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Sex-specific negative affect-like behaviour and parabrachial nucleus activation induced by BNST stimulation in adult mice with adolescent alcohol history

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Abstract

Adolescent alcohol use is a strong predictor for the subsequent development of alcohol use disorders later in life. Additionally, adolescence is a critical period for the onset of affective disorders, which can contribute to problematic drinking behaviours and relapse, particularly in females. Previous studies from our laboratory have shown that exposure to adolescent intermittent ethanol (AIE) vapour alters glutamatergic transmission in the bed nucleus of the stria terminalis (BNST) and, when combined with adult stress, elicits sex-specific changes in glutamatergic plasticity and negative affect-like behaviours in mice. Building on these findings, the current work investigated whether BNST stimulation could substitute for stress exposure to increase the latency to consume a palatable food in a novel context (hyponeophagia) and promote social avoidance in adult mice with AIE history. Given the dense connections between the BNST and the parabrachial nucleus (PBN), a region involved in mediating threat assessment and feeding behaviours, we hypothesized that increased negative affect-like behaviours would be associated with PBN activation. Our results revealed that the chemogenetic stimulation of the dorsolateral BNST induced hyponeophagia in females with AIE history, but not in female controls or males of either group. Social interaction remained unaffected in both sexes. Notably, this behavioural phenotype was associated with higher activation of calcitonin gene-related peptide and dynorphin cells in the PBN. These findings provide new insights into the neurobiological mechanisms underlying the development of negative affect in females and highlight the potential involvement of the BNST-PBN circuitry in regulating emotional responses to alcohol-related stimuli.

KEYWORDS
adolescent, alcohol, bed nucleus of the stria terminalis, hyponeophagia, parabrachial nucleus, social interaction

1 | INTRODUCTION

Adolescence is a sensitive developmental period during which the brain undergoes substantial maturation[1] and is especially vulnerable to different insults including alcohol. Early-age episodic heavy alcohol drinking in adolescents has been linked to increased risk of developing alcohol use disorders later in life[2–4] and experiencing several adverse psychosocial consequences that can have long-lasting effects.[5] While definitive causal relationships are yet to be established, it is widely accepted that binge patterns of alcohol consumption early in adolescence can alter the behavioural response to subsequent stressors during adulthood, which may over time contribute to the development of stress-related disorders.[6]

Behavioural consequences linked to alcohol binge drinking involve persistent changes in brain-stress circuits within the extended amygdala, a network of interconnected brain regions implicated in emotion processing and development of substance abuse.[7–9] Among these regions, the bed nucleus of the stria terminalis (BNST) plays a crucial role in mediating the negative affective state associated with chronic alcohol use and withdrawal.[10] Moreover, the BNST is a sexually dimorphic region, displaying differences in anatomy, connectivity and receptor subpopulations.[11,12] Therefore, alcohol-induced changes in BNST plasticity may underlie the molecular mechanism of sex differences in negative affective phenotypes that emerge during adolescence.

Several studies have reported that the parabrachial nucleus (PBN) exerts significant influence on threat assessment and food-related behaviours.[13–16] Anatomical evidence indicates that the PBN receives input from regions that encode affective information, including the BNST.[17–19] Recent findings demonstrated that the BNST conveys sensory and affective information to two key neuropeptide-containing populations within the PBN, calcitonin gene-related peptide (CGRP), and dynorphin neurons, influencing anxiety-like behaviours and feeding.[20] However, further investigation of these systems under distinct internal states imposed by adverse experiences is necessary to better understand their contributions to emotional regulation.

In a previous study, our laboratory demonstrated that male and female mice exposed to adolescent intermittent ethanol (AIE) vapour exhibit increased glutamatergic transmission in the dorsolateral BNST (dlBNST) during acute alcohol withdrawal compared to air control mice.[21] More recently, we found that AIE elicits sex-dependent expression of stress-induced negative affect-like behaviours in adulthood. Notably, AIE did not induce any changes in baseline anxiety-like behaviour in the absence of stress. However, female mice exposed to AIE displayed an increased latency to consume an appetitive reinforcer in a novel and mildly anxiogenic environment (hyponeophagia) during the novelty-induced hypophagia (NIH) test after exposure to restraint stress. In contrast, male mice exposed to AIE showed increased freezing behaviour in response to low-intensity foot shocks.[22]

The aim of the present study was to expand upon these findings and examine the involvement of the BNST in the expression of hyponeophagia and social avoidance in adult mice with AIE history. We substituted restraint stress with chemogenetic stimulation of the dlBNST using designer receptors exclusively activated by designer drugs (DREADDs) in adult mice with AIE history or air controls. Given that the PBN receives dense inputs from the BNST, we hypothesized that an increase in negative affect-like behaviour resulting from BNST stimulation would be accompanied by PBN activation. Specifically, we evaluated the activation of CGRP- and dynorphin-expressing neurons in the medial and lateral aspects of the PBN.

2 | METHODS AND MATERIAL

2.1 | Animals

A total of 94 three-week-old male and female C57BL/6 J mice (Jackson Laboratories; Bar Harbour, ME, USA) were used in this study. Animals were housed in groups of 2–3 in a humidity- and temperature-controlled (22°C) vivarium on a 12-h light–dark cycle (lights on at 7:00 AM). They had ad libitum access to food and water throughout the experiments. All procedures were approved by the Institutional Animal Care and Use Committee of the Louisiana State University Health Sciences Center and were in accordance with the National Institutes of Health guide for the care and use of Laboratory animals (NIH Publications No. 8023, revised 1978).

2.2 | Ethanol vapour exposure

Mice were exposed to AIE following the protocol described in previous studies.[21–23] In brief, the exposure to ethanol vapour occurred from 4:00 PM to 8:00 AM the following day, allowing for the consistent attainment of blood ethanol concentrations ranging from 200 to 250 mg/dL. The AIE protocol consisted of two cycles, each lasting 4 days, with 16 h of in-chamber sessions followed by 8 h of out-of-chamber sessions. These cycles were separated by 3 days of no exposure, starting from postnatal day (PND) 27, and ending on PND 38. Prior to each ethanol vapour session, mice received an injection of pyrazole (air control groups, 1 mmol/kg) or pyrazole + ethanol (AIE groups, 1 mmol/kg + 0.8 g/kg). Male and female mice were exposed separately in dedicated vapour chambers. Control animals underwent an identical regimen, with the exception that they were exposed to water vapour instead of ethanol.

2.3 | Stereotaxic surgery

Seven to ten days following the final AIE exposure, mice were anesthetized using isoflurane and positioned on an Angle Two Small Animal Stereotaxic Instrument (Leica Microsystems; Wetzlar, Germany). They received a subcutaneous injection of Meloxicam-SR (4 mg/kg) for pain relief. The stereotaxic coordinates for targeting the dlBNST were as follows: 0.14 mm anterior to bregma, ±0.8 mm

lateral to the midline and 4.14 mm ventral to the skull.[24] Viral constructs were infused at a rate of 100 nl/min, with a total volume of 200 nl per side. Following infusion, injectors were left in place for 5 min to allow for virus diffusion. To ensure adequate viral expression, experimental manipulations were performed at least 4 weeks after injections.

2.4 | Drug and viruses administration

The alcohol dehydrogenase inhibitor pyrazole (1 mmol/kg; Sigma-Aldrich; Saint Louis, MO, USA) was dissolved in 0.9% saline or saline + ethanol.[21] Viral vectors AAV5-CAMKIIa-mCherry (Plasmid #114469; titre ≥ 7×10^{12} vg/mL) and AAV5-CAMKIIa-hM3D(Gq)-mCherry (Plasmid #50476; titre ≥ 2×10^{12} vg/mL) were obtained from Addgene (Watertown, MA, USA). The CaMKII promoter has been used as a forebrain-predominant excitatory neuron-specific promoter.[25] However, recent reports showed that CaMKII promoter can transduce both excitatory and inhibitory neurons, suggesting that it may be non-specific to excitatory neurons in certain brain regions.[26] Clozapine *N*-oxide dihydrochloride (CNO, 3 mg/kg; Tocris; Minneapolis, MN, USA) was dissolved in 0.9% saline.[19]

2.5 | NIH test

On PND 78, mice were given 2-h access to 50-mL bottles containing Ensure vanilla-flavoured shake in their home cage in a dimly lit room (50 lx). The following day, home cage testing was conducted to measure the latency to consume Ensure. Mice that did not drink during the 30-min home cage testing were included in the experiment but excluded from further NIH analysis (n = 12 mice). On the novel cage test day (PND 80), mice were injected intraperitoneally with CNO and returned to their home cage for 1 h. Mice were then individually placed into new clean cages without shavings, with bottles containing Ensure positioned. Novel cage testing took place under bright light (420 lx), and the testing lasted for 30 min.[22,23]

2.6 | Social interaction test

On PND 83, social behaviour was assessed using a three-compartment apparatus made of transparent Plexiglas (length: 60 cm, width: 40 cm, height: 22 cm). The apparatus was divided into three chambers (20 cm in length each). The two lateral chambers contained wire cup-like containers (length: 8.2 cm, width: 8.2 cm, height: 9.5 cm) that allow breathing and nose pokes. Prior to the start of the experiment, mice were brought into the testing room and given a 30-min acclimation period. Each test mouse received an intraperitoneal injection of CNO and, after 1 h, the mouse was placed in the central chamber for a 5-min habituation period. An unfamiliar mouse of the same sex, age and strain was placed under one of the wire containers, while the other remained empty. A total of eight unfamiliar mice (four each sex) were used throughout the experiment and systematically rotate among the experimental mice. Following the introduction of the unfamiliar mouse, the experimental mouse had 10 min to explore the three chambers. Behaviour was recorded using an overhead video camera, and subsequent analysis was conducted manually. The primary measure of social behaviour was the time spent in close proximity to the wire container with the unfamiliar mouse. After each testing session, the apparatus and wire containers were cleaned using 70% ethanol. The placement of the unfamiliar mouse under the containers was counterbalanced across experimental animals.[27]

2.7 | Histological confirmation and fluorescent in situ hybridization

One to two weeks after the social interaction test (PND 90+), mice received an intraperitoneal injection of CNO and, after 1 h, they were euthanized by decapitation under isoflurane anaesthesia. Brains were dissected, snap-frozen in −30°C isopentane and stored at −80°C until sectioning. Coronal sections of 12 µm (PBN) or 30 µm (BNST) were obtained using a cryostat and mounted on Super Frost Plus slides (Thermo Fisher Scientific; Waltham, MA, USA). The sections containing the BNST were subjected to fluorescence examination, and only mice in which the majority of the fluorescence signal was localized within the dlBNST were considered for data analysis (n = 6 mice were excluded).

The RNAscope Multiplex Fluorescent Reagent Kit v2 with TSA Vivid Dyes (Cat. No. 323270; Advanced Cell Diagnostics; Newark, CA, USA) was utilized to conduct in situ hybridization in sections containing the PBN, following the manufacturer's guidelines for fresh frozen tissue. Given that increased negative affect-like behaviour resulting from BNST stimulation was observed exclusively in female mice, RNAscope staining was limited to female brains for further analysis. The following probes were used: mm-Fos (Cat. No. 316921) as a marker of cell activation, mm-Calca-tv2tv3-C2 (Cat. No. 420361-C2) as a marker of CGRP neurons, and mm-Pdyn-C3 (Cat. No. 318771-C3) as a marker of dynorphin neurons. Slides were mounted using ProLong Gold Antifade Mountant medium with DAPI (Thermo Fisher Scientific) and stored at 4°C until imaging. Negative controls were included and processed in the same manner. The negative control probe targeted the DapB gene from a soil bacterium (Cat. No. 310043).

2.8 | Imaging processing and cell quantification

Images were captured using the ZEISS AxioScan.Z1 slide scanner (Oberkochen, Germany) and analysed with QuPath 0.4.3. software. Images were acquired as a z-stack of six steps, each with a thickness of 0.85 µm. All imaging parameters were maintained consistently across sections, and the analysis was performed in a blind manner to

mitigate potential bias. Individual cells were identified based on DAPI staining of the nucleus. Prior to analysing the experimental images, the negative controls were quantified. For each cell, we quantified the total number of fluorescent puncta in each channel. The threshold was determined using the mean plus one standard deviation. PBN images were captured bilaterally, and the average was calculated for quantification purposes. To determine the percentage of cells expressing a specific mRNA, we divided the number of positive cells by the total number of DAPI-labelled nuclei.

2.9 | Statistical analysis

Data are presented as mean and standard error of the mean (SEM). Statistical analysis and graph construction were conducted using GraphPad Prism 10 (GraphPad; La Jolla-CA, USA). Body weight on PND 38 was analysed using a two-way analysis of variance (ANOVA) with the variables of sex and alcohol exposure (AIE vs. air controls). For each sex, separate two-way ANOVAs were performed to analyse body weight on PND 78, change in latency to consume on the NIH test, time exploring the container with a mouse and the empty container in the social interaction test, percentage of double-labelling c-Fos- and CGRP-positive cells in the medial and lateral PBN and percentage of double-labelling c-Fos- and dynorphin-positive cells in the medial and lateral PBN. The variables in all these cases were virus injection (control vs. hM3D(Gq) virus) and alcohol exposure (AIE vs. air controls). In instances where significant interactions were identified, Tukey's post-hoc comparisons were conducted. The statistical significance threshold was set at $p < 0.05$.

3 | RESULTS

3.1 | AIE does not induce persistent alterations in body weight

The general timeline of the experimental procedure is presented in Figure 1A. Following the last exposure to AIE (PND 38), mice displayed a significantly reduced body weight gain compared to the air control group, regardless of sex (Figure 1B; alcohol exposure effect: $F(1, 76) = 8.46$, $p = 0.0048$). As expected, there was a significant sex difference in body weight (Figure 1B; sex effect: $F(1, 76) = 148.40$, $p < 0.0001$). In adulthood prior to the NIH test (PND 78), there were no significant differences in body weight observed between the AIE and air control groups, or between mice injected with the control or hM3D(Gq) virus, for both females (Figure 1C; alcohol exposure effect: $F(1, 36) = 0.02$, $p = 0.8874$; virus effect: $F(1, 36) = 0.007$, $p = 0.9323$) and males (Figure 1D; alcohol exposure effect: $F(1, 36) = 3.71$, $p = 0.0621$; virus effect: $F(1, 36) = 2.59$, $p = 0.1163$). These results demonstrate that adolescent mice exhibit a temporary blunted weight gain immediately after the AIE treatment, but subsequently regain the lost weight by adulthood.

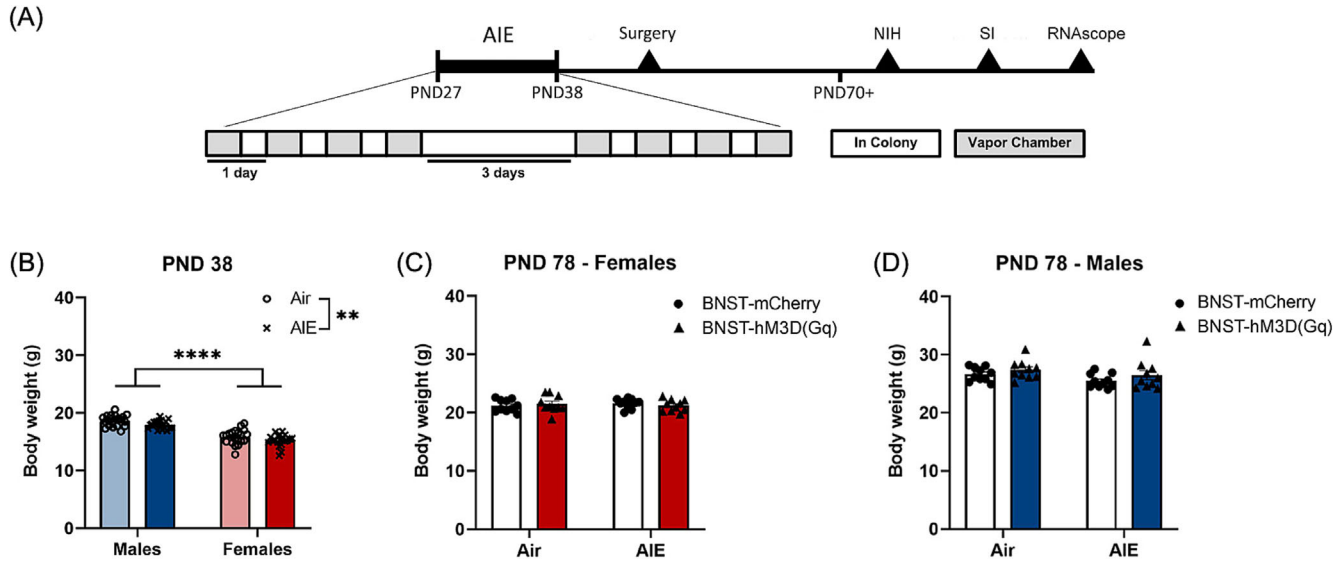

FIGURE 1 Adolescent intermittent ethanol (AIE) vapour exposure does not promote permanent alterations in body weight. (A) Experimental timeline: adolescent mice underwent two 4-day cycles of 16-h ethanol vapour exposure per day, separated by 3 days of no exposure, from postnatal day (PND) 27 to 38. Following AIE, viral vectors were administered into the dorsolateral BNST. Mice were then left undisturbed until adulthood (PND 70+) and subjected to the novelty-induced hypophagia (NIH) test, social interaction (SI) test, and had the brains collected for histological confirmation and RNAscope in situ hybridization processing. (B) Body weight after the last AIE session (PND 38) in adolescent male and female mice (n = 20 mice/group). (C) Body weight in adult female mice prior to the NIH test (PND 78; n = 10 mice/group). (D) Body weight in adult male mice prior to the NIH test (PND 78; n = 10 mice/group). Data presented as mean ± SEM. ** denotes $p < 0.01$; **** denotes $p < 0.0001$

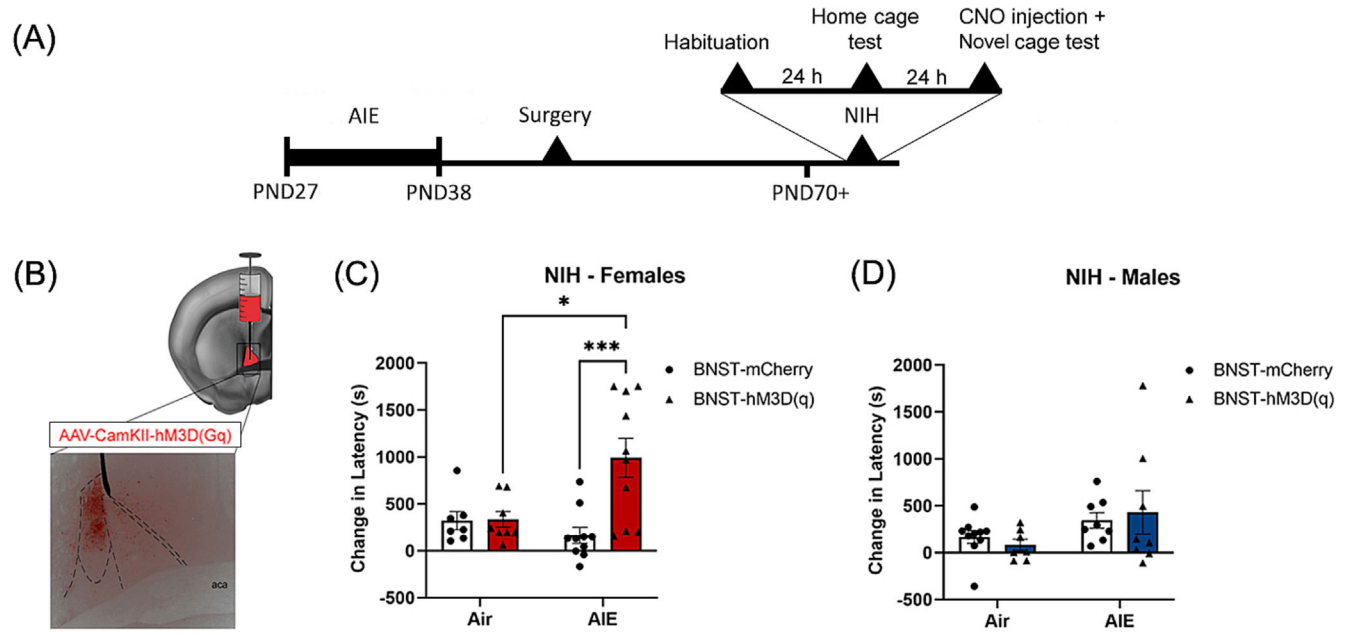

FIGURE 2 Bed nucleus of the stria terminalis (BNST) stimulation induces hyponeophagia in adult female mice exposed to adolescent intermittent ethanol (AIE) vapour. (A) Experimental timeline: on postnatal day (PND) 78, mice underwent a habituation phase with access to an appetitive reinforcer for 2 h. The following day, home cage testing was conducted to measure the latency to consume. On the novel cage test day, mice received an injection of clozapine *N*-oxide dihydrochloride (CNO, 3 mg/kg, i.p.) and, after 1 h, were placed in a new cage under bright light. The latency to consume the reinforcer was measured for 30 min. (B) Schematic of viral injection and representative image showing mCherry expression in the dorsolateral BNST. (C) Change in latency to consume the appetitive reinforcer, calculated as the difference between the latency to drink during the novel cage test and the latency to drink during the home cage test, following CNO injection in female mice. (D) Change in latency to consume the appetitive reinforcer following CNO injection in male mice. Aca, anterior commissure, anterior part. Data presented as mean ± SEM. N = 7–10 mice/group. * denotes $p < 0.05$; *** denotes $p < 0.001$

3.2 | BNST stimulation induces hyponeophagia in adult females with AIE history

The NIH test timeline is presented in Figure 2A. Figure 2B displays a representative photomicrograph of the virus injection into the dlBNST. Change in latency was calculated as the latency to consume during the novel cage test minus the latency to consume during the home cage test, measured in seconds. A two-way ANOVA revealed significant main virus effect ($F(1, 31) = 8.76$, $p = 0.0059$) and an interaction between alcohol exposure and virus ($F(1, 31) = 8.25$, $p = 0.0073$) on the latency to consume the palatable reinforcer in females. Tukey's post-hoc comparisons indicated that, following CNO injection, females with a history of AIE that received the hM3D(Gq) virus exhibited a higher latency to consume compared to air controls that received the hM3D (Gq) virus ($p = 0.0111$) and AIE females that received the control virus ($p = 0.0006$) (Figure 2C). Male mice with AIE history showed a non-significant increase in latency to consume (alcohol exposure effect: $F(1, 29) = 4.16$, $p = 0.0506$). However, there was no virus effect ($F(1, 29) = 6.356e\text{-}005$, $p = 0.9937$) or interaction between alcohol exposure and virus on the latency to consume the palatable reinforcer ($F(1, 29) = 0.44$, $p = 0.5142$) in males (Figure 2D). These results show that BNST stimulation using DREADDs elicits hyponeophagia in adult females with AIE history but has no effect on male mice.

3.3 | BNST stimulation does not induce social avoidance in adult mice with AIE history

The social interaction test timeline is presented in Figure 3A. In female mice, a two-way ANOVA revealed no significant effects of alcohol exposure or virus on the time exploring the wire container with a mouse (Figure 3B; alcohol exposure effect: $F(1, 36) = 3.68$, $p = 0.063$; virus effect: $F(1, 36) = 1.54$, $p = 0.2225$) or on the time exploring the empty wire container (Figure 3D; alcohol exposure effect: $F(1, 36) = 0.037$, $p = 0.8483$; virus effect: $F(1, 36) = 0.048$, $p = 0.8284$). In male mice, a two-way ANOVA revealed no significant effects of alcohol exposure or virus on the time exploring the wire container with a mouse (Figure 3C; alcohol exposure effect: ($F(1, 35) = 0.28$, $p = 0.5977$; virus effect: ($F(1, 35) = 0.53$, $p = 0.4699$). However, there was a significant virus effect on the time exploring the empty container (Figure 3E; $F(1, 35) = 5.67$, $p = 0.0229$). These results indicate that BNST stimulation leads to a reduction in exploratory behaviour in male mice regardless of AIE history but does not induce social avoidance in either male or female mice.

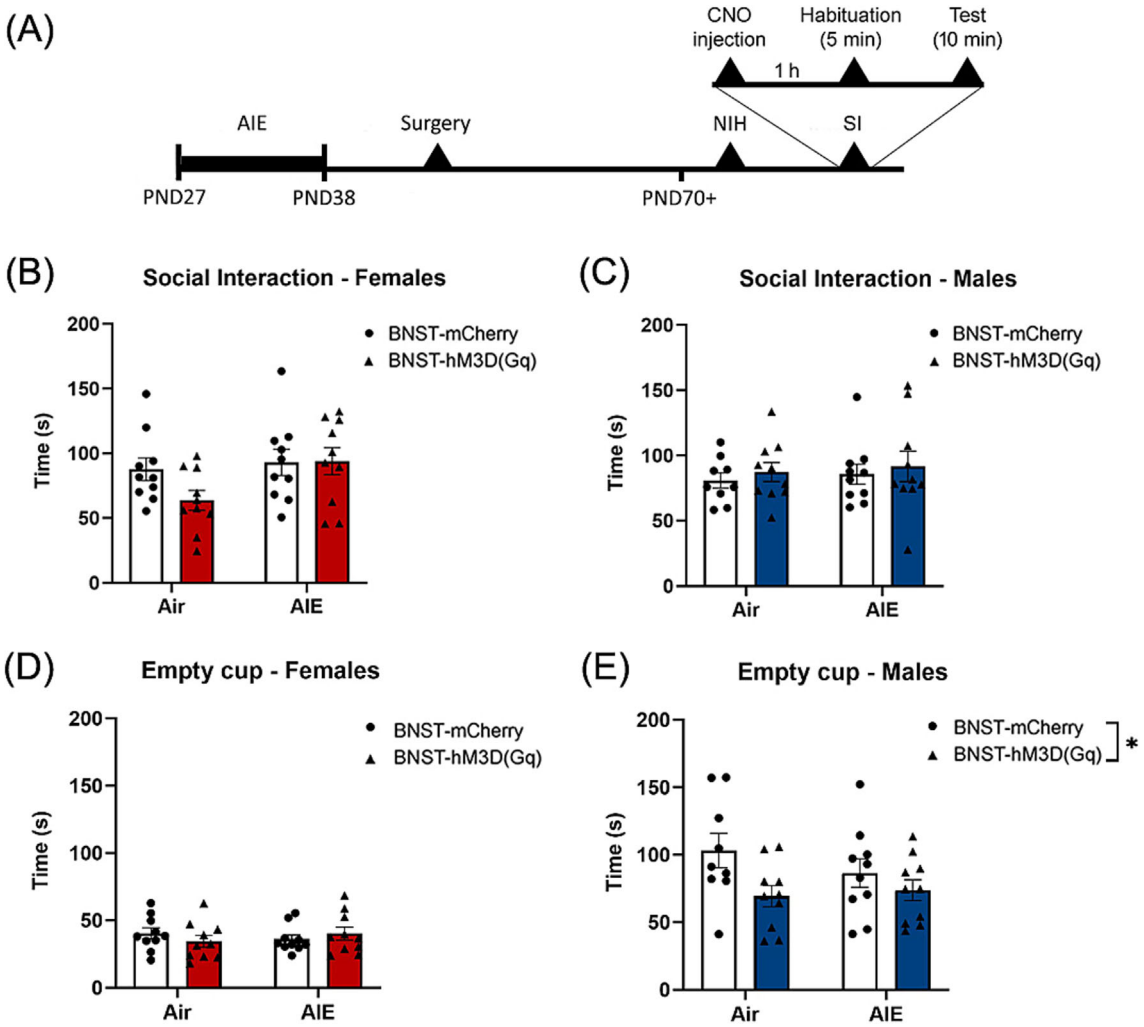

FIGURE 3 Bed nucleus of the stria terminalis (BNST) stimulation does not induce social avoidance in adult mice exposed to adolescent intermittent ethanol (AIE) vapour. (A) Experimental timeline: on postnatal day (PND) 83, mice received an injection of clozapine *N*-oxide dihydrochloride (CNO, 3 mg/kg, i.p.) and, after 1 h, were placed in the social interaction (SI) chamber for a 5-min habituation period. An unfamiliar mouse was placed under a wire container, while the other container remained empty. The experimental mouse had 10 min to freely explore the chamber. (B) Time exploring the wire container with an unfamiliar mouse following CNO injection in female mice. (C) Time exploring the wire container with an unfamiliar mouse following CNO injection in male mice. (D) Time exploring the empty wire container following CNO injection in female mice. (E) Time exploring the empty wire container following CNO injection in male mice. Data presented as mean ± SEM. N = 9–10 mice/group. * denotes $p < 0.05$

3.4 | BNST stimulation promotes the activation of CGRP cells in the PBN of adult female mice with AIE history

The RNAscope in situ hybridization timeline is presented in Figure 4A, and this experiment includes only female mice. Figure 4B depicts a representative negative control image. Cell nuclei are stained with DAPI (blue). Figure 4C shows a representative photomicrograph illustrating the presence of c-Fos mRNA (green) and CGRP mRNA (red) in the PBN. Cell nuclei are stained with DAPI (blue). A two-way ANOVA indicated significant main effects of alcohol exposure ($F(1, 32) = 5.87$, $p = 0.0212$), virus ($F(1, 32) = 15.22$, $p = 0.0005$), and an interaction between alcohol exposure and virus ($F(1, 32) = 15.56$, $p = 0.0004$) on the percentage of double-labelling c-Fos- and CGRP-positive cells in the medial PBN of female mice. Tukey's post-hoc comparisons revealed that, following CNO injection, females with AIE history that received the hM3D(Gq) virus exhibited a higher percentage of double-labelling c-Fos- and CGRP-positive cells in the medial PBN compared to air controls that received the hM3D(Gq) virus ($p = 0.0003$) and AIE females that received the control virus ($p < 0.0001$) (Figure 4E).

Regarding the lateral PBN, a two-way ANOVA showed significant main effects of virus ($F(1, 32) = 6.25$, $p = 0.0178$), as well as an interaction between alcohol exposure and virus ($F(1, 32) = 4.44$, $p = 0.0430$) on the percentage of double-labelling c-Fos- and CGRP-positive cells in female mice. Tukey's post-hoc comparisons indicated that, following CNO injection, females with AIE history that received the hM3D(Gq) virus exhibited a higher percentage of double-labelling

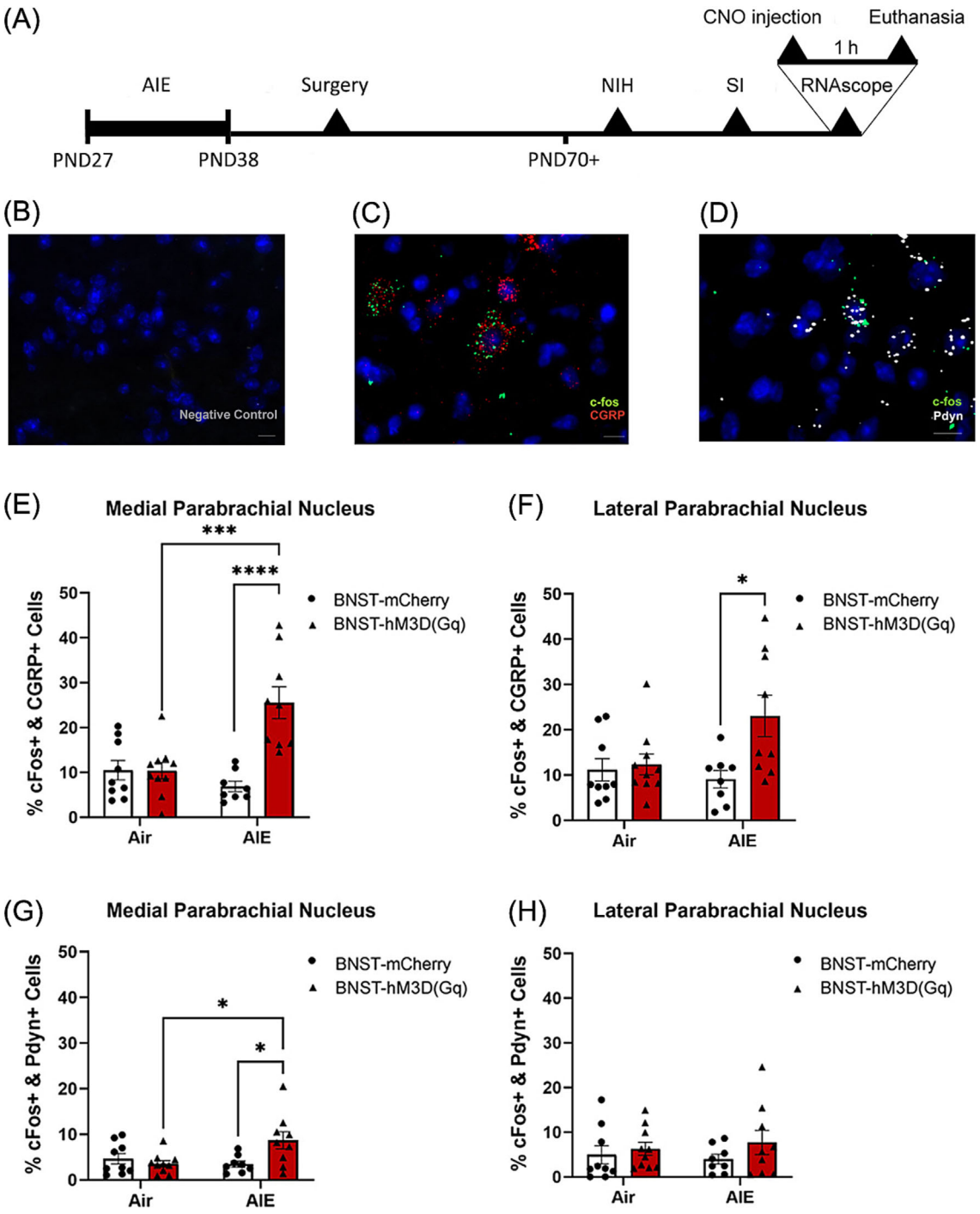

FIGURE 4 Bed nucleus of the stria terminalis (BNST) stimulation promotes neuronal activation of the parabrachial nucleus (PBN) in adult female mice exposed to adolescent intermittent ethanol (AIE) vapour. (A) Experimental timeline: on postnatal day (PND) 90+, mice received an injection of clozapine N-oxide dihydrochloride (CNO, 3 mg/kg, i.p.) and, after 1 h, were euthanized by decapitation. Female brains were removed and processed for RNAscope in situ hybridization. (B) Fluorescent in situ hybridization of the negative control probe in the PBN. Scale bar, 10 μm. (C) Fluorescent in situ hybridization of c-Fos (green) and calcitonin gene-related peptide (CGRP, red) in the PBN. Scale bar, 10 μm. (D) Fluorescent in situ hybridization of c-Fos (green) and dynorphin (Pdyn, white) in the PBN. Scale bar, 10 μm. (E) Percentage of double-labelled c-Fos- and CGRP-positive neurons following CNO injection in the medial PBN of female mice. (F) Percentage of double-labelled c-Fos- and CGRP-positive neurons following CNO injection in the lateral PBN of female mice. (G) Percentage of double-labelled c-Fos- and Pdyn-positive neurons following CNO injection in the medial PBN of female mice. (H) Percentage of double-labelled c-Fos- and Pdyn-positive neurons following CNO injection in the lateral PBN of female mice. Data presented as mean ± SEM. N = 8–10 mice/group. * denotes $p < 0.05$; *** denotes $p < 0.001$; **** denotes $p < 0.0001$

c-Fos- and CGRP-positive cells in the lateral PBN compared to AIE females that received the control virus ($p = 0.0168$) (Figure 4F). These results demonstrate that BNST stimulation promotes the activation of CGRP cells in both the medial and lateral PBN of adult female mice with AIE history.

3.5 | BNST stimulation promotes the activation of dynorphin cells in the medial PBN of adult female mice with AIE history

Figure 4D depicts a representative photomicrograph illustrating the presence of c-Fos mRNA (green) and dynorphin mRNA (white) in the PBN. Cell nuclei are stained with DAPI (blue). A two-way ANOVA indicated significant interaction between alcohol exposure and virus ($F(1, 32) = 6.67$, $p = 0.0146$) on the percentage of double-labelling c-Fos- and dynorphin-positive cells in the medial PBN of female mice. Tukey's post-hoc comparisons revealed that, following CNO injection, females with a history of AIE that received the hM3D(Gq) virus exhibited a higher percentage of double-labelling c-Fos- and dynorphin-positive cells in the medial PBN compared to air controls that received the hM3D(Gq) virus ($p = 0.0223$) and AIE females that received the control virus ($p = 0.0295$) (Figure 4G).

Regarding the lateral PBN, a two-way ANOVA showed no significant effects of alcohol exposure ($F(1, 32) = 0.01$, $p = 0.9075$) or virus ($F(1, 32) = 1.72$, $p = 0.1992$) on the percentage of double-labelling c-Fos- and dynorphin-positive cells in female mice (Figure 4H). These results show that BNST stimulation promotes the activation of dynorphin cells in the medial PBN of adult female mice with AIE history.

4 | DISCUSSION

Although stress, negative affect and psychiatric disorders are strongly linked with unhealthy alcohol use in both women and men, these factors appear to be particularly relevant for women.[28] The current data demonstrate that the stimulation of the dlBNST using DREADDs elicits hyponeophagia in adult females with AIE history, but not in males. This behavioural phenotype was associated with increased activity of CGRP neurons in both the medial and lateral PBN, as well as dynorphin neurons in the medial PBN. The stimulation of the BNST did not induce social avoidance in male or female mice, thereby highlighting the involvement of the BNST in certain aspects of alcohol-induced negative affect-like behaviours, particularly in females.

In previous work from our laboratory, we demonstrated that adult female mice exposed to AIE exhibit hyponeophagia following exposure to restraint stress.[22] The present data reveal that the chemogenetic stimulation of the dlBNST increased the latency to consume an appetitive reinforcer specifically in females with AIE history, thus mimicking the behavioural phenotype produced by restraint stress exposure. Although AIE mice showed reduced weight gain after the final vapour session (PND 38), the changes in latency to consume cannot be attributed to alterations in body weight. Both male and female mice recovered weight gain by adulthood and exhibited body weights similar to their air control counterparts prior to the NIH test at PND 78.

In both our previous study following restraint stress[22] and the present study following BNST stimulation, adult males with AIE history did not exhibit hyponeophagia. However, our group has demonstrated that male mice show AIE-induced changes in other behavioural paradigms.[22] Similar investigations using the novelty-suppressed feeding test (NSFT) have reported a significant increase in latency to eat in adult mice undergoing withdrawal from voluntary ethanol exposure. Notably, increased latency manifests during a relatively brief period of abstinence in males but requires more extended periods of abstinence in females.[29–33] Thus, negative affect-like behaviours might manifest as more immediate and transient in males. Interestingly, chemogenetic manipulations of BNST altered the expression of active avoidance in male but not female rats,[34] suggesting that BNST might have sex-specific roles in distinct behavioural coping responses.

The stimulation of the BNST promoted decreased exploration of the empty cup during the social interaction test in males, regardless of an AIE history. However, it did not induce social avoidance in either male or female mice. Our investigations were limited to stimulation of dlBNST neurons, and we did not target ventral BNST cells, which could potentially have a distinct impact on these behavioural phenotypes. In a study using adult California mice, social stress had long-term effects on oxytocin neurons within the ventral BNST in females but not in males.[35] Additionally, the stimulation of somatostatin-expressing GABAergic neurons in the dorsal BNST significantly influenced binge alcohol consumption in female mice but not in males. This stimulation, however, did not result in any changes in anxiety-like behaviour in either sex.[36] Interestingly, chemogenetic manipulation of corticotropin-releasing factor and protein kinase C-δ neurons in the BNST had opposing effects on anxiety-like behaviours, indicating that functional specificity may be attributed to distinct subdivisions and specific cell types within the BNST.[37]

The PBN has been proposed to play a role in appetite suppression during unfavourable eating conditions.[15] Additionally, emerging research has revealed that the PBN modulates behaviour in response to diverse threat-like contexts through dense reciprocal connections with the BNST and the central nucleus of the amygdala.[38] The PBN is composed of diverse cell populations that contribute to distinct aspects of feeding and aversive behaviours. Among these cell types, CGRP-expressing neurons have garnered significant attention due to their involvement in appetite regulation and the transmission of signals associated with real or potential threats.[15,39] On the other hand, dynorphin-expressing neurons in the PBN have been primarily associated with thermoregulation.[40,41] Recent findings, however, suggest that dynorphin cells also play a critical role on threat assessment and anxiety.[20]

In the current study, following BNST stimulation, females with AIE history exhibited hyponeophagia and neuronal activation of the PBN. These mice displayed activation of CGRP-expressing neurons in both the medial and lateral PBN, as well as dynorphin-expressing neurons in the medial PBN. In a previous study, the optogenetic stimulation of BNST-PBN projections bidirectionally modulates feeding behaviour in

mice, with GABAergic projections increasing consumption of normal chow under sated conditions and glutamatergic projections decreasing consumption of normal chow after food deprivation.[20] Interestingly, these opposing circuits appear to drive feeding behaviours in part via the attribution of threat assessment and the inherent valence of the circuit itself.[20] Therefore, it is possible that these circuits adapt under varying internal states induced by adverse experiences such as alcohol withdrawal or stress. Notably, the chemogenetic stimulation of PBN-BNST projections induces hyponeophagia specifically in female mice exposed to the NSFT,[42] suggesting a sex-specific effect within the reciprocal projections between the BNST and PBN.

Our findings suggest that CGRP-expressing neurons in the PBN may play a role in mediating threat assessment in adult females with AIE history, but they are likely not the sole neuropeptide population responsible for regulating feeding and aversion. Dynorphin-expressing cells in the medial PBN also appear to be involved in mediating these behaviours. Previous studies suggest that these cells respond to mechanosensory feedback and pain signals from the body, contributing to aversive learning and nocifensive behaviours, potentially through communication with CGRP neurons.[43–45] In line with our findings, a previous study[20] revealed that dynorphin-expressing neurons in the medial PBN respond to noxious stimuli and environmental threats.

It is important to recognize that the dlBNST has multiple targets beyond the PBN that may also be influenced by CNO injection and potentially impact feeding behaviour. Recent research has demonstrated that the optogenetic activation of lateral hypothalamus-projecting BNST neurons reduces consumption of regular chow while not affecting high-fat diet consumption in male mice.[46] In contrast, the photoactivation of ventrolateral periaqueductal grey-projecting BNST neurons promotes high-fat diet intake without influencing normal chow consumption.[46] Future studies employing selective chemogenetic approaches and Cre driver lines will be instrumental in gaining a deeper understanding of how BNST projections to the PBN, as well as specific neuropeptide populations within the BNST, regulate negative affect-like behaviour in the context of alcohol addiction.

Limitations of the current study should be acknowledged. Firstly, because we did not directly compare the dlBNST stimulation with exposure to stressors, we cannot ascertain if stress would produce a similar pattern of PBN cells activation. Additionally, because we hypothesized that increased negative affect-like behaviours would be associated with PBN activation, only female brains were processed for RNAscope in situ hybridization. Therefore, it remains unclear whether PBN activation is specific to females. Lastly, the current findings cannot be extrapolated to other alcohol exposure paradigms, such as gavage or voluntary consumption. We employed the intermittent ethanol vapour exposure model because it is well established in our laboratory, and it is considered the gold standard for investigating alcohol dependence.[47]

In conclusion, these findings expanded our previous work by demonstrating that the stimulation of the dlBNST using DREADDs can substitute for adult stress to elicit hyponeophagia specifically in adult females with AIE history. This behavioural phenotype is associated with an increase in activity in CGRP and dynorphin cells in the PBN, suggesting that a history of adolescent alcohol exposure may lead to sex-dependent changes in BNST-PBN circuitry. These insights contribute to advancing our understanding of the neurobiological mechanisms underlying alcohol-induced neuroadaptations and provide a foundation for further exploration of the specific contributions of BNST pathways and PBN cell populations on the development of negative affect in individuals with a history of adolescent alcohol use.

AUTHOR CONTRIBUTIONS

Lucas Albrechet-Souza: Conceptualization; data curation; investigation; formal analysis; visualization; writing—original draft preparation. **Chelsea R. Kasten:** Conceptualization; writing—review and editing. **Natalia B. Bertagna:** Investigation; writing—review and editing. **Tiffany A. Wills:** Conceptualization; supervision; methodology; resources; writing—review and editing; funding acquisition. All authors read and approved the final manuscript.

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CONFLICT OF INTEREST STATEMENT

The authors report no conflicts of interest pertaining to this work.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon request.

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