

## Mixing energy drinks and alcohol during adolescence impairs brain function: A study of rat hippocampal plasticity

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### ABSTRACT

In the last decades, the consumption of energy drinks has risen dramatically, especially among young people, adolescents and athletes, driven by the constant search for ergogenic effects, such as the increase in physical and cognitive performance. In parallel, mixed consumption of energy drinks and ethanol, under a binge drinking modality, under a binge drinking modality, has similarly grown among adolescents. However, little is known whether the combined consumption of these drinks, during adolescence, may have long-term effects on central function, raising the question of the risks of this habit on brain maturation. Our study was designed to evaluate, by behavioral, electrophysiological and molecular approaches, the long-term effects on hippocampal plasticity of ethanol (EtOH), energy drinks (EDs), or alcohol mixed with energy drinks (AMED) in a rat model of binge-like drinking adolescent administration. The results show that AMED binge-like administration produces adaptive hippocampal changes at the molecular level, associated with electrophysiological and behavioral alterations, which develop during the adolescence and are still detectable in adult animals. Overall, the study indicates that binge-like drinking AMED adolescent exposure represents a habit that may affect permanently hippocampal plasticity.

### 1. Introduction

Epidemiological data indicate an escalation in alcohol (EtOH) use among adolescents, ranging from low to heavy use to pathological abuse (Gutierrez and Sher, 2015; Lees et al., 2020). Additionally, young consumers concentrate their consumption of alcoholic beverages in a very restricted time frame with a phenomenon known as binge drinking (Chung et al., 2018). Thus, binge drinking in adolescent EtOH users represents a prominent health risk factor (Hill et al., 2000; Mokdad et al., 2016), which needs effective prevention strategies (Hawkins et al., 1992; Toumbourou et al., 2019).

Moreover, EtOH consumption is often associated with the intake of energy drinks (EDs) that are similar to soft drinks but characterized by

high concentrations of stimulants such as caffeine with additional ingredients (taurine and vitamins). EDs have become very popular due to the advertised effects that have a particular appeal to young users. In fact, besides the pleasant taste, the manufacturers claim these drinks can have several “positive” actions, including increased physical energy, concentration, athletic performance, metabolism, mental activity, and alertness (Vercammen et al., 2019; Verster et al., 2012, 2015). Young adults, but particularly many adolescent students, drink significant amounts of EDs, with the expectation that it will improve their concentration and cognitive performance (Smit and Rogers, 2000; Specterman et al., 2005), help them stay awake and reduce physical tiredness, reportedly driven to drink EDs with the expectation to counteract the effects of daily stress related to study commitments (Mahoney

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et al., 2019). From data analyses reported in several studies, it is not clear whether the consumption of EDs associated with EtOH is beneficial or detrimental to brain function (Alsunni, 2015; Arria et al., 2011; Brunborg et al., 2022; Cadoni and Peana, 2023; De Giorgi et al., 2022; Petribu et al., 2023). Indeed, important risk factors for EtOH consumption during adolescence could be represented by its association with an energy drink (ED), i.e. Alcohol Mixed with ED (AMED) (Acquas et al., 2023; Sefen et al., 2022). It is plausible to think that some of the substances contained in EDs, such as caffeine and taurine, that may influence the brain reward system on their own (Vargiu et al., 2021), may also contribute to altered sensitivity to EtOH by influencing the brain reward system and increasing the risk of harmful alcohol consumption (Dazzi et al., 2024; Hsu et al., 2009; Lubman et al., 2007; Yasuma et al., 2021). Several experimental evidence suggests that the combination of EtOH and caffeine during adolescence may increase the potential for EtOH abuse (Arria et al., 2011; O'Brien et al., 2008; Peacock et al., 2012; Thombs et al., 2011), but very little is known about the impact of AMED consumption.

Adolescence is a period of development characterized by cognitive, emotional, neurobiological maturation (Dahl, 2004), and the neurotrophin brain-derived neurotrophic factor (BDNF) plays crucial roles in these processes (Cohen-Cory et al., 2010). Adolescence is also characterized by highly risky decision-making and increased vulnerability to alcohol abuse (Crews et al., 2007). The impact of binge drinking among adolescents has been extensively studied in rodent models. Several studies demonstrated that such early exposures to EtOH cause impaired cognitive function and flexibility, increased social anxiety, behavioral disinhibition, and impulsivity (Beaudet et al., 2016; Coleman et al., 2011, 2014), which persist into adulthood. Moreover, repeated exposure to EtOH during adolescence also increases motivation for its consumption (Spear, 2018) and it seems that changes in BDNF are evident after the adolescent regularly consumes alcohol (Cutuli and Sampedro-Piquero, 2022). As the adolescent brain is undergoing significant neurological maturation, EtOH consumption during this critical period has the potential to interfere with normal development and produce persistent neurological changes and functional deficits (Spear, 2000, 2018). While clinical evidence are available on the behavioral and functional consequences of AMED (Roemer and Stockwell, 2017), and despite the compelling need to better understand costs on health and to design preventive strategies to reduce the negative outcomes of this teenage habit, most preclinical studies focused on the use of high doses of EtOH and caffeine (Fritz et al., 2014) as a model to characterize the impact of adolescent AMED binge drinking in adulthood, disregarding the combined consumption of a whole ED (with all its pharmacologically active ingredients) with an EtOH-based drink, especially in adolescence.

To provide a still missing translational background for a better understanding of the consequences of binge drinking alcoholic EDs (i.e., AMED), with particular attention to the transition from adolescence to adulthood, in this study we used a multidisciplinary approach to investigate if the early adolescent binge-like drinking administration of EtOH, ED or AMED does impact on the brain function of young and adult rats. In this regards, our previous study reported that the combined consumption of ED, EtOH or AMED under an adolescent binge-like drinking administration has long-lasting detrimental effects on the prefrontal cortex (Dazzi et al., 2024).

To mimic a close to the real situation in human adolescents that consume moderate amounts of EtOH (Eckardt et al., 1998), we choose to administer, as done in our previous study (Dazzi et al., 2024), each animal, on each treatment session, with an amount of EtOH that corresponds to 1.5–2 drinks containing about 12 g of EtOH (Eckardt et al., 1998; Kalant, 1975), in order to ascertain further whether such binge-like drinking protocol of adolescent AMED administration could affect the brain either in adulthood and adolescence, a critical period of development where neuronal growth factors are crucial for neurobiological maturation playing important roles during brain development participating in the formation of appropriate synaptic connections in the

brain (Cohen-Cory et al., 2010). Therefore, we studied the EtOH-, ED-, or AMED-induced behavioral effects on locomotion, learning and memory, as well as the electrophysiological and molecular changes involved in synaptic plasticity, such as expression of BDNF and its tyrosine-kinase receptor trkB in the hippocampus of adolescent, and in different cohorts, of adult rats. Moreover, corticosterone plasma levels were also measured in order to evaluate a possible long-term dysregulation of the hypothalamic-pituitary-adrenal axis induced by adolescent binge-like drinking exposure to EtOH, ED, and AMED.

## 2. Materials and methods

### 2.1. Animals

Male Sprague-Dawley CD rats (Charles River, Como, Italy) were bred in the animal facility of the University of Cagliari (Ce.S.A.St.) and maintained under an artificial 12 h-light, 12 h-dark cycle (lights on from 8:00 to 20:00 h) at a constant temperature of  $22 \pm 2$  °C and relative humidity of 65%. The animals had ad libitum access to water and standard laboratory food. According to the 3R principles, all possible efforts were made to minimize animal suffering and to reduce the number of animals used. Experimental protocols were reviewed and approved by the Italian Ministry of Health (authorization #371/2020-PR) in compliance with the European Communities Council Directives (86/609/EEC and 63/2010) and the Italian law (D.L. 26/2014) for care and use of experimental animals and with policies issued by the Organism for Animal Welfare (OPBA) of the University of Cagliari.

### 2.2. Binge-like drinking protocol

At weaning [Post Natal Day 21 (PND21)], animals were housed in groups of 3–4 per cage, randomly divided into four experimental groups, and subjected to binge drinking (Bertola et al., 2013; Jeanblanc et al., 2019) using a modified previously validated binge-like drinking protocol (Coleman et al., 2011). Distinct batteries of animals were treated for the different experimental protocols. In particular, male adolescent rats of the indicated experimental groups were subjected to intragastric gavage administration (volume 10 ml/kg of body weight) of the different solutions: tap water for control (CO); 3.2 g/kg of a 20% v/v ethanol solution (EtOH) equivalent to a moderate drinking of about 1.5–2 drinks containing 12 g of EtOH for a 70 kg human individual; the ED commercially available Red Bull® (2 CanEq/kg, i.e., the equivalent of 2 cans for a 70 kg human individual; or a combination of a 20% v/v EtOH solution and Red Bull® (Alcohol Mixed with Red Bull Energy Drink®, AMED). Animals were treated from PND28 to PND37 once a day with an intermittent protocol (2 days ON/2 days OFF) on PND28–29, PND32–33, and PND36–37.

The amount of EtOH administered by gavage (3.2 g/kg of body mass) has been estimated, from published data (Bloom et al., 1982; Eckardt et al., 1998; Liang et al., 2007; Livy et al., 2003; Smith et al., 2004), to produce an initial rapid rise followed by a gradual rise in blood ethanol concentration (BEC) to reach a measured peak after 60 min leading to a relatively low BEC (74 mg/dl, about 16–20 mM) and then a gradual decline to a BEC close to zero at the 450-min sampling point (Livy et al., 2003). Instead, the amount of ED administered corresponds to an amount of caffeine of 2.5 mg/kg and taurine of 30 mg/kg of body mass mimicking an appropriate caffeine/taurine blood concentration of 10–20 µM and 100–300 µM, respectively (Ghandforoush-Sattari et al., 2010; White et al., 2016). Behavioral, electrophysiological, biochemical, and molecular analyses were performed at least in two of the following different time points, 3 (PND40), 23 (PND60), and 53 (PND90) days after the termination (PND37) of the pharmacological treatment.

Red Bull (Red Bull Energy Drink®) was chosen for the study as reference for ED treatment and contained the following ingredients: water, sugars (11 g/100 ml), caffeine (80 mg/250 ml), taurine (1 g/250 ml), B-group vitamins [niacinamide (vitamin B3), pantothenic acid

(vitamin B5), vitamin B6, and vitamin B12 in not declared amounts], (<http://www.redbull.com/int-en/energydrink/red-bull-energy-drink-ingredients-list>).

### 2.3. Experimental design

After the binge-like administration protocol during adolescence (PND 28 to PND 37, see Sect. 2.2), rats from the four treatment groups (i. e., controls, EtOH, ED, or AMED) were divided into five different cohorts. Each cohort of animals was used independently for behavioral, electrophysiological, molecular and, neurochemical experiments. In detail, rats of the cohort 1 were used sequentially for the evaluation of both motor activity and the NOR test. Rats of the cohort 2 were used for all Morris water maze tests: learning, memory, and behavioral flexibility. Rats of the cohort 3 were used for all electrophysiology experiments. Rats of the cohort 4 were used for molecular analyses in brain extracts and those of cohort 5 for neurochemical measurements in plasma.

### 2.4. Spontaneous locomotor activity

Before the beginning of treatments, each rat underwent one habituation session that lasted for 2 h in order to prevent the influence of novelty factors linked to the experimental procedure and motility apparatus during the experimental sessions and in order to obtain basal values of motor activity. Then, rats were assigned to treatment groups in a pseudorandom sequence in order to avoid differences in motor performances among groups. After the completion of the treatment protocol, each rat was tested for spontaneous locomotor activity at PND40, PND60, and PND90. Locomotor activity was measured as already described (Angioni et al., 2016; Bharatiya et al., 2020). Rats were individually tested under standardized environmental conditions (in a soundproof room with a light level of 30 lux) with a Digiscan Animal Activity Analyzer (Omnitech Electronics, Columbus, Ohio). Each cage (42 cm × 42 cm × 63 cm) had two sets of 16 photocells located at right angles to each other, projecting horizontal infrared beams 2.5 cm apart and 2 cm above the cage floor and a further set of 16 horizontal beams whose height was adapted to the size of the animals. Horizontal and vertical activities were measured as total number of sequential infrared beam breaks (counts) in the horizontal or vertical sensors, recorded every 5 min, beginning immediately after placing the animals into the cage, over a test period of 30 min.

### 2.5. Novel object recognition test

The novel object-recognition test was used to assess cognitive alterations associated with the treatments done (Bharatiya et al., 2020) and was performed on the same cohort of animals used for the locomotor activity assessment. Training in the object recognition task took place in an open field arena (45 × 45 × 30 cm), with the floor covered with sawdust. Rats (one animal at a time) were first habituated for 30 min in the open field arena, in a dimly lit quiet room, to acclimatize with the new arena environment, and then put back to their home cage. Twenty-four hours after habituation session, the rats were placed again in the open field arena; two identical objects (made of plastic or metal and devoid of any natural significance) were positioned in two adjacent corners of the open field arena 9 cm apart from the walls and the rats were placed in remaining corner, facing towards the wall of the apparatus, and left to familiarized with the objects for 10 min (Trial 1). One h after familiarization, rats explored the open field arena for other 10 min in the presence of a familiar object (F) (identical to those used in Trial 1) and a novel object (N) (different in shape from those used in Trial 1 but of the same plastic or metal nature) positioned in similar way (Trial 2). The exploration of the objects (defined as sniffing or touching the object with the nose and/or forepaws but not by turning around or sitting on the object) was recorded by means of a digital video-camera, and

video-files stored in a backup device for the following analyses. A recognition index expressed by the ratio  $TN/(TF + TN)$  [ $TN$  = time spent exploring the novel object  $N$ ;  $TF$  = time spent exploring the familiar object  $F$ ] was first calculated for each animal. As this measure can be biased by differences in overall levels of exploration, the discrimination index (DI) expressed by the ratio  $(TN - TF)/(TN + TF)$  was also calculated (Cavoy and Delacour, 1993). Between trials, the objects were carefully washed with 20%  $H_2O_2$  solution to avoid olfactive cues. Repeated measurements were performed; therefore, each rat was tested at PND40, PND60, and PND90.

### 2.6. Morris water maze test: learning, memory and behavioral flexibility

Spatial learning and memory, as well as behavioral flexibility were assessed in the Morris water maze test in PND40 and PND90 male rats, as previously described (Boi et al., 2022; Pisu et al., 2019). The Morris water maze consisted of a circular pool (150 cm in diameter, 60 cm in depth) whose interior was painted black. It was located in the center of a room dedicated to the assessments in this behavioral paradigm. The room was illuminated (120 lx) by four light bulbs aimed at the ceiling to avoid light reflections in the water. The water temperature was maintained at  $25 \pm 2$  °C with the use of a submersible digital water-heating system. The pool was divided into four virtual quadrants, and a removable circular escape platform (10 cm in diameter, 32 cm in height) was introduced into one of the quadrants (target quadrant), at a depth of 2 cm below the water surface, for all the 4 days of training. Visual cues were present on the walls of the room. The initial assessment of spatial learning occurred on 4 consecutive days (days 1–4), followed by a probe trial on day 5; 48 h later, behavioral flexibility was assessed by performing three additional days of training in the reversal learning test (days 8–10). Each rat ( $n = 9$  to 12 rats/experimental group) was subjected to four training trials on each of four consecutive days. On each training trial, an animal was released in the maze from one of four equally spaced starting positions around the perimeter of the pool, always turning left from the start location (i.e., the quadrant next to the target quadrant), to locate the escape platform. Once the animal had climbed onto the platform, it was allowed to remain there for 15 s before the next trial; if the animal did not locate the platform within 120 s on any trial, the experimenter gently guided it to the platform and allowed to rest there for 15 s. Cumulative search error (CSE), i.e., the corrected cumulative distance from the platform that represents deviation from an optimal search, that is, from a direct path to the goal (Gallagher et al., 1993) and speed were recorded on training trials. On day 5, 24 h after the last training trial, each rat was subjected to a probe trial, in which the escape platform was removed from the pool and the animal was released from the quadrant opposite to the original platform location and allowed to freely swim for 60 s. Behavioral flexibility was assessed during a reversal learning. Forty-eight hours after the probe trial, rats were subjected to a new 3-day training session, starting from day 8; the platform was placed in the opposite side of the initial target quadrant (i. e., from the East to West quadrant), and rats had to relearn the new platform location. Performance in this reversal learning session was indexed by CSE. All behavioral data were acquired and analyzed using an automated tracking system (Ethovision XT 5.0, Noldus, Wageningen, The Netherlands). Using this software, the precise rat location (in  $x$ ,  $y$  coordinates) was recorded throughout the probe test (capture rate: 10 frames/s). From this spatial distribution, the proximity measure from target (i.e., the average distance from the target location) or opposite (average distance from a comparable location in the opposite quadrant of the maze, that is,  $180^\circ$  from the target) was calculated automatically (Gallagher et al., 1993).

### 2.7. Brain slices preparation and electrophysiology experiments

Brain slices containing the hippocampus were prepared from rats of the different experimental groups at three different ages (PND40–45,

60–65, and 90–95), as previously described (Talani et al., 2016). Briefly, after deep anesthesia with 5% isoflurane, animals were sacrificed. Brains were rapidly removed from the skull and transferred to a modified artificial cerebrospinal fluid (aCSF) containing (in mM): 220 sucrose, 2 KCl, 0.2 CaCl<sub>2</sub>, 6 MgSO<sub>4</sub>, 26 NaHCO<sub>3</sub>, 1.3 NaH<sub>2</sub>PO<sub>4</sub>, and 10 D-glucose (pH 7.4, set by aeration with 95% O<sub>2</sub> and 5% CO<sub>2</sub>). Coronal brain slices (thickness, 400 μm) were cut using a vibratome (Leica, Germany) and then immediately transferred to a nylon net submerged in standard aCSF containing (in mM): 126 NaCl, 3 KCl, 2 CaCl<sub>2</sub>, 1 MgCl<sub>2</sub>, 26 NaHCO<sub>3</sub>, 1.25 NaH<sub>2</sub>PO<sub>4</sub>, and 10 D-glucose (pH 7.4, set by aeration with 95% O<sub>2</sub> and 5% CO<sub>2</sub>), at a controlled temperature of 35 °C, for at least 40 min. After subsequent incubation for at least 1 h at room temperature, hemi-slices were transferred to a recording chamber with a constant flow rate of ~2 ml/min of aCSF at a controlled temperature of 33 °C.

Extracellular recordings of field excitatory postsynaptic potentials (fEPSPs) were recorded at dendritic level of CA1 subfield placing both stimulating and recording electrodes in the middle of the *stratum radiatum* and stimulating the Schaffer collateral afferents as previously described (Sanna et al., 2011; Talani et al., 2011). fEPSPs were recorded by filling the recording electrode with KCl (3 M) and were triggered digitally every 20 s by application of a constant current pulse of 0.2–0.4 mA with a duration of 60 μs, which usually yielded a half-maximal response, using a bipolar concentric electrode (FHC, ME, USA) and a stimulator (Digitimer Ltd, UK). Before every single recording, an input-output (I–O) curve was constructed by stimulating the Schaffer's collateral afferents with increasing current intensity (from 0 to 1.0 mA). Quantitative analysis was performed by measuring the slope of each fEPSP. To evaluate the long-term synaptic plasticity, after 10 min of stable baseline, where fEPSPs were evoked every 20 s with a stimulation intensity that triggered about 50% of the maximal response, a high frequency stimulation (HFS, 100 stimuli at 250 Hz) was applied, and recording was continued for additional 60 min, with fEPSPs again evoked every 20 s. The extent of LTP was calculated by averaging the slope of fEPSPs during the interval between the 55th and 60th min post-HFS and comparing this value to the pre-HFS baseline.

## 2.8. Measurement of BDNF mRNA levels by qRT-PCR

Brains were rapidly dissected and left and right hippocampi were removed and placed in 1.5-ml RNase-free plastic tubes. Liquid nitrogen was used to snap-freeze the dissected tissues; frozen tissues were then stored at –80 °C until the day of analysis. Total RNA was extracted from hippocampal tissue using AllPrep DNA/RNA/Protein Mini Kit (QIAGEN GmbH, Hilden, Germany) according to the manufacturer's instructions. The RNA concentration of each sample was determined by measuring the absorbance at 260 nm with a NanoDrop (Thermo Scientific, Madison, WI, U.S.A.). Template RNA (100 ng/reaction) was used in One-Step RT-PCR named QuantiFast SYBER Green RT-PCR kit (QIAGEN) according to the manufacturer's instructions. The primer assay QuantiTect (QIAGEN) was used with the following primers Brain-derived neurotrophic factor (BDNF) (NM\_012513; QIAGEN), glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (NM\_017008; QIAGEN), β-actin (NM\_031144; QIAGEN). Real-Time Cycler conditions consisted of one reverse transcription cycle at 50 °C with a 10 min hold. The amplification program consisted of one activation step at 95 °C with a 5 min hold, followed by 40 cycles of denaturation at 95 °C with a 10-s hold, and annealing/extension at 60 °C with a 5-s hold. A negative control without cDNA template was run with each assay to assess the overall specificity. Each assay included duplicate reactions. Obtained gene-expression values were normalized using housekeeping β-actin and GAPDH genes as internal control, the Livak method was used to calculate the  $2^{-\Delta\Delta Ct}$  values (Livak and Schmittgen, 2001).

## 2.9. Measurement of BDNF and trkB proteins by western blot

Determination of total protein concentrations was carried out

according to the (Lowry et al., 1951). Proteins from each hippocampal tissue homogenate (40 μg), diluted 3:1 in 4X loading buffer (NuPAGE LDS Sample Buffer 4X, Cat# NP0008, Novex by Life Technologies, Carlsbad, CA, USA), were then heated to 95 °C for 7 min and separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) using precast polyacrylamide gradient gel (NuPAGE 4–12 % Bis-Tris Gel Midi, Cat# NP0321, Novex by Life Technologies) in the XCell4 Sure Lock™ Midi-Cell chamber (Life Technologies). Internal molecular weight (MW) standards (Precision Plus Protein Western C Standards, Cat# 161–0376, Bio-Rad, Hercules, CA, USA) were run in parallel. Blots were blocked by immersion in 20 mM Tris-base and 137 mM sodium chloride (TBS) containing 5% milk powder and 0.1% Tween 20 (TBS-T), for 60 min at room temperature. Rabbit polyclonal antisera against BDNF (Cat# R-017, RRID: AB\_2492693, Biosensis Pty Ltd., Thebarton, Australia), diluted 1:1000, and tyrosine kinase receptor B (trkB) [Cat# (794) sc-12, RRID: AB\_632557, Santa Cruz Biotechnology, Dallas, TX, USA) diluted 1:1000 in TBS containing 5% milk powder and 0.02% sodium azide, were used as primary antibody. Equal loading of the wells was checked by using a mouse monoclonal antibody directed against GAPDH (MAB374, RRID: AB\_2107445, EMD Millipore, Darmstadt, Germany), diluted 1:1000 as the primary antiserum. Incubations with primary antibodies were performed at 4 °C and lasted two nights. Blots were then rinsed in TBS/T, incubated for 60 min, at room temperature, with peroxidase-conjugated goat anti-rabbit serum (Cat#9169, RRID: AB\_258434, Sigma Aldrich, St Louis, MO, USA), diluted 1:10,000, and goat anti-mouse serum (AP124P, RRID: AB\_90456, Millipore, Darmstadt, Germany), diluted 1:5000 in TBS/T, as secondary antiserum. To control for nonspecific staining, blots were stripped and incubated with the relevant secondary antiserum. After TBS/T rinse, protein bands were developed using the Clarity Max ECL Substrate (Cat# 1705062, Bio-Rad, Hercules, CA, USA), according to the protocol provided by the company, and visualized with ImageQuant LAS-4000 (GE Healthcare, Little Chalfont, UK) or by Geliance 600 Chemi Imaging System (PerkinElmer, Monza, Italy). Approximate MW and relative optical density (O.D.) of labeled protein bands were evaluated by a blinded examiner. Values for BDNF and trkB were normalized using GAPDH as a housekeeping gene for internal control. Image Studio Lite Software (RRID: SCR\_014211, Li-Cor, [http://www.licor.com/bio/products/software/image\\_studio\\_lite/](http://www.licor.com/bio/products/software/image_studio_lite/)) was used to quantify the O.D. of each sample.

## 2.10. Corticosterone assay

PND40, PND60 and PND90 rats (n = 10 rats/experimental group) were sacrificed by decapitation. Blood was collected from the trunk upon decapitation into EDTA-coated tubes and centrifuged at 900×g for 10 min at 4 °C. The resulting plasma supernatant was frozen at –20 °C until use. Plasma corticosterone levels were measured as previously described (Boero et al., 2018) using an enzyme-linked immunosorbent assay (ELISA #RE52211; IBL International, Hamburg, Germany). The ELISA assay was performed according to the manufacturer's instructions using a 96-well plate pre-coated with a polyclonal antibody against an antigenic site on the corticosterone molecule. The kit also provided a six-point standard curve using two-fold serial dilutions. Each sample was run in duplicate.

## 2.11. Statistical analyses

Behavioral and molecular measurements were statistically evaluated using one- or two-way Analysis of Variance (ANOVA) followed by either Bonferroni's correction or Tukey's *post-hoc* test with the significance level set at  $p < 0.05$ . As regards the electrophysiology, quantitative data were obtained by calculating the mean percent change of each fEPSP slope ± SEM from baseline. In detail, motility scores and NOR indexes were analyzed with repeated measures two-way ANOVAs, with the age of rats (i.e., PND) as within-subject factor and treatment as between-subject factor. Pairwise comparisons were performed by using

Bonferroni's corrected *t*-test. As for the Morris water maze performances, CSE and speed were analyzed with repeated measures one-way ANOVA, considering time as within-subject factor and treatment as between-subjects factor; the average proximity in the probe trial was analyzed by two-way ANOVA considering zone specificity (target vs. opposite) as within-subject factor and treatment as between-subject factor. Pairwise comparisons were performed by using Tukey's corrected *t*-test. One-way ANOVA followed by the Tukey's post-hoc test was used to analyze BDNF mRNA, relative BDNF and trkB protein levels, and plasma corticosterone. All the statistical analyses were carried out with PRISM, GraphPad 6 Software (San Diego, CA, USA).

### 3. Results

#### 3.1. Effects of EtOH, ED and AMED on spontaneous locomotor activity

As shown in Fig. 1, all treatment groups displayed an increase in the amount of both horizontal and vertical locomotor activity when passing from adolescence (PND40) to adulthood (PND90). Accordingly, two-way ANOVA revealed a significant main effect of age in both horizontal [ $F_{(2,96)} = 32.45$ ;  $p < 0.0001$ ] and vertical activity [ $F_{(2,96)} = 12.56$ ;  $p < 0.0001$ ] as shown in Fig. 1A and B, respectively. The effect of age was also confirmed by Bonferroni's pairwise comparisons that detected similar age-related differences in all the treatment groups, although in the ED group no significant differences were observed among the three timepoints in vertical activity nor differences between PND40 and PND60 in horizontal activity. However, regardless of these age-related effects, two-way ANOVA also revealed a significant effect of treatment for both horizontal [ $F_{(3,48)} = 5.78$ ;  $p = 0.0019$ ] and vertical activity [ $F_{(3,48)} = 4.99$ ;  $p = 0.0043$ ]. Accordingly, Bonferroni's pairwise comparisons revealed greater values of horizontal activity in the ED-treated group compared with all the other groups at PND40 and a significant difference in the same direction between the ED-treated group and the vehicle- and AMED-treated groups at PND90 (see Fig. 1A for individual points of statistical significance).

#### 3.2. Effects of EtOH, ED and AMED on novel object recognition

As shown in Fig. 2A and B, all treatment groups displayed age-related differences in both recognition (RI) and discrimination (DI) indexes of the NOR test when passing from adolescence (PND40) to adulthood (PND90). Accordingly, two-way ANOVA revealed a significant main effect of age in both the RI [ $F_{(2,96)} = 3.310$ ;  $p = 0.0407$ ] and the DI [ $F_{(2,96)} = 3.312$ ;  $p = 0.0407$ ] as shown in Fig. 2A and B, respectively. However, at variance from all the other treatment groups, that displayed the higher values in adolescence and a constant decrease in both indexes when passing from adolescence to adulthood, ED-treated rats displayed

a different trend and significantly lower values in both RI and DI of the NOR test at PND40 when compared to age matched vehicle- ( $55.23 \pm 3.14$  vs  $68.92 \pm 2.21$  and  $10.54 \pm 6.25$  vs  $37.71 \pm 4.37$ ,  $p = 0.0417$  and  $p = 0.0435$ , for RI and DI, respectively) and AMED-treated rats ( $55.23 \pm 3.14$  vs  $68.85 \pm 2.82$  and  $10.54 \pm 6.25$  vs  $37.38 \pm 5.62$ ,  $p = 0.0433$  and  $p = 0.0472$ , for RI and DI, respectively) (see Fig. 2A and B for individual points of statistical significance).

#### 3.3. Effects of EtOH, ED, and AMED on learning in the Morris water maze test

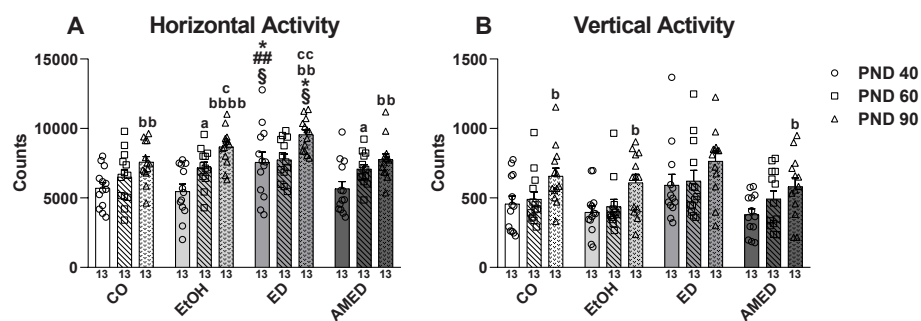
The Morris water maze test was used to assess learning and memory in adolescent (PND40) and adult (PND90) rats. During the four days of training no difference in locating the hidden platform was found among the experimental groups. PND40: As shown in Fig. 3, panels A and B, the CSE and speed decreased over time in all groups at PND 40. Specifically, a significant effect of time was observed for CSE and speed [ $F_{(3,528)} = 59.70$ ;  $p < 0.001$  and  $F_{(3,528)} = 43.85$ ;  $p < 0.001$ , respectively] The post-hoc analysis showed a significant decrease in CSE and speed in all experimental groups, starting on day 2 (Fig. 3A and B). Likewise, at PND90 the CSE decreased over time in all groups; ANOVA revealed a significant effect of time [ $F_{(3,456)} = 94.68$ ;  $p < 0.001$ ], and the post-hoc analysis showed that such significant decrease was apparent in all experimental groups, starting on day 2 (Fig. 3C). ANOVA also revealed a significant effect of time for speed [ $F_{(3,456)} = 14.13$ ;  $p < 0.001$ ], although no significant differences among experimental groups were found in the post-hoc test (Fig. 3D).

#### 3.4. Effects of EtOH, ED, and AMED on spatial memory in the Morris water maze test

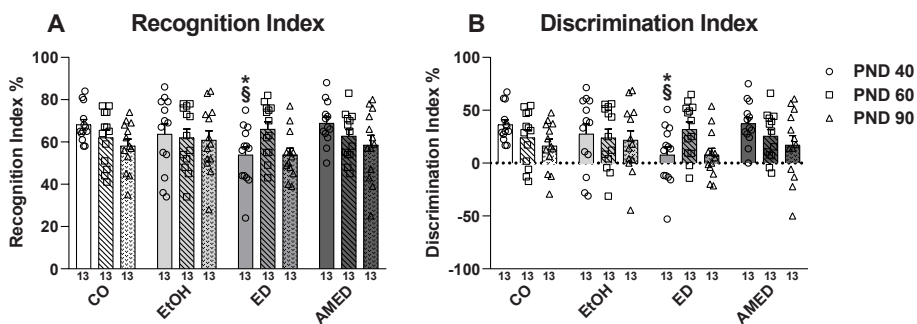
To evaluate the spatial memory, a probe trial was performed on the fifth day of the Morris water maze test in both PND40 and PND90 groups. Average proximity of the target compared to the opposite location of the platform was used to evaluate spatial memory. ANOVA revealed a significant effect of platform location [ $F_{(1,82)} = 16.91$ ;  $p < 0.001$ ] and [ $F_{(1,70)} = 29.89$ ;  $p < 0.001$ ] (Fig. 4A and B). A significant reduction was observed in the average proximity of the target compared to the opposite location for all experimental groups at both PND40 (Fig. 4A;  $p < 0.05$ ) and PND90 (Fig. 4B;  $p < 0.01$ ) except for the EtOH group at both ages.

#### 3.5. Effects of EtOH, ED, and AMED on behavioral flexibility in the Morris water maze test

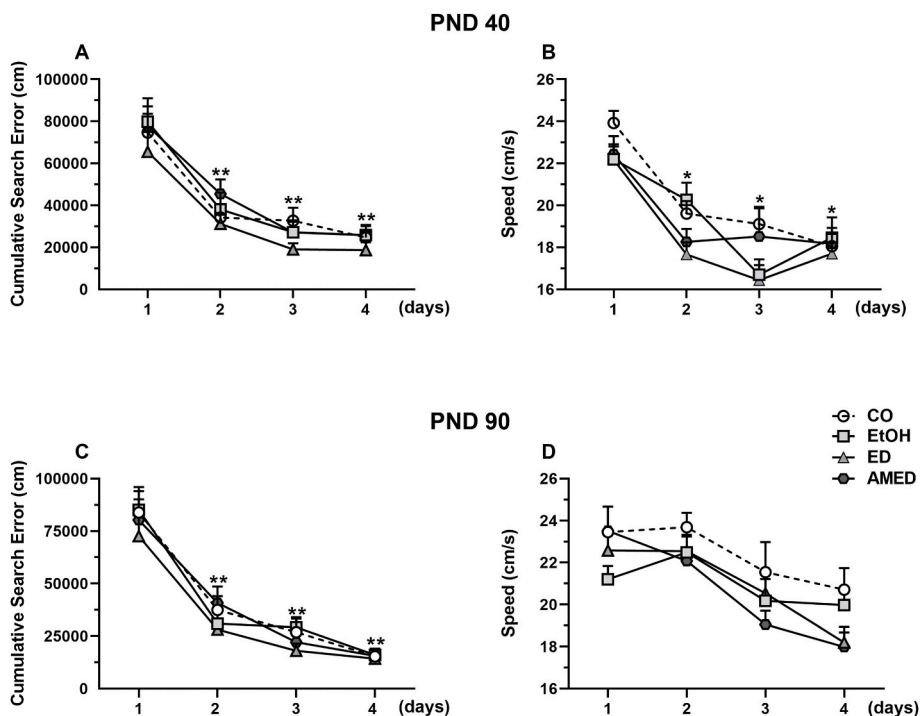
Behavioral flexibility was assessed through the reversal learning test in the Morris water maze, where rats had to learn the new platform



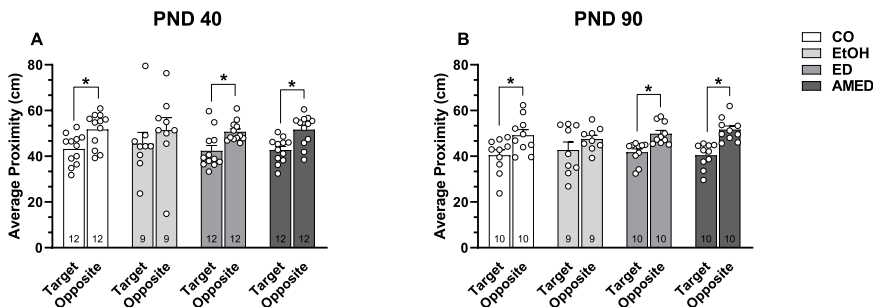
**Fig. 1. Effects of EtOH, ED and AMED treatment on spontaneous locomotor activity.** Total counts of horizontal (A) and vertical (B) locomotor activity in the 30 min of test for rats of the indicated experimental groups at PND40, PND60, and PND90 are reported. Dot plots are represented by open circles, squares, or triangles, and the numbers at the base of the columns indicate the number of animals for each experimental group. Values are expressed as means  $\pm$  SEM of the indicated numbers of rats/group. \* $p < 0.05$  vs. age-matched control-treated rats; # $p < 0.05$  vs. age-matched EtOH-treated rats;  $\ddagger p < 0.05$  vs. age-matched AMED-treated rats;  $\text{a}^p < 0.05$  PND40 vs. PND60;  $\text{b}^p < 0.05$ ,  $\text{bb}^p < 0.01$ ,  $\text{bbbb}^p < 0.0001$  PND40 vs. PND90;  $\text{c}^p < 0.05$ ,  $\text{cc}^p < 0.01$  PND60 vs. PND90 (two-way ANOVA followed by Bonferroni's pairwise comparisons).



**Fig. 2.** Effects of EtOH, ED and AMED treatment on Novel Object Recognition (NOR) test. Recognition (A) and discrimination (B) index for rats of the indicated experimental groups at PND40, PND60, and PND90 are reported. Dot plots are represented by open circles, squares, or triangles, and the numbers at the base of the columns indicate the number of animals for each experimental group. Values are expressed as means  $\pm$  SEM of the indicated numbers of rats/group. \* $p < 0.05$  vs. age-matched control-treated rats;  $^{\#}p < 0.05$  vs. age-matched AMED-treated rats (two-way ANOVA followed by Bonferroni's pairwise comparisons).



**Fig. 3.** Effects of EtOH, ED and AMED treatment on the learning performance in the Morris water maze test in PND40 (A) and PND90 rats (B). Cumulative Search Error (panels A, C) and Speed (panels B, D) were measured in rats of the indicated experimental groups during the four days of training in PND40 and PND90 animals. Data represent the mean  $\pm$  SEM of the indicated numbers of rats/group as indicated in Fig. 4. \* $p < 0.05$ ; \*\* $p < 0.01$  vs. the respective first day of training.



**Fig. 4.** Effects of EtOH, ED and AMED treatment on spatial memory performance. Spatial memory performance was measured as average proximity, in the Morris water maze test in PND40 (A) and PND90 rats (B) of the indicated experimental groups. Dot plots are represented by open circles and the numbers at the base of the columns indicate the number of animals for each experimental group. Data represent the mean  $\pm$  SEM of the indicated numbers of rats/group. \* $p < 0.05$  vs. the respective target location.

location that was moved from the East to the West quadrant of the maze. As shown in Fig. 5, a reduction in CSE was observed over test days in all experimental groups (Fig. 5A for PND40 and 3B for PND90); repeated measures ANOVA revealed a significant effect of time for PND40 [ $F_{(2,352)} = 30.681$ ;  $p < 0.0001$ , Fig. 5A] and PND90 [ $F_{(2,304)} = 23.50$ ;  $p < 0.0001$ , Fig. 5B]. The post-hoc analysis showed a significant reduction in CSE starting on day 2 in all groups.

### 3.6. Effects of EtOH, ED, and AMED on synaptic plasticity in CA1 pyramidal neurons

To explore whether the treatment with EtOH, ED, and their association AMED, under a protocol of binge-like drinking adolescent administration, could alter the function and long-term plasticity of hippocampal excitatory glutamatergic synapses in CA1 pyramidal neurons, electrophysiological experiments were performed. Rats from the different experimental groups were tested starting from 3 (PND40), 23 (PND60), and 53 (PND90) days after the end of the pharmacological treatment. Field excitatory postsynaptic potentials (fEPSPs) were recorded extracellularly and generated through the activation of glutamatergic ionotropic receptors of the AMPA/kainate subtype, as they were completely suppressed by the antagonist NBQX (data not shown).

We first constructed input-output (I–O) curves in brain slices by gradually increasing the stimulation intensity from 0.0 to 1.0 mA, with intervals of 0.1 mA. Subsequently, we extrapolated the stimulation intensity required to evoke an fEPSP whose slope value was 50% of the maximal response, defined as  $SI_{50}$ . The results indicate that the  $SI_{50}$  value in control (CO) rats ( $0.51 \pm 0.02$  mA,  $n = 6$ ) was not significantly altered by the treatment with EtOH ( $0.58 \pm 0.01$  mA,  $n = 5$ ), ED ( $0.50 \pm 0.01$  mA,  $n = 12$ ), or AMED ( $0.57 \pm 0.02$  mA,  $n = 6$ ) at PND40 (Fig. 6 A). Two-way ANOVA revealed an effect of the stimulation [ $F_{(10, 250)} = 454.9$ ,  $p < 0.0001$ ] but not of the treatment ( $p = 0.51$ ). Similar outcome was obtained in PND60 rats, where the  $SI_{50}$  value found in CO animals ( $0.56 \pm 0.01$  mA,  $n = 6$ ) did not differ in response to the treatment with EtOH ( $0.53 \pm 0.01$  mA,  $n = 11$ ), ED ( $0.51 \pm 0.01$  mA,  $n = 11$ ), or AMED ( $0.58 \pm 0.01$  mA,  $n = 7$ ) (Fig. 6 D). Again, two-way ANOVA shows a significant effect of the stimulation intensity [ $F_{(10, 310)} = 772.8$ ,  $p < 0.0001$ ] with no change caused by the treatments ( $p = 0.47$ ). There was no significant change in the I–O curve also in PND90 tested rats, with the  $SI_{50}$  measured in CO animals ( $0.61 \pm 0.01$  mA,  $n = 11$ ) not different from that of EtOH ( $0.68 \pm 0.01$  mA,  $n = 11$ ), ED ( $0.63 \pm 0.01$  mA,  $n = 15$ ), or AMED ( $0.65 \pm 0.01$  mA,  $n = 14$ ) (Fig. 6 G). Two-way ANOVA shows a significant effect of the stimulation intensity [ $F_{(10, 550)} = 425.3$ ,  $p < 0.0001$ ] with no changes caused by the treatment ( $p = 0.20$ ). These results suggest that the basal excitatory glutamatergic transmission, at any time-point tested, is not affected by the various drug treatments. We then focused our experiments on determining the effects of the different treatments on the long-term plasticity of glutamatergic synapses in CA1 pyramidal cells. To this end, we induced LTP by applying a single

episode of high-frequency electrical stimulation (HFS, 100 stimuli at 250 Hz) to the Schaffer's presynaptic fibers. As observed previously (see Albensi et al., 2007 for review), one single train of 100 stimuli at 250 Hz was able to evoke an LTP with a consistent magnitude. In particular, in CO rats tested at the different time points (PND40–60–90), HFS was able to promote a significant LTP at all age tested (PND40,  $48 \pm 11.5\%$ ,  $n = 5$ ; PND60,  $89 \pm 17.9\%$ ,  $n = 5$ ; PND90,  $65 \pm 4.9\%$ ,  $n = 6$ ) (Fig. 6B–I). Moreover, when comparing the levels of LTP at different age (first column of graphs C, F, I) we did not observe a significant age-dependent difference of LTP magnitude [ $F_{(2, 13)} = 2,820$ ,  $p = 0.09$ ]. At PND40, the treatment with EtOH did not significantly alter the value of LTP observed in CO animals, with an increase in fEPSP slope of  $52 \pm 8.3\%$  ( $n = 5$ ), while in both ED and AMED treated rats, LTP was increased with values of  $120 \pm 19.1\%$  ( $n = 8$ ) and  $125 \pm 28.2$  ( $n = 5$ ), respectively (Fig. 6 B and C). One-way ANOVA revealed a significant effect of the treatment [ $F_{(3, 19)} = 5,72$ ,  $p = 0.005$ ], with a difference between the LTP values measured in ED ( $p = 0.03$ ) and AMED ( $p = 0.036$ ) treated rats and those found in CO animals.

In rats tested at PND60, LTP was significantly reduced in all experimental groups compared to CO animals [ $F_{(3, 19)} = 3,95$ ,  $p = 0.02$ ]. Post hoc analysis revealed a significant reduction of LTP magnitude in EtOH ( $p = 0.02$ ), ED ( $p = 0.0009$ ) and AMED ( $p = 0.002$ ) animals (Fig. 6 E and F).

Similarly, in rats tested at PND90, the magnitude of LTP was significantly reduced in all experimental groups compared with CO animals [ $F_{(3, 33)} = 8.18$ ,  $p = 0.0003$ ]. Post hoc analysis revealed a significant reduction of LTP extent in EtOH ( $p = 0.0001$ ), ED ( $p = 0.007$ ) and AMED ( $p = 0.03$ ) treated animals (Fig. 6 H and I). We have to report that we observed in both ED and AMED group at PND40 as well as in CO group at PND60 a sort of run-up of LTP formation that appear different from its stable effect observed in other experimental groups. However, this observation occurs randomly, suggesting that it does not appear to be treatment- or age-dependent.

### 3.7. Effects of EtOH, ED, and AMED on BDNF mRNA abundance in the hippocampus

BDNF gene expression was evaluated in the hippocampus of both adolescent (PND40) and adult (PND60) rats by qRT-PCR. The comparison of BDNF mRNA levels measured in the four experimental groups at the two ages is shown in Fig. 7. Statistical analysis of the values, obtained in each qRT-PCR, demonstrates that nor EtOH or ED administration, alone or in combination, significantly altered the abundance of BDNF mRNA at PND40 (Fig. 7 A) or PND60 (Fig. 7 B). One-way ANOVA revealed no significant effects between control group and any of the treatment [ $F_{(3, 28)} = 0.6827$ ;  $p = 0,5701$ ] and [ $F_{(3, 26)} = 2.323$ ;  $p = 0.0983$ ] for PND40 and PND60 respectively.

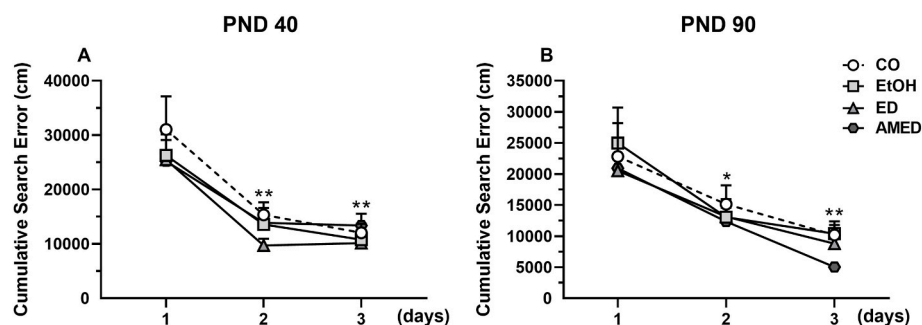
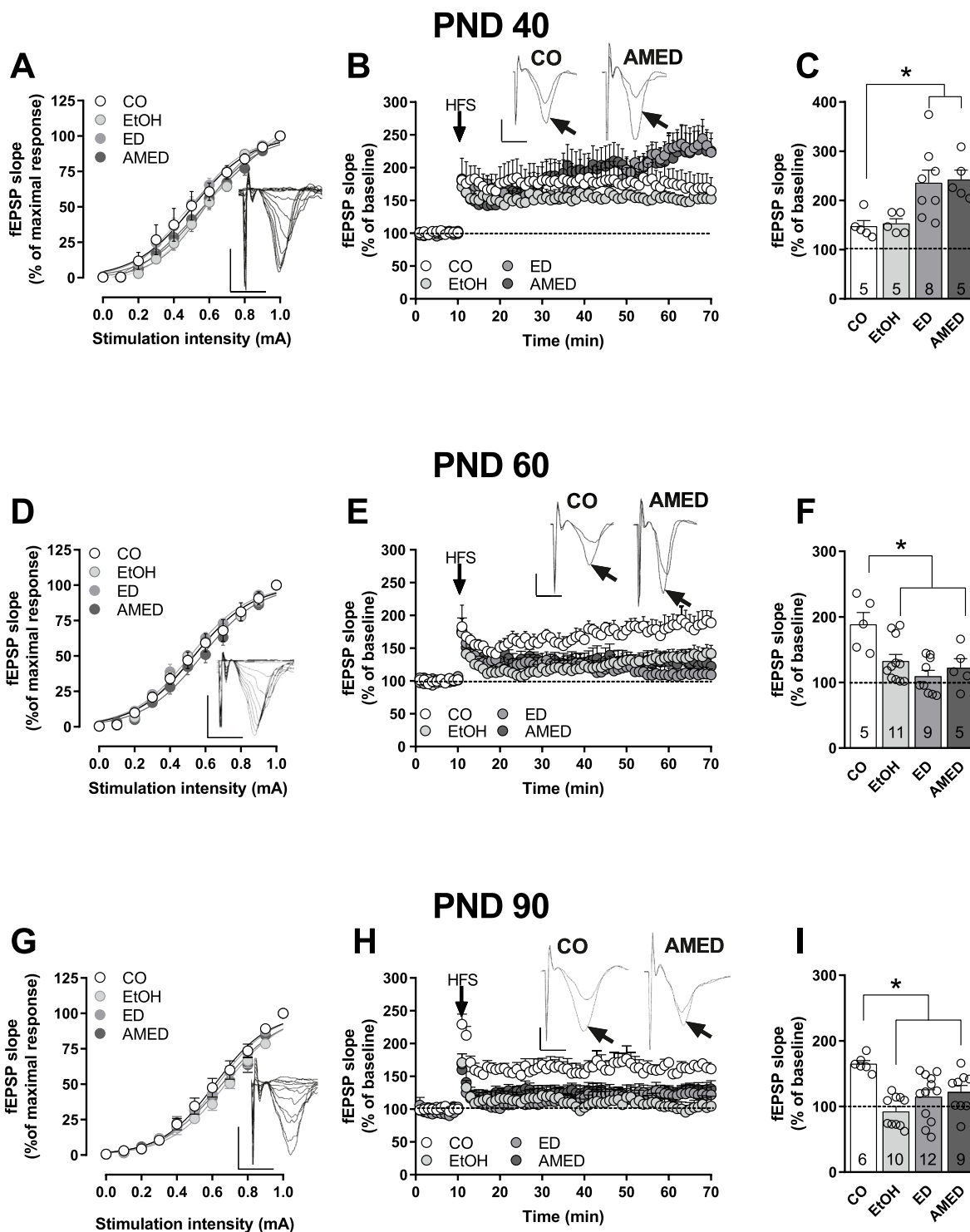
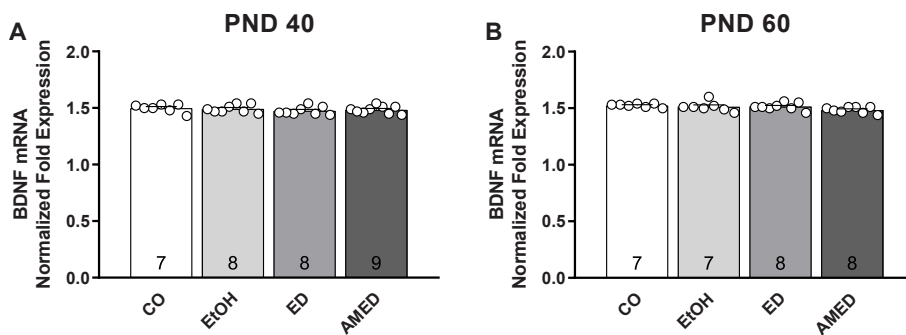


Fig. 5. Effects of EtOH, ED and AMED treatment on behavioral flexibility in the Morris water maze test in PND40 (A) and PND90 rats (B). Male rats of the indicated experimental groups were tested and Cumulative Search Error was measured over the three days of the reversal learning test. The data represent the mean  $\pm$  SEM of the values of the indicated numbers of rats/group as indicated in Fig. 4. \* $p < 0.05$ ; \*\* $p < 0.01$  vs. the respective first day of training.



**Fig. 6.** Effects of EtOH, ED and AMED treatment on the function and plasticity of glutamatergic synapses in hippocampal CA1 pyramidal neurons. (A-D-G) Scatter plot representing the I–O curves obtained in rats of the indicated experimental groups at different ages (PND40, 60 and 90). The insets show the representative traces of evoked glutamatergic field excitatory postsynaptic potentials (fEPSPs) recorded in the dendritic region of hippocampal CA1 neurons in response to increasing electrical stimulation steps (0.0–1.0 mA) of Shaffer collaterals; scale bar, 1 mV/5 ms. (B, E, H) Scatter plot representing the percentage of change in fEPSP slope values induced by high frequency stimulation (HFS) with respect to the baseline, in rats subjected to different treatments and at different ages. The insets show the representative traces of fEPSPs recorded in the dendritic portion of CA1 neurons. Traces are recorded before and after (black arrow) LTP conditioning (HFS, 100 stimuli at 250 Hz); scale bar 1 mV/5 ms. (C, F, I) The graphs in C, F, and I, obtained from graphs B, E, and H, respectively, illustrate the percent change in fEPSP slope (calculated in the period from the 55th to the 60th min after HFS) compared to the baseline value. The numbers in each bar indicates the number of recordings obtained in the following number of animals: PND40, CO = 4, EtOH = 4, ED = 5, AMED = 4; PND60, CO = 4, EtOH = 5, ED = 5, AMED = 5; PND90, CO = 5, EtOH = 5, ED = 5, AMED = 5. Data are expressed as mean percent change of fEPSP slope  $\pm$  SEM from baseline. \*p < 0.05 vs CTRL.



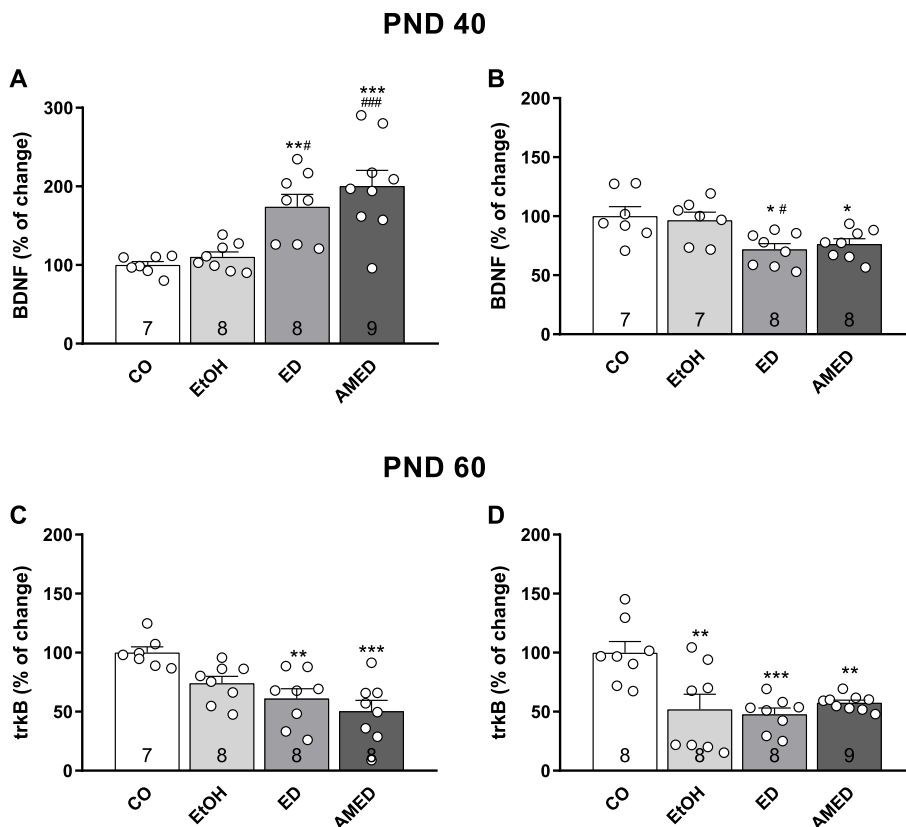


**Fig. 7.** Effects of EtOH, ED and AMED treatment on BDNF mRNA abundance in the hippocampus of adolescent and adult male rats. BDNF mRNA expression in the hippocampus of male rats at PND40 (A) and PND60 (B) of the indicated experimental groups. Dot plots are represented by open circles and the numbers at the base of the columns indicate the number of animals for each experimental group. Quantitative data are means  $\pm$  SEM of values obtained from the indicated number of rats. One-Way Analysis of Variance (ANOVA) followed by the Tukey's post-hoc test (Graph Pad 8 Software, PRISM); all comparisons resulted not significant with  $p$  values  $>$  0.05.

**3.8. Effects of EtOH, ED, and AMED on BDNF and trkB proteins in the hippocampus**

BDNF and trkB relative protein levels were evaluated at PND40 and PND60 by immunoblot analysis. The antibodies against BDNF and trkB recognized, respectively, a band with a relative molecular weight (MW) of  $\sim$ 13 kDa, in agreement with the reported MW of the monomeric form of the BDNF protein (Rosenthal et al., 1991), and a band of  $\sim$ 140 kDa, consistent with the reported MW of the trkB receptor protein (Klein et al., 1989). As shown in Fig. 8, the administration of EtOH or ED alone, as well as their combination in the AMED experimental group, induced

specific changes in the relative levels of both BDNF and trkB, depending on the rat age. At PND40 we found a significant increase of BDNF relative protein levels in animals exposed to ED alone ( $+74 \pm 15.8\%$ ;  $p < 0.005$ ; Fig. 8 A) or AMED treatment ( $+100 \pm 20.1\%$ ;  $p < 0.0005$ ; Fig. 8 A) when compared with the control or the EtOH treated groups. One-way ANOVA revealed a significant effect of treatment [ $F_{(3, 28)} = 11, 67$ ;  $p = 0.0001$ ]. On the contrary, at PND60 we observed a significant decrease of BDNF protein in both ED ( $-28 \pm 5\%$ ,  $p < 0.05$ ; Fig. 8 C) and AMED experimental groups ( $-24 \pm 4.4\%$ ,  $p < 0.05$ ; Fig. 8 C) compared to the control group. Nevertheless, only ED experimental group was significantly different compared to EtOH experimental group ( $p < 0.05$ ;



**Fig. 8.** Effects of EtOH, ED and AMED treatment on BDNF and trkB protein expression in the hippocampus of adolescent and adult male rats. BDNF (A–B) and trkB (C–D) protein determination in the hippocampus of male rats of the indicated experimental groups at PND40 (A, C) and PND60 (B, D). Dot plots are represented by open circles and the numbers at the base of the columns indicate the number of animals for each experimental group. Quantitative data are means  $\pm$  SEM of values obtained from the indicated number of rats for each experimental group. One-Way Analysis of Variance (ANOVA) followed by Tukey's post-hoc test (Graph Pad 8 Software, PRISM); \* $p < 0.05$ ; \*\* $p < 0.01$  and \*\*\* $p < 0.001$  vs. CO; ## $p < 0.01$  and ### $p < 0.001$  vs EtOH.

Fig. 8 C), as demonstrated by One-way ANOVA that revealed a significant effect of treatment [ $F_{(3, 26)} = 5.4$ ;  $p = 0.0048$ ].

In the same way, the assessment of densitometric values followed by one-way ANOVA revealed statistically significant effects on *trkB* levels at both PND40 [ $F_{(3, 27)} = 8.03$ ;  $p = 0.0006$ ] and PND60 [ $F_{(3, 29)} = 8.45$ ;  $p = 0.0003$ ], as shown in Fig. 8 B and D, respectively. Moreover, pairwise comparisons revealed that, at PND40, both ED and AMED induced a statistically significant reduction of the *trkB* protein levels, amounting to  $-38.88\% \pm 10.61\%$  ( $p < 0.01$ ) and to  $-49.57 \pm 10.61\%$  ( $p < 0.001$ ), respectively (Fig. 8 B). At PND60, post-hoc tests revealed that all three condition treatments induced a statistically significant reduction of the *trkB* relative protein levels, amounting to  $-48.08 \pm 11.76\%$  ( $p < 0.01$ ) in the EtOH,  $-52.20\% \pm 11.76\%$  ( $p < 0.001$ ) in the ED, and to  $-42.45\%$  ( $p < 0.01$ ) in the AMED experimental group (Fig. 8 D).

### 3.9. Effects of EtOH, ED, and AMED on plasma corticosterone levels

Corticosterone levels were measured in plasma samples from PND40, PND60, and PND90 rats subjected to EtOH, ED, and AMED binge-like drinking treatment during adolescence. As shown in Table 1, corticosterone levels were not significantly altered following EtOH, ED, or AMED treatment at any of the age groups examined, PND 40 [ $F_{(3, 36)} = 0.79$ ;  $p = 0.506$ ], PND 60 [ $F_{(3, 36)} = 0.97$ ;  $p = 0.419$ ] and PND 90 [ $F_{(3, 36)} = 0.005$ ;  $p = 0.999$ ].

## 4. Discussion

### 4.1. Age-related effect of AMED on locomotion and memory related behaviors

ED-treated rats displayed significantly higher spontaneous locomotor activity than the other three groups at PND40 and the vehicle- and AMED-treated groups at PND90, indicating that adolescent rats undergoing binge-like drinking protocol of ED administration are hyperactive not only in the short-term (i.e., at PND40) but also display long-lasting effects in adulthood (i.e., at PND90). The ED stimulatory effects resemble those observed in mice after acute ED treatment, which also prevented the depressant effects induced by EtOH when mixed with it (Ferreira et al., 2004). This concurs with our data, suggesting that the ED-induced hyperactivity was counteracted by the simultaneous presence of EtOH. This is a key point if considering that ED consumption in humans leads to a reduced subjective perception of EtOH intoxication, though not offsetting its negative effects (Ferreira et al., 2004, 2006; Riesselmann et al., 1996) that still affect the individual's judgment, and might increase the probability of being involved in EtOH-related high-risk situations.

As regards the NOR test, a significant difference was observed between the ED- and the control- and AMED-treated groups at PND40, with the former displaying worse memory performance and the latter showing no significant difference, suggesting that the concomitant presence of EtOH and ED likely reverted the negative effects induced by the ED alone. To date, only a few studies investigated the effect of EDs or

**Table 1**

Effects of EtOH, ED and AMED treatment on plasma corticosterone levels in PND40, PND60 and PND90 rats.

	PND40	PND60	PND90
CO	104.5 ± 38.0	96.5 ± 23.4	94.4 ± 19.0
EtOH	60.0 ± 16.1	257.4 ± 84.6	99.2 ± 32.1
ED	53.7 ± 16.8	153.3 ± 53.3	95.2 ± 36.4
AMED	87.2 ± 28.8	306.7 ± 135.0	96.5 ± 24.6

Adolescent male rats were treated as described in the methods for the different experimental groups and sacrificed at the indicated age for measurement of plasma corticosterone levels. Data are expressed as ng/ml of plasma and represent the mean ± SEM of 10 animals per experimental group.

AMED on object memory; male adult rats (>PND60) treated for six days and tested after 48–72 h displayed impaired discrimination index in the EtOH- and AMED-treated groups, but not in the ED-treated group (Takahashi et al., 2015), while (Costa-Valle et al., 2022) found no effects by ED or AMED on short-term memory at PND60 in rats treated for 3 day at PND42/44. Although the methodological differences could account for the discrepant results (Acquas et al., 2023; Petribu et al., 2023), some hypotheses emerge: for instance, the ED-induced effects on locomotor activity and object memory are both detectable at PND40, decreasing at PND60. Hence, the ED-induced memory impairment could be partially attributable to attentional impairments due to hyperactivity. Several studies highlighted the detrimental effects of sustained ED consumption on attention and executive functions in humans (Schwartz et al., 2015).

With respect to spatial memory EtOH but not ED or AMED treatments impaired performance in both PND40 and PND90. Accordingly, exposure to EtOH during adolescence affects cognitive processes in humans and animal models (Seemiller and Gould, 2020). Spatial learning and behavioral flexibility were not affected by any of the treatments at both PND40 and PND90. Nonetheless, the effects of binge-like EtOH consumption during adolescence on learning and behavioral flexibility yielded mixed results depending on species, behavioral tests, and EtOH administration protocols. Our finding that EtOH exposure does not alter spatial learning agrees with previous studies in rats and mice subjected to the Barnes maze or the Morris water maze (Coleman et al., 2011, 2014; Vetreno and Crews, 2012). However, and in contrast to our results, these studies showed an EtOH-induced deficit in reversal learning, suggestive of altered behavioral flexibility. This discrepancy might be attributable to the lower EtOH dose (3.2 g/kg versus 5 g/kg), or the different rat strains and/or species tested. Unlike to EtOH, EDs improve cognitive performance in several tasks and both animal models and humans (Costa-Valle et al., 2018; Wesnes et al., 2017). These actions were thought to be attributable to caffeine, although its effects on cognitive performance are not so clear-cut (Zhang and Madan, 2021). Our findings do not support a cognitive-enhancing effect of the ED alone concerning control rats for spatial learning, memory, and behavioral flexibility. Differences in the behavioral tests used to assess cognitive performance, in EDs treatment protocols, age, and species used might account for the discrepancies. Likewise, AMED treatment, unlike EtOH alone, does not alter memory performance compared to control or ED treatment in PND40 and PND90 rats. This finding suggests that the active components of ED might prevent the memory deficits induced by binge-like EtOH administration during adolescence. In support of this hypothesis, taurine has been shown to prevent EtOH-induced amnesia in zebrafish (Bertoncello et al., 2019). However, the exact mechanisms behind this action remain unclear and might not be generalized. In fact, ED does not protect against binge-like EtOH-induced cognitive deficits in young adult rats tested 48–72 h after the last drug administration (Takahashi et al., 2015). Likewise, human studies showed that co-administration of ED and EtOH under laboratory settings did not alter the EtOH-induced cognitive impairment in non-EtOH-dependent young adults (Benson et al., 2019; Marczynski et al., 2012). Thus, further studies are needed to test these hypotheses to elucidate the effects of AMED on cognitive performance.

### 4.2. Age-related effect of AMED on hippocampal plasticity and plasma corticosterone levels

*Ex-vivo* electrophysiological recordings in hippocampal slices revealed an age- and treatment-dependent effect on the magnitude of LTP without any impairment of basal glutamatergic synaptic excitability. LTP is crucial for spatial recognition from early life (Ashby et al., 2021) and when evaluated at PND40, it was significantly enhanced in ED- and AMED-, but not EtOH-treated rats compared to controls, while there was a marked decrease in LTP in EtOH-, ED- and AMED-treated rats at PND60 and PND90, suggestive of a profound alteration in

glutamatergic plasticity due to the administration of these beverages during adolescence. The results obtained at PND40, showing an increase in LTP in ED- and AMED-treated rats, are consistent with our measurements of BDNF levels, which are known to play a crucial role in learning and memory processes by facilitating synaptic plasticity and LTP formation in the hippocampus (Bekinschtein et al., 2014; Miranda et al., 2019). In a previous study (Martín and Buño, 2003), caffeine was found that to increase glutamate release in the CA1 region, by interacting with presynaptic P1 and P2 purinergic receptors and ryanodine receptors and to produce a form of presynaptic LTP ( $CA_1$ LTP) that does not require NMDA receptor activation or high-frequency stimulation of Schaffer collaterals. Thus, it cannot be excluded that the mechanisms described by Martín and Buño contribute to the increase in LTP observed in ED- and AMED-treated PND40 rats tested shortly after treatment. Nevertheless, it is important to point out that such a presynaptic effect of caffeine on glutamate release should have been detected in the input-output curves in which presynaptic glutamate release was electrically evoked, but no significant change was observed in treated animals compared to controls. Thus, the mechanism underlying the increase in LTP in PND40 rats treated with ED and AMED requires further investigation. In contrast, LTP was found to be consistently reduced in both PND60 and PND90 rats that were treated with EtOH, ED, or AMED compared to controls. These effects appear consistent with the changes in the BDNF and trkB levels discussed below. Accordingly, chronic caffeine consumption produced a marked reduction of hippocampal LTP in freely moving rats (Blaise et al., 2018), an effect that could be consequential in the upregulation of adenosine receptors as compensation for caffeine's blockade of adenosine. The same authors also hinted at a caffeine-induced elevation of superoxide dismutase that, in turn, may regulate negatively hippocampal LTP. In addition, another possible mechanism suggested by the same authors consists of the caffeine ability to induce a downregulation of BDNF, which may disrupt synaptic plasticity in hippocampal neuronal networks. Our electrophysiological results are also in line with reviewed studies (Avshalumov and Mandyam, 2020) in the hippocampus, showing that both acute and chronic EtOH exposure, are capable of dysregulating glutamatergic transmission, producing consistent impairments in long-term synaptic plasticity and hippocampal-dependent learning and memory functions that may be the result in regulation of gene expression. Accordingly, ED and AMED groups revealed age-related specific changes in BDNF and trkB protein levels, while EtOH affected the trkB levels at PND60 only. Nevertheless, *Bdnf* transcription and translation were not affected in the same way, but this is not surprising and is consistent with the complex regulation of this gene (You and Lu, 2023). Thus, EtOH during development may have differential effects on the expression of *Bdnf* (Boschen et al., 2015; Davis, 2008; Scheidt et al., 2015; Shojaei et al., 2015) in the rat hippocampus, and further investigations are required to fully understand the complex *Bdnf* regulation in our experimental model. For example, the measurement of the different transcripts may help to understand the mechanisms since we now measured only the coding region. Therefore, the possibility exists that expression of the different splice variants of the non-coding region with the 13 different transcripts, located in distinct neuronal districts, could be differently regulated at the translational level. In fact, ED treatment induced age-related changes that go opposite directions for BDNF and trkB, with an apparent classical ligand-induced receptor downregulation, and a general boosting effect of ED alone or AMED on the BDNF regulation. Thus, at PND40, i.e., just 3 days after drug administration, BDNF and trkB proteins are not affected by EtOH alone, underlying the possibility of adaptive modifications induced by the main active components of ED, such as caffeine and taurine, as we also speculated above for behavioral and functional data. By contrast, at PND60, i.e., 20 days after drug administration, decrements of BDNF and trkB in the ED, and AMED groups, and EtOH-induced trkB downregulation occurred. This may suggest that, in the first 20 days after the end of treatment a remodeling of the BDNF/trkB system, contemplating a lower release of BDNF

suitable to balance the downregulation of trkB receptor, could occur, in agreement with the increase in LTP magnitude. This interpretation may suggest that the exposure to ED, EtOH, and AMED influences the activity-dependent-BDNF secretion, by enhancing it at PND40 (except EtOH group) and depressing it after 20 days of drug washout, with LTP impairment that persists up to 30 days after the end of treatment at PND90. A previous study on the effects of ED alone or AMED on the hippocampal BDNF and trkB showed that chronic ED treatment of young adult rats was detrimental to the maintenance of cerebral cortex structure and associated with decreased BDNF levels (Sayed, 2021). Different studies report effects of caffeine (Ardais et al., 2014; Connolly and Kingsbury, 2010; Costa et al., 2008; Diógenes et al., 2007, 2011; Han et al., 2013; Mioranza et al., 2014; Sallaberry et al., 2013) and taurine (Caletti et al., 2015) on BDNF-induced neuronal function. In particular, *in vivo* studies show that chronic caffeine has different dose-dependent effects on the BDNF trophic system in the brain of adolescent rats (Ardais et al., 2014); thus, doses of moderate/high caffeine solutions, at 1 mg/mL concentration) decrease the BDNF levels in the hippocampus, while moderate concentrations (0.3 mg/mL) increase the BDNF levels in the cerebral cortex; by contrast, no caffeine-induced effect on TrkB was observed.

Our data also suggest that the hypothalamic-pituitary-adrenal axis activity is not affected by AMED. No significant alterations in plasma corticosterone levels after EtOH, ED or AMED treatment measured at PND40, PND60, and PND90. With respect to EtOH, this result was partly unexpected given that several findings indicate that acute or chronic binge EtOH differentially alters corticosterone plasma/serum levels, even though differences in doses, administration protocols, age, sex, and species could account for the dissimilar results. Another important difference is the elapsed time between the treatment end and the corticosterone assessment: 3, 23 and 53 days in our study vs. hours in the majority of the studies. To the best of our knowledge, no data are available about the effect of ED on plasma corticosterone. By contrast, chronic treatment with caffeine or taurine has been shown to decrease or increase, respectively, plasma corticosterone (Ryu and Roh, 2019; Sajid et al., 2017). Hence, the effects of their combination, with the other ingredients in the ED might result in a lack of changes in corticosterone levels.

#### 4.3. Conclusions

In summary, starting from the assumption that binge-like consumption of EtOH as well as ED during adolescence, may cause impaired cognitive, behavioral impairments, and changes in hippocampal plasticity which persist in adulthood, it was crucial to deeply evaluate the effect of early adolescent binge-like drinking administration of EtOH, ED or AMED in young and adult rats. Our results show that AMED consumption during the peri-adolescent period produces adaptive hippocampal changes at the electrophysiological and molecular levels, associated with behavioral alterations, which are already detectable during adolescence and persist in adulthood. This conclusion is strengthened by the long-lasting impairment of prefrontal cortical function disclosed by the identical protocol of binge-like AMED administration of our previous study (Dazzi et al., 2024). Overall, the analysis of the whole set of data obtained strongly suggests that AMED, during adolescence, may have upshots that are not necessarily the sum of those observed with EtOH or ED alone and permanently affect hippocampal plasticity. However, it must be kept in mind that these results may be limited in interpretation considering sex differences. Although the analysis of putative sex differences deserves attention, it was beyond the scope of our study and would have introduced the additional variable of hormonal fluctuations during the estrous cycle. It is well known that gonadal hormones and their neuroactive metabolites modulate various neurotransmission systems involved in the response to EtOH and contribute to sex differences in the effects of alcohol on the central nervous system (Finn, 2023). Therefore, their fluctuations during the

estrous cycle could contribute to greater variability and the need to use a greater number of animals for experimentation in order to have a precise evaluation that can be considered in future studies.

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## CRediT authorship contribution statement

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## Declaration of competing interest

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

## Data availability

Data will be made available on request.

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