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RESEARCH ARTICLE

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Cannabidiol tempers alcohol intake and neuroendocrine and behavioural correlates in alcohol binge drinking adolescent rats. Focus on calcitonin gene-related peptide's brain levels

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Abstract

Alcohol binge drinking is common among adolescents and may challenge the signalling systems that process affective stimuli, including calcitonin gene-related peptide (CGRP) signalling. Here, we employed a rat model of adolescent binge drinking to evaluate reward-, social- and aversion-related behaviour, glucocorticoid output and CGRP levels in affect-related brain regions. As a potential rescue, the effect of the phytocannabinoid cannabidiol was explored. Adolescent male rats underwent the intermittent 20% alcohol two-bottle choice paradigm; at the binge day (BD) and the 24 h withdrawal day (WD), we assessed CGRP expression in medial prefrontal cortex (mPFC), nucleus accumbens (NAc), amygdala, hypothalamus and brainstem; in addition, we evaluated sucrose preference, social motivation and drive, nociceptive response, and serum corticosterone levels. Cannabidiol (40 mg/kg, i.p.) was administered before each drinking session, and its effect was measured on the above-mentioned readouts. At BD and WD, rats displayed decreased CGRP expression in mPFC, NAc and amygdala; increased CGRP levels in the brainstem; increased response to rewarding- and nociceptive stimuli and decreased social drive; reduced serum corticosterone levels. Cannabidiol reduced alcohol consumption and preference; normalised the abnormal corticolimbic CGRP expression, and the reward and aversion-related hyper-responsivity, as well as glucocorticoid levels in alcohol binge-like drinking rats. Overall, CGRP can represent both a mediator and a target of alcohol binge-like drinking and provides a further piece in the intricate puzzle of alcohol-induced behavioural and neuroendocrine sequelae. CBD shows promising effects in limiting adolescent alcohol binge drinking and rebalancing the bio-behavioural abnormalities.

Abbreviations: AMY, amygdala; BD, binge day; CBD, cannabidiol; CGRP, calcitonin gene-related peptide; CORT, corticosterone; CRH, Corticotropin-releasing hormone; HPA, hypothalamus–pituitary–adrenal axis; HYP, hypothalamus; IA2BC, intermittent alcohol two-bottle choice paradigm; mPFC, medial prefrontal cortex; NAc, nucleus accumbens; WD, withdrawal day.

Giuseppe Tringali and Gianluca Lavanco equally contributed to this work as co-first authors.

KEYWORDS
adolescent alcohol binge drinking, cannabidiol, CGRP, corticosterone, reward/aversion

1 | INTRODUCTION

Despite a decline in underage alcohol use, binge drinking, that is, the episodic consumption of 4–5 drinks per occasion (NIAAA, 2004), displays a significant increase among adolescent heavy drinkers in several European Countries (Loy et al., 2021; Pape et al., 2018). Recent data from this lab shows that, unlike moderate alcohol consumption, alcohol binge drinking among high school students has a predictive value in the occurrence of clinical syndromes and personality disorders within the affective dimension (Castelli et al., 2022). When adolescents consume alcohol in a binge-like pattern, they experience repeated alcohol intoxications and withdrawals (Hiller-Sturmhöfel & Spear, 2018), which may represent a further challenge for the still-maturing brain. Indeed, besides exerting highly reinforcing effects, alcohol intake during adolescence can affect the brain's maturational processes and jeopardise the correct neurodevelopmental trajectory across species, promoting abnormal behavioural and neurobiological outcomes (Crews et al., 2016; Pascual et al., 2009; Spear, 2011; Tetteh-Quarshie & Risher, 2023).

Previous evidence from this group shows that at 10-day withdrawal from alcohol binge-like exposure in adolescence, rats show abnormal behavioural and neuroendocrine response to psychosocial stress, accompanied by disturbances in the mesolimbic dopaminergic transmission (Brancato et al., 2021, 2022, 2023). In addition, following prolonged withdrawals from an intermittent alcohol diet, Wills et al. (2009) reported behavioural sensitisation to social avoidance in adolescent rats, which was prevented by the pharmacological blockade of the stress axis' central response.

In cooperation with classical neurotransmitters, neuropeptides contribute to long-lasting neuron-to-neuron communication and play an ideal role as neuromodulators of the affective states that guide the behavioural responses to diverse environmental stimuli (Brancato & Cannizzaro, 2018; Merighi et al., 2011). Among them, calcitonin gene-related peptide (CGRP), whose role is well-established in the regulation of autonomic functions and the formation and transmission of nociceptive signals in the periphery (van Rossum et al., 1997), is emerging as a player in the central integration of the affective state (Carboni et al., 2022; Kang et al., 2022; Neugebauer et al., 2020). This abundant 37-amino acid neuropeptide is produced in neurones predominantly located in the medial hindbrain, including the parabrachial nucleus, the hypothalamus (HYP) and the posterior thalamus. From there, CGRP is released in several areas of the forebrain, such as the cortico-limbic regions (Warfvinge & Edvinsson, 2019), where it conveys multi-sensory innate threat information (Bowen et al., 2023; Kang et al., 2022) and promotes anxiety and fear-related behaviour (Carboni et al., 2022; Poore & Helmstetter, 1996). As a matter of fact, central CGRP signalling participates in the stress response: when CGRP is administered into the lateral brain ventricle, a dose-dependent increase in plasma corticosterone level is observed (Brown & Gray, 1988; Kovács et al., 1995), thus playing a central role as a mediator of negative affect (Sink et al., 2013).

Early studies show that the central CGRP system is also implicated in alcohol preference and withdrawal-induced sequelae in adult rodents (Ehlers et al., 1999; Hwang et al., 1995). More recently, studies have shown that after voluntary alcohol drinking, Sardinian alcohol-preferring rats display a marked increase in CGRP expression in the extended amygdala (Rossetti et al., 2019), suggesting that CGRP is implicated in the proneness to excessive alcohol consumption. Recently, we observed an increase in CGRP levels in the medial prefrontal cortex (mPFC), nucleus accumbens (NAc) and HYP in long-term withdrawn rats with a history of alcohol binge-like exposure in adolescence; this was associated with persistent modifications in affect-related behaviour, including social avoidance, abnormal coping with social and environmental stress and decreased food consumption. What is still unclear, however, is whether the CGRP system is also affected by the dynamic transitions produced by the functionally different time points of the binge-like drinking pattern, namely bingeing episodes and withdrawal, during adolescence. Notably, if several reports show that repeated exposure to 20% alcohol and 24 h withdrawal promotes excessive alcohol consumption and behavioural disruptions in adult rodents (Carnicella et al., 2014), less evidence is available in adolescent animals. And adolescence is a time of spectacular plasticity and adaptive potential that can promote ongoing vulnerability, but also allows healing processes.

Therefore, this research originally aimed at a wide-spread evaluation of diverse variables in male rats exposed to alcohol binge-like drinking in the intermittent alcohol two-bottle choice paradigm (IA2BC) during adolescence. In detail, we measured: voluntary drinking pattern; affective behaviour—in terms of social interaction, sucrose preference and sensitivity to an aversive threat; stress axis activity—as to corticosterone levels; and the dynamic changes in CGRP levels in discrete alcohol-salient brain regions, during functionally different time points of the alcohol binge-like drinking paradigm, that is, binge day (BD) and acute withdrawal day (WD). Previous work has highlighted the promising potential of cannabidiol (CBD), a major phytocannabinoid with no abuse liability, in alcohol-related outcomes (Nona et al., 2019). We have recently shown that CBD can offset the behavioural, neuroendocrine and neuroplastic maladaptations to social and environmental stress associated with withdrawal from adolescent alcohol binge-like exposure in adult rats (Brancato et al., 2021, 2022). However, the effects of CBD on voluntary alcohol drinking in adolescence have not been explored yet, not to mention the interaction with central CGRP levels. To this aim, CBD was administered before the drinking sessions during the IA2BC paradigm, and its impact on alcohol consumption and preference was evaluated. In addition, the effect of the repeated CBD administration was assessed

on the region-specific CGRP expression, the responsivity to rewarding, social and aversive stimuli, and serum corticosterone levels.

2 | EXPERIMENTAL PROCEDURES

2.1 | Animals

Male Wistar rats from Envigo (Italy) arrived on postnatal day (PND) 21 and were gently handled for 7 days before starting the experimental procedure. They were 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 conducted in accordance with protocols approved by the Committee for the Protection and Use of Animals of the University of Palermo and the Italian Ministry of Health (1119/2016-PR), in adherence to the current Italian and European regulations (D.L. 26/2014; Directive 2010/63/EU) on care and use of laboratory animals. Every effort was made to minimise animal suffering and reduce the number of animals used.

2.2 | Drugs

Alcohol (96%; J.T. Baker) was diluted with tap water to the final concentration (20% v/v). Cannabidiol (2-[(1R, 6R)-6-Isopropenyl-3-methylcyclohex-2-en-1-yl]-5pentylbenzene-1,3-diol) (CBD), purity (NMR) >99%, was extracted by the Forensic Laboratory of Biologically Active Substances of the University of Chemistry and Technology of Prague (Hložek et al., 2017). CBD was dissolved in DMSO (1%, Sigma Aldrich), Tween 80 (1%, Sigma Aldrich) and saline and administered intraperitoneally (i.p.) at the dose of 40 mg/kg before each alcohol drinking session. The dose was modified from previous studies in mice (Viudez-Martínez et al., 2018) and our previous evaluations in late adolescent rats (Brancato et al., 2021, 2022).

2.3 | Experimental procedures

Adolescent rats (PND 35; Spear, 2015) were randomly assigned to the water-drinking control (CTRL) group or the intermittent access to 20% alcohol in the two-bottle choice drinking paradigm (IA2BC) group.

In experiment 1, rats' drinking behaviour was evaluated under undisturbed conditions for nine drinking sessions; then, alcohol-drinking rats were matched for drinking pattern and assigned to two subgroups, which were tested for response to a natural reward in the sucrose preference test, social motivation and drive in the modified social interaction test, and sensitivity to an aversive threat in the tail-flick test, either at the end of the binge-like drinking session (BD) or 24 h of alcohol withdrawal (WD), in comparison with water-drinking CTRL rats. Rats were sacrificed at the same time points, following a further cycle of BD and WD, in order to rule out any influence on the measures of serum corticosterone levels and CGRP expression in discrete brain regions due to behavioural testing.

In experiment 2, rats exposed to the IA2BC paradigm were administered intraperitoneally with CBD (40 mg/kg) or vehicle (1% DMSO, 1% Tween 80, saline) before each drinking session, and CBD effects on alcohol drinking pattern were measured along nine drinking sessions. Then, the effects of CBD on sucrose preference, social motivation and drive in the modified social interaction test, sensitivity to an aversive threat, serum corticosterone levels and CGRP expression in discrete brain regions were explored at BD.

2.3.1 | IA2BC paradigm

The procedure was conducted as previously described (Stuber et al., 2008). Briefly, rats were given two 250 mL graduated bottles, one containing 20% alcohol (v/v) and the other tap water, in their home cage, every other day, 3 days a week (i.e., Monday, Wednesday, Friday). The bottle position in the wire top of the cage was alternated to avoid side preference. Two bottles of tap water were available on the intervening days (i.e., Tuesday, Thursday, Saturday and Sunday). Bottles were weighed before their presentation and after 24 h of alcohol availability. Rats were weighed three times a week before the drinking session, and alcohol intake was expressed as g/kg. Alcohol preference was computed as follows: ml of 20% alcohol consumed/(mL of 20% alcohol + mL water consumed) × 100. CTRL rats were given two bottles of tap water and manipulated on the same days as the IA2BC group.

2.3.2 | Sucrose preference test

The response to a natural reward was evaluated in the sucrose preference test (Brancato et al., 2021). Briefly, rats had a free choice between two bottles, one filled with a 1% sucrose solution and the other containing tap water, for 24 h, in their home cage. The bottles were weighed before and after the test. Sucrose preference was calculated by dividing the consumed sucrose solution by the total fluids and multiplying this value by 100.

2.3.3 | Social interaction test

Rats were tested in the modified social interaction test as previously described (Brancato et al., 2021). The testing apparatus was a Plexiglas® box (45 × 30 × 30 cm) partially divided into two equally sized compartments by a Plexiglas® partition, which allowed movements of the animals between the compartments. On the test day, animals were taken from their home cage and habituated to the testing apparatus for 30 min. A conspecific social partner of the same age and sex was then introduced for 10-min. Social stimulus rats were

experimentally naïve, unfamiliar with the apparatus and the experimental rat, and not socially deprived before the test. Weight differences between test subjects and their partners were minimised and did not exceed 20 g. The test session was recorded for later scoring. All testing procedures were conducted between 10:00 and 14:00 under dim light (15–20 lux). Time spent in the two compartments and the number of crossovers toward and away from the compartment occupied by the social partner were measured. The social motivation score was computed as the percentage of time spent by the experimental subject in the compartment occupied by the social partner out of the total time. As a measure of the energetic drive to social interaction, we employed the social drive index, computed as follows: (crossovers to the partner − crossovers away from the partner)/(total number of crossovers) × 100. The total number of crossovers was considered a measure of locomotor activity.

2.3.4 | Tail-flick test

The sensitivity to the aversive threat was explored in the hot-water immersion tail-flick test (Brancato et al., 2016). Briefly, 2 cm of the rat tail was immersed in a water-bath apparatus, maintained at 52 ± 0.5°C. The response was determined by measuring the latency for the rat to display a vigorous tail flick. A cut-off time of 10 s was imposed to minimise tissue damage.

2.4 | Tissue collection

Rats were sacrificed under anaesthesia (medetomidine 0.5 mg/kg /tiletamine-zolazepam 40 mg/kg, i.m.). Trunk blood was collected, kept for 1 h at room temperature and centrifuged at 1500 rpm for 10 min at 4°C. The serum was separated and stored at −20°C. The brain of each rat was quickly isolated on ice from the skull and snap-frozen in liquid nitrogen before being stored at −80°C. Tissues were kept under these conditions until the dissection of the brain regions of interest and their subsequent homogenisation. In detail, the brainstem was rapidly isolated; the entire HYP was dissected, while mPFC, NAc and AMY were dissected bilaterally employing an acrylic rat brain matrix on ice, according to the rat brain atlas, considering the following rostral-caudal coordinates: mPFC, +4.0 to +2.0 mm from bregma; NAc, +3.0 to +1.0 mm from bregma; AMY, −2.0 to −3.0 mm from bregma (Paxinos & Watson, 1998).

Subsequently, brain tissues were washed in ice-cold PBS and weighed before proceeding with homogenisation. The latter was performed in fixed volumes (100 mg wet tissue/1 mL) of fresh lysis buffer (Tris–HCl 50 mM [Sigma Chemicals Co., St. Louis, MO, USA], pH 7.4, containing 0.2% bovine serum albumin [BSA; Sigma Chemicals Co., St. Louis, MO, USA] and 40 IU/mL of aprotinin [Sigma Chemicals Co., St. Louis, MO, USA]) by a Teflon glass homogeniser on ice (DuPont Co., Wilmington, NC, USA). The homogenates were subsequently centrifuged at 20,000 rpm for 20 min at 4°C, and the supernatant was separated and used for radioimmunoassay reactions in duplicate, according to the manufacturer's instructions.

2.5 | CGRP measurement

To quantify CGRP levels in brain tissues, a commercially available radioimmunoassay kit (Cat. # Rk-015-09; Phoenix Pharmaceutical Inc, Burlingame, CA, USA) was used. It was used as a rabbit polyclonal antibody against CGRP, which has 100% cross-reactivity with rat α-CGRP, 78% with rat β-CGRP, 35% and 16% with human α- and β-CGRP respectively, whereas no cross-reactivity with amylin (rat and human), rat calcitonin and somatostatin-14. The standard curve (in duplicate) ranged from 10 to 1280 pg of α-CGRP/mL. The assay sensitivity was 32 pg/tube. CGRP amounts in the brain areas were expressed as pg/mL and reported as relative percentages, with reference to CTRL levels.

2.6 | Blood alcohol concentration

Alcohol concentration was measured in the serum collected at BD by employing a commercially available colourimetric assay kit (STA-620; Cell Biolabs, Inc., San Diego, CA), in duplicate, according to the protocol supplied by the manufacturer (Brancato et al., 2021). Two samples were removed from the analysis due to hemolysis.

2.7 | Corticosterone determination

Serum corticosterone levels (CORT, ng/mL) were measured in duplicate using a commercially available enzyme-linked immunosorbent assay kit (Cat. # DEV9922, Demeditec Diagnostics GmbH, Kiel, Germany), according to the manufacturer's instructions (Brancato et al., 2021; Castelli et al., 2022).

2.8 | Data analysis

Animals were randomly assigned to an experimental group. During the behavioural manipulations and data interpretation, researchers were blind to the treatment each animal had received. All data are presented as means ± standard error (SEM). Group size for the individual experiments is shown in the corresponding figure legends. The analysis was performed by the software GraphPad Prism v. 9.1. Student's *t*-test (two-tails) or analysis of variance (ANOVA)—for repeated measures when appropriate—followed by Bonferroni's multiple comparison post hoc test was employed. When the data did not show normal distribution, equal variance or sample size, mixed effect- or nonparametric analysis (Kruskal–Wallis and Dunn's tests) were performed. Pearson's r was employed for performing the correlation analysis. The significance threshold was set at $p < 0.05$ and a trend was indicated for $p < 0.1$.

3 | RESULTS

3.1 | Experiment 1

3.1.1 | Adolescent rats displayed alcohol binge-like drinking in the IA2BC paradigm

Alcohol binge drinking in adolescence was modelled in rats employing the IA2BC paradigm (Figure 1a). Alcohol drinking behaviour was rapidly acquired, and no significant differences among the nine sessions of the paradigm were highlighted in alcohol consumption ($F_{(8,117)} = 1.436$, $p = 0.1887$) (Figure 1b) and alcohol preference ($p = 0.2197$) (Figure 1b). In detail, the mean alcohol consumption was 5.5 ± 0.5 g/kg/24 h during the first week, 5.2 ± 0.5 g/kg/24 h during the second week and 4.1 ± 0.3 g/kg/24 h during the third week. Mean blood alcohol concentration levels at the last BD were 113.2 ± 25.6 mg/dL and correlated with alcohol intake ($r = 0.9195$, $p = 0.0271$) (Figure 1c). The exposure to the IA2BC paradigm during adolescence had no significant effect on rat body weight ($F_{(1,19)} = 3.55$, $p = 0.075$), while a significant effect of drinking sessions ($F_{(1.03,26.65)} = 830.5$, $p < 0.001$) and their interaction ($F_{(8,152)} = 2.585$, $p = 0.0113$) was observed. The post hoc test, however, did not highlight significant differences within the drinking sessions (Figure 1d). The analysis of water consumption indicated a significant main effect of the IA2BC paradigm ($F_{(1,19)} = 5.539$, $p = 0.0295$), drinking session ($F_{(1.768,33.59)} = 37.33$, $p < 0.001$) and their interaction ($F_{(8,152)} = 10.76$, $p < 0.001$). IA2BC-exposed rats showed decreased water consumption in session 1 ($t = 7.248$, $p < 0.001$), 2 ($t = 3.666$, $p = 0.0149$) and 6 ($t = 4.282$, $p = 0.0065$) when compared to the CTRL counterparts (Figure 1e).

3.1.2 | Adolescent alcohol binge-like drinking displayed altered CGRP levels in discrete brain regions, paralleled by abnormal affective behaviour and stress axis activity

Adolescent rats that resulted homogeneous for alcohol intake over the 3 weeks of the IA2BC paradigm (time point: $F_{(1,12)} = 0.2367$, $p = 0.6354$; week: $F_{(2,24)} = 4.695$, $p = 0.0190$; interaction: $F_{(2,24)} = 0.01101$, $p = 0.9891$), were assessed for CGRP levels in brain regions relevant to the processing of affective stimuli and multisensory aversive threats, complemented with the evaluation of affective behaviour and corticosterone levels, respectively at BD- and WD-time-points (Figure 2a).

Alcohol binge-like drinking rats showed significant alterations in CGRP levels in the cortico-limbic network. In detail, significant differences were observed in the mPFC ($F_{(2,6)} = 62.56$, $p < 0.001$); here, at BD and WD, rats displayed decreased CGRP levels in comparison with CTRL rats ($t = 10.93$, $p < 0.001$; $t = 7.52$, $p = 0.0009$); in addition, at WD, rats displayed a significant increase when compared to BD ($t = 3.412$, $p = 0.0429$) (Figure 2b).

Furthermore, different CGRP levels were observed in the NAc ($F_{(2,9)} = 7.885$, $p = 0.0105$), where a significant decrease was highlighted in rats at BD ($t = 3.540$, $p = 0.0189$) and WD ($t = 3.328$, $p = 0.0265$) when compared to CTRLs; no difference was indicated

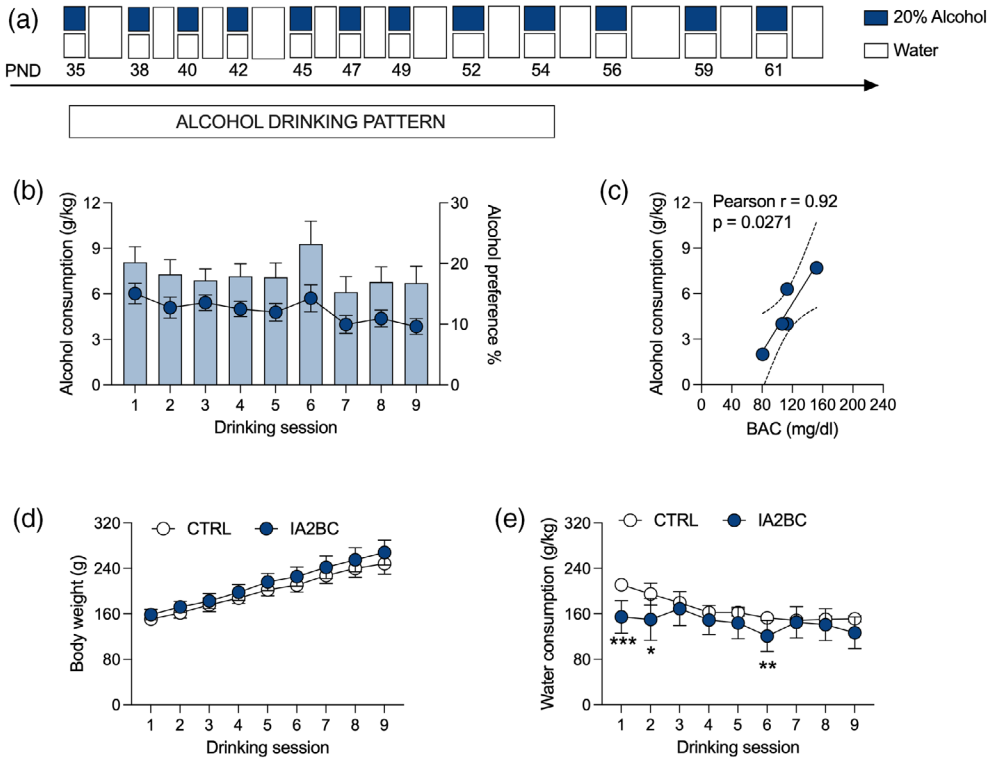

FIGURE 1 Alcohol binge drinking in adolescence was modelled in rats employing the IA2BC paradigm. (a) Schematic timeline of the experiment. (b) Alcohol consumption (bars) and preference (dots) measured during the first 3 weeks of the intermittent alcohol 2-bottle choice paradigm. Bars and dots refer to the mean ± SEM of 14 rats. (c) Blood alcohol levels correlated with alcohol intake during the binge-like drinking session. Dots refer to individual values. (d) Body weight and (e) water consumption of rats exposed to the IA2BC paradigm were compared to CTRL counterparts. Dots represent the mean ± SEM of 7–14 rats. IA2BC, rats exposed to the intermittent alcohol 2-bottle choice paradigm; CTRL, control rats; PND, postnatal day. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

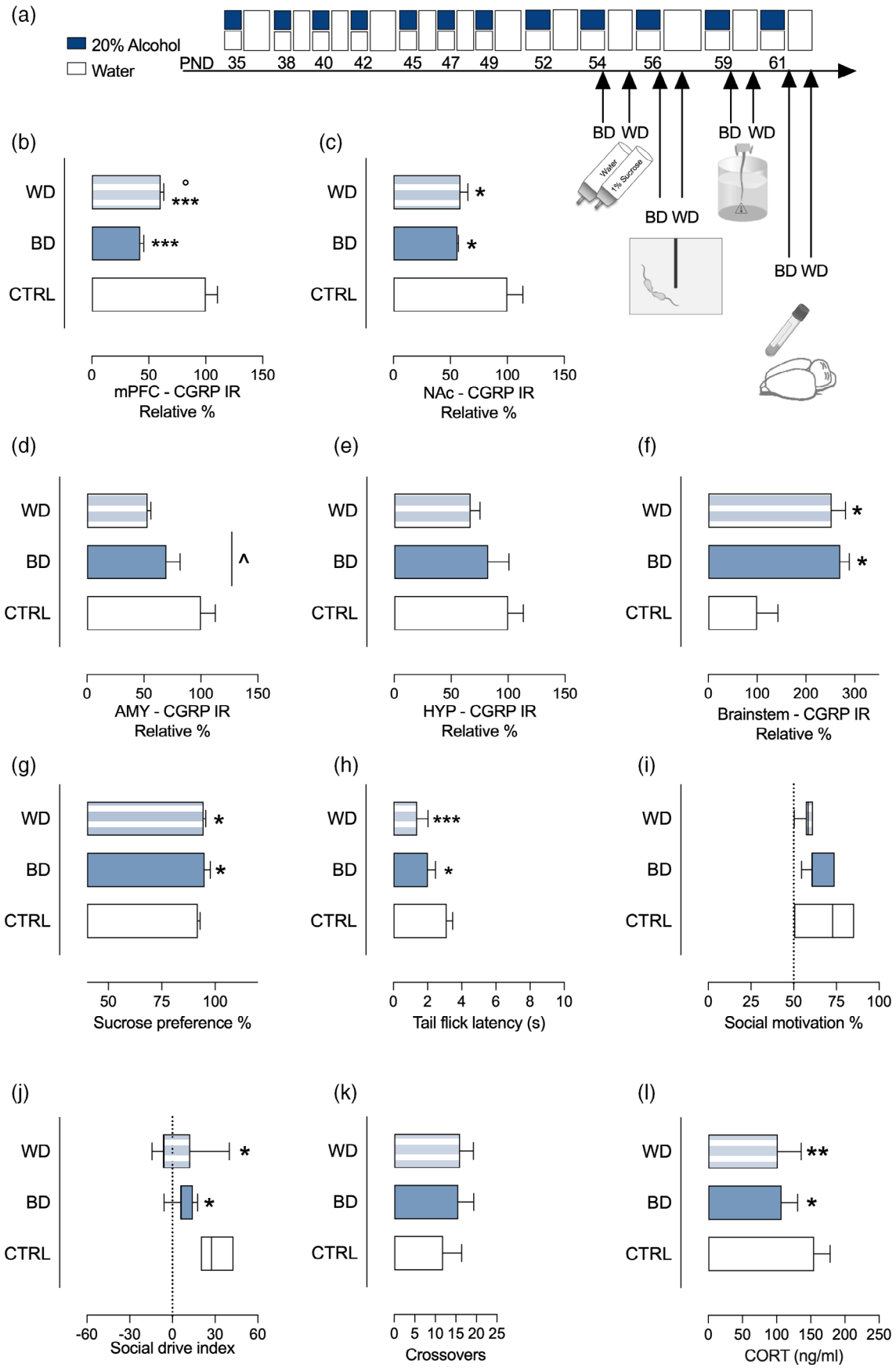

FIGURE 2 Legend on next page.

between the alcohol-exposed groups ($t = 0.2124$, $p > 0.999$) (Figure 2c).

Moreover, significantly different CGRP levels were measured in the AMY ($F_{(2,7)} = 4.921$, $p = 0.0463$), where a lower expression was observed in intermittent alcohol-exposed rats (Figure 2d).

No significant difference in CGRP levels was observed in the HYP ($F_{(2,7)} = 1.467$, $p = 0.2937$) (Figure 2e). Besides, the analysis of CGRP levels in the brainstem revealed different expressions among the groups ($F_{(2,11)} = 7.250$, $p = 0.0098$). In detail, BD and WD rats displayed increased levels of CGRP in comparison with CTRL rats ($t = 3.347$, $p = 0.0196$; $t = 3.004$, $p = 0.0360$). No difference was indicated between the alcohol-exposed groups ($t = 0.3130$, $p > 0.999$) (Figure 2f).

When the response to a natural reward in the sucrose preference test was analysed, CTRL rats and alcohol-drinking rats at BD and WD preferred the sucrose solution to water. Significant differences in sucrose preference were, however, observed ($F_{(2,18)} = 6.601$, $p = 0.0071$), with a significant increase in rats tested at BD ($t = 3.376$, $p = 0.0101$) and WD ($t = 2.851$, $p = 0.0318$) when compared to CTRL. No difference was observed between BD- and WD time points ($p > 0.999$) (Figure 2g).

As far as the response to the aversive threat is concerned, the analysis of tail-flick latency showed significant differences among the groups ($p < 0.001$). Dunn's post hoc test revealed a significant decrease in tail-flick latency in rats at BD ($p = 0.0253$) and WD ($p = 0.0006$) compared with the CTRL counterpart. No difference was indicated between BD- and WD time points ($p = 0.840$) (Figure 2h).

The analysis of data from the modified social interaction test showed no significant difference among the groups in social motivation, as to the preference for the social compartment ($F_{(2,18)} = 2.247$, $p = 0.1345$) (Figure 2i). In contrast, significant differences were highlighted in data from the social drive index ($F_{(2,18)} = 6.34$, $p = 0.0082$), with a significant decrease in rats at BD ($t = 2.893$, $p = 0.0291$) and WD ($t = 3.245$, $p = 0.0135$) when compared with CTRL counterpart; no difference in social drive index was observed between BD and WD time points ($p > 0.999$) (Figure 2j). The groups did not differ for the number of crossovers ($F_{(2,18)} = 2.382$, $p = 0.1209$) (Figure 2k).

When the effects of alcohol binge-like drinking were explored on neuroendocrine stress axis activity, significant differences in CORT levels were indicated ($F_{(2,18)} = 7.878$, $p = 0.0035$). In detail, Bonferroni post hoc test showed decreased levels in rats at BD ($t = 3.237$, $p = 0.0137$) and WD ($t = 3.608$, $p = 0.0060$) compared with CTRLs, and no difference between BD and WD time points ($t = 0.3703$, $p > 0.999$) (Figure 2l).

3.2 | Experiment 2

3.2.1 | Repeated CBD administration reduced alcohol intake in adolescent rats exposed to the binge-like drinking paradigm

CBD was administered to adolescent rats before each drinking session of the IA2BC paradigm (Figure 3a). The analysis of data from alcohol consumption showed a significant effect of CBD administration in decreasing alcohol intake over the paradigm ($F_{(1,12)} = 5.079$, $p = 0.0437$), a significant effect of the drinking session ($F_{(8,96)} = 2.236$, $p = 0.0311$), and no interaction between the two factors ($F_{(8,96)} = 0.952$, $p = 0.4781$) (Figure 3b). In detail, the mean alcohol consumption displayed by CB-administered rats was 4.5 ± 0.6 g/kg/24 h during the first week, 3.4 ± 0.2 g/kg/24 h during the second week and 3.1 ± 0.3 g/kg/24 h during the third week. When preference data were considered, the analysis indicated a significant main effect of CBD in decreasing rat preference for 20% alcohol (CBD: $F_{(1,12)} = 4.839$, $p = 0.0482$); moreover, a significant effect of the drinking session was highlighted ($F_{(8,96)} = 2.552$, $p = 0.00145$), with no significant interaction between the statistical factors ($F_{(8,96)} = 1.633$, $p = 0.1254$) (Figure 3c). The repeated CBD administration did not affect adolescent rat development in terms of body weight (CBD: $F_{(1,12)} = 0.6396$, $p = 0.4394$; drinking session: $F_{(8,96)} = 538.3$, $p < 0.001$; interaction: $F_{(8,96)} = 0.7175$, $p = 0.6756$) (Figure 3d). When water consumption was analysed, the results showed no significant effect of CBD ($F_{(1,12)} = 0.2323$, $p = 0.6385$) and a significant effect of the drinking session ($F_{(8,96)} = 26.44$, $p < 0.001$) and the interaction between CBD and drinking session ($F_{(8,96)} = 2.701$, $p = 0.0100$). Bonferroni post hoc test did not reveal any significant difference among CBD- and vehicle-administered groups within the same drinking session (Figure 3e).

FIGURE 2 Adolescent alcohol binge-like drinking altered CGRP levels, affective behaviour and corticosterone in rats. Rats exposed to the IA2BC paradigm during adolescence were tested (a) at binge day (BD) and withdrawal day (WD). They showed: significant differences in CGRP levels in (b) mPFC; (c) NAc; (d) AMY; (f) brainstem. No significant difference was observed in the (e) HYP. In addition, rats displayed: (g) an increased hedonic response in the sucrose preference test at BD and WD, with respect to CTL rats; (h) an increased response to the aversive threat, with decreased latency in the tail-flick test at BD and WD, in comparison with CTRL rats; (i) no difference in social motivation, as to the preference for the social compartment, (j) decreased social drive index at BD and WD and (k) no difference in locomotor activity, as to the total number of crossovers, with respect to CTRL rats, in the modified social interaction test. Moreover, (l) at BD and WD, rats displayed decreased corticosterone levels in comparison with the CTRL counterpart. BD, rats tested on binge day; WD, rats tested on withdrawal day; CTRL, control rats; CORT, corticosterone; mPFC, medial prefrontal cortex; NAc, nucleus accumbens; AMY, amygdala; HYP, hypothalamus. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$ versus CTRL; ^main effect of treatment, $p < 0.0.05$; $^{\circ}p < 0.05$ versus BD. The bars and box plots refer to the mean ± SEM of 3–7 rats.

3.2.2 | Repeated CBD administration prevented the alterations of region-specific CGRP expression, affective behaviour and corticosterone levels in binge-like drinking adolescent rats

The effects of CBD administration in adolescent rats before the drinking sessions of the IA2BC paradigm were explored on CGRP levels in discrete brain regions and complemented with the evaluation of affective behaviour and corticosterone levels (Figure 4a). Data analysis revealed brain region-specific differences in CGRP immunoreactivity. In detail, CBD-administered rats displayed increased CGRP immunopositivity in the mPFC ($t = 4.882$, $p = 0.002761$), NAc ($t = 2.962$, $p = 0.02105$) and HYP ($t = 2.367$, $p = 0.04979$) with respect to the vehicle-administered group. No significant differences were highlighted in the AMY ($t = 0.5824$, $p = 0.5786$) and brainstem ($t = 0.04$, $p = 0.9969$) (Figure 4b).

Besides, rats were tested for hedonic response, sensitivity to aversive threat, social motivation and drive. Data analysis indicated that CBD administration in IA2BC-exposed rats decreased sucrose preference ($t = 3.890$, $p = 0.0022$) (Figure 4c) and increased nociceptive latency in the tail-flick test ($t = 2.529$, $p = 0.0265$) (Figure 4d) with respect to the vehicle-administered rats. In addition, the analysis of data from the modified social interaction test indicated that CBD administration did not change social motivation ($t = 1.141$, $p = 0.2759$) (Figure 4e); exerted a trending increase on the social drive index ($t = 1.802$, $p = 0.0968$) (Figure 4f); and did not affect the number of crossovers ($t = 0.4035$, $p = 0.6937$) (Figure 4g) in alcohol binge-like drinking rats, with respect to vehicle administration.

When CORT levels were considered, CBD-administered IA2BC-exposed rats displayed significantly higher levels than their vehicle-administered counterpart ($t = 9.226$, $p < 0.001$) (Figure 4h).

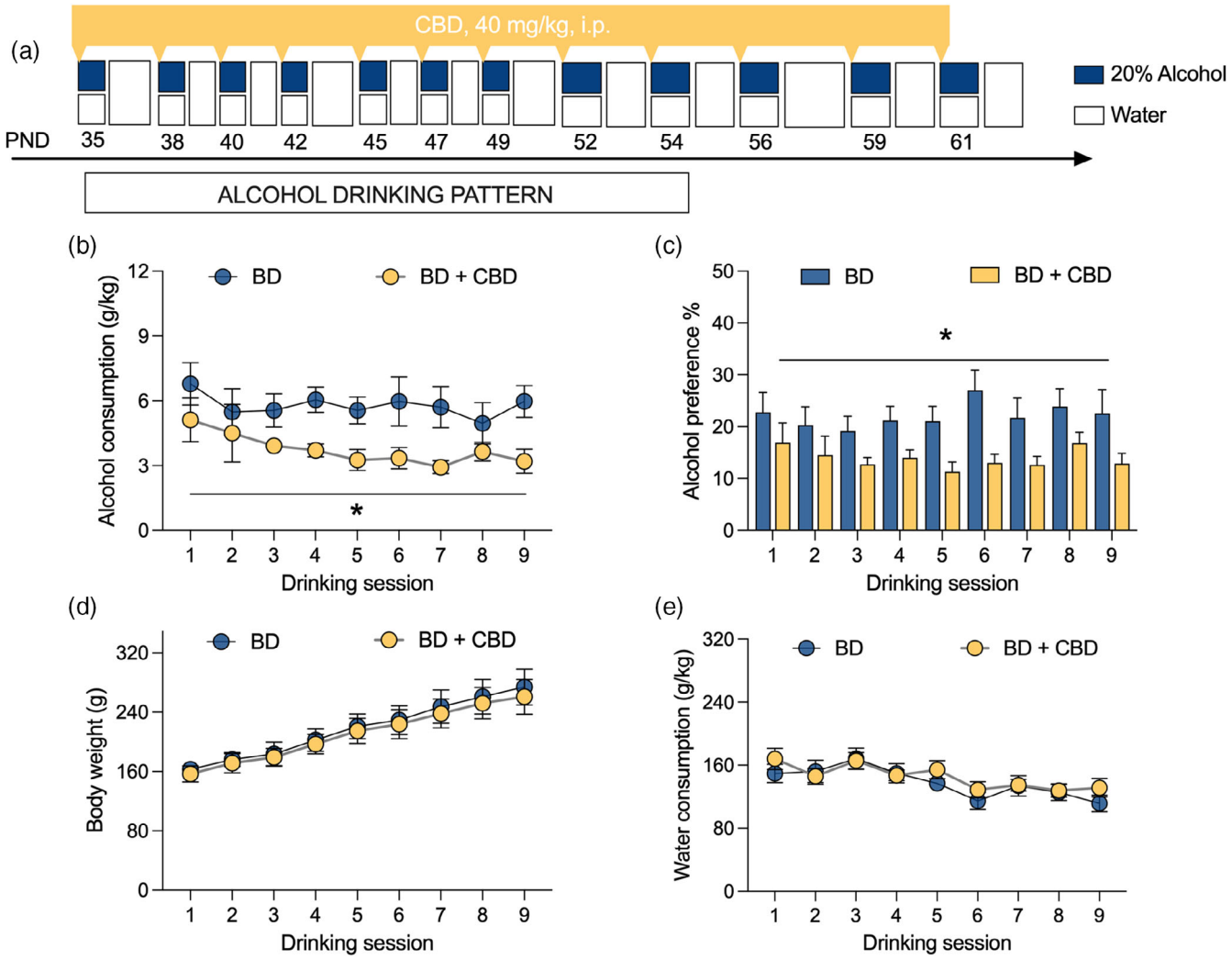

FIGURE 3 CBD decreased alcohol binge-like drinking in adolescent rats. (a) When administered before each binge day (BD) of the IA2BC paradigm, CBD (b) decreased alcohol consumption and (c) alcohol preference in adolescent rats. Repeated CBD administration (d) did not affect body weight and (e) water consumption. BD, rats exposed to the intermittent alcohol 2-bottle choice paradigm; CBD, cannabidiol. *main effect of CBD, $p < 0.05$. Bars and dots refer to the mean ± SEM of seven rats.

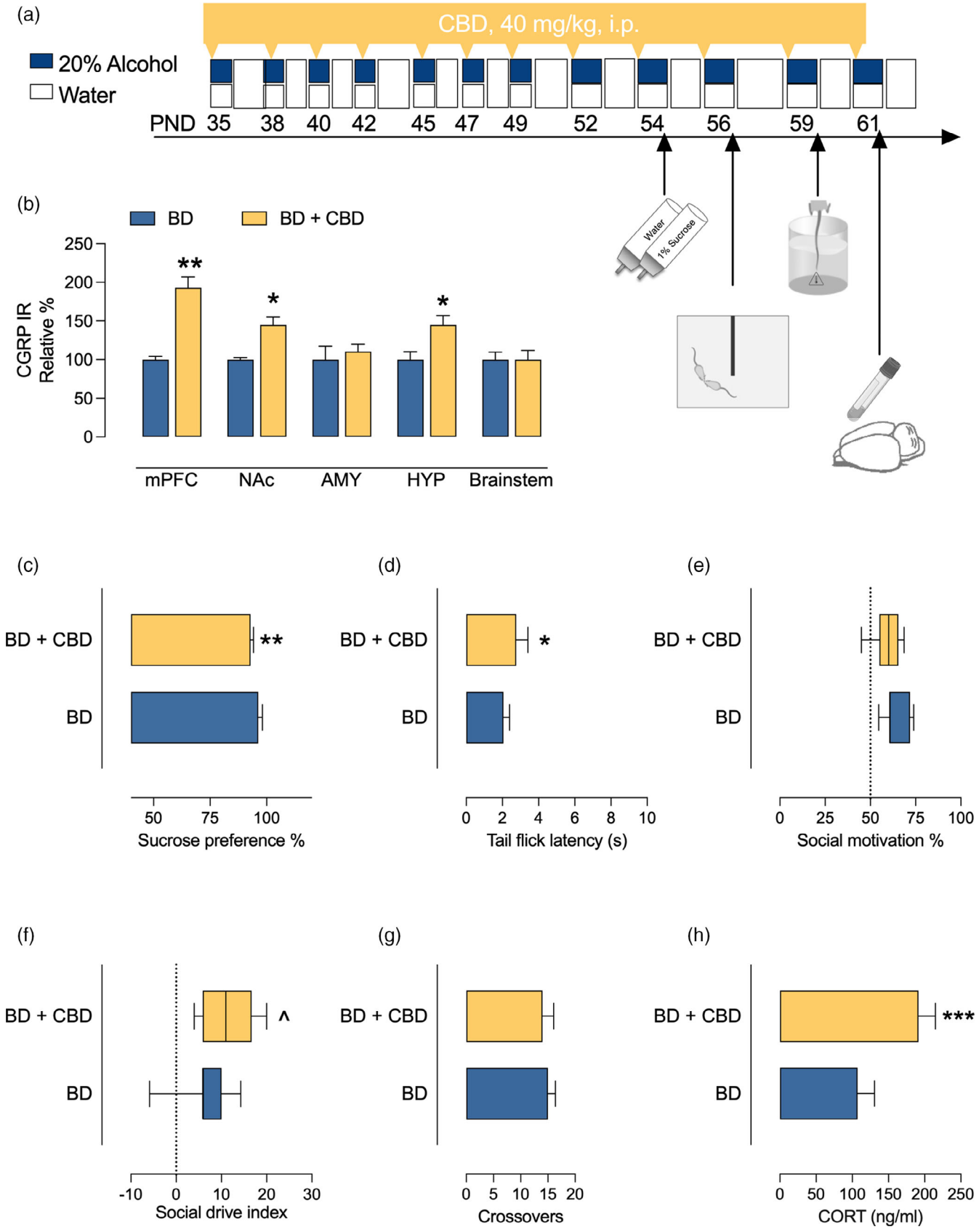

FIGURE 4 Legend on next page.

4 | DISCUSSION

This research shows that, when exposed to a binge-like drinking paradigm, adolescent rats displayed excessive alcohol drinking and region-specific alterations in brain CGRP expression. These findings were associated with aberrant responses to rewarding and aversive stimuli and decreased glucocorticoid levels. Notably, repeated CBD administration reduced alcohol consumption in adolescent rats and rebalanced alcohol-related bio-behavioural outcomes.

The analysis of the drinking pattern indicates that adolescent rats exhibited high levels of alcohol intake in the IA2BC paradigm since the first week of exposure. Based on the mean alcohol consumption, adolescent rats could be classified as high drinkers (Ehinger et al., 2021): the average alcohol intake was higher than 3 g/kg/24 h, and corresponded to the binge-like blood alcohol levels measured at the end of the alcohol-drinking sessions (>80 mg/dL, NIAAA, 2004). Notably, none of the rats displayed low levels of alcohol intake in the IA2BC paradigm, in accordance with previous reports indicating high drinking as the prevalent drinking pattern when adolescent rats undergo voluntary drinking of 20% alcohol in an every-other-day availability pattern (Pajser et al., 2019). In addition, the adolescent rats seem to drink higher levels of alcohol than the adults exposed to the same paradigm (Schramm-Sapyta et al., 2014), confirming that adolescents are more sensitive to alcohol's reinforcing properties and less sensitive to alcohol's conditioned aversive effects than adults (Spear, 2011). Indeed, previous research on the effects of intermittent alcohol drinking in adolescence found a correlation between prior great alcohol consumption and increased hedonic valence of alcohol, even at concentrations that are basally perceived as aversive, thus paving the way for a greater vulnerability to alcohol abuse (Wukitsch & Cain, 2021). Notably, rats' alcohol intake did not show an increase over the weeks of the paradigm, in contrast with the escalation in alcohol drinking displayed by adult rats in the same experimental conditions (Brancato et al., 2018; Carnicella et al., 2014; Stuber et al., 2008). This data suggests that young binge-like drinking rats do not develop a tolerance to alcohol's rewarding effects, opposite to adults, due to the peculiar characteristics of the adolescent brain. Indeed, it has been previously indicated that developmental differences in specific neural circuits, namely those mediating responses to aversive experiences, contribute to the initially high drinking levels observed in all adolescents (Schramm-Sapyta et al., 2014).

In the brain, the classical neurotransmitters act in concert with neuropeptides to modulate synaptic plasticity and recruit the neuronal networks required for integrated and complex functions (Eiden et al., 2022). In this diverse landscape, CGRP signalling provides the integration of multisensory aversive afferents and affective responses, which lead to the timely initiation of behavioural outputs to threats (Kang et al., 2022). Limited evidence exists on the relationship between alcohol drinking, acute withdrawal and central CGRP levels (Rossetti et al., 2019). For the first time, here we report region-specific brain modifications of CGRP expression in adolescent binge-like drinking rats. In particular, at BD and WD, rats showed decreased CGRP levels in the corticolimbic network, that is, mPFC, NAc and AMY, where CGRP-releasing terminals from the hindbrain nuclei are directed and involved in the integration of the multisensory threat perception and response (Bowen et al., 2023; Campos et al., 2018; Kang et al., 2022). Noteworthy, limbic CGRP signalling is associated with negative affect (Brancato et al., 2023) and a prevalent D2 receptor-mediated dopamine transmission in the NAc (Kovács & Telegdy, 1995). Indeed, it is generally assumed that NAc D2 receptor-positive medium spiny neurones are avoidance-promoting, whereas NAc D1 receptor-expressing ones are approach-promoting and associated with an increased hedonic response (Hikida et al., 2013; Tang et al., 2022). Intriguingly, here we found that the decreased expression of adolescent rats' corticolimbic CGRP at BD and WD was paralleled by an increased response to the rewarding stimuli, with respect to controls. Indeed, binge-like drinking rats displayed an increased hedonic response in the sucrose preference test, in line with previous evidence from other research groups (Wukitsch & Cain, 2021). Notably, the increased responsivity to the hedonic stimulus seems to be a feature of the alcohol binge-like drinking pattern, as it was observed both during alcohol intoxication and acute withdrawal. Actually, we have previously observed in adult rats exposed in adolescence to a non-contingent paradigm of alcohol binge-like intake that an increased sucrose preference was associated with a distinguished pattern of dopamine signalling in the NAc: in detail, D1 receptors were overexpressed, and D2 receptor mRNA displayed a significant reduction, at BD and WD in comparison with controls (Brancato et al., 2021). The low CGRP expression observed in the NAc might contribute to maintaining a high response to reinforcing stimuli, such as alcohol, in alcohol binge-like drinking adolescent rats, occluding the development of tolerance to alcohol-induced rewarding effects.

As a matter of fact, CGRP neurones are required for multisensory threat perception and innate defensive behaviour (Kang et al., 2022). Thus, the decreased CGRP levels in the corticolimbic regions observed at BD and WD suggest a reduced activation of the CGRP system in response to the anxiogenic environmental cues, likely as a result of alcohol binge-like drinking. Accordingly, at BD and WD,

FIGURE 4 CBD prevented the alterations of region-specific CGRP levels, affective behaviour, and neuroendocrine activity in rats exposed to the IA2BC. (a) When administered before the drinking sessions of the IA2BC paradigm, (b) CBD increased CGRP levels in mPFC, NAc and HYP with respect to vehicle-administered rats. In addition, CBD administration normalised the response to (c) rewarding and (d) aversive stimuli in rats exposed to the IA2BC paradigm and tested at BD. In the modified social interaction test, CBD administration in IA2BC rats (e) did not affect social motivation; (f) induced a trending increase in the social drive index; (g) did not alter locomotor activity. In addition, (h) CBD-administered rats displayed increased serum corticosterone levels. IA2BC: intermittent alcohol two-bottle choice paradigm; CBD, cannabidiol; BD, binge day; mPFC, medial prefrontal cortex; NAc, nucleus accumbens; AMY, amygdala; HYP, hypothalamus. $*p < 0.05$; $**p < 0.01$; $***p < 0.001$; $\hat{}p < 0.1$ versus vehicle. The bars and box plots refer to the mean ± SEM of 3–7 rats.

rats did not show social aversion during the encounter with a peer in a novel environment, as they displayed the same social motivation as control rats. Indeed, although social interaction has been considered a reinforcing stimulus in young animals, the rat makes predictions about the valence of the eventual interaction with the new companion and adapts its behaviour to either initiate or avoid social contact in a novel environment (Solié et al., 2022). In the present study, we did not observe significant alterations in the rat attribution of a positive valence to the social stimulus at BD and WD. Thus, the typical positive valence of the social stimulus in adolescence is not weakened during the binge-like drinking paradigm and prevails over the anxiogenic novel environment. Accordingly, evidence in humans shows that alcohol exerts a socially appealing effect, even in socially anxious adolescents, in accordance with the tension-reducing hypothesis (Cappell & Herman, 1972; Carrigan & Randall, 2003; Castelli et al., 2022).

Notably, a deeper analysis showed a decreased social drive index at BD and WD in terms of the differential number of approaches to the social compartment over the total number of crosses in light of the same time spent with the social partner. This data would suggest a weakening in the drive to go, but a similar salience attribution to the social interaction.

Many aspects of social behaviour, including specific forms of social contact, can be affected by the hypothalamus–pituitary–adrenal (HPA) axis activity, especially during adolescence (Barik et al., 2013; Romeo et al., 2006). Interestingly, a blunted HPA axis activity in adolescent rats is associated with a reduced energetic drive in response to environmental demands (Myers et al., 2014), whereas a recent study reports that a significant decrease in glucocorticoid signalling within the mesocorticolimbic system can prevent social aversion (Barik et al., 2013). Notably, at BD and WD, rats did display decreased levels of CORT; a low glucocorticoid signalling is indeed suggestive of a decreased energetic drive to interact, consistent with decreased social motivation. Interestingly, CGRP has emerged as an upstream modulator of the neuroendocrine stress axis. CGRP administration in freely behaving rats stimulates Corticotropin-releasing hormone (CRH)-mediated CORT release: CGRP increases the CRH mRNA expression both in the paraventricular HYP nucleus and in central AMY (Bowe et al., 2008), where direct synaptic contacts between CGRP terminals and CHR-positive neurons occur (Harrigan et al., 1994). Thus, the decreased corticolimbic- CGRP levels observed at BD and WD are consistent with the reduced serum corticosterone levels displayed at the same time points, suggesting that alcohol binge-like drinking affected the integrated control of physiological functioning and energetic activation to meet changing environmental (including social) demands in adolescent rats.

Besides, at BD and WD, rats displayed an increased sensitivity to the aversive thermal stimulus, as compared to controls, in accordance with previous studies highlighting thermal hyperalgesia during the early stages of withdrawal from continuous alcohol drinking (Gatch & Lal, 1999; Li et al., 2019) and chronic intermittent-alcohol consumption (Gregor et al., 2019). Interestingly, we observed a significant CGRP increase in the brainstem of BD and WD rats. In this brain region, CGRP mediates the transmission of afferent nociceptive signals (Capuano et al., 2011; Greco et al., 2016; Tringali & Navarra, 2019). Thus, as alcohol has been previously shown to evoke the release of CGRP in the trigeminal ganglia directly (Nicoletti et al., 2008), our data suggest that repeated cycles of binge and short-term withdrawal during adolescence may have sensitised the CGRP brainstem neurotransmission, which conveys increased nociceptive stimuli to the central nervous system (Dina et al., 2006; Gregor et al., 2019).

Our previous data, however, show that the decrease in corticolimbic CGRP levels observed at BD and acute WD is followed by a marked increase in the same brain network after 10-day withdrawal from adolescent alcohol binge-like exposure, in line with evidence of no thermal hyperalgesia starting from 36 h of withdrawal (Gatch & Lal, 1999).

Overall, CGRP can represent both a mediator and a target of alcohol drinking, and this study hopefully provides a further piece in the intricate puzzle of alcohol-induced behavioural and neuroendocrine sequelae. Obviously, besides CGRP and CORT, numerous other systems participate in the activity exerted by alcohol in the brain and could be targeted by several compounds in order to counterbalance alcohol-induced abnormalities. Thus, we explored the opportunity to exploit both the peculiar plasticity of the adolescent brain and the wide-spectrum activity of CBD, and relevant findings emerged. When administered at 40 mg/kg before each drinking session of the paradigm, CBD was able to curb excessive alcohol drinking in the IA2BC model, decreasing alcohol intake and preference. These data confirm and extend previous evidence indicating the potential of CBD in attenuating discrete alcohol-related behaviours in adult rodents (Nona et al., 2019). Indeed, CBD reduced alcohol reinforcement and consummatory behaviour in the two-bottle choice paradigm, alcohol motivational properties and reinstatement in operant tasks, both in outbred rodents and models of genetically determined excessive alcohol drinking (Gonzalez-Cuevas et al., 2018; Maccioni et al., 2022; Viudez-Martínez et al., 2018). Notably, CBD administration was associated with a lower occurrence of high drinking in adolescent rats that decreased their consummatory behaviour for alcohol up to intermediate amounts (Ehinger et al., 2021), thus showing a promising effect in preventing vulnerability to excessive alcohol drinking. CBD did not affect water intake and body weight, indicating that the curbing effect of CBD on alcohol self-administration was specific and, at the dose and regimen employed in this study, not associated with adverse outcomes. As a matter of fact, CBD has shown a favourable safety and tolerability profile in preclinical and clinical studies (Chesney et al., 2020; Viudez-Martínez et al., 2019). In particular, based on a recent metanalysis, which collected preclinical and clinical data to provide a between-species translatability of CBD effects, the dose we administered in rats would result in similar plasma levels as in humans after a chronic CBD administration at 3000 mg CBD per day (Kwee et al., 2022). This dose is considered safe and well-tolerated in humans due to mild adverse effects (Chesney et al., 2020; Laux et al., 2019; Taylor et al., 2018). Many mechanisms have been involved to mechanistically explain the CBD-related curbing effect on alcohol

drinking. These include agonism at the serotonin 5-HT1A receptors, negative allosteric modulation of type 1 cannabinoid receptors and blockade of the hydrolysis of the endocannabinoid anandamide, which are relevant pharmacological mechanisms for decreasing alcohol binge-like drinking in rodents (Agoglia et al., 2016; Belmer et al., 2022; Zhou et al., 2017). Recently it has been suggested that the activation of the TRPV1 may also contribute to the CBD mechanism of action in decreasing the rewarding effects of drugs of abuse (Galaj & Xi, 2020). At this stage, it would be hasty to propose a mechanistic interpretation; rather, the multitarget activity of CBD could exert a synergistic effect and produce an attenuation of alcohol binge-like drinking in adolescent rats.

Interestingly, CBD repeated administration during the IA2BC paradigm was associated with an augmentation of CGRP levels in mPFC and NAc of alcohol binge-like drinking rats, up to the values measured in CTRL rats. This data was paralleled by rectification of the heightened response to sucrose, suggesting a re-programming of the reward set point in accordance with the observed CBD-induced decrease in alcohol binge-like consumption in adolescent rats. Indeed, CBD can modulate reward processing in the mesolimbic system, interfering with dopamine synthesis and postsynaptic signalling (Brancato et al., 2021; Viudez-Martínez et al., 2020; Metz et al., 2021). Notably, CGRP can modulate reward-aversion-related behaviours (Kovács & Telegdy, 1995). Therefore, the increased CGRP levels in the NAc of CBD-administered rats could contribute to the modification of the reward processing observed here. In addition, CBD administration was associated with a significant increase in CGRP levels in the HYP. The increased corticolimbic CGRP is consistent with increased CORT levels, suggesting an augmented activity of the corticolimbic-neuroendocrine axis. It is reported that moderate arousal is able to ameliorate physiological and behavioural performances (Maniaci et al., 2015; Myers et al., 2014); in accordance with that, CBD counterbalanced the deficit in the social drive index, with no non-specific effect on locomotor activity.

CBD has already been associated with a concentration-dependent release of CGRP from cultured rat dorsal root ganglion neurons, depending on extracellular calcium and the activation of TRPV channels (Qin et al., 2008). This, however, is the first study to show a CBD-related modulation of CGRP levels in multiple brain regions in adolescent binge-like alcohol-drinking rats.

In our experimental conditions, CBD did not modify the higher CGRP immunoreactivity observed in the brainstem of alcohol binge-like drinking rats, although a normalisation in response to the aversive stimulus was observed. This is consistent with the anti-hyperalgesic action of repeated administration of CBD at a wide dose range (10–100 mg/kg), which could be ascribed to a progressively desensitising effect on nociceptive targets, such as the TRPV receptors (Etemad et al., 2022).

Overall, CBD prevented binge-like alcohol-associated deficits in central CGRP transmission and prompted CGRP activity in the control booth of glucocorticoid output, thus promoting functional signalling in the integrated affective-neuroendocrine response.

In conclusion, our data shows for the first time that voluntary alcohol binge-like drinking in adolescence produces - region-specific alterations in CGRP levels associated with abnormal affective behavioural responses. The present evidence advances our previous data and shows that corticolimbic CGRP levels respond dynamically to adolescent binge-like alcohol exposure. This response is consistent with an altered glucocorticoid output, and further investigation is needed to characterise the relationship between CGRP deficit, occurring at the intoxication and acute withdrawal phase of the binge-like drinking pattern, and alcohol-induced dysregulation of the stress response. Thus, CGRP neurotransmission emerges as a player of the (meta)plastic changes induced by alcohol binge drinking in the adolescent brain and a potential pharmacological target for contrasting the detrimental consequences of-binge drinking in the adolescents. Besides, for the -first time, this study provides evidence of an interplay between adolescent alcohol binge-like drinking, central CGRP signalling and CBD pharmacological modulation. Indeed, repeated CBD administration during the unique window of plasticity of adolescence exerted a protective effect toward binge drinking vulnerability, decreasing excessive alcohol consumption and rescuing the behavioural and neuroendocrine correlates. Overall, if our findings support CBD's potential for limiting binge alcohol-related vulnerability in adolescence, a broader investigation is needed to characterise its complex and still puzzling activity on the developing brain.

AUTHOR CONTRIBUTIONS

Giuseppe Tringali: Conceptualization; data curation; formal analysis; investigation; supervision; writing – review and editing. **Gianluca Lavanco:** Formal analysis; investigation; writing – review and editing. **Valentina Castelli:** Investigation; visualization; writing – review and editing. **Giuseppe Pizzolanti:** Methodology; resources; writing – review and editing. **Martin Kuchar:** Methodology; resources; writing – review and editing. **Diego Currò:** Methodology; resources. **Carla Cannizzaro:** Conceptualization; funding acquisition; project administration; resources; supervision; validation; writing – review and editing. **Anna Brancato:** Conceptualization; data curation; formal analysis; investigation; supervision; writing – original draft; writing – review and editing.

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CONFLICT OF INTEREST STATEMENT

All authors declare that they have no conflict of interest.

DATA AVAILABILITY STATEMENT

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

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