Title:

Adolescent binge-like alcohol exposure dysregulates NPY and CGRP in rats: behavioural and immunochemical evidence

Authors:
Anna Brancato[1]*, Valentina Castelli[2], Carla Cannizzaro[2#] and Giuseppe Tringali[#3, 4]

Affiliations:
[1]University of Palermo, Dept. of Health Promotion, Mother and Child Care, Internal Medicine and Medical Specialties of Excellence "G. D'Alessandro", piazza delle Cliniche 2, 90127 Palermo, Italy

[2]University of Palermo, Dept. of Biomedicine, Neuroscience and Advanced Diagnostics, via del Vespro 129, 90127 Palermo, Italy

[3]Pharmacology Section, Department of Health Care Surveillance and Bioethics, Università Cattolica del Sacro Cuore, Largo F. Vito 1, 00168 Rome, Italy

[4]Fondazione Policlinico Universitario A. Gemelli IRCSS, 00168 Rome, Italy.

[#]*These Authors contributed equally as Last Authors*

Corresponding author:
*Anna Brancato, Ph.D.
Laboratory of Neuropsychopharmacology, Ed.11D, via del Vespro 129, 90127 Palermo, Italy
+3909123865724
anna.brancato@unipa.it

Abstract

Alcohol binge drinking during adolescence impacts affective behaviour, possibly impinging on developing neural substrates processing affective states, including calcitonin gene-related peptide (CGRP) and neuropeptide Y (NPY). Here, we modelled binge-like alcohol exposure in adolescence, by administering 3.5 g/kg alcohol per os, within 1 hour time, to male adolescent rats every other day, from postnatal day 35 to 54. The effects on positive and negative affective behaviour during abstinence were explored, including consummatory behaviour and weight gain; social preference and motivation in a modified social interaction test; nociception in the tail-flick test; psychosocial stress coping in the resident-intruder paradigm. Moreover, CGRP and NPY levels were evaluated in functionally relevant brain regions. Our data shows that binge-like intermittent high-dose alcohol administration during adolescence decreased weight gain, social preference and motivation, nociception, and active psychosocial stress coping during abstinence. In addition, intermittent alcohol-exposed rats displayed increased expression of CGRP and NPY in the prefrontal cortex and nucleus accumbens; decreased NPY levels in the amygdala; and opposite changes in CGRP levels in the hypothalamus and brainstem. Overall, our data shows that adolescent binge-like alcohol exposure, through the allostatic load of alternate intoxication and withdrawal, jeopardises sensory and affective states and dysregulated complementary neuropeptidergic systems. Thus, neuropeptide-targeted interventions have promising potential for addressing the incubation of negative affect during prolonged withdrawal in young subjects.

Keywords: adolescence; alcohol; affective behaviour; NPY; CGRP

Abbreviations
NPY: Neuropeptide Y; CGRP: Calcitonin gene-related peptide

1. Introduction

Although a substantial decline in underage alcohol use has been reported, the last findings from the European School Survey Project on Alcohol and Other Drugs (ESPAD, 1999–2019) indicate a diverging trend in alcohol consumption, with steady- or a significant increase in binge drinking amid heavy adolescent drinkers (Pape et al., 2018; Loy et al., 2021). Alcohol binge drinking, i.e., the consumption of 4-5 drinks per occasion in about two hours (National Institute on Alcohol Abuse and Alcoholism, 2004), exerts detrimental consequences on the whole body, but impacts primarily on the extensive remodelling occurring during adolescence, which involves the mesocorticolimbic reward system and the peptidergic stress signalling Brenhouse and Andersen, 2011; Viau et al., 2005).

The developmental period of adolescence shares several similarities across mammals: as in humans, adolescence in rodents spans from the early post-pubertal period through physiological maturity and is characterised by adolescent-typical behaviours, such as novelty seeking and high social interactions and sensitivity to social stress (Schneider, 2013). Notably, when consuming alcohol amounts that lead to intoxicating blood alcohol concentrations, adolescents display a lower sensitivity than adults to motor impairment, sedation or hangover; however, persistent negative affective state and increased perception of psychophysical distress occur during alcohol withdrawal (Towner and Varlinskaya, 2020). Indeed, evidence from preclinical models reveals the occurrence of anxiety-like behaviour in the dark-light box and elevated plus maze, decreased approach behaviour in the novelty-suppressed feeding test, and increased vulnerability to acute swim stress in the aftermath of adolescent binge-like alcohol exposure (Towner and Varlinskaya, 2020; Kyzar et al., 2019; Varlinskaya et al., 2020; Brancato et al., 2021). Noteworthy, impaired social behaviour has also been consistently highlighted (Towner et al., 2022; Brancato et al., 2021; Varlinksaya et al., 2020; Varlinskya et al., 2014). As far as we have investigated, binge-like intermittent alcohol exposure during adolescence negatively impacts the development of the nucleus accumbens (NAc), where time-dependent changes in the expression of dopamine- and glutamate- related proteins reflect the development of affective modifications, especially evident during late withdrawal, 10 days after the last alcohol exposure (Brancato et al., 2021). In this regard, we highlighted an anxiety-prone phenotype during late abstinence, which was associated with augmented corticotropin-releasing hormone (CRH) expression and serum corticosterone levels, together with limited dopamine availability and dysfunctional excitatory synaptic remodelling in the NAc (Brancato et al., 2021). Scaling up at a circuit level, Towner and colleagues have recently shown that alcohol binge-induced social impairment is negatively correlated to the activation of “social brain” regions, including prelimbic and infralimbic medial prefrontal cortex (PFC), NAc, shell and core, central and basolateral amygdala (AMY), and anterior hypothalamus (HYP) (Towner et al., 2022).

When it comes to circuit-driven behavioural modifications, more than classic neurotransmitters, neuropeptides persistently shape long-lasting neuron-to-neuron communication and affective behavioural responses, due to their unique characteristics - i.e. high receptor binding affinity, diffusion over relatively long distances and extended extracellular half-life compared with classical neurotransmitters (Merighi et al., 2011; Tringali et al., 2012; Brancato and Cannizzaro, 2018). In the assessment of alcohol-related consequences, a growing interest is focusing on the contribution of the intersecting and antithetical systems of calcitonin gene-related peptide (CGRP) and neuropeptide Y (NPY), which modulate behaviour across the nuanced affective spectrum. On the one hand, the 37-aminoacid neuropeptide CGRP is involved in sensory information processing – encoding hyperalgesia and anorexia - and avoidance behaviour - inducing anxiogenic and fear-related responses (Greco et al., 2014; Lutz et al., 1997, Sink et al., 2013; Poore and Helmstetter, 1996). Consistently, CGRP promotes the stress response, stimulating CRH (Sink et al., 2013; Harrigan et al., 1994), the entire hypothalamic-pituitary-adrenocortical axis (Kovács et al., 1995) and the sympathetic system in rats (Brown and Gray, 1988). Given the close interplay between stress and alcohol, it comes with no

Abbreviations

NPY: Neuropeptide Y; CGRP: Calcitonin gene-related peptide

surprise that rat lines selectively bred for high alcohol preference and non-selected rats subjected to chronic alcohol exposure and long-term abstinence display discrete alterations in forebrain CGRP levels (Hwang et al., 1995; Ehlers et al., 1999; Rossetti et al., 2019).
On the other hand, the widely-distributed NPY exerts well-established opposite effects on feeding behaviour and emotionality – promoting food intake, and exerting anxiolytic-like effects and stress-buffering activity (Marcos and Coveñas, 2022; Heilig et al., 1989; Antonijevic et al., 2000). In addition, NPY promotes a robust reduction in alcohol taking and craving playing a role in the recovery from withdrawal (Robinson and Thiele, 2017; Olling et al., 2007; Plescia et al., 2014).
Given their overall opposite physiological roles and their contribution to alcohol-related effects, in this study, we aimed at assessing the involvement of NPY and CGRP in the occurrence of signs of negative affect in rats with a history of adolescent binge-like intermittent alcohol exposure.
Here, we extended our previous evaluation of behavioural and molecular consequences occurring during late withdrawal from binge-like alcohol exposure during adolescence in rats (Brancato et al., 2021), exploring different dimensions of affective behaviour such as social preference and motivation in the modified social interaction test (Varlinskaya et al., 2014), psychosocial stress coping in the resident-intruder paradigm (Burke and Miczek, 2015) and response to aversive stimuli, i.e., nociception, employing the tail-flick test (Brancato et al., 2016). Afterwards, CGRP levels and NPY immunoreactivity were evaluated in limbic brain regions, including PFC, NAc, AMY and HYP (Towner et al., 2022), but also in the functionally relevant brainstem (Capuano et al., 2014), in the attempt to find new pieces of the complex behavioural and molecular puzzle resulting from early binge-like alcohol exposure.

2. Materials and Methods

2.1. Animals

Male Wistar rats from Envigo (Italy) arrived on postnatal day (PND) 21 and were gently handled for seven days before starting the experimental procedure. They were pair-housed in standard polycarbonate cages with standard bedding, maintained at 22 ± 2 °C temperature, 55 ± 5% humidity, on a 12 h light/dark cycle (lights on at 08:00 AM), *ad libitum* laboratory rodent chow (Mucedola, Italy) and tap water. All the procedures were carried out in strict adherence to the current Italian regulation (D.L. 26/2014) and the European directive (2010/63/EU) on laboratory animal care and use. Accordingly, the research project was approved by the Animal Welfare Board of the University of Palermo and received authorisation from the Institutional Review Board of the Italian Ministry of Health (1119/2016-PR to Carla Cannizzaro). Every effort was made to minimise animal suffering and reduce the number of animals used. Moreover, the authors complied with the ARRIVE guidelines.

2.2. Binge-like Intermittent Alcohol Exposure

Rats were randomly assigned to binge alcohol withdrawn- (BAW) and control (CTRL) experimental groups (n=7 per group). BAW rats were exposed to alcohol in a binge-like intermittent alcohol paradigm during adolescence (PND 35–54) (Spear, 2000), where the experimenters gently and patiently administered the rats with alcohol at the dosage of 3.5 g/kg (Varlinskaya et al., 2014), every other day, for nine exposures (fig. 1A). To this aim, alcohol (96%; Carlo Erba Reagenti, Milan, Italy) was diluted in tap water to a 25% solution, before each administration. Rats were weighed every other day and 25% alcohol was administered *per os* as previously described (Brancato et al., 2021; 2022; Turner et al., 2011). Briefly, the administration was performed by gently and step-by-step introducing the calculated amount of solution (from about 1 ml to 3 ml, according to the adolescent rat development) in the rat's mouth through a laboratory pipette, ensuring the complete deglutition of the solution. Rats were gently handled and habituated to the oral administration procedure from PND 28 onwards. This procedure aimed to avoid the distress of gavage in adolescent rats, employ the

Abbreviations
NPY: Neuropeptide Y; CGRP: Calcitonin gene-related peptide

common administration route of alcohol consumption in humans, and ensure accurate dosing (Turner et al., 2011). CTRL rats were given an isovolumetric amount of tap water on the same exposure days.

2.3. Body weight and weight gain

Animals exposed to binge-like intermittent alcohol or water, as previously described, were monitored for body weight and food intake every other day, from the start of the binge-like intermittent alcohol exposure (PND 35) until 10 days after the last alcohol administration (PND 64) and followed up 30 days after the last alcohol administration (second cohort). Weight gain during binge-like intermittent alcohol exposure and abstinence was computed for each subject as a percentage change from baseline. Mean daily food intake during abstinence was calculated as the average food consumption in each rat cage.

2.4. Social interaction test

Social testing occurred during young adulthood, 10 days after the last alcohol exposure (i.e., on PND 64). On test day, rats were taken from their home cage and placed individually in the testing apparatus for 2 min. The testing apparatus was a Plexiglas® arena (45 × 30 × 30 cm) incompletely divided into two equally sized compartments by a partition, allowing rats to move between compartments (Varlinskaya et al., 2014; Brancato et al., 2021). A social partner, of the same age and sex of the experimental rat, was then introduced for a 10-min test period. Partners were always unfamiliar with the test apparatus and the experimental animal and were not socially deprived before the test. The social partner was marked with a green line across the tail to identify experimental animals and their social partners during the test. The behaviour of the animals was recorded for later scoring (Boris v.7.9.15). All testing procedures were conducted between 8:00 AM and 2:00 PM under dim light (15–20 lux).

Social preference was analysed by separately measuring the percentage of time the experimental subject spent in the compartment occupied by the social partner. In addition, social motivation was assessed using the preference/ avoidance coefficient %, computed as indicated: crossovers to the partner - crossovers away from the partner)/ (total number of crosses both to and away from the partner) x 100 (Varlinskaya et al., 2014).

2.5. Tail-flick test

Nociception was explored by measuring tail-flick latency in the hot-water immersion tail-flick test (Brancato et al., 2013). Briefly, 2 cm of the rat tail was immersed in a water-bath apparatus maintained at 52 ± 0.5°C. The nociceptive response was determined by a vigorous tail flick. A cut-off time of 10 seconds was imposed to minimise tissue damage.

2.6. Resident intruder paradigm

In order to minimize the distress of repeated testing and the exposure to stressful procedures, a separate cohort (n=7 per group) of rats was randomly assigned to binge-like alcohol withdrawn - (BAW) or CTRL- group and tested as intruders in the resident intruder paradigm (RIP), at PND 55 (session 1), 57 (session 2), 61 (session 3), and 64 (session 4).

The RIP was carried out as previously indicated (Burke and Miczek, 2015). Briefly, retired breeder male rats were identified as reliably aggressive residents by the confrontation for three consecutive days with nonexperimental adolescent rats before the RIP. A female rat was housed with the resident rat and removed a few minutes before the encounter. The most consistently aggressive residents were selected based on latency to attack, aggressive postures, and frequency of attacks/bites.

Abbreviations

NPY: Neuropeptide Y; CGRP: Calcitonin gene-related peptide

On each day of the RIP, the intruder rat was placed into the resident's home cage for the confrontation. The experimenter recorded the latency and frequency of bites, the duration of each supine posture, and the total duration of the interaction. The confrontation was terminated 5 minutes after the first attack bite, or earlier if the intruder displayed a submissive supine posture for more than 4 s, or if more than 12 attack bites occurred. If no attack bite was observed within 5 min, the intruder was placed into a different resident's cage. After that, the intruder was placed behind a transparent perforated plexiglass partition, inside the resident's home cage, allowing further sensory contact for 10 min. Intruders were exposed to a different resident during each session of the paradigm.
RIP occurred between 9:00 AM and 1:00 PM under dim light (15–20 lux). The sessions were recorded and scored by a trained observer, blind to the treatment. Intruder defensive behaviour was scored, as to flight, submissive posture, upright defensive posture, freezing, and quantified in terms of duration and number of episodes during the confrontation, by using Boris v. 7.9.4. The defense score, calculated as the sum of the percentage of time spent on flight, defensive upright posture, submission, and freezing was calculated for data reduction. In addition, the active defense score, including the sum of the percentage of time spent on flight, defensive upright posture, and submission, was also calculated.

2.7. Tissue collection

Rats of the first cohort were anesthetised (medetomidine 0.5 mg/kg associated with tiletamine-zolazepam 40 mg/kg, i.m.) and sacrificed at the end of the behavioural assessment in the early afternoon (1:00–3:00 PM). Brains were rapidly removed on ice and divided into two sagittal halves by employing a brain matrix. One half was immediately immersed in cold 4% paraformaldehyde in phosphate-buffered saline (PBS; pH 7.4) for 24 h fixation at 4 °C, and then stored at 4° C in 0.02% sodium azide in PBS until the preparation of the sections for immunofluorescence. The second half was immediately frozen on liquid nitrogen and stored at −80 °C, until brain areas dissection and until subsequent radioimmunoassay analysis. Brain hemispheres were counter-balanced among experiments.

2.8. CGRP measurement

The assay was performed as previously described, with minor modifications (Capuano et al., 2014). The brainstem was quickly isolated, and the PFC, NAc, AMY and HYP were dissected by employing an acrylic rat brain matrix on ice, according to the brain atlas (Paxinos and Watson, 1998). The homogenisation was performed in fixed volumes (100 mg wet tissue/1 ml) of Tris–HCl 50 mM (Sigma Chemicals Co., St. Louis, MO, USA), pH 7.4, containing 0.2% bovine serum albumin (Sigma Chemicals Co., St. Louis, MO, USA) and 40 IU/ml of aprotinin (Sigma Chemicals Co., St. Louis, MO, USA). Tissues were homogenised using a Teflon glass homogeniser (DuPont Co., Wilmington, NC, USA), and centrifuged at 20,000 rpm for 30 min at 4 °C, and the supernatant was utilized for the CGRP immunoreactivity assay. CGRP was measured in duplicate samples by a specific radioimmunoassay (RIA) technique (Capuano et al., 2011). Briefly, the RIA was performed as follows: 100 µl of the sample or standard solution was diluted three-fold into RIA buffer containing anti-hα-CGRP (kindly provided by Prof. D. Currò, who developed and validated the assay) at a final dilution of 1:120,000. After 24 hours at 4 °C, 100 µl of [125I]-hα-CGRP (4000 cpm/tube) were added, and incubation continued at 4 °C for 48 hours. The standard curve (in duplicate) ranged from 1.95 to 1000 pg of rα-CGRP/tube. Separation of free from bound α-CGRP was performed by double-antibody technique combined with polyethylene, and the pellet was counted in a γ-counter. The detection limit of the assay was 19.5 pg/ml, with intra- and inter-assay coefficients of variation of 1.27% and 13.7%, respectively. CGRP

Abbreviations
NPY: Neuropeptide Y; CGRP: Calcitonin gene-related peptide

amounts in the brain areas were expressed as pg/ml and reported as relative percentages, with reference to CTRL levels.

2.9. NPY Immunofluorescence

The procedure was performed as previously described, with minor modifications (Brancato et al., 2020). Briefly, brains were coronally sectioned at a thickness of 35 µm using a microtome (Campden Instruments, Loughborough, UK). Serial sections were collected through the rostral-caudal dimensions (every sixth slice) and stored at 4° C in 0.02% sodium azide in PBS until the immunofluorescence staining.

Five sections per rat, including the PFC (3.20 mm to 2.20 mm from the bregma), NAc (2.20 mm to 1.60 mm from the bregma), AMY (−1.80 mm to −2.30 mm from the bregma), HYP (-1.80 to -2.30 mm from the bregma), brainstem (-11.60 to -14.08 mm from the bregma), were identified according to the brain atlas and selected references (Paxinos and Watson, 1998; Brancato et al., 2020; Gumbs et al., 2019), washed in PBS for 30 minutes and incubated in blocking solution (3% Normal Goat Serum (NGS), 0.3% Triton X-100 in PBS) for two hours at room temperature under gentle shaking. Afterwards, sections were incubated in primary antibody solution (3% NGS, 0.3% Tween-20 in PBS) with rabbit anti-NPY antibody (1:1000, T-4070 Peninsula Laboratories International, Inc., San Carlos, CA) for 72 hours. Subsequently, sections were washed in PBS solution for one hour, and incubated in secondary antibody solution (Alexa Fluor 488 AffiniPure Goat Anti-Rabbit IgG; 1:200; Jackson ImmunoResearch, West Grove, PA) for two hours under gentle shaking. After washing for one hour, slices were briefly incubated with DAPI (1 µg/mL). Sections were mounted onto Superfrost Plus slides (Thermo Fisher Scientific, Waltham, MA) and coverslipped using Vectashield HardSetTM Antifade mounting medium. Images were acquired at 40x magnification using an epifluorescence microscope (Meji Techno, Saitama, Japan) and Deltapix Insight software (Denmark). NPY-positive immunofluorescence over the threshold was quantified as integrated density using Image J and verified by a trained experimenter. Data was expressed as relative immunofluorescence percentages, with reference to CTRL levels.

2.10. Statistics

The number of animals was calculated using an a priori power analysis based on effect sizes (Cohen's d) observed in our previous published work (Brancato et al., 2021). Data was presented as means ± standard error (SEM). Data was analyzed with Graphpad Prism v. 9.1, using the two-tailed Student's t test and two-way ANOVA for repeated measures (RM 2-way ANOVA), when data displayed a normal distribution and equality of variances. Welch's t test was employed when data did not present equal variance. Nonparametric analysis was performed when the data did not follow these requirements (Mann-Whitney test). Significance was set at $p < 0.05$.

3. Results

3.1. Binge-like intermittent alcohol exposure during adolescence altered affective responses during late withdrawal

Rats were exposed to alcohol in an intermittent binge-like alcohol exposure paradigm during adolescence (PND 35–54) at the dosage of 3.5 g/kg, every other day, for 9 exposures (fig. 1 a). This procedure allows the achievement of binge-like intoxicating blood alcohol levels (>80mg/dl) since blood alcohol concentrations of 193 ± 19 mg/dl were previously measured 1 hour after the last alcohol administration (Brancato et al., 2021).

Abbreviations

NPY: Neuropeptide Y; CGRP: Calcitonin gene-related peptide

Overall, adolescent rats did not show significant differences in body weight over the binge-like alcohol exposure (RM 2-way ANOVA, main effect of time: $F_{(8, 96)} = 2170$, $p<0.0001$; main effect of binge-like alcohol: $F_{(1, 12)} = 0.1165$, $p=0.7388$; interaction: $F_{(8, 96)} = 0.5452$, $p=0.8196$) and during withdrawal (RM 2-way ANOVA, main effect of time: $F_{(3, 36)} = 213.9$, $p<0.0001$; main effect of binge-like alcohol: $F_{(1, 12)} = 0.3447$, $p=0.5680$; interaction: $F_{(3, 36)} = 0.3565$, $p=0.7847$) when compared with water-exposed counterparts (suppl. fig. 1 a, b). No difference was observed at 30 days from the last administration.
When weight gain was considered, binge-like alcohol-exposed rats did not display differences during the three-week exposure paradigm, however, they gained significantly less weight during the withdrawal period than CTRL rats (RM 2-way ANOVA, main effect of period: $F_{(1, 12)} = 456.1$, $p<0.0001$; main effect of binge-like alcohol exposure: $F_{(1, 12} = 8.086$, $p=0.0148$; interaction: $F_{(1, 12)} = 1.185$, $p=0.2977$. Bonferroni post hoc test for between-group differences: binge-like intermittent alcohol exposure: $t=1.120$, $p=0.548$; WDL: $t=2.724$, $p=0.0237$) (fig. 1 b). In addition, binge-like alcohol-exposed rats showed a significant decrease in mean food consumption during withdrawal (Student's t test, $t=3.720$, $p=0.0029$) (fig. 1 c). No difference was observed at 30 days from the last administration.
Ten days after the last alcohol administration, rats were tested in the modified social interaction test, which allows the assessment of social preference and motivation for social interaction. Here, rats with a history of binge-like alcohol exposure during adolescence displayed a decreased preference for the compartment occupied by the social partner (Student's t test, $t=3.899$, $p=0.0021$) (fig. 1 d) and decreased social motivation, in terms of preference/avoidance coefficient (Student's t test, $t=2.965$, $p=0.0118$) (fig. 1 e), when compared with CTRL rats. No difference was observed in locomotion, as to the total number of crossings was similar in the two groups (Student's t test, $t=0.9496$, $p=0.3610$) (fig. 1 f).
Previous work has suggested a bidirectional association between emotional regulation and pain sensitivity, which is sensitive to alcohol-related negative affect (Kopera et al., 2021).
When rats were evaluated for nociception, binge-like alcohol-exposed rats displayed decreased response to the nociceptive stimulus, since the latency to tail-flick response increased with respect to CTRL rats (Student's t test, $t=2.909$, $p= 0.0131$) (fig. 1 g).
Besides, defensive behaviour in response to a proximal threat is sensitive to altered emotional regulation, and discrete defensive responses have been associated with affect-related psychopathological traits and clinical syndromes (Mesquita et al., 2011).
Thus, the behavioural response to a social threat was explored, and a different cohort of rats was evaluated for psychosocial coping in repeated RIP during abstinence (fig. 1 h). The analysis of the defence score, including the complete set of defensive behaviours, did not show significant differences in the intensity of the behavioural response between the two groups (RM two-way ANOVA, session: $F_{(3, 36)}=3.168$, $p=0.0360$; binge-like alcohol exposure: $F_{(1, 12)}=0.6808$, $p=0.4254$; interaction: $F_{(3, 36)}=2.455$, $p=0.0789$); however, when we evaluated the direction of the rat's defensive response facing the aversive resident, the analysis the active defense score indicated a significant main effect of session ($F_{(3, 36)}= 3.520$, $p=0.0246$), indicating that the coping strategy evolved over the four RIP sessions, but also a significant effect of binge-like alcohol exposure ($F_{(1, 12)}= 54.76$, $p<0.0001$), with decreased active defense score in BAW rats with respect to their CTRL counterparts (fig. 1 i).

Figure 1

Abbreviations
NPY: Neuropeptide Y; CGRP: Calcitonin gene-related peptide

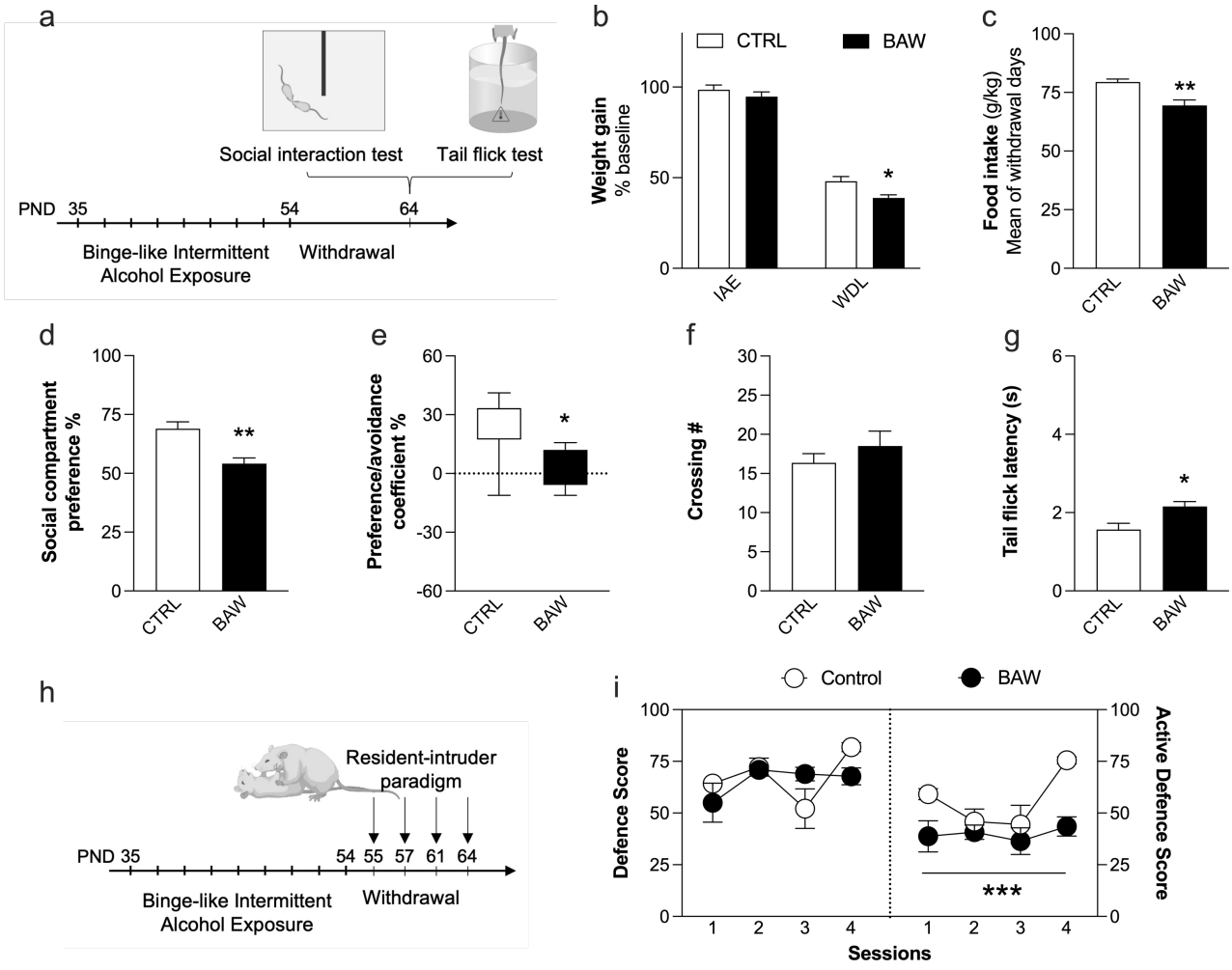

Figure 1. Binge-like alcohol exposure during adolescence altered affective responses during late withdrawal. **a)** Rats were exposed to alcohol in an intermittent binge-like alcohol paradigm during adolescence (PND 35–54) at the dosage of 3.5 g/kg, every other day, for 9 exposures. **b)** BAW rats did not display differences in weight gain during the three-week drinking paradigm, however, they gained significantly less weight than controls and **c)** displayed decreased food intake during the withdrawal period. When tested in the modified social interaction test, BAW rats displayed **d)** decreased preference for the compartment occupied by the social partner and **e)** decreased social motivation, in terms of preference/avoidance coefficient, **f)** with no differences in the number of crossings, as a measure of locomotion. In addition, **g)** BAW rats displayed decreased nociception in the tail-flick test with respect to CTRL rats.

h) A parallel cohort of rats was exposed to binge-like alcohol during adolescence and evaluated for coping abilities in four sessions of the resident-intruder paradigm during withdrawal, in comparison with CTRL counterparts. **i)** BAW rats did not display significant differences in the intensity of the behavioural response, in terms of total defense score; however, they showed decreased active defense score with respect to their CTRL counterparts.

CTRL: water-exposed control group; BAW: binge-like alcohol withdrawn rats; IAE: intermittent alcohol exposure; WDL: withdrawal. Each bar, each box plot and each circle represent the mean of n = 7 rats; error bars indicate SEM. * $p<0.05$; ** $p<0.01$; ***$p<0.001$. Cartoons were created with BioRender.com.

Abbreviations

NPY: Neuropeptide Y; CGRP: Calcitonin gene-related peptide

3.2. Binge-like alcohol exposure during adolescence altered CGRP and NPY levels in a region-specific manner

CGRP levels were assessed in brain regions relevant to its modulatory effects on the processing of sensory and affective aversive stimuli (Greco et al., 2014, 2016; Lutz et al., 1997; Sink et al., 2013; Poore and Helmstetter, 1996; Harrigan et al., 1994; Kovács et al., 1995; Brown and Gray, 1988, Capuano et al., 2014) and alcohol-related effects (Hwang et al., 1995; Ehlers et al., 1999; Rossetti et al., 2019).

Rats with a history of binge-like alcohol exposure during adolescence showed increased CGRP levels in limbic brain regions involved in affective state and alcohol's properties, such as the PFC (Welch's t test, $t=4.186$, $p=0.0053$) (fig. 2 a) and the NAc (Student's t test, $t=4.63$, $p=0.0063$) (fig. 2 b), with respect to CTRL rats. No differences were observed in CGRP levels in the AMY, where BAW rats displayed similar levels to CTRL rats ones (Student's t test, $t=0.238$, $p=0.8159$) (fig. 2 c).

On the other hand, hypothalamic CGRP levels, involved in anorectic and neuroendocrine effects (Lutz et al., 1997; Brown and Gray, 1988), were increased in BAW rats with respect to CTRLs (Student's t test, $t=3.23$, $p=0.0072$) (fig. 2 d). As to CGRP levels in the brainstem, where it mediates the gating of nociceptive stimuli (Greco et al., 2014, 2016; Capuano et al., 2014), binge-like alcohol-exposed rats showed decreased CGRP levels when compared with their CTRL counterparts (Student's t test, $t=3.756$, $p=0.0027$ (fig. 2 e).

On the other hand, NPY-positive immunofluorescence was quantified in brain regions relevant to its broad modulatory role on affective behaviour, including food- (Marcos and Coveñas, 2022), social- and non-social emotional responses (Heilig et al., 1989, Antonijevic et al., 2000), and alcohol-related behaviours (Robinson and Thiele, 2017; Olling et al., 2007; Plescia et al., 2014).

Rats exposed to binge-like alcohol in adolescence displayed a significant increase in NPY levels in the PFC (Student's t test, $t=9.276$, $p<0.001$) (fig. 3 a-c) and in the NAc (shell: Welch's t test, $t=6.531$, $p=0.0004$; core: Welch's t test, $t=5.928$, $p=0.0004$) (fig. 4 a-f) with respect to CTRL rats.

As to NPY levels in the AMY, binge-like alcohol-exposed rats showed decreased NPY-positive immunofluorescence (BLA: Student's t test, $t=4.864$, $p=0.0004$; CeA: Student's t test, $t=10.39$, $p<0.001$) when compared with their CTRL counterparts (fig. 5 a-f).

No significant difference was observed when considering NPY levels in the HYP, where the relative percentages of NPY-positive immunofluorescence (mean ± standard error) were: CTRL: 100 ± 16; BAW: 95 ± 16; Student's t test, $t=0.2370$, $p=0.8167$); and brainstem, where the relative percentages of NPY-positive immunofluorescence were CTRL: 100 ± 17; BAW: 98 ± 11; Student's t test, $t=0.07828$, $p=0.9389$).

Figure 2

Abbreviations

NPY: Neuropeptide Y; CGRP: Calcitonin gene-related peptide

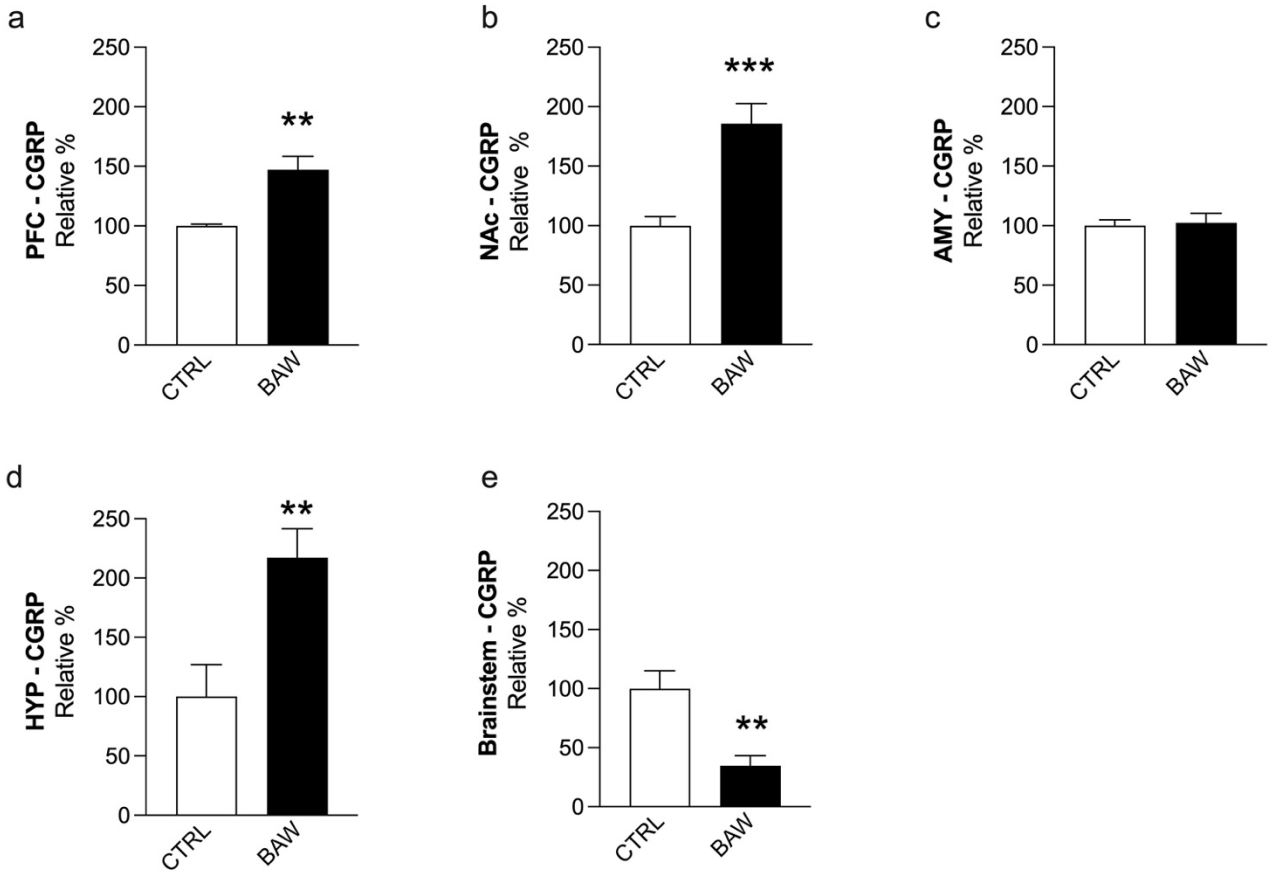

Figure 2. Binge-like alcohol exposure during adolescence altered CGRP levels in a region-specific manner. Rats with a history of binge-like alcohol during adolescence showed increased CGRP levels in limbic brain regions involved in affective state and alcohol's properties, including the **a)** PFC and **b)** the NAc, with respect to control rats. **c)** No differences were observed in CGRP levels in the AMY, where CGRP levels were similar to those measured in CTRL rats. In addition, while **d)** hypothalamic CGRP levels were increased in BAW rats with respect to CTRL counterparts, **e)** Moreover, decreased CGRP levels in the brainstem were observed in binge-like alcohol-exposed rats, compared with CTRL rats. CTRL: water-exposed control group; BAW: binge-like alcohol withdrawn rats; PFC: prefrontal cortex; NAc nucleus accumbens; AMY: amygdala; HYP: hypothalamus. Each bar represents the mean of n = 7 rats; error bars indicate SEM. ** $p<0.01$; ***$p<0.001$.

Figure 3

Abbreviations

NPY: Neuropeptide Y; CGRP: Calcitonin gene-related peptide

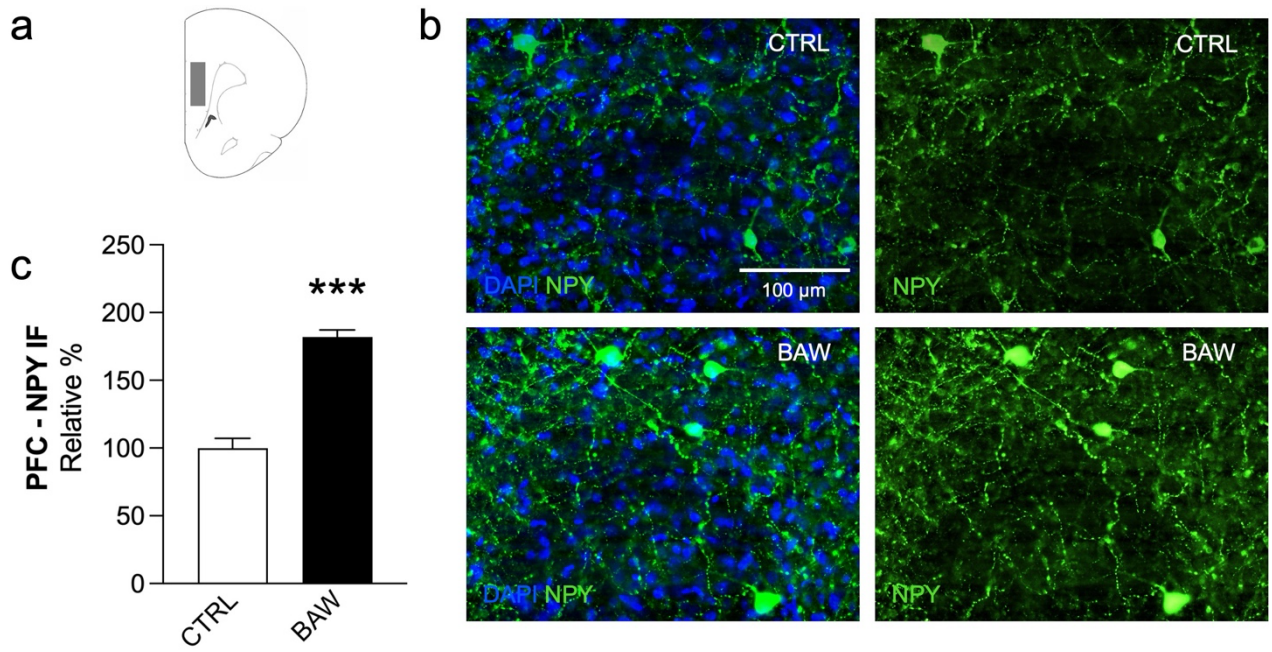

Figure 3. Binge-like alcohol exposure during adolescence increased NPY levels in the prefrontal cortex.

When we assessed **a)** the prefrontal cortex, brain region relevant to affective behaviour and alcohol-related effects, the assessment of **b)** NPY-positive immunofluorescence showed that **c)** rats exposed to binge-like alcohol in adolescence displayed a significant increase in NPY levels with respect to control rats. CTRL: water-exposed control group; BAW: binge-like alcohol withdrawn rats; PFC: prefrontal cortex, DAPI: nuclear staining with 4',6-diamidino-2- phenylindole, NPY: Neuropeptide Y. Each bar represents the mean of n = 7 rats; error bars indicate SEM. ***p<0.001.

Figure 4

Abbreviations

NPY: Neuropeptide Y; CGRP: Calcitonin gene-related peptide

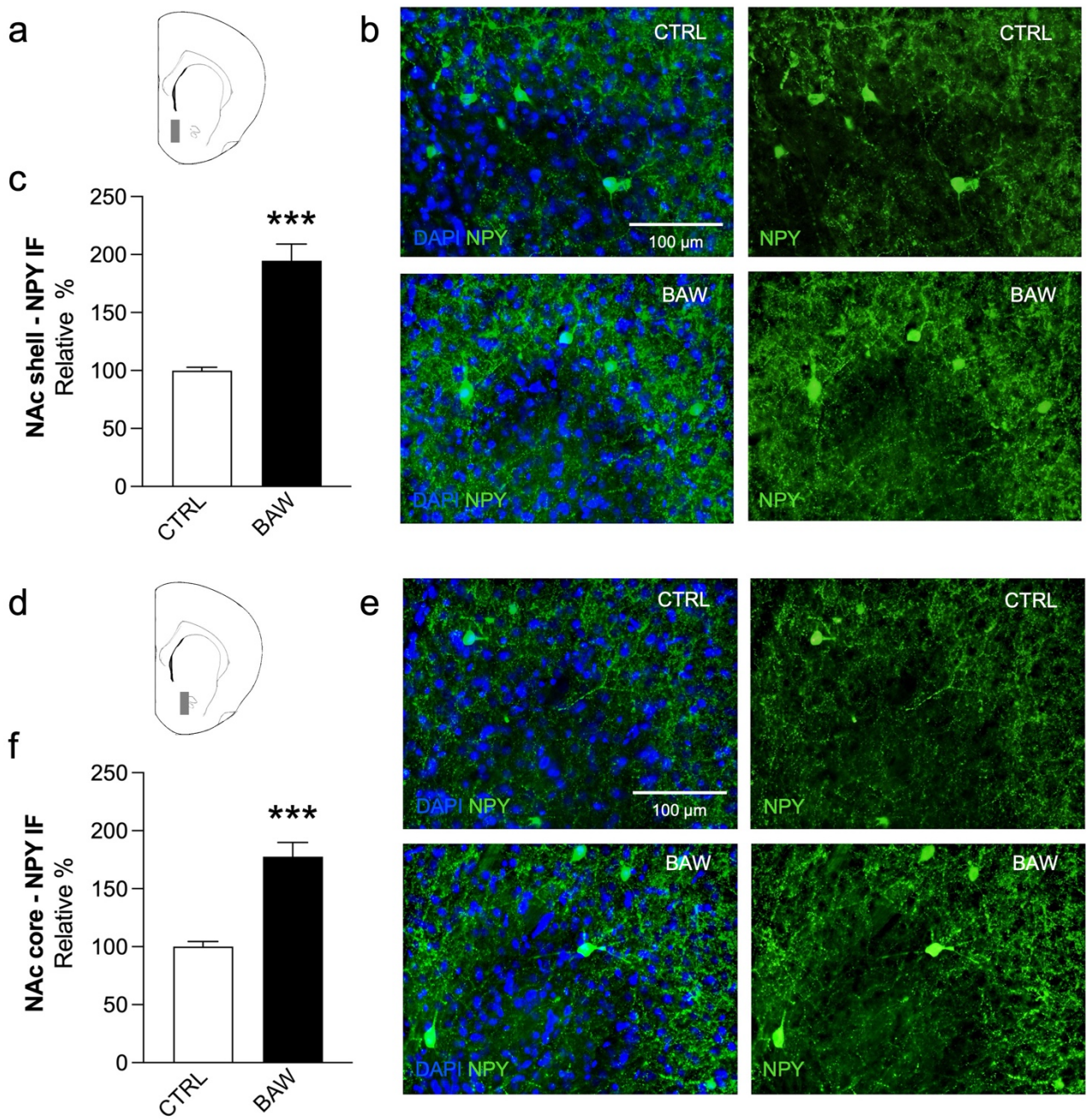

Figure 4. Binge-like alcohol exposure during adolescence increased NPY levels in the nucleus accumbens.

When we focused on the nucleus accumbens, brain region relevant to affective behaviour and alcohol-related effects, the assessment of **a)** NAc shell **b)** NPY-positive immunofluorescence showed that **c)** rats exposed to binge-like alcohol in adolescence displayed a significant increase in NPY levels with respect to control rats. Similarly, the assessment of **d)** NAc core **e)** NPY-positive immunofluorescence showed that **f)** BAW displayed a significant increase in NPY levels with respect to control rats.

CTRL: water-exposed control group; BAW: binge-like alcohol withdrawn rats; NAc: Nucleus accumbens; DAPI: nuclear staining with 4',6-diamidino-2- phenylindole, NPY: Neuropeptide Y. Each bar represents the mean of n = 7 rats; error bars indicate SEM. ***p<0.001.

Figure 5

Abbreviations

NPY: Neuropeptide Y; CGRP: Calcitonin gene-related peptide

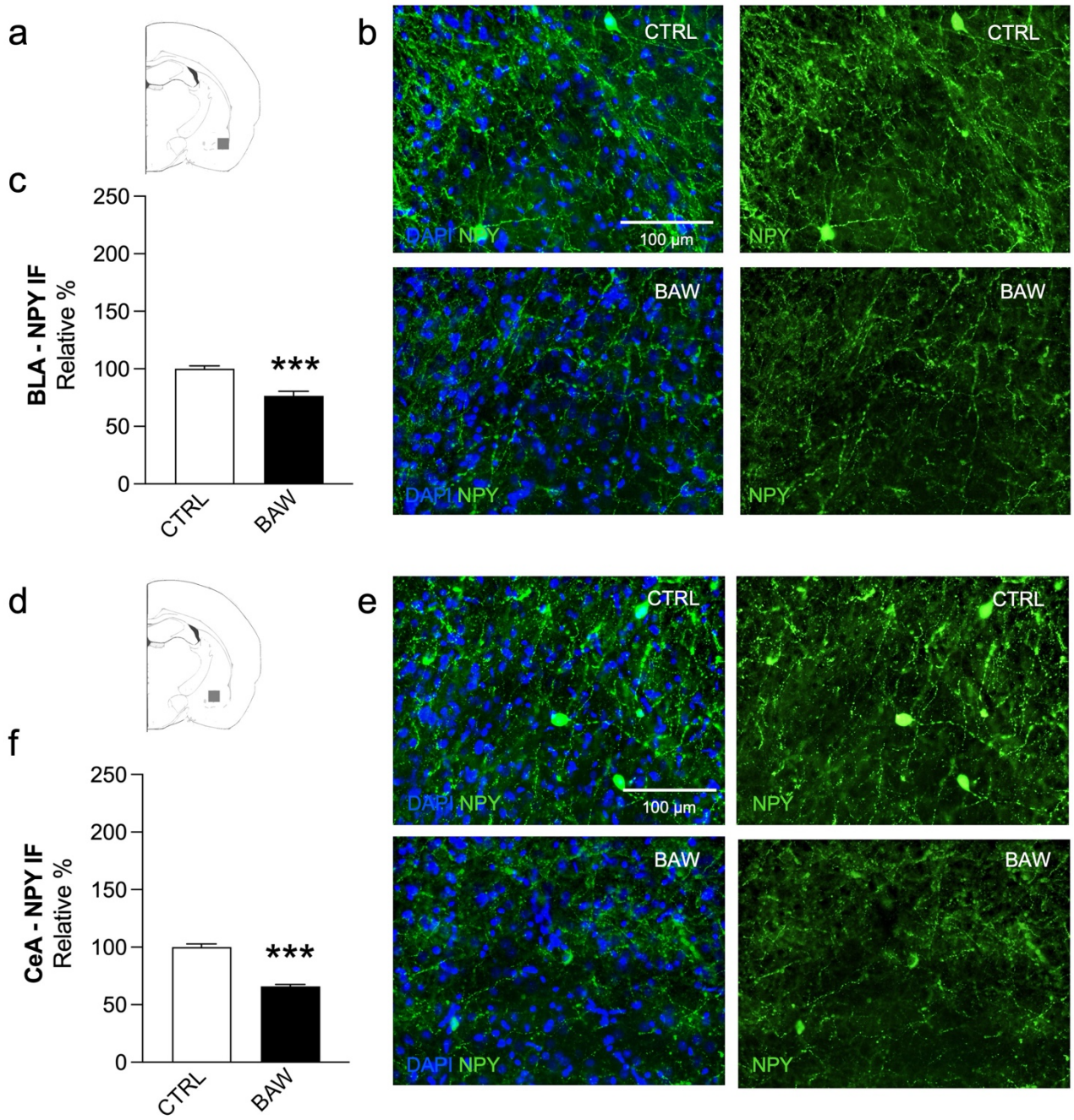

Figure 5. Binge-like alcohol exposure during adolescence decreased NPY levels in the amygdala.
When we focused on the amygdala, brain region relevant to affective behaviour and alcohol-related effects, the assessment of **a)** basolateral **b)** NPY-positive immunofluorescence showed that **c)** rats exposed to binge-like alcohol in adolescence displayed a significant decrease in NPY levels with respect to control rats. Similarly, the assessment of **d)** central amygdala **e)** NPY-positive immunofluorescence showed that **f)** BAW rats displayed a significant decrease in NPY levels with respect to control rats.
CTRL: water-exposed control group; BAW: binge-like alcohol withdrawn rats; BLA: basolateral amygdala; CeA: central amygdala DAPI: nuclear staining with 4',6-diamidino-2- phenylindole, NPY: Neuropeptide Y. Each bar represents the mean of n = 7 rats; error bars indicate SEM. ***p<0.001.

Abbreviations
NPY: Neuropeptide Y; CGRP: Calcitonin gene-related peptide

4. Discussion

In this study, we aimed at evaluating in young male rats the consequences of binge-like intermittent alcohol exposure during adolescence on discrete behavioural correlates of affective behaviour during late withdrawal, including social preference and motivation, vulnerability to psychosocial stress, nociception, feeding behaviour, together with the expression of NPY and CGRP in functionally relevant brain regions. Our hypothesis was that withdrawal from binge-like alcohol exposure is associated with a dysregulation in two antithetic (stress-related) neuropeptidergic systems, which may contribute to the occurrence of critical signs of negative affect.

Only a few studies have investigated NPY and CGRP levels during withdrawal from alcohol exposure (Hwang et al., 1995; Ehlers et al., 1999; Olling et al., 2007; Plescia et al., 2014) and no previous evidence is available on the effects of alcohol binging during adolescence.

Thus, we modelled the exposure to intoxicating alcohol levels in adolescent rats through a binge-like alcohol paradigm. The every-other-day consumption of high alcohol doses did not result in differences in body weight, compared to controls, although a slight but significant reduction in food intake and in weight gain was recorded. If the binge-like alcohol exposure did not cause major harm to the adolescent rats' growth (Anji and Kumari, 2008), the decreased weight gain might mirror a withdrawal induced-abnormal aversive state, consistent with behavioural observation of anxiety-like state and increased latency to feed in the same experimental conditions (Brancato et al., 2021). Indeed, feeding is driven by a combination of internal and external factors, where neuropeptidergic signalling integrates nutritional balance, environmental cues, and conflicting motivational states (Phua et al., 2021), therefore a significant imbalance in hypothalamic orexigenic and anorexigenic signalling can play a relevant role in it, as discussed later on.

In order to further explore the long-lasting consequences of binge-like intermittent alcohol exposure during adolescence, after ten days from the last alcohol administration rats were tested in the modified social interaction test. In young rodents, social interactions with a conspecific have a positive emotional effect and elicit conditioned place preference (Trezza et al., 2010). The social interaction test here employed allows the assessment of motivation for social interaction via the preference/avoidance coefficient, which measures the number of crossings toward or away from the non-manipulated social partner in a 2-compartment apparatus. In accordance with our previous data (Brancato et al., 2021), social preference and motivation for social interaction were reduced in alcohol withdrawn rats, without any motor alteration. On the basis of what we observed earlier, the reduction in social motivation could result from the alterations in dopamine signalling in the NAc associated with late withdrawal from binge-like alcohol exposure, which is evocative of a hypodopaminergic state (Brancato et al., 2010). However, since interactive social behaviours are sensitive to anxiogenic manipulations of the internal and the external milieu (Varlinksaya et al., 2014), the decrease in social preference measured in this setting might also reflect an increased emotionality under social circumstances. This is in agreement with increased anxiety-like behaviour in the novelty suppressed feeding test and indexes of abnormal stress response occurring during late withdrawal, previously reported by this and other groups (Towner and Varlinskaya, 2020; Kyzar et al., 2019; Varlinskaya et al., 2020, 2014; Towner et al., 2022).

Notably, similarly to social interaction, coping behaviour with psychosocial stress appeared impaired. The RIP is a valuable tool for studying functional aspects of social threat response in rodents, as young intruders implement and adjust their defensive behaviour when they face repeated encounters with an isolated older aggressive resident rat (Burke and Miczek, 2015). As defensive behaviour includes a complex and hierarchical set of responses to threatening situations, the choice of the most appropriate defensive strategy for each threat depends mainly on the distance from the threat, the ambiguity of the antagonist, and the availability of an escape route or a hiding place (Blanchard et al., 2001). When the distance from the threat decreases, upright defensive postures and submissive

Abbreviations

NPY: Neuropeptide Y; CGRP: Calcitonin gene-related peptide

postures are the potential alternatives; when at a certain distance from the threat, the animal will choose to flee, if a flight route is available. In the absence of the possibility of flight, freezing would represent another option (Blanchard et al., 2001). Throughout the interactive sessions, intruder rats developed defensive behavioural and physiological responses aimed at reducing the resident's offensive threat. Binge alcohol exposure in adolescence did not affect the intensity of the defensive behaviour, in agreement with the relative stability of the defensive response to social threats (Blanchard et al., 2001; Niermann et al., 2018). However, when the direction of the defensive response was considered, rats with a history of binge-like alcohol exposure showed a decrease in active behavioural responses. As a matter of fact, this data implies a relative increase in freezing behaviour, a behavioural response generally considered as a preparative stage, allowing perception, risk assessment, and action preparation, integral to selecting the most appropriate proactive defensive strategy, i.e., fight-or-flight reaction (Roelofs, 2017). Notably, the behavioural response to social threat has been associated with the affective state, and increased freezing has been considered a predictor for internalising symptoms in adolescence (Mesquita et al., 2011; Nierman et al., 2018). Overall, the abnormal defensive behaviour, displayed by binge-like alcohol-exposed rats, suggests a deficit in the perception of the threat and aberrant processing of the environmental information, where an emotional overload impairs the recruitment of the appropriate instinctive repertoire in response to aggression, producing the inability to develop an adaptive defensive strategy.

Overall, our data indicates that exposure to repeated cycles of alcohol intoxication and withdrawal during adolescence produces a progressive and enduring shift away from homeostatic motivation- and stress-related responses, suggesting the onset of an allostasis-like negative emotional state already conceptualised for addictive disorders (Edwards and Koob, 2010).

Notably, the aversive emotional state produced by late alcohol withdrawal was associated with a reduction in nociception. Although hyperalgesia at 6 and 12 hours after withdrawal has been reported (Gatch and Lal, 1999), the assessment of pain perception in mice exposed to chronic intermittent alcohol during adolescence did not reveal a difference in the hot plate test 6 days after the last administration (Monteleón et al., 2020). Moreover, clinical observations in young adult binge drinkers did not find hyperalgesia within 48 hours after a drinking episode (You et al., 2020). While differences in species, time point and age of exposure may account for inconsistent results, a significant insight may be provided by the assessment of neuropeptidergic signalling. Indeed, the multifaceted behavioural evidence of this study, from pain perception to anxiety-like behaviour, to reduced social motivation and social stress coping, was paralleled by with differences in CGRP and NPY expression in relevant brain regions.

Indeed, CGRP neurons located in the parabrachial nucleus of the brainstem are considered major players transmitting nociceptive signals to higher brain regions of the extended AMY, that integrate and interpret the sensory aversive information (Phua et al., 2021; Tringali and Navarra 2019). Consistent with the decreased nociceptive response observed, we highlighted a reduction in CGRP levels in the brainstem of alcohol withdrawn rats, suggesting that the reduced pain perception could be mediated by enduring adaptations in nociceptive signal processing, which may involve a blunted pontine parabrachial nucleus output to the upper brain regions (Phua et al., 2021).

At this stage, we cannot dissect the individual components contributing to the hypoalgesia here reported. However, alcohol interacts with the nociceptive signalling to increase the release of sensory neuropeptides, such as CGRP, from sensory ganglia, through a Ca2+- and TRPV1-dependent mechanism (Nicoletti et al., 2008). Thus, repeated binge-like alcohol exposure may produce adapting modifications in the CGRP system that result in decreased CGRP levels at late withdrawal. The levels of NPY in the brainstem of alcohol withdrawn rats were not different from the control's, indicating no direct involvement of NPY signalling.

Abbreviations

NPY: Neuropeptide Y; CGRP: Calcitonin gene-related peptide

In addition, we observed an increase in CGRP levels in the HYP, which may be linked to decreased feeding, thus decreased weight gain, during withdrawal from binge-like alcohol exposure, whereas NPY was not affected. Actually, hypothalamic CGRP and NPY affect food intake, playing opposite roles (Lutz et al., 1997; Marcos and Coveñas, 2022). Thus, it is intuitive to hypothesise that the relative CGRP prominence observed in binge-like alcohol-exposed rats could have influenced their consummatory behaviour, promoting an anorectic-like attitude, as shown by decreased weight gain during withdrawal. Increased hypothalamic CGRP levels were also reported in adult rats after a 4-week withdrawal from chronic alcohol exposure (Ehlers et al., 1999). Interestingly, the same alcohol exposure paradigm in adult rats was associated with a significant increase in hypothalamic NPY immunoreactivity (Ehlers et al., 1998), confirming that different alcohol drinking pattern and age of exposure can account for different effects and degrees of alcohol vulnerability.
Notably, the HYP is also part of the brain regions that control social behaviour, together with the PFC, the NAc and the AMY, outlined as the social brain network (Towner et al., 2022). In this regard, we observed increased levels of both CGRP and NPY in the PFC and NAc, and decreased NPY levels in the AMY.
NPY has been reported to exert region-specific effects promoting social behaviour (Sajdyk et al., 2002); on the other hand, although CGRP overexpressing transgenic mice did not show any difference in social interaction compared to wild-type counterparts (Hashikawa-Hobara et al., 2021), no region-specific evidence has linked CGRP to social behaviour so far. In our experimental conditions, the decrease in social preference and motivation observed in binge-like alcohol withdrawn rats suggests that increased CGRP and NPY expression in the PFC may abnormally modulate discrete PFC neural ensembles exerting an opposite role on social behaviour (Minami et al., 2017; Huang et al., 2020). Moreover, the altered neuropeptide levels observed in the PFC output brain regions, i.e., NAc and AMY, may contribute to an unbalance in the activation of distinct social-behaviour-related circuits, producing maladaptive behavioural responses to positive social stimuli and social stress.
Indeed, increased levels of both NPY and CGRP in the NAc of binge-like alcohol-exposed rats may interfere with pre-and post-synaptic dopamine signalling. On the one hand, NPY increases extracellular outputs of accumbal monoamines, mainly norepinephrine, but also dopamine (Quarta et al., 2011). On the other hand, CGRP receptors are localised on dopaminoceptive neurons such that, when administered intracerebroventricularly, CGRP occludes dopamine-related behaviours inducing hypomotility, and increasing haloperidol-induced catalepsy (Clementi et al., 1992). Thus, a dysregulation of dopamine tone and postsynaptic signalling in the NAc could be implicated in the altered motivational processing and stress coping highlighted in this work.

Noteworthy, although no difference emerged in CGRP levels in the AMY, where CGRP terminals densely innervate and stimulate CRH neurons (Harrigan et al., 1994; Kovács et al., 1995), the decreased NPY-positive immunofluorescence observed in both central and basolateral AMY is consistent with previous reports on prolonged withdrawal from intermittent alcohol exposure during adolescence (Sakharkar et al., 2019). Notably, besides playing a key role in down-regulating anxiety and alcohol drinking (Sakharkar et al., 2019), when infused into the BLA, NPY exerts a prosocial effect in rats (Sajdyk et al., 2002) and, within the CeA, promotes active coping with social stress, decreasing the expression of social fear in mice (Kornhuber and Zoicas, 2021). Thus, the peptidergic imbalance in the AMY in favour of CGRP may contribute to the social anxiety-like behaviour and the abnormal coping with psychosocial stress observed in binge-like alcohol-exposed rats.
Although the mechanisms underlying the effects of adolescent binge-like alcohol exposure and withdrawal on CGRP and NPY peptidergic transmission are still unknown, the long-lasting dysregulation in dopamine transmission during late withdrawal from binge-like adolescent alcohol exposure (Brancato et al., 2021) may have affected neuropeptide expression.

Abbreviations
NPY: Neuropeptide Y; CGRP: Calcitonin gene-related peptide

Indeed, CGRP has been implicated in several neurological and psychiatric dopamine-related disorders, including dementia, major depression and Parkinson's disease, and increased CGRP levels have been reported in the frontal cortex, AMY, and HYP in Flinders sensitive rat line, a model of depression (Angelucci et al., 2019; Svenningsson et al., 2017).
In addition, increased NPY mRNA expression has been observed in the striatum in Parkinson's disease-affected patients (Cannizzaro et al., 2003), and increased NPY and CGRP levels were measured in the cerebrospinal fluid in patients affected by Parkinson's Disease with comorbid depression, versus patients with just major depressive disorder (Svenningsson et al., 2017). Thus, previous and our present evidence suggest an inverse relationship with hypofunctional dopamine neurotransmission.

5. Conclusions

Overall, adolescent binge-like alcohol exposure, through an allostatic load due to alternate alcohol intoxication and withdrawal episodes, can dysregulate the complementary CGRP and NPY systems to engender altered sensory and affective negative states. Importantly, for the first time, our data points to potential neuropeptide-targeted intervention as a novel strategy to decrease negative affect during withdrawal in young adults, and long-term consequences of adolescent-onset alcohol binge drinking.

Abbreviations

NPY: Neuropeptide Y; CGRP: Calcitonin gene-related peptide

6. Reference List

Angelucci, F., Ellenbroek, B.A., El Khoury, A., Mathé, A.A. CGRP in a gene-environment interaction model for depression: effects of antidepressant treatment. *Acta Neuropsychiatr*. **31**(2), 93-99 (2019)

Anji, A., Kumari, M. Supplementing the liquid alcohol diet with chow enhances alcohol intake in C57 BL/6 mice. *Drug Alcohol Depend.* **97** (1-2), 86-93 (2008).

Antonijevic, I. A., et al. Neuropeptide Y promotes sleep and inhibits ACTH and cortisol release in young men. *Neuropharmacology.* **39** (8), 1474–81 (2000).

Blanchard, D. C., Griebel, G., Blanchard, R. J. Mouse defensive behaviors: pharmacological and behavioral assays for anxiety and panic. *Neurosci Biobehav Rev.* **25,** 205-218 (2001).

Brancato, A., Lavanco, G., Cavallaro, A., Plescia, F., Cannizzaro, C. The use of the Emotional-Object Recognition as an assay to assess learning and memory associated to an aversive stimulus in rodents. *J Neurosci Methods.* **274,** 106-115 (2016).

Brancato, A., & Cannizzaro, C. Mothering under the influence: how perinatal drugs of abuse alter the mother-infant interaction. *Rev Neurosci.* **29** (3), 283-294 (2018).

Brancato, A., Castelli, V., Lavanco, G., Marino, R. A. M., Cannizzaro, C. In utero Δ9-tetrahydrocannabinol exposure confers vulnerability towards cognitive impairments and alcohol drinking in the adolescent offspring: Is there a role for neuropeptide Y? *J Psychopharmacol.* **34** (6), 663-679 (2020).

Brancato, A., et al. Binge-like Alcohol Exposure in Adolescence: Behavioural, Neuroendocrine and Molecular Evidence of Abnormal Neuroplasticity… and Return. *Biomedicines*. **9** (9), 1161 (2021).

Brancato, A., et al. Social stress under binge-like alcohol withdrawal in adolescence: evidence of cannabidiol effect on maladaptive plasticity in rats. *Psychol Med*. 1-132022. (2022) doi: 10.1017/S0033291722002744. Epub ahead of print.

Brenhouse, H. C., & Andersen, S. L. Developmental trajectories during adolescence in males and females: a cross-species understanding of underlying brain changes. *Neurosci Biobehav Rev.* **35(8),** 1687–1703 (2011).

Brown, M. R., & Gray, T.S. Peptide injections into the amygdala of conscious rats: effects on blood pressure, heart rate and plasma catecholamines. *Regul Pept.* **21**(1-2), 95-106 (1988).

Burke, A. R., & Miczek, K. A. Escalation of cocaine self-administration in adulthood after social defeat of adolescent rats: role of social experience and adaptive coping behavior. *Psychopharmacology (Berl).* **232** (16), 3067-79 (2015).

Cannizzaro, C., Tel, B. C., Rose, S., Zeng, B. Y., Jenner, P. Increased neuropeptide Y mRNA expression in striatum in Parkinson's disease. *Brain Res Mol Brain Res*. **110** (2), 169-76 (2003).

Abbreviations

NPY: Neuropeptide Y; CGRP: Calcitonin gene-related peptide

Capuano, A., Currò, D., Navarra, P., Tringali, G. Cortistatin modulates calcitonin gene-related peptide release from neuronal tissues of rat. Comparison with somatostatin. *Peptides*. **32** (1), 138-43 (2011).

Capuano, A., Greco, M. C., Navarra, P., Tringali, G. Correlation between algogenic effects of calcitonin-gene-related peptide (CGRP) and activation of trigeminal vascular system, in an in vivo experimental model of nitroglycerin-induced sensitization. *Eur J Pharmacol.* **740,** 97-102 (2014).

Clementi, G., et al. Effects of calcitonin gene-related peptide on extrapyramidal motor system. Pharmacol Biochem Behav. **42** (3), 545-8 (1992).

Edwards, S., & Koob, G. F. Neurobiology of dysregulated motivational systems in drug addiction. *Future Neurol.* **5** (3), 393-401 (2010).

Ehlers, C. L., et al. Neuropeptide Y levels in ethanol-naive alcohol-preferring and non-preferring rats and in Wistar rats after ethanol exposure. *Alcohol Clin Exp Res*. **22** (8), 1778-82 (1998).

Ehlers, C. L., et al. Calcitonin gene-related peptide (CGRP) levels and alcohol. *Int J Neuropsychopharmacol.* **2** (3), 173-179 (1999).

Gatch, M. B., & Lal, H. Effects of ethanol and ethanol withdrawal on nociception in rats. *Alcohol Clin Exp Res.* **23** (2), 328-33 (1999).

Greco, M. C., Lisi, L., Currò, D., Navarra, P., Tringali, G. Tapentadol inhibits calcitonin gene-related peptide release from rat brainstem in vitro. *Peptides.* **56,** 8-13 (2014).

Greco, M. C., Navarra, P., Tringali, G. The analgesic agent tapentadol inhibits calcitonin gene-related peptide release from isolated rat brainstem via a serotonergic mechanism. *Life Sci.* **145,** 161-5 (2016).

Gumbs, M. C. R., et al. Afferent neuropeptide Y projections to the ventral tegmental area in normal-weight male Wistar rats. *J Comp Neurol*. **527** (16), 2659-2674 (2019).

Harrigan, E. A., Magnuson, D. J., Thunstedt, G. M., Gray, T. S. Corticotropin releasing factor neurons are innervated by calcitonin gene-related peptide terminals in the rat central amygdaloid nucleus. *Brain Res Bull.* **33** (5),529-34 (1994).

Hashikawa-Hobara, N., Otsuka, A., Okujima, C., Hashikawa, N. CGRP overexpression does not alter depression-like behavior in mice. *PeerJ.* **9,** e11720 (2021).

Heilig, M., Söderpalm, B., Engel, J. A., Widerlöv, E. Centrally administered neuropeptide Y (NPY) produces anxiolytic-like effects in animal anxiety models. *Psychopharmacology (Berl)*. **98** (4), 524-9 (1989).

Huang, W. C., Zucca, A., Levy, J., Page, D. T. Social Behavior Is Modulated by Valence-Encoding mPFC-Amygdala Sub-circuitry. *Cell Rep*. **32** (2), 107899 (2020).

Abbreviations
NPY: Neuropeptide Y; CGRP: Calcitonin gene-related peptide

Hwang, B. H., Kunkler, P. E., Lumeng, L., Li, T. K. Calcitonin gene-related peptide (CGRP) content and CGRP receptor binding sites in discrete forebrain regions of alcohol-preferring vs. -nonpreferring rats, and high alcohol-drinking vs. low alcohol-drinking rats. *Brain Res.* **690** (2), 249-53 (1995).

Kopera, M., et al. Pain Sensitivity, Negative Affect, and Alcohol Use Disorder Status: A Moderated Mediation Study of Emotion Dysregulation. *J Clin Med.* **10** (6), 1321 (2021).

Kornhuber, J., & Zoicas, I. Neuropeptide Y Reduces Social Fear in Male Mice: Involvement of Y1 and Y2 Receptors in the Dorsolateral Septum and Central Amygdala. *Int J Mol Sci*. **22** (18), 10142 (2021).

Kovács, A., Biró, E., Szeleczky, I., Telegdy, G. Role of endogenous CRF in the mediation of neuroendocrine and behavioral responses to calcitonin gene-related peptide in rats. *Neuroendocrinology.* **62** (4), 418-24 (1995).

Kyzar, E. J., Zhang, H., Pandey, S. C. Adolescent Alcohol Exposure Epigenetically Suppresses Amygdala Arc Enhancer RNA Expression to Confer Adult Anxiety Susceptibility. *Biol Psychiatry*. **85** (11), 904-914 (2019)

Loy, J. K., et al. Trends in alcohol consumption among adolescents in Europe: Do changes occur in concert? *Drug Alcohol Depend.* **228,** 109020 (2021).

Lutz, T. A., Rossi, R., Althaus, J., Del Prete, E., Scharrer, E. Evidence for a physiological role of central calcitonin gene-related peptide (CGRP) receptors in the control of food intake in rats. *Neurosci Lett.* **230** (3), 159-62 (1997).

Marcos, P., & Coveñas, R. Regulation of Homeostasis by Neuropeptide Y: Involvement in Food Intake. *Curr Med Chem.* **29** (23), 4026-4049 (2022).

Merighi, A., Salio, C., Ferrini, F., Lossi, L. Neuromodulatory function of neuropeptides in the normal CNS. *J Chem Neuroanat*. **42**(4), 276-87 (2011).

Mesquita, S. C., et al. The response of social anxiety disorder patients to threat scenarios differs from that of healthy controls. *Braz J Med Biol Res.* **44** (12), 1261-8 (2011).

Minami, C., Shimizu, T., Mitani, A. Neural activity in the prelimbic and infralimbic cortices of freely moving rats during social interaction: Effect of isolation rearing. *PLoS One.* **12** (5), e0176740 (2017).

Monleón, S., Duque, A., Vinader-Caerols, C. Emotional memory impairment produced by binge drinking in mice is counteracted by the anti-inflammatory indomethacin. *Behav Brain Res.* **381,** 112457 (2020).

National Institute on Alcohol Abuse and Alcoholism (NIAAA). NIAAA Council Approves Definition of Binge Drinking. NIAAA Newsletter; Rockville, MD, USA (2004).

Abbreviations

NPY: Neuropeptide Y; CGRP: Calcitonin gene-related peptide

Nicoletti, P., et al. Ethanol causes neurogenic vasodilation by TRPV1 activation and CGRP release in the trigeminovascular system of the guinea pig. *Cephalalgia*. **28** (1), 9-17 (2008).

Niermann, H. C. M., Figner, B., Tyborowska, A., Cillessen, A. H. N., Roelofs, K. Investigation of the Stability of Human Freezing-Like Responses to Social Threat From Mid to Late Adolescence. *Front Behav Neurosci.* **12,** 97 (2018).

Olling, J. D., et al. Decreased gene expression of neuropeptide Y and its receptors in hippocampal regions during ethanol withdrawal in rats. *Neurosci Lett* **424** (3), 160–4 (2007).

Pape, H., Rossow, I. & Brunborg, G. S. Adolescents drink less: How, who and why? A review of the recent research literature. *Drug Alcohol Rev.*;**37(1),** S98-S114 (2018).

Paxinos, G. & Watson, C. The Rat Brain in Stereotaxic Coordinates. San Diego, CA: Academic Press. (1998)

Phua, S. C., et al. A distinct parabrachial-to-lateral hypothalamus circuit for motivational suppression of feeding by nociception. *Sci Adv.* **7** (19), eabe4323 (2021).

Plescia, F., et al. Effect of Acetaldehyde Intoxication and Withdrawal on NPY Expression: Focus on Endocannabinoidergic System Involvement. *Front Psychiatry.* **5,** 138 (2014).

Poore, L. H, & Helmstetter, F. J. The effects of central injections of calcitonin gene-related peptide on fear-related behaviour. *Neurobiol Learn Mem.* **66** (2), 241-5 (1996).

Quarta, D., Leslie, C. P., Carletti, R., Valerio, E., Caberlotto, L. Central administration of NPY or an NPY-Y5 selective agonist increase in vivo extracellular monoamine levels in mesocorticolimbic projecting areas. *Neuropharmacology.* **60** (2-3), 328-35 (2011).

Robinson, S. L., Thiele, T. E. The Role of Neuropeptide Y (NPY) in Alcohol and Drug Abuse Disorders. *Int Rev Neurobiol.* **136,** 177-197 (2017).

Roelofs K. Freeze for action: neurobiological mechanisms in animal and human freezing. *Phil. Trans. R. Soc. B* **372**, 20160206 (2017)

Rossetti, I., et al. Predisposition to Alcohol Drinking and Alcohol Consumption Alter Expression of Calcitonin Gene-Related Peptide, Neuropeptide Y, and Microglia in Bed Nucleus of Stria Terminalis in a Subnucleus-Specific Manner. *Front Cell Neurosci.* **13**, 158 (2019).

Sajdyk, T. J., Schober, D. A., Gehlert, D. R. Neuropeptide Y receptor subtypes in the basolateral nucleus of the amygdala modulate anxiogenic responses in rats. *Neuropharmacology*. **43** (7),1165-72 (2002).

Sakharkar, A. J., et al. Altered amygdala DNA methylation mechanisms after adolescent alcohol exposure contribute to adult anxiety and alcohol drinking. *Neuropharmacology*. **157**, 10767 (2019).

Abbreviations
NPY: Neuropeptide Y; CGRP: Calcitonin gene-related peptide

Schneider, M. Adolescence as a vulnerable period to alter rodent behavior. *Cell Tissue Res* **354,** 99–106 (2013).

Sink, K. S., Chung, A., Ressler, K. J., Davis, M., Walker, D. L. Anxiogenic effects of CGRP within the BNST may be mediated by CRF acting at BNST CRFR1 receptors. *Behav Brain Res*. **243**, 286-93 (2013).

Spear LP. The adolescent brain and age-related behavioral manifestations. *Neurosci Biobehav Rev.* **24**(4), 417-63 (2000)

Svenningsson, P., Pålhagen, S., Mathé, A. A. Neuropeptide Y and Calcitonin Gene-Related Peptide in Cerebrospinal Fluid in Parkinson's Disease with Comorbid Depression versus Patients with Major Depressive Disorder. *Front Psychiatry* **8**, 102 (2017).

Towner, T. T., & Varlinskaya, E. I. Adolescent Ethanol Exposure: Anxiety-Like Behavioral Alterations, Ethanol Intake, and Sensitivity. *Front Behav Neurosci*. **14,** 45 (2020).

Towner, T. T., Applegate, D. T., Varlinskaya, E. I., Werner, D. F. Impact of Adolescent Intermittent Ethanol Exposure on Social Investigation, Social Preference, and Neuronal Activation in cFos-LacZ Male and Female Rats. *Front Pharmacol.* **13,** 841657 (2022).

Trezza, V., Baarendse, P. J., Vanderschuren, L. J. The pleasures of play: pharmacological insights into social reward mechanisms. *Trends Pharmacol Sci.* **31** (10), 463-9 (2010).

Tringali, G., Greco, M. C., Lisi, L., Pozzoli, G., Navarra, P. Cortistatin modulates the expression and release of corticotrophin releasing hormone in rat brain. Comparison with somatostatin and octreotide. *Peptides*. **34** (2), 353-9 (2012).

Tringali, G., & Navarra, P. Anti-CGRP and anti-CGRP receptor monoclonal antibodies as antimigraine agents. Potential differences in safety profile postulated on a pathophysiological basis. *Peptides.* **116,** 16-21 (2019).

Turner, P.V.; Brabb, T.; Pekow, C.; Vasbinder, M.A. Administration of Substances to Laboratory Animals: Routes of Administration and Factors to Consider. *J. Am. Assoc. Lab. Anim. Sci*. **50**, 600–613 (2011)

Varlinskaya, E. I., Truxell, E., Spear, L. P. Chronic intermittent ethanol exposure during adolescence: effects on social behavior and ethanol sensitivity in adulthood. *Alcohol.* **48**(5), 433-44 (2014).

Varlinskaya, E. I., Hosová, D., Towner, T., Werner, D. F., Spear, L. P. Effects of chronic intermittent ethanol exposure during early and late adolescence on anxiety-like behaviors and behavioral flexibility in adulthood. *Behav Brain Res.* **378,** 112292 (2020).

Viau et al. Gender and Puberty Interact on the Stress-Induced Activation of Parvocellular Neurosecretory Neurons and Corticotropin-Releasing Hormone Messenger Ribonucleic Acid Expression in the Rat. *Endocrinology* **146 (1),** 137–146 (2005).

Abbreviations

NPY: Neuropeptide Y; CGRP: Calcitonin gene-related peptide

You, D. S., Hahn, H. A., Welsh, T. H., Meagher, M. W. Hyperalgesia after a Drinking Episode in Young Adult Binge Drinkers: A Cross-Sectional Study. *Alcohol Alcohol.* **55** (6), 608-615 (2020).

Abbreviations
NPY: Neuropeptide Y; CGRP: Calcitonin gene-related peptide

7. Funding

This research was funded by the European Foundation for Alcohol Research—ERAB (EA 16 42 to C.C.), Fondazione Zardi Gori (post-doctoral fellowship to A.B.) and internal funding (Fondi di Ateneo D1: 2020-2021 to G.T.; FFR 2021 to C.C. and A.B.).

8. Author Contributions

AB, GT, and VC conducted the experiments, AB and GT analyzed the data. CC and GT conceived and oversaw the study, interpreted data and secured funding. All authors contributed to the writing and editing of the manuscript.

9. Data availability

The datasets generated and analysed during the current study are available from the corresponding author on reasonable request.

10. Competing interests

The authors declare no competing interests. The funders had no role in the design of the study; in the collection, analysis, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

11. Ethical approval

All the procedures were carried out in strict adherence to the current Italian regulation (D.L. 26/2014) and the European directive (2010/63/EU) on laboratory animal care and use. Accordingly, the research project was approved by the Animal Welfare Board of the University of Palermo and received authorisation from the Institutional Review Board of the Italian Ministry of Health (1119/2016-PR to Carla Cannizzaro). Every effort was made to minimise animal suffering and reduce the number of animals used.

Abbreviations

NPY: Neuropeptide Y; CGRP: Calcitonin gene-related peptide