE and stress in a sex-specific manner.

AIE does not alter the response of NTS TH neurons to acute alcohol exposure in adulthood

Our previous work and that from others indicates that acute EtOH exposure increases cfos expression in NTS TH neurons [1,44,45], although potential sex differences in adult EtOH sensitivity of NTS TH in animal models with a history of AIE has not previously been examined. Therefore, we next sought to determine if AIE may alter EtOH activation of NTS TH neurons in a sex-specific manner (Fig 4). Male and female mice underwent AIE or Air exposure on PND28-42 and then were injected intraperitoneally with EtOH (0, 0.5, 2 or 4g/kg) on PND 70. Mice were then euthanized 30 min later with NTS sections prepared for IHC. Three-way ANOVA indicated a significant main effect of EtOH concentration on NTS cfos expression ($F_{(3, 58)} = 16.09$, $p<0.0001$), with no significant main effects of sex, AIE or interaction effects ($p>0.05$), suggesting increasing concentrations of EtOH increases cfos expression in NTS cells. Three-way ANOVA further indicated significant effects of EtOH concentration on cfos/TH colocalization in the NTS ($F_{(3, 58)} = 2.917$, $p=0.0417$) with no significant effects of AIE, sex, or any interaction effects. Overall, these findings indicate that neither AIE, sex, nor their interaction alter EtOH induced activation of NTS TH neurons.

AIE does not alter acquisition of EtOH Conditioned Place Preference (CPP)

Noradrenergic neurons in the NTS are critical for regulation of behavioral and physiologic responses to stress [34] and for acquisition of reward preference in the CPP model [26]. Since AIE altered the development of NTS TH mRNA expression, it is possible that AIE-

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exposed mice may have an altered place preference behavior for EtOH, but acute effects of EtOH on NTS TH neurons were not altered by AIE. Therefore, we sought to examine if AIE might alter acquisition of EtOH preference in the CPP paradigm, potentially in a sex-specific manner. Mice underwent the CPP assay beginning at PND70 using 2g/kg EtOH I.P. (Fig 5), a dose previously shown to induce place preference in mouse models [14]. Prior to EtOH exposure, Two-way ANOVA showed no significant sex, AIE, or sex by AIE interaction effects in time spent in the side to be paired with EtOH ($p>0.05$). Repeated measures three-way ANOVA indicated training resulted in a significant increase in time spent in the EtOH paired side ($F_{(1,22)} = 14.14$, $p=0.0011$) and a significant main effect of sex ($F_{(1, 22)} = 4.943$, $p=0.0368$), with no significant main effect of AIE and no interaction effects with any variables ($p>0.05$). These data suggest that AIE does not alter acquisition of EtOH CPP but that sex differences may occur.

Discussion

Adolescent EtOH exposure is a critical risk factor for adult AUD development and one potential mechanism for this may be increased stress-susceptibility. In these studies, we show that both male and female mice exposed to AIE during early adolescence are more sensitive to acute stress as adults, an effect that may be more pronounced in females. This alteration in stress sensitivity may reflect sex-specific alteration of NTS TH mRNA expression during the AIE period. These behaviors do not seem to be mediated by altered cfos activation of NTS TH neurons following acute stress or EtOH. Previous work shows that genetic manipulations resulting in disrupted NTS TH mRNA expression during adolescent development can alter TH innervation of various brain regions and increased EtOH intake [23–25,42]. Since NTS TH cells are targets of acute EtOH exposure [1,44,45], these findings suggest that AIE alterations to NTS TH developmental trajectories may be a critical factor for altered adult stress- and EtOH-related behaviors. As the NTS does not contain dopamine producing neurons, AIE disruptions to NTS TH expression likely indicate disrupted noradrenergic signaling that may result in altered NE innervation of ascending brain regions. Such altered NE innervation would be expected to be related to the behavioral differences seen in the current studies, although this was not directly tested. The NTS is also critical in regulating central control of autonomic functions, suggesting AIE alterations of NTS TH neurons may set the stage for both central and peripheral nervous system dysfunction. Future work will need to address these hypotheses more directly.

The current data indicate that AIE alters NTS TH mRNA expression across adolescent development. This may be due to previously established sensitivity of NTS TH neurons to alcohol exposure [1,44,45]. These novel findings add to the burgeoning literature indicating the NTS as a potential critical locus of EtOH induced central dysfunction in various aspects of AUD [19]. These data further show that EtOH induced NTS TH neuron alterations in adolescence may lead to life-long disruptions to NTS-dependent functions. Future experiments will be needed to verify these results and determine if AIE might have altered other aspects of NTS anatomy such as AIE effects on overall NTS cell number and volume, and other NTS dependent functions not examined here. It is important to note that the NTS is a component of the dorsal vagal complex and acts to coordinate central and peripheral functions to interoceptive and exteroceptive stimuli [5–7,19,34,35]. Therefore, AIE induced

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alterations to NTS TH neuron function may lead to long lasting changes in many central and peripheral functions, and potentially changes to other neurotransmitter and peptide signaling mechanisms in the NTS and the dorsal vagal complex [13,27]. While more work will be needed to fully assess which central and peripheral functions are altered by EtOH both in adolescence and adulthood, these data show that such developmental NTS TH disruptions do not alter NTS TH neuron cfos activation induced by stress or acute alcohol stimuli in adulthood. A limitation to these studies is that we were not able to directly assess NTS TH neuron activity *in vivo* during behavioral assessments, which should be examined in future studies. Combined, these findings suggest that NTS TH disruptions may lead to alterations in TH innervation of multiple brain regions to disrupt stress and EtOH related behaviors [42].

AIE has previously been shown to enhance anxiety-like behaviors in multiple models [36]. Intriguingly, AIE via vapor exposure, as used in this study, results in enhanced anxiety-like behavior in female mice if they experience an additional stressor before testing [17]. In the current study, AIE enhanced stress-related behaviors as assessed by the light-dark box, particularly in response to forced swim stress. Overall, our data indicate that female mice may be more sensitive to both AIE and acute stress induced modulation of anxiety-like behavior. Within-sex statistical analysis indicated subtle behavioral differences in male vs female mice in this model, while multiple linear regression analysis incorporating potential latent variables relevant to the behavioral repertoire between sexes within the model suggested AIE-sex-stress interactions. Such information can be used to further refine available models and optimize future experiments when examining sex differences. Together, these data suggest sex differences in AIE-induced alterations in light-dark box behavior may not be fully represented solely by time in the light side. We posit that such subtle differences may be due to sex differences in stress related behaviors in general that may be further impacted by EtOH in age- and sex-specific manners, a hypothesis supported by the previous literature [3,10,12,32,37]. Different stressors may also influence NTS TH neurons to different extents, suggesting AIE may modulate adult stress-sensitivity differently depending on the type of stress model used. Such questions should be addressed in future studies. It should also be noted that some findings indicate sex differences in AIE effects on adult anxiety-like behavior may be different in rats [16]. Overall further work is needed to fully understand the impact of AIE on adult stress sensitivity in relation to stress modulation of EtOH intake in multiple species.

Previous work indicates that NTS TH neuron activity is required for expression of CPP, at least to morphine [26]. Our data indicate that EtOH can increase cfos expression in NTS TH neurons in a dose-dependent manner, an effect that is not altered by AIE. The data here indicate that AIE does not alter the acquisition of EtOH CPP in adult mice. Together, these findings suggest that adult EtOH sensitivity was not altered by AIE and that AIE does not alter development of CPP in adulthood. While saline conditioning was used within the groups here, a potential limitation of the current study is the lack of a saline-only control group. It will be important in future work to examine the impact of AIE on extinction and stress-induced reinstatement behaviors in the EtOH CPP model. Interestingly, previous work indicates that AIE impact on EtOH preference may be dissociable from intake. For instance, in rats, adolescent EtOH exposure increased EtOH-reinforcing effects in both sexes [15], but

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males may be more likely to develop long-term changes to EtOH sensitivity, which could drive increased intake seen in previous studies [36]. Future work will be needed to examine whether AIE induced changes to NTS TH expression may alter EtOH self-administration vs CPP in mice.

Combined, the data here indicate that AIE induced alterations to NTS TH development leading to disruptions in stress behaviors but did not alter NTS TH neuron sensitivity to acute EtOH or stress in adulthood or CPP acquisition. Overall, these data suggest that NTS TH neuron disruptions by AIE may lead to altered NE innervation of ascending and descending brain regions regulating central and peripheral nervous system functions. One brain region that is highly innervated by NTS noradrenergic inputs, important for behavioral and physiologic responses to acute and chronic stressors, and critical to stress-induced reinstatement to EtOH seeking and other reinforcers in the CPP model, is the BNST [21,29–31,39,48]. BNST noradrenergic signaling, particularly noradrenergic regulation of CRF neurons and resultant alterations in BNST glutamatergic signaling, has been shown to be critical to behavioral responses to both stress and EtOH [39]. Recent work indicates that AIE alters glutamatergic plasticity in the BNST [8,17,18]. Future experiments will be needed to determine how AIE may alter noradrenergic innervation of the BNST as well as how this might alter the expression of the various noradrenergic receptor subtypes in the BNST.

In conclusion, we found that AIE alters NTS TH development, increased behavioral sensitivity to stress in adulthood, particularly in females, and maintained NTS TH neuron responsiveness to acute stress and EtOH as measured by cfos. Together, these findings may suggest that altered NTS TH connectivity with other regions may sensitize stress-related circuits to increasing function, potentially due to increased NTS TH neuron output, increased ascending noradrenergic input to brain regions like the BNST, alteration to pre- and post-synaptic glutamatergic and noradrenergic receptor function in these brain regions, or some combination of these possibilities. A limitation of the current studies is that we did not attempt to reverse behavioral changes by directly altering or targeting NTS TH neurons or their projections to the upstream brain regions. Such work will be needed in future studies. Future work will also be needed to fully elucidate the mechanisms by which AIE alters stress sensitivity and if this alters reinstatement to EtOH CPP. Even with these limitations, the current work may have numerous implications as stress is known to be a major factor in relapse to AUDs in treatment seeking individuals [4], although there is very little data on differences in stress-induced relapse rates in adult AUD patients with and without a history of adolescent drinking [2,43]. Given the heterogeneous nature of AUD development, effective treatments for adult AUD patients with a history of adolescent drinking may be different than treatments for adult AUD patients without adolescent drinking histories, especially in terms of stress-induced relapse. Therefore, understanding the mechanisms involved in stress-induced relapse in AUD patients with and without adolescent drinking histories may lead to the development of more effective treatments for both subpopulations.

Supplementary Material

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Data availability

Data will be made available on request.

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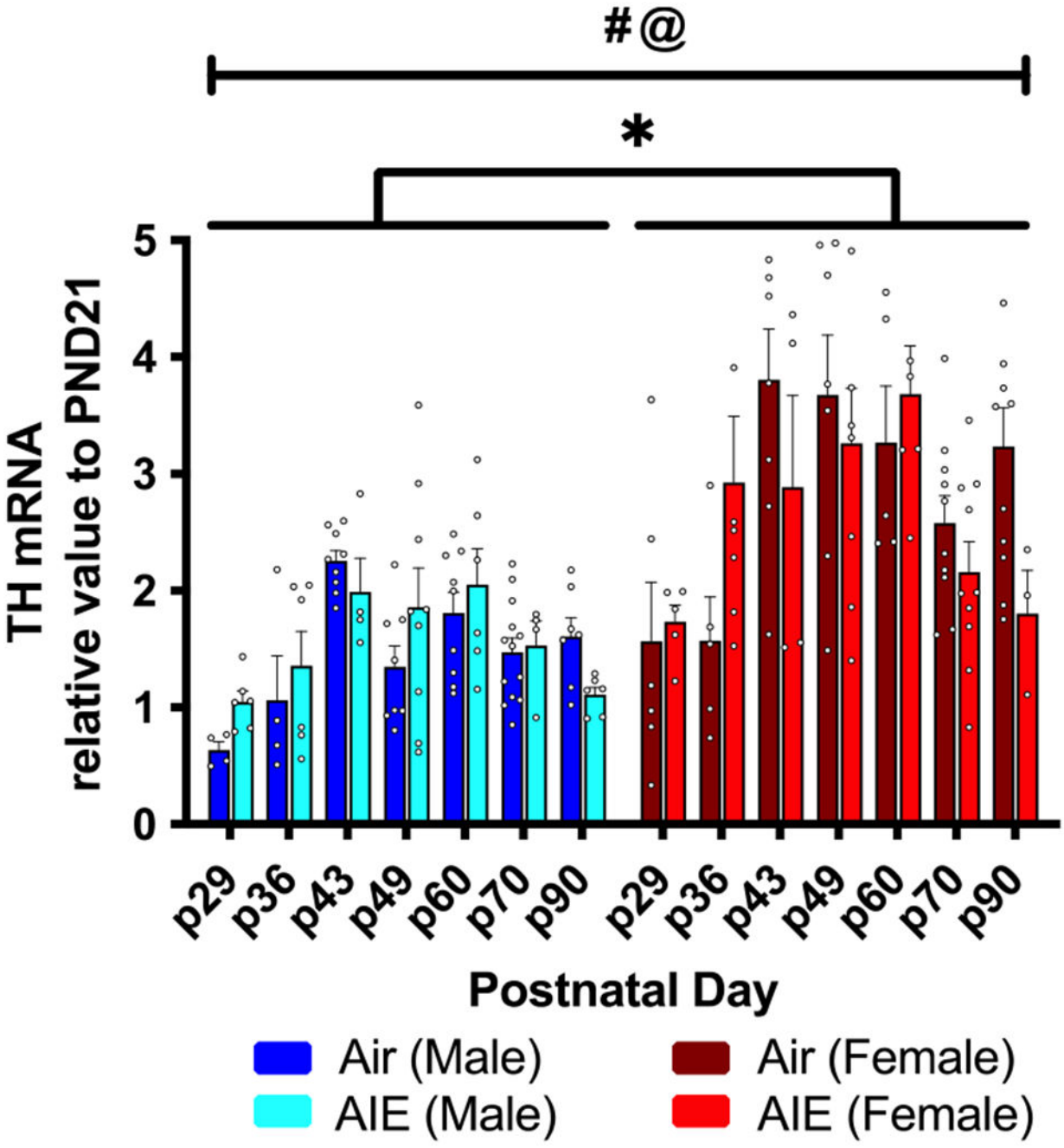

Fig. 1. AIE alters the timeline of NTS TH mRNA expression in a sex-specific manner.

Bar graph effect of AIE on NTS TH mRNA expression across ages in male and female mice. * indicates significant effect of sex, @ indicates significant overall effect of age, and # indicates significant Age by AIE interaction effect as determined by three-way ANOVA ($p<0.05$).

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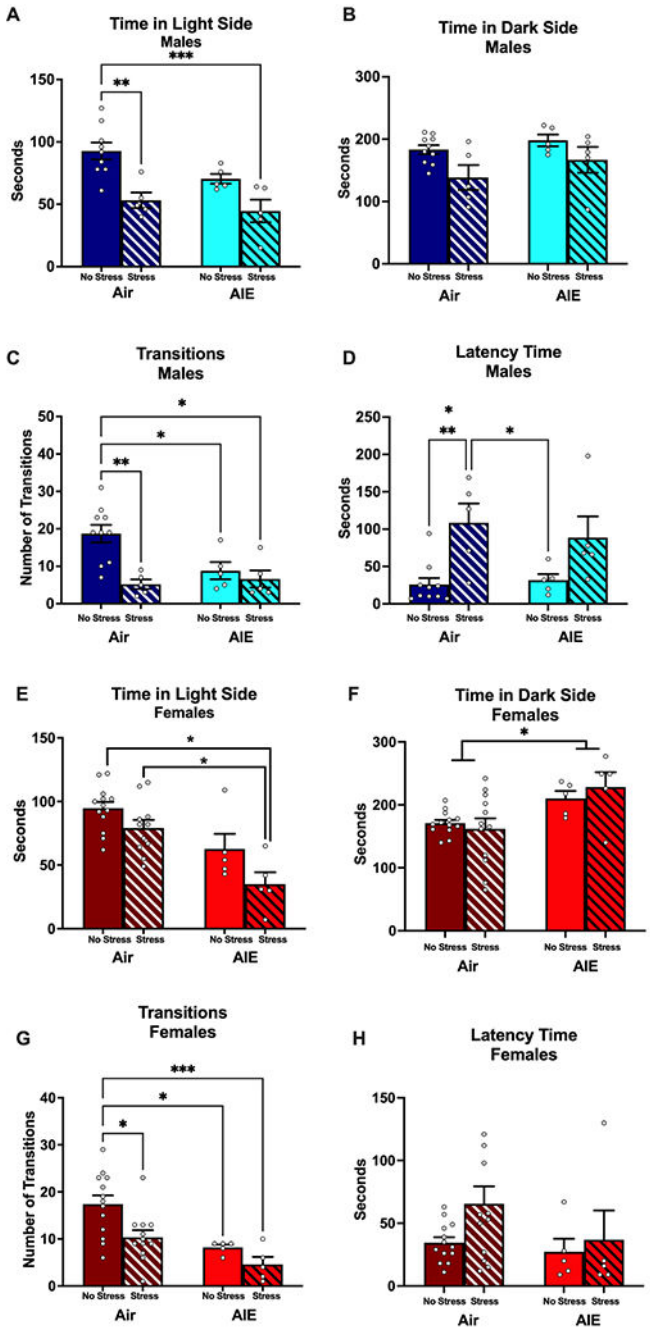

Fig. 2. AIE induces sex-specific changes in anxiety-like behaviors in the light-dark box.
A-D) Summary data in male mice showing effects of AIE and stress on A) time in the light side B) time in the dark side C) transitions and D) latency to first enter the dark side. Male:Air- dark blue (Air-no stress, n=9; Air-stress, n=5) Male:AIE-light blue (AIE-no stress, n=5; AIE-stress, n=5). **E-F)** Summary data in female mice showing effects of AIE and stress on E) time in the light side F) time in the dark side G) transitions and H) latency to first enter the dark side. Female:Air-dark red (Air-no stress, n= 13; Air-stress, n=11), Female: AIE-light red (AIE-no stress, n=5, AIE-stress n=5). Significant differences between

groups as determined by Tukey's multiple comparison tests are indicated. *=p<0.05, **=p<0.01, ***= p<0.005. For panel F, * indicates a significant main effect of AIE vs Air as determined by two-way ANOVA, p<0.05

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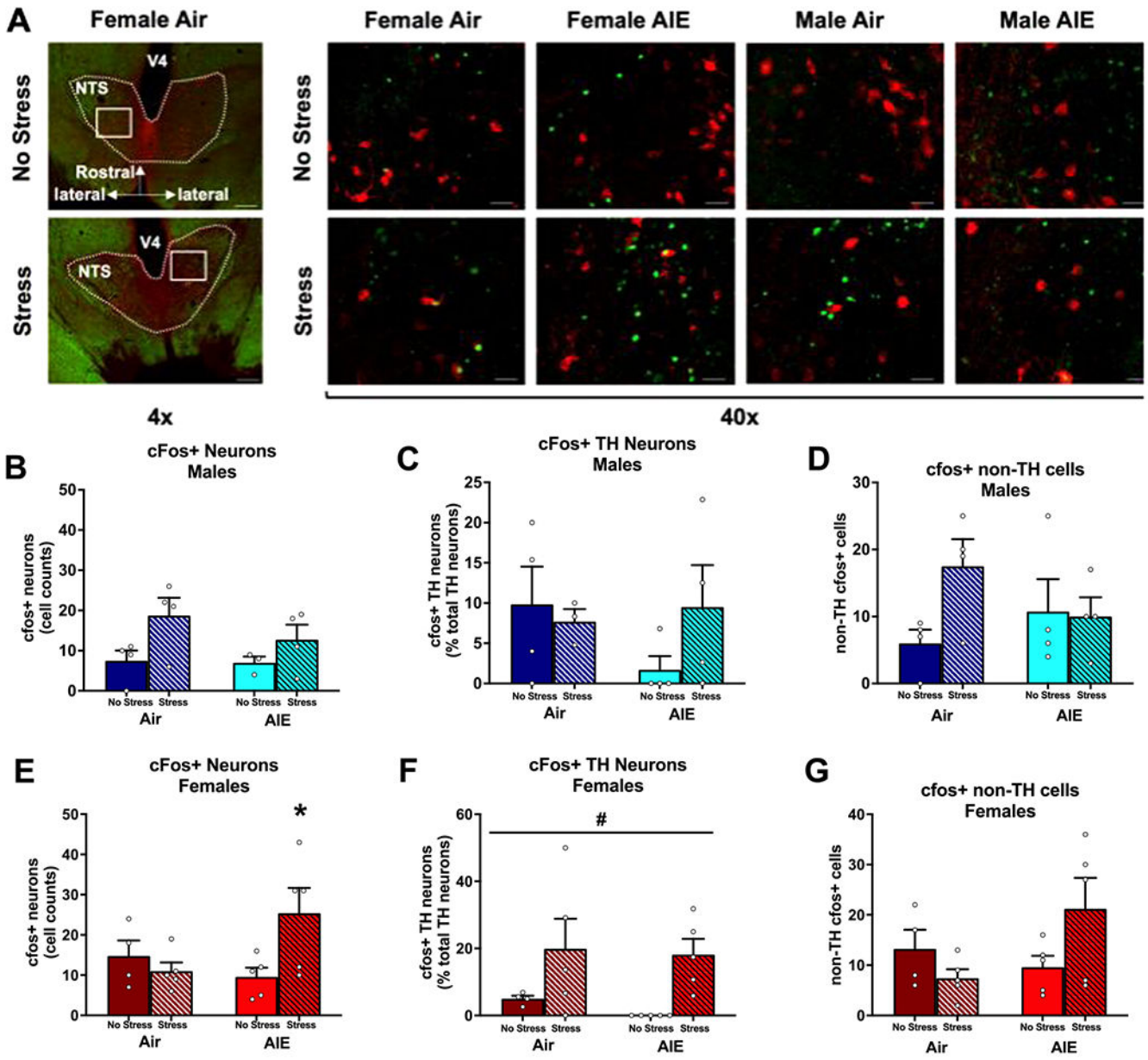

Fig. 3. AIE increases stress induced NTS cfos expression in a sex-specific manner.

A-left) Example 4x fluorescent images of immunohistochemistry to label of cfos (green) and TH (red) in the NTS (outlined in gray dotted lines) in female Air mice with and without stress exposure. Scale bar: 200 um, V4= fourth ventricle. Box indicates area for 40x image shown at right. A-right) Example 40x fluorescent images of immunohistochemistry to label of cfos (green) and TH (red) in the NTS in all groups. Scale bar= 20 um. B-E) Summary data of overall cfos expression, cfos/TH colocalization, and cfos expression in non-TH cells in males (B-D) and females (E-G). * indicates significant difference between female AIE-stress and male Air-no stress mice as determined by three-way ANOVA with Tukey's post hoc analysis, $p<0.05$. # indicates significant main effect of stress on cfos/TH colocalization in female mice as determined by within sex two-way ANOVA, $p<0.05$.

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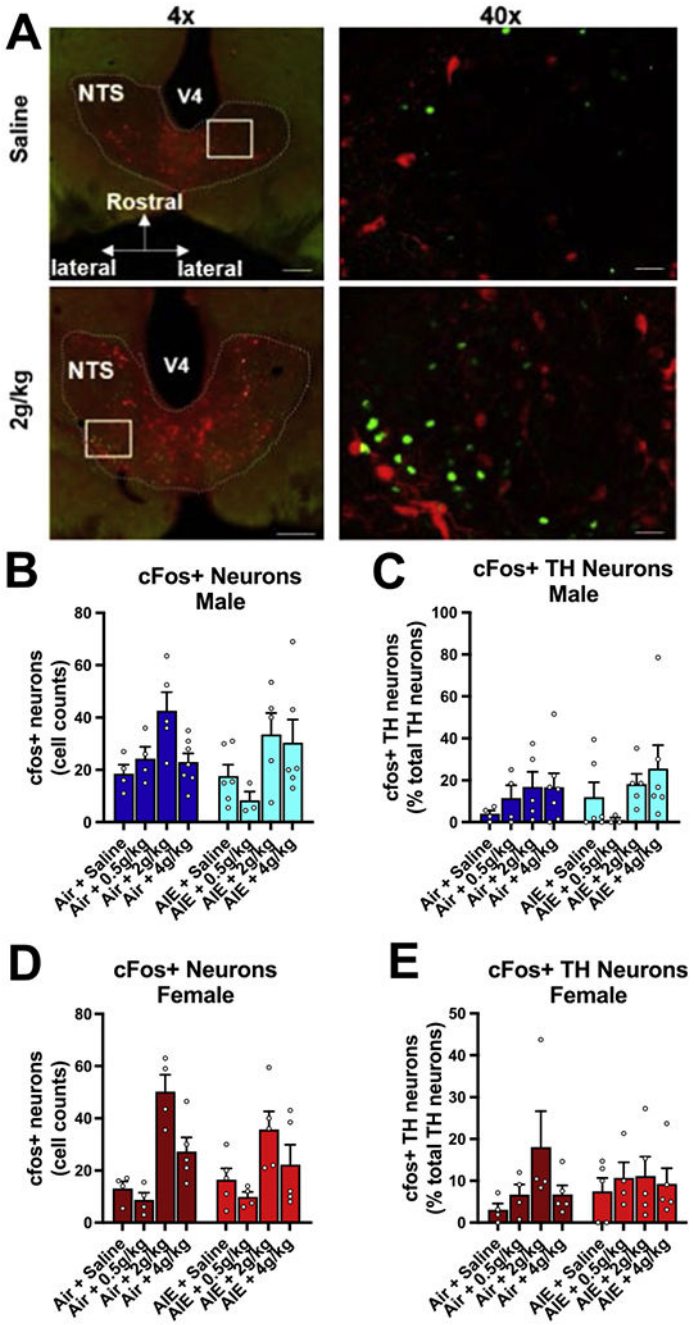

Fig. 4. Acute EtOH exposure increases cfos expression in NTS similarly in adult male and female mice with and without AIE history

A) Example 4x and 40x fluorescent images of immunohistochemistry to label of cfos (green) and TH (red) in the NTS (outlined in gray dotted lines). Scale bars: 4x=200 um, 40x= 20 um. V4= fourth ventricle. B-E) Summary bar graphs showing effects of various EtOH concentrations (0-4 g/kg) on total cfos expression and cfos/TH colocalization in male and female mice with and without AIE history. Three-way ANOVA indicates a significant overall effect of EtOH concentration ($p<0.05$, see, main text) with no significant effects of sex, AIE, or any interaction effects for both total cfos expression and cfos/TH colocalization.

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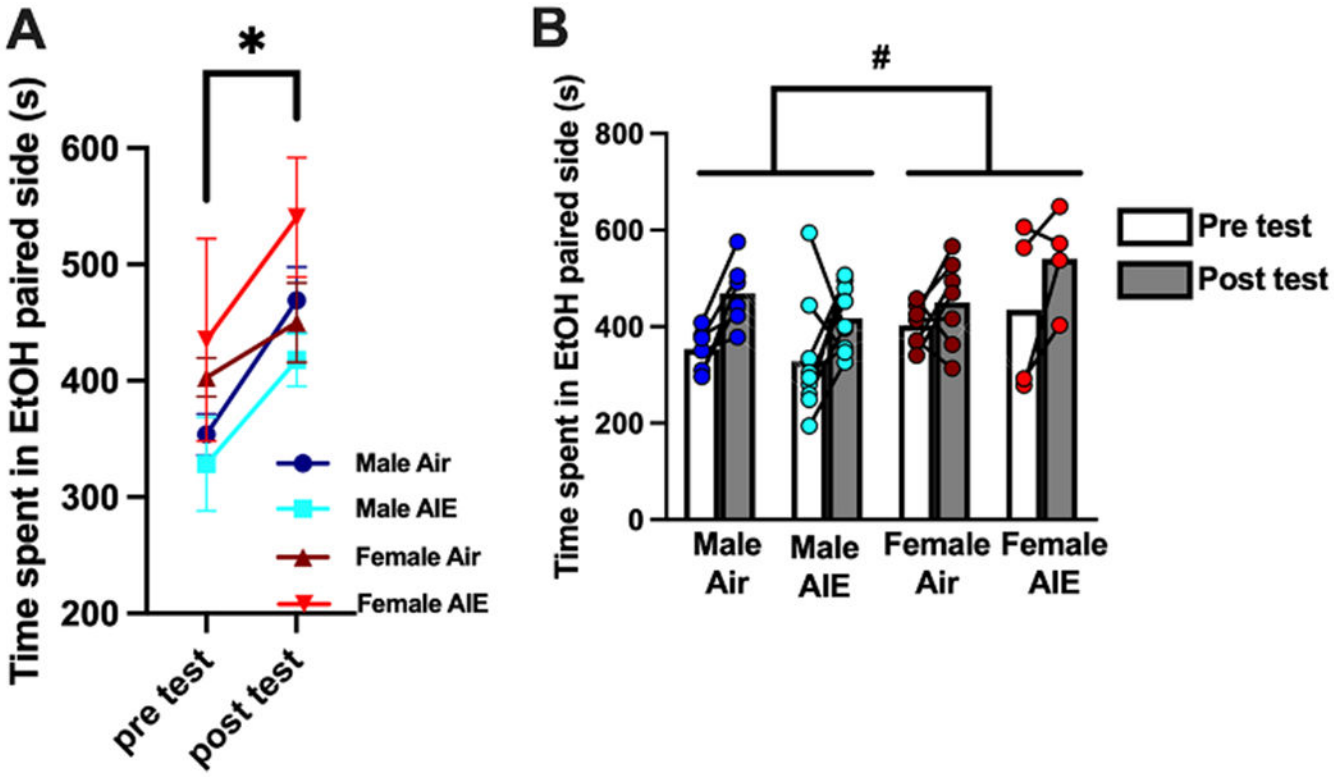

Fig. 5. AIE does not alter CPP acquisition.

A) Line graph showing time spent in the EtOH paired chamber before and after CPP acquisition in male and female mice with and without AIE history. * indicates significant main effect of CPP acquisition on time in the EtOH paired side between pre-test and post-test as determined by three-way ANOVA ($p<0.05$). B) Individual data points from the group data in A. # indicates significant main effect of sex on time in the EtOH paired side between pre-test and post-test as determined by three-way ANOVA ($p<0.05$). N/group: Male Air (n=6), Male AIE (n=9), Female Air (n=7), and Female AIE (n=4).