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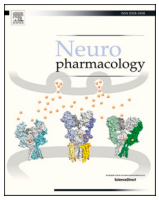

Binge-like administration of alcohol mixed to energy drinks to male adolescent rats severely impacts on mesocortical dopaminergic function in adulthood: A behavioral, neurochemical and electrophysiological study

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ABSTRACT

A growing body of evidence indicates that the practice of consuming alcohol mixed with energy drinks (ED) (AMED) in a binge drinking pattern is significantly diffusing among the adolescent population. This behavior, aimed at increasing the intake of alcohol, raises serious concerns about its long-term effects. Epidemiological studies suggest that AMED consumption might increase vulnerability to alcohol abuse and have a gating effect on the use of illicit drugs.

The medial prefrontal cortex (mPFC) is involved in the modulation of the reinforcing effects of alcohol and of impulsive behavior and plays a key role in the development of addiction. In our study, we used a binge-like protocol of administration of alcohol, ED, or AMED in male adolescent rats, to mimic the binge-like intake behavior observed in humans, in order to evaluate whether these treatments could differentially affect the function of mesocortical dopaminergic neurons in adulthood. We did so by measuring: i) physiological sensorimotor gating; ii) voluntary alcohol consumption and dopamine transmission before, during, and after presentation of alcohol; iii) electrophysiological activity of VTA dopaminergic neurons and their sensitivity to a challenge with alcohol. Our results indicate that exposure to alcohol, ED, or AMED during adolescence induces differential adaptive changes in the function of mesocortical dopaminergic neurons and, in particular, that AMED exposure decreases their sensitivity to external stimuli, possibly laying the foundation for the altered behaviors observed in adulthood.

1. Introduction

Alcohol is one of the most consumed and harmful drugs worldwide (Morgan et al., 2013), and its toxic effects considerably increase when it is heavily consumed during adolescence (Brocato and Wolstenholme, 2021). Epidemiological studies from different countries are increasingly reporting that alcohol is frequently mixed with alcohol-free recreational beverages containing high dosages of caffeine, other stimulants such as ginseng and guaranà, taurine, glucose, and vitamins, i.e. Energy Drinks (EDs), in an attempt to attenuate the sedative effect of alcohol and/or reach higher level of intoxication in shorter periods of time (O'Brien et al., 2008; Higgins et al., 2010). The content of such ingredients significantly differs between the numerous brands available in the market; thus, as an example, caffeine content may range from 50 to 500 mg/330 ml can (Patrick and Maggs, 2014).

The consumption of EDs on their own has grown exponentially worldwide, especially among adolescents (Reissig et al., 2009), and in parallel, has risen the risk of consuming alcohol mixed with them (Alcohol Mixed Energy Drinks, AMED) as supported by a significant increase of the admissions in the Emergency Room of drinkers often

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underage, with high level of intoxication (Cleary et al., 2012). AMED consumption peaks over the weekends and founds its roots in, and is boosted by, the false belief that EDs are safe and could prevent some of the adverse effects of alcohol, allowing its consumption at higher and intoxicating amounts (Marczinski and Fillmore, 2006, 2014; Arria et al., 2011). However, EDs though shown to increase alcohol intake and shorten the time to reach intoxication and alcohol-induced sedation (Thombs et al., 2011), fail to alter the Breath Alcohol Concentration (BRAC) as well as the alcohol-induced motor impairments (Ferreira et al., 2006; Marczinski and Fillmore, 2006, 2014). In addition, when compared with alcohol-only consumers, AMED consumers are reported to engage more frequently in risky behaviors (Mallett et al., 2014, 2015; Miller, 2012; Thombs et al., 2010; Snipes and Benotsch, 2013), possibly due to an increased impulsive attitude coupled to lower behavioral inhibition, that makes them more likely to pursue dangerous situations such as driving when drunk, having unprotected and/or promiscuous sex or consuming illicit drugs. Moreover, longitudinal studies also suggest that AMED consumption during adolescence can increase alcohol intake and its potential for abuse during adulthood (Arria et al., 2011; Miller, 2008a,b).

The mesocorticolimbic dopaminergic pathway is part of the reward and motivation circuitry that has been proven to be crucial in the modulation of impulsive behavior and all the stages of addiction (Kalivas and Volkow, 2005; Koob and Volkow, 2010; Jentsch et al., 2014). Accordingly, an imbalanced function of this neural circuit has been associated with increased impulsivity, loss of control associated with compulsive drug seeking and intake, and the development of addiction (Jentsch et al., 2014; Koob et al., 2014). In this scenario, the medial prefrontal cortex (mPFC) is thought to play a pivotal role in driving the maladaptive changes that characterize the dynamics of addiction. In particular, this takes place through a hyper-responsivity to drug-associated stimuli combined with the loss of control on the hijacked mesolimbic activity that underlies drug-seeking behavior (Kalivas and Volkow, 2005). Accordingly, dysregulation in the function of the mPFC could be considered an early index of neuroadaptation in alcohol addiction (Koob et al., 2014).

Interestingly, despite a robust literature on the clinical and epidemiological evidence on AMED effects (McKetin et al., 2015; Roemer and Stockwell, 2017; Peacock et al., 2013; Acquas et al., 2023), the experimental approach to model AMED consumption, especially from a developmental perspective from adolescence to adulthood, still suffers from the limitation that most of the studies were set to investigate the consequences of the combined administration of caffeine and alcohol without taking into account the peculiarities of AMED consumption that refer to *i)* adolescent binge-like episodic intake of *ii)* whole energy drinks in combination with drinks with high alcohol content (Petribu et al., 2023).

Based on these premises, this study attempted to accomplish multiple targets. Firstly we evaluated whether exposure of male Sprague-Dawley rats during adolescence (postnatal days 28–37) to high, intoxicating, doses of alcohol or to a commercially available energy drink (RedBull®), or to their combination (AMED) under a binge-like protocol of administration (BLPA) could impact on physiological sensorimotor gating in adulthood, as an index of precognitive mPFC-dependent functioning. Secondly, in separate cohorts of animals, we studied the effects of BLPA on voluntary alcohol consumption in adulthood and dopamine (DA) transmission responsivity in the mPFC assessed by *in vivo* brain microdialysis at different stages (anticipation, presentation, and post-removal) of the protocol of voluntary consumption. Finally, we studied in VTA DA neurons of adult rats exposed during adolescence to the BLPA, the basal electrophysiological activity and sensitivity to an acute challenge with alcohol.

2. Experimental procedures

2.1. Animals

Male Sprague-Dawley CD rats (Charles River, Como, Italy) were bred in the animal facility of the University of Cagliari (CeSASt) 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 ± 2 °C and relative humidity of 65%. The animals had *ad libitum* access to water and standard laboratory food. According to the 3R principles governing animal experimentation, all possible efforts were made to minimize animal suffering and 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 protocol of administration (BLPA)

At weaning [Post Natal Day (PND) 21], the animals were housed in groups of 4 per cage, randomly divided into four experimental groups, and subjected to an administration protocol aimed at mimicking a previously validated binge-like drinking protocol (Coleman et al., 2011). In particular, male adolescent rats were administered via gavage (volume of administration: 10 ml/kg of body weight) as follows: 1) tap water (**CTRL**); 2) 3.2 g/kg of alcohol (20% solution, v/v) (**EtOH**); 3) energy drink (2 CanEq of Red Bull®/kg, i.e., the equivalent of 2 cans for a 70 kg individual) (**ED**); or 4) a combination of alcohol and Red Bull® (3.2 g/kg of alcohol and 2 CanEq of Red Bull®) (Alcohol Mixed Energy Drink, **AMED**). Animals were treated from PND 28 to PND 37 once a day with an intermittent protocol (2 days ON at PND 28–29, 32–33, and 36–37, and 2 days OFF at PND 30–31, 34–35, and 38–39. The dose of alcohol was chosen based on the protocol described by Coleman et al. (2011) and adapted for the rat; it corresponds, according to the conversion calculation of Nair and Jacob (2016), to a human equivalent dose of 0.52 g/kg. For a 70 kg subject, this dose equals 36.4 g of pure alcohol, corresponding to 2.6 standard USA drinking units.

Behavioral, neurochemical, and electrophysiological experiments were carried out between PND40 and PND90 in dedicated cohorts of animals as described in Fig. 1.

Red Bull (Red Bull Energy Drink®) was chosen as the energy drink of reference for the study and its composition was as follows: water, caffeine (80 mg/250 ml), B-group vitamins [niacinamide (vitamin B3), pantothenic acid (vitamin B5), vitamin B6, and vitamin B12 in not declared amounts], taurine (1 g/250 ml), sugars (11 g/100 ml) (http://www.redbull.com/int-en/energydrink/red-bull-energy-drink-ingredients-list).

2.3. Prepulse inhibition (PPI) test

On the test day (PND 40 or 60 or 90), rats were transferred to their home cages in a testing room under controlled environmental conditions. PPI testing was performed with minor modifications as previously described (Noli et al., 2017). The apparatus used for the detection of startle reflexes (Med Associates, St Albans, VT, USA) consisted of four standard cages placed in sound-attenuated chambers with fan ventilation. Each cage consisted of a Plexiglas cylinder of 9 cm diameter, mounted on a piezoelectric accelerometric platform connected to an analog/digital converter. Two separate speakers conveyed background noise and acoustic bursts. Both speakers and startle cages were connected to a main computer, which detected and analyzed all chamber variables with a dedicated software. Before each testing session, acoustic stimuli and mechanical responses were calibrated. The test began after an acclimatization phase (5 min), with a 70 dB background white noise,

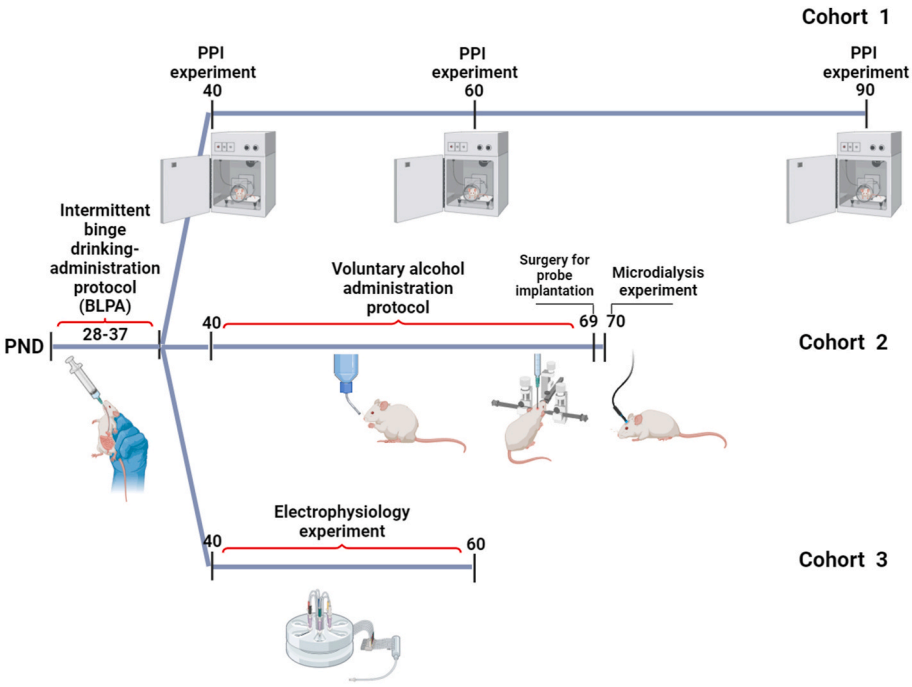

Fig. 1. Experimental timeline and cohorts used for behavioral, neurochemical, and electrophysiological assessment. After the binge-like protocol of administration (BLPA) during adolescence (from PND 28 to PND 37, see Material and Methods section), rats from the four treatment groups (i.e., CTRL, EtOH, ED or AMED) were divided into three different Cohorts and dedicated to the behavioral, neurochemical or electrophysiological experiments. In detail, rats of Cohort 1 were used for the PPI experiments performed at PND 40, PND 60 and PND 90. Rats of Cohort 2 were used for the studies on voluntary alcohol intake (from PND 40 to PND 68) that ended with the microdialysis surgery (PND 69) and experiment in the mPFC at PND 70. Finally, rats of the Cohort 3 were sacrificed between PND 40 and PND 60, and brain slices containing the VTA used for the electrophysiological experiments.

continuing for the entire session. This period was followed by three blocks, comprising five pulse-alone trials of 130 dB (the first and the third), while the second includes a pseudorandom sequence of 50 trials, containing 12 pulse-alone trials, 30 trials of pulse preceded by 68-, 70-, or 80-dB pre-pulses (10 for each pre-pulse loudness level), and eight no-pulse trials, where exclusively the background noise was delivered. Time between two consecutive trials (inter-trial intervals) was arbitrarily selected between 10 s and 15 s. The % PPI was measured through the formula: % PPI = (100—mean startle amplitude for pre-pulse trials/mean startle for pulse alone trial) X 100.

2.4. Alcohol voluntary consumption protocol

Right after the BLPA, and starting at PND 40, all experimental groups were exposed to a four week-long session of voluntary alcohol consumption in which a bottle containing an alcohol solution was made available for voluntary consumption daily for 2 h (from 11:00 to 13:00 h). To instigate alcohol consumption without recurring to food or fluid deprivation, we used a procedure that involved sucrose in the alcohol solution, as previously described (Lallai et al., 2016). Sucrose concentration was progressively decreased, keeping constant that of alcohol, according to the following paradigm: (days 1–2) 5% (v/v) alcohol + 5% sucrose; (days 3–4) 5% alcohol+ 4% sucrose; (days 5–6) 5% alcohol + 3% sucrose; (days 7–8) 5% alcohol + 2% sucrose; from day 9 the composition of the solution was kept constant at 5% alcohol (v/v) and 1% sucrose until the end of the treatment. Animals of all experimental groups were placed in individual cages for the 2 h of daily exposure to alcohol to allow a precise measure of alcohol consumption. Water was available *ad libitum* for all the animals for the remaining 22 h. At the end of each daily session, the animals' body weight , the amount of fluid (water and alcohol), and food intake were monitored.

2.5. Surgery and brain microdialysis experimental procedures

At the end of the voluntary alcohol consumption protocol, at PND 69, rats were anesthetized with isoflurane, and a concentric dialysis probe was inserted at the level of the mPFC (A +3.2, ML +0.8, V −5.3 relative to the bregma), according to the rat brain atlas (Paxinos and Watson, 2004). The active length of the dialysis membrane was restricted to 4 mm, allowing sampling from infralimbic and prelimbic cortices (Dazzi et al., 2014). Experiments were performed in freely moving rats 24 h after probe implantation (PND 70) to allow recovery from surgical procedures. Ringer's solution [3 mM KCl, 125 mM NaCl, 1.3 mM $CaCl_2$, 1 mM $MgCl_2$, 23 mM $NaHCO_3$, 1.5 mM potassium phosphate (pH 7.3)] was pumped through the dialysis probe at a constant rate of 2 μl/min. Dialysate samples were collected every 20 min from 8:30 to 15:00 h. To avoid that different amounts of alcohol voluntarily assumed by the animals might differentially affect DA extracellular concentration during the experiment, at 11:00 h, when the animals were supposed to receive alcohol, an empty bottle was presented as a stimulus to all the experimental groups. Samples were immediately analyzed for DA by high-performance liquid chromatography (HPLC) with electrochemical detection as previously described (Dazzi et al., 2002). The detection limit for DA was 2 fmol per injection. The average concentration of DA in the first two samples was taken as 100% (basal), and all subsequent values were expressed as mean ± SEM relative to the basal value. All probes were tested for DA recovery before implantation, and those with a recovery value outside a mean range of 15 ± 3% were not used. The absolute concentration of DA was not corrected for this value. The correct placement of each probe was verified histologically at the end of the experiment.

2.6. Preparation of VTA slices

Brain slices were prepared as previously described (Talani et al., 2016). In brief, starting at PND40 up to PND60, animals were

decapitated under 5% isoflurane deep anesthesia. Brains were rapidly removed from the skull and transferred into a modified artificial cerebrospinal fluid (aCSF) solution containing (in mM): 220 sucrose, 2 KCl, 0.2 $CaCl_2$, 6 $MgSO_4$, 26 $NaHCO_3$, 1.3 NaH_2PO_4, and 10 D-glucose (pH 7.4, set by aeration with 95% O_2 and 5% CO_2). Horizontal brain slices containing the VTA (thickness, 260 μm) were cut in ice-cold modified aCSF with the use of a Leica VT1200S vibratome (Leica, Heidelberg, Germany). The slices were then transferred immediately to a nylon net submerged in standard aCSF containing (in mM): 126 NaCl, 3 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 26 $NaHCO_3$, 1.25 NaH_2PO_4, and 10 D-glucose (pH 7.4, set by aeration with 95% O_2 and 5% CO_2). After incubation for at least 40 min at the controlled temperature of 35 °C and a subsequent waiting for at least 1 h at room temperature, hemi-slices were transferred to the recording chamber and continuously perfused with standard aCSF at a constant flow rate of ~2 ml/min. For all recordings, the temperature of the bath was maintained at 33 °C.

2.7. Patch-clamp recordings

Patch-clamp recordings from VTA dopaminergic neurons were performed as previously described (Dazzi et al., 2014). Recording pipettes were prepared from borosilicate capillaries with an internal filament using a P-97 Flaming Brown micropipette puller (Sutter Instruments, Novato, CA, USA). Resistance of the pipettes ranged from 4.5 to 6.0 MΩ when they were filled with the following solution (in mM): 135 potassium gluconate, 10 $MgCl_2$, 0.1 $CaCl_2$, 1 EGTA, 10 Hepes-KOH (pH 7.3), and 2 ATP (disodium salt). Signals were recorded using of an Axopatch 200-B amplifier (Axon Instruments Inc., San Jose, CA, USA), filtered at 2 kHz, and digitized at 5 kHz. The pClamp 9.2 software (Molecular Devices, Union City, CA, USA) was used to measure and analyze the firing rate and other membrane kinetic parameters of VTA neurons and the occurrence of HCN-mediated I_h currents (see below). The cell-attached configuration was used for monitoring the spontaneous firing rate in the control condition (baseline) and during and after 60 mM EtOH application. After obtaining a pipette-membrane seal with a GΩ resistance, at least 10 min were allowed prior recording to have a stable and regular baseline firing rate. At the end of each recording, the whole-cell configuration was obtained to determine the presence of I_h currents to confirm the identity of VTA DA neurons. In fact, as also previously reported (Grace and Onn, 1989; Margolis et al., 2006), DA neurons in the VTA were identified by the presence of both a robust I_h current evoked in response to a single hyperpolarizing voltage step from −65 to −115 mV and a regular firing rate of spontaneous action potentials.

2.8. Statistical analyses

All data are reported as mean ± SEM. Before running the ANOVA analyses, normal data distribution was evaluated by inspection of skewness and kurtosis, and homoscedasticity by the Bartlett test. For the PPI experiment, data of 7–8 rats/group were analyzed by two-way ANOVA for repeated measures with the treatment as between groups factor and time points (i.e., age of rats, PND 40, 60 and 90) as within-subjects factor. Because no significant differences in PPI responses to the three different pre-pulse intensities were detected, data from the three different pre-pulse intensities were put together to calculate individual averaged values for each rat which were used for statistical analyses and reported in Fig. 2.

For voluntary alcohol intake and microdialysis experiments, comparisons among experimental groups (at least 9 rats/group) have been performed by two-way ANOVA for repeated measures, with the Treatment as between groups factor and Time points as within-subjects factor. For voluntary alcohol intake and microdialysis experiments, the raw values of alcohol consumption (g/kg/session) and DA concentrations (see Results), respectively, were used for statistical analysis.

When significant differences in the variances of a data set were found, data were analyzed with ANOVAs and Geisser-Greenhouse correction.

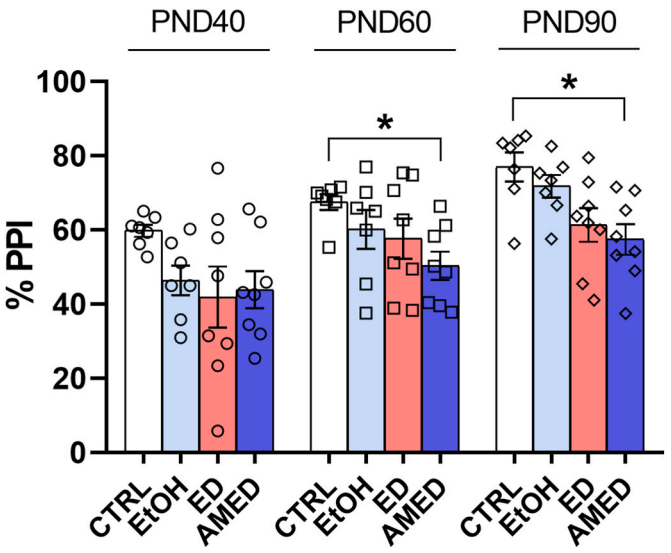

Fig. 2. Effect of BLPA in adolescence with EtOH, ED or AMED on pre-pulse inhibition of the startle response in adulthood. Rats underwent an intermittent BLPA with water (CTRL), alcohol (EtOH), ED, or AMED between PND 28 and PND 37 and thereafter were tested at PND40, 60 and 90 for sensorimotor gating function (for details see the Material and Methods section). Data are expressed as percentage and are mean ± SEM of 7–8 rats/group. Individual averaged values of the three different pre-pulse intensities are reported for each rat (see Material and Methods section). *p < 0.05 compared to age-matched control group (CTRL).

For electrophysiological recordings, comparisons among experimental groups (at least n = 4 rats/group) have been performed by one-way analysis of variance (ANOVA) or *t*-Test where indicated.

Post-hoc comparisons were performed with Bonferroni's corrected pairwise contrasts in the case of PPI, microdialysis and alcohol intake data and with the Newman-Keuls test for electrophysiology data.

All the statistical analyses were performed using PRISM, Graph Pad 8 Software (San Diego, SA, USA), and the significance level was set for all the experiments at $p < 0.05$.

3. Results

3.1. Effects of BLPA during adolescence on PPI in adulthood

This experiment aimed to investigate if a BLPA with alcohol, ED, or their combination (AMED) applied in adolescence was able to affect the sensorimotor gating by using the PPI paradigm.

As shown in Fig. 2, the BLPA with alcohol, ED, or AMED applied to adolescent rats from PND 28 to PND 37 induced, at different stages of adulthood (PND 40, 60, and 90), significant age- and treatment-dependent modifications of the PPI. Accordingly, repeated measures two-way ANOVA detected a significant effect of Time [$F_{(1.52,\ 39.54)} = 16.65$; $p < 0.0001$] and of Treatment [$F_{(3,\ 26)} = 7.55$; $p < 0.0009$], but not a significant Time × Treatment interaction [$F_{(6,\ 52)} = 0.44$; $p > 0.05$]. Moreover, pairwise comparisons detected a significant difference between **CTRL** and **AMED** groups both at PND 60 (67.50 ± 2.10 vs. 50.30 ± 3.81, $p < 0.02$) and PND 90 (76.97 ± 3.94 vs. 57.42 ± 4.09, $p = 0.02$), indicating a poorer performance of the AMED group compared to vehicle-treated rats (see Fig. 2 for single points of statistical significance). A similar trend was also observed for **EtOH** and **ED** groups, but in both cases, the comparison with the control group did not reach statistical significance.

3.2. Effects of BLPA during adolescence on alcohol voluntary consumption in adulthood

To evaluate whether exposure to alcohol, ED, or AMED by the BLPA during adolescence might affect voluntary alcohol consumption in adulthood, we exposed the animals of all experimental groups to daily sessions of a voluntary alcohol drinking as previously reported (Lallai et al., 2016).

The body weight of the animals from the different experimental groups was monitored throughout the protocol. Two-way ANOVA revealed a non significant effect of the experimental group [$F(_{3,24}) = 0.8536$; $p = 0.4784$], a significant effect of time [$F(_{2.257,54.16}) = 1971$; $p < 0.0001$], and a non significant interaction between factors [$F(_{18,144}) = 0.4670$; $p = 0.9680$] suggesting that the animals from the different experimental groups increased their body weight over time with no differences due to the treatment received during adolescence.

For the training period (Fig. 3 Panel A) two-way ANOVA revealed a significant main effect of the experimental group [$F(_{3,95}) = 10.55$; $p < 0.0001$], a significant main effect of time [$F(_{2.234,212.2}) = 34.78$; $p < 0.0001$], and a significant interaction between factors [$F(_{27,855}) = 2.716$; $p < 0.0001$]. In particular, during the training period (from day 1 to day 10; Panel A), animals from all the experimental groups showed their highest consumption of the alcohol solution, possibly due to the presence of sugar. It is noteworthy that the rats from the different experimental groups showed different drinking behavior; animals from the **EtOH** group consumed a significantly lower amount of alcohol from day 2 to day 10 of training (1.18 ± 0.34 g/kg compared to an average intake of 2.05 ± 0.30 g/kg of the other experimental groups, p values ranging from 0.0438 to <0.0001), while **ED** animals showed the highest intake particularly in the first 4 days, when the concentration of sugar in the solution was the highest (3.48 ± 0.52 g/kg; $p = 0.0117$ for Day1) (see Fig. 3 Panel A for single points of statistical significance).

For the period after training (Fig. 3, Panel B), two-way ANOVA revealed a significant main effect of the experimental group [$F(_{3,95}) = 5.735$; $p = 0.0012$], a significant main effect of time [$F(_{8.252,783.9}) = 3.817$; $p = 0.0002$], and a significant interaction between factors [$F(_{51,1615}) = 2.223$; $p < 0.0001$]. The intake of alcohol tended to be more stable over days in all the experimental groups. Rats from the **EtOH** group still showed a tendency to drink less alcohol than CTRLs although the difference was not statistically significant except for the first two days ($p = 0.0045$ Day 1; $p = 0.0067$ Day 2; Day 3–10: p ranging from 0.2797 to >0.999). Interestingly, rats of the **AMED** group showed a significantly greater voluntary intake of alcohol from Day 13 to the end of the experiment (1.87 ± 0.23 g/kg vs. 0.89 ± 0.10 g/kg of control rats; p values ranging from 0.0002 to 0.112) (see Fig. 3 Panel B for single points of statistical significance).

3.3. Effects of BLPA during adolescence on mPFC DA in adulthood

We have previously shown that activation of mesocortical dopaminergic neurons negatively correlates with alcohol consumption, consistent with the role of mPFC DA in controlling compulsive behavior (Lallai et al., 2016). Therefore, to evaluate whether exposure of adolescent rats to alcohol, ED, or AMED under a BLPA might alter the sensitivity of mesocortical dopaminergic neurons to voluntary alcohol intake anticipation, alcohol presentation and after its removal, at the end of the voluntary alcohol intake protocol (28 days treatment, at PND 70) we measured the extracellular concentration of DA in the mPFC of all the animals in basal conditions and during the 2-h periods before alcohol presentation, during bottle presentation and after its removal.

As for basal concentrations, one-way ANOVA revealed a non significant difference between the experimental groups [$F(3,28) = 0.4946$; $P = 0.6889$] suggesting that prolonged voluntary alcohol intake did not significantly modify the basal extracellular concentration of DA in the mPFC of rats in the **CTRL, EtOH**, **ED** or **AMED** groups (Table 1).

At variance, when analyzing the sensitivity of mesocortical dopaminergic neurons to alcohol presentation, two-way ANOVA revealed a non significant effect of experimental groups [$F(_{3,28}) = 0.4454$; $p = 0.7225$], a significant main effect of time [$F(_{18,\ 504}) = 8.611$; $p < 0.005$], and a significant interaction between factors [$F(_{54,504}) = 4.806$; $p < 0.0001$]. As shown in Fig. 4, in fact, in the **CTRL** group, DA extracellular concentration increased as early as 80 min before alcohol presentation (+90% over basal values), remaining significantly elevated when the bottle was available (+100%), and returning to basal values 60 min after its removal (p values ranging from 0.035 to 0.02). In the **EtOH** group,

Table 1
Basal extracellular concentration of DA in the mPFC of rats exposed to a BLPA during adolescence of water or alcohol or RedBull® or alcohol mixed with RedBull®.

Experimental Group	CTRL	EtOH	ED	AMED
DA concentration (pmol/sample)	1.640 ± 0.195	1.465 ± 0.282	1.516 ± 0.405	1.559 ± 0.577

Male rats, after undergoing an intermittent BLPA with water (CTRL), alcohol (EtOH), RedBull (ED), or alcohol mixed with RedBull (AMED) between PND 28 and PND 37, were trained in a protocol for voluntary intake of alcohol for 28 days starting on PND 40, then tested for DA extracellular content in the mPFC at PND 70. Data are expressed in pmol of DA/20 min sample and are mean ± SEM of 8 rats/group.

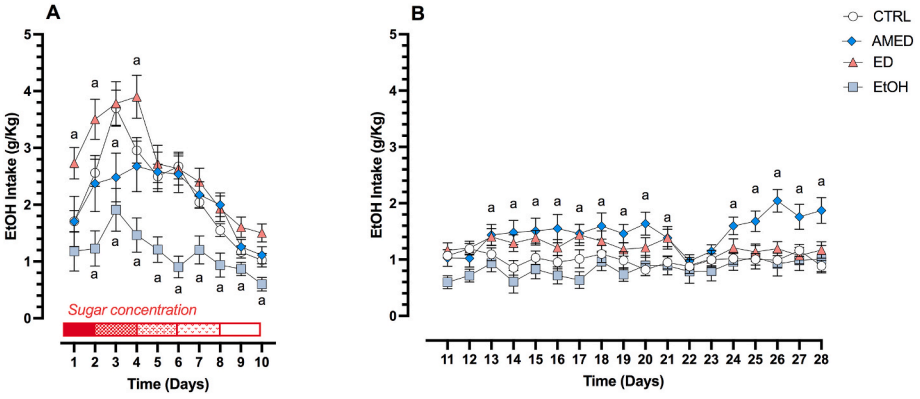

Fig. 3. Effect of BLPA in adolescence with alcohol, ED or AMED on the voluntary consumption of alcohol in adulthood. Rats underwent an intermittent BLPA with water (CTRL), alcohol (EtOH), ED, or AMED between PND 28 and PND 37. From PND 40 to PND 50 (training period; Panel A) they were trained to self-administer an alcohol solution (5% v/v) containing a decreasing amount of sugar (see Methods for details) that was made available for 2 h a day (from 11.00 to 13.00). During (PND 40–50) and after training (PND 51–68; Panel B) alcohol intake was measured daily (for details see Methods). Data are expressed in g of alcohol/kg of body weight and are mean ± SEM of 7–8 rats/group. [a]$p < 0.05$ vs. the time-matched point of CTRL.

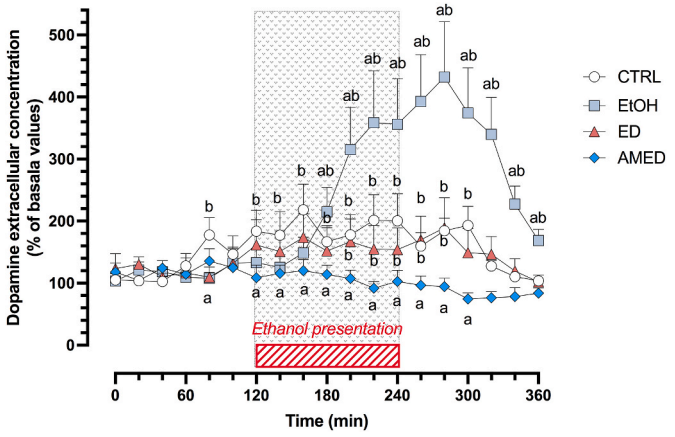

Fig. 4. Effect of BLPA in adolescence with alcohol, ED or AMED and alcohol voluntary consumption in adulthood on mPFC DA transmission, before, during and after alcohol presentation in adulthood. Male rats underwent an intermittent BLPA with water (CTRL), alcohol (EtOH), ED, or AMED between PND 28 and PND 37. From PND 40 they were trained to self-administer an alcohol solution (5% v/v) that was made available for 2 h a day (from 11.00 to 13.00). At the end of the alcohol voluntary intake protocol each animal underwent surgery for the insertion of a microdialysis probe at the mPFC and, on the following day, DA extracellular concentration was measured in the 2h preceding alcohol presentation (anticipation), during the 2 h in which the bottle was presented (presentation) and in the 2 h after bottle removal (post-removal) (for details see the Material and Methods section). Data are expressed as percentage of basal values and are mean ± SEM of 7–8 rats/group. [a]$p < 0.05$ vs. the time-matched point of CTRL; [b]$p < 0.05$ vs. basal values.

extracellular DA concentration increased when the bottle was presented (+50% over basal values), showed a further increase 60 min after bottle presentation, and raised even more consistently after its removal (+200% and +350% over basal values, respectively) (p values ranging from 0.0366 to 0.0050). Animals of the **ED** group showed a non significant increase (+30% over basal values) in extracellular DA only during alcohol anticipation (p values ranging from 0.2207 to 0.6407). At the same time, this parameter did not significantly change with respect to basal values when the bottle was available. In the **AMED** group, on the contrary, DA extracellular concentration did not change significantly during anticipation or presentation of the bottle nor after its removal (all $p > 0.9999$) (see Fig. 4 for single points of statistical significance).

3.4. Effects of BLPA during adolescence on basal and alcohol-stimulated firing rate of VTA DA neurons in adulthood

Electrophysiological experiments were aimed at evaluating the effects of BLPA of water (**CTRL**), alcohol (**EtOH**), RedBull® (**ED**) or alcohol + RedBull® (**AMED**), during adolescence, on basal activity and responsiveness to alcohol of VTA DA neurons from adult rats. Identification of single DA neurons in acute mesencephalic slices containing the VTA was accomplished by confirming, in agreement with previous studies (Grace and Onn, 1989; Margolis et al., 2006; Ungless and Grace, 2012), the presence of both HCN-mediated I_h currents evoked by hyperpolarizing the cell membrane from −65 to 115 mV (amplitude, −185 ± 43 pA, n = 15) (data not shown) and a regular spontaneous firing activity (4.2 ± 0.7 Hz, n = 15, Fig. 5A and B). As shown in Fig. 5A and B, the basal spontaneous firing rate of DA neurons was not significantly different when compared between experimental groups [$F_{(3,28)} = 0.2659$; $p = 0.8494$].

Subsequently, we tested whether the different treatments during adolescence may have an impact on the well-known (Bassareo et al., 2019; Brodie et al., 1990; Brodie and Appel, 1998; Xiao and Ye, 2008; Xiao et al., 2009) capability of alcohol to increase the firing rate of VTA DA neurons when perfused acutely in the slice. As expected, perfusion of VTA slices with 60 mM EtOH for 5 min elicited a significant increase above baseline (62 ± 15.3%; *t*-Test, t = 4.050, df = 6, p = 0.007, n = 7) in the firing rate in slices from rats of the **CTRL** group (Fig. 5C–E), but not in the other treatment groups where such stimulatory action of alcohol showed a trend toward reduction in slices from **EtOH** (42 ± 15.1%, n = 11) or **ED** groups (16 ± 9.7%, n = 6), though not reaching statistical significance. On the contrary, the stimulatory effect of alcohol was entirely abolished in VTA DA neurons from rats of the **AMED** group (2 ± 4.4 %, n = 6). This difference among treatment groups in response to the EtOH challenge was also confirmed by one-way ANOVA [$F_{(3,26)} = 3.354$; $p = 0.0341$] that detected a significant difference in the percent of response (i.e., variation above baseline) after the EtOH challenge between the **CTRL** and the **AMED** groups ($p = 0.037$) but not between the **CTRL** and **EtOH** ($p = 0.69$) or the **CTRL** and **ED** groups ($p = 0.15$) (Fig. 5E).

4. Discussion

The phenomenon of AMED consumption has been widely investigated in humans from clinical and epidemiological perspectives (Ferreira et al., 2006; O'Brien et al., 2008; Mallett et al., 2014), raising an increasing concern for its effects on the behavior and the health of the individuals who indulge in this consumption (Cleary et al., 2012; Mallett et al., 2015; Marczinski and Fillmore, 2006, 2014; Miller, 2008a, 2008b, 2012). However, relatively few preclinical studies have evaluated the effects of the combined administration of alcohol and EDs, and most of them have addressed the issue by investigating the effects of the combination of alcohol with the main components of EDs, caffeine and taurine.

The present study was set to evaluate in adulthood the effects of adolescent exposure to alcohol, or ED or AMED on basal and alcohol-stimulated mesocortical DA function by determining gate sensory control (PPI experiments), voluntary alcohol intake, mPFC DA concentration by microdialysis measurements (behavioral and neurochemical experiments), and VTA DA neuron's function and sensitivity to alcohol (electrophysiological experiments).

Our data disclose that BLPA of AMED during adolescence significantly decreased PPI during adulthood in an age-dependent manner. Sensorimotor gating provides a precognitive attentional filter that prevents sensory overload ensuring the proper processing of relevant sensory information, thus allowing correct cognitive functioning and behavioral responses to environmental stimuli (Braff and Geyer, 1990). A broad body of literature, including clinical and/or preclinical studies, invariably indicates that alterations in mPFC function lead to PPI impairments. Accordingly, sensorimotor gating has been consistently reported to be compromised in several pathological conditions ranging from schizophrenia to drug addiction, characterized by a weakened and or altered PFC functioning, known as hypofrontality (Rassnick et al., 1992; Hazlett and Buchsbaum, 2001; Day-Wilson et al., 2006; Dissanayake et al., 2013).

Preclinical studies investigating the effects of adolescent alcohol exposure on PPI are generally inconclusive. Studies in mice observed a slight or no-significant reduction in PPI in adult mice repeatedly treated with alcohol in adolescence (Coleman et al., 2011; Ledesma et al., 2017) or no reduction at all (Barnett et al., 2022), while in rats, both reductions (Ehlers et al., 2013) and increases were observed (Slawecki and Ehlers, 2005). In particular, our results are coherent with those of Coleman and colleagues (Coleman et al., 2011) that observed slightly significant reductions in PPI during adulthood as a consequence of a similar alcohol binge protocol in adolescence, although their experiments were performed in mice, under a 10-day continuous administration protocol (from PND 28 to PND 37), with an alcohol dose of 5 g/kg, and PPI was assessed only once in young adults (i.e., between PND 60 and 72). On the other hand, Slawecki and Ehlers (2005) observed an increase in PPI in rats treated during adolescence (starting at PND 26–28), but not when treated during adulthood, with a continuous 14-day protocol of ethanol

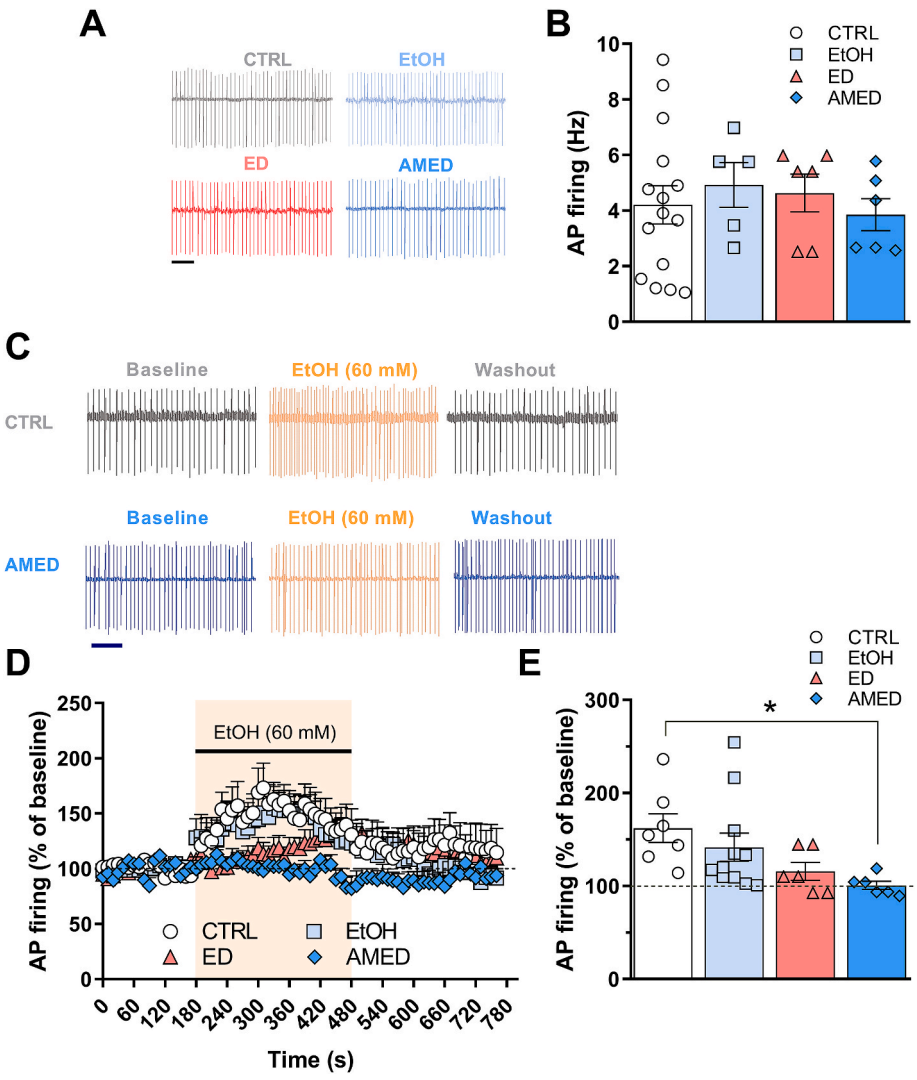

Fig. 5. Effect of BLPA in adolescence with alcohol, ED or AMED on basal and EtOH-stimulated firing rate of VTA DA neurons. (A) Electrophysiological traces of spontaneous firing recorded in the cell-attached configuration from representative VTA DA neurons of rats from all experimental groups. Scale bar, 2s. (B) Bar graph summarizing the basal firing rate measured in all experimental groups. Data are expressed as absolute values (in Hz) and are mean ± SEM of 5–15 neurons from 6 rats per group. (C) Electrophysiological traces of spontaneous firing recorded in the cell-attached configuration from representative VTA DA neurons from the CTRL and AMED groups before, during and after bath perfusion of 60 mM EtOH. Scale bar, 2s. (D) The graph summarizes the averaged changes in the firing rate of VTA DA neurons before (baseline), during and after (washout) bath perfusion of 60 mM EtOH. Data are expressed as the percent change in firing rate from baseline and are mean ± SEM of 6–11 neurons from 4 rats per group. (E) Bar graph representing the maximal effect of alcohol application on firing rate recorded from VTA DA neurons, as calculated from (D). Data are expressed as the percent change from baseline and are mean ± SEM of 6–11 neurons obtained from 4 rats per each experimental group. *p < 0.05 vs CTRL group. (D)

vapor exposure and tested for sensorimotor gating six days after the cessation of the treatment (i.e., around PND 40–42). However, the significant difference in the route of administration and the lack of a subsequent assessment in the same animals in later adulthood makes very difficult any direct comparison between the Slawecki and Ehlers (2005) and the present study. Overall, experimental protocol differences, including alcohol doses, timing and route of administration, age of administration and/or testing, and the specific treatment schedule used (e.g., continuous vs. intermittent administration) could be at the basis of the differences observed in literature regarding the effects of adolescent alcohol exposure on PPI.

Similarly, both caffeine and taurine *per se* have been observed in preclinical studies to produce different effects on PPI, ranging from disruption or no effect to potentiation, depending on the specific doses and administration protocols, making difficult a direct comparison of our present results with those of previous studies (see, for instance, Giongo et al., 2023; Dubroqua et al., 2014; Oral and Göktalay, 2021).

To the best of our knowledge there are no published studies so far on the effects of AMED beverages on PPI neither in adolescence nor in adulthood. Our data indicate that administration of alcohol and ED together have an additive detrimental effect on PPI and, perhaps more importantly, that these effects are long-lasting and still present in adulthood (at PND 90), suggesting that the early intermittent exposure of the BLPA used here can long-lastingly modify the function of one or more of the neuronal systems involved in the PPI process.

Several preclinical studies suggest that early exposure to AMED might enhance the vulnerability to alcohol addiction (Arria et al., 2011). To evaluate this possibility, immediately from the end of BLPA of water, alcohol, ED, or their combination (AMED), we trained animals of these experimental groups to self-administer an alcohol solution made available to them for 2 h every day for the following 28 days. Our data show that in the last days of this self-administration period, rats of the AMED group significantly escalated their voluntary intake of alcohol compared to animals that received water or alcohol or ED only in adolescence.

Notably, our *in vivo* microdialysis study disclosed that basal DA extracellular concentrations did not significantly differ between groups. However, the response of mPFC DA transmission to alcohol presentation in animals trained to consume alcohol voluntarily was dramatically blunted in rats of the AMED group. In fact, in these animals, DA extracellular concentration in the mPFC did not significantly change during any of the phases (anticipation, presentation, post-removal) during DA monitoring. On the contrary, this parameter increased significantly and long-lastingly in rats of the EtOH group, who consumed a significantly lower amount of alcohol. This observation appears to support the tenet that some substances, such as caffeine, can reduce some effects of alcohol, as anedoctically reported for decades. On the contrary, it should be firmly kept in mind that such supposed influence does not take place as a consequence of a contingent interaction between these pharmacologically active substances but, instead, is the expression of a long-term consequence, detected in adulthood, of an intermittent, binge-like adolescent exposure to high dosages of both substances (AMED group).

The blunted response of mesocortical dopaminergic neurons to the presentation of alcohol is in line with our previous data showing that an increase in its voluntary consumption in socially isolated rats was accompanied by a decreased sensitivity of these neurons to alcohol presentation (Lallai et al., 2016). As in our previous paper (Lallai et al., 2016) in fact, the response of mesocortical dopaminergic neurons to alcohol presentation is inversely correlated to its consumption. Thus, in the AMED group, where the amount of alcohol voluntarily assumed is the highest, the extracellular DA concentration in the mPFC did not significantly change during anticipation, consumption, and satiety for alcohol. This inverse correlation strongly supports the attribution of a crucial role of a dysfunctional mPFC in the development of addiction as well as that of dopaminergic hypofrontality as the pivotal factor in altering the balance between mesocortical and mesolimbic dopaminergic pathways, thus having the potential of triggering the switch between use and abuse of substances. A decreased sensitivity to alcohol of mesocortical dopaminergic neurons of rats of the AMED group might therefore be responsible for the loss of inhibitory control and the development of compulsive use (Jentsch and Taylor, 1999; Jentsch et al., 2014).

Accordingly, our electrophysiology experiments show that DA neurons recorded in the posterior portion of the VTA from adult rats are susceptible to the association of alcohol and ED (AMED in adolescence), as a complete suppression of the acute alcohol-induced stimulation of the firing rate was found in VTA slices from these animals. In further agreement with the microdialysis data, DA neuron firing activity, on the contrary, was not altered, with respect to controls, in response to alcohol in slices from the EtOH group (alcohol in adolescence), whereas firing resulted partially reduced in animals treated with ED alone, although this latter effect did not reach statistical significance. Finally, we found no significant alterations in the basal firing activity of these DA neurons in slices from rats of all experimental groups. This finding also aligns with the microdialysis data showing no differences in basal DA concentrations in the mPFC (Table 1). The acute stimulatory effect of alcohol on the firing of VTA DA neurons is thought to be associated with the rewarding and reinforcing properties of alcohol as well as other substances of abuse (Morikawa and Morrisett, 2010). The concentration of alcohol used in our study (60 mM) was in the range of those used in previous reports in which the effects of alcohol on VTA DA neurons were examined (Brodie et al., 1990; Brodie and Appel, 1998; Okamoto et al., 2006), and which are attainable upon systemic administration of doses between 1 and 2 g/kg, the same achieved in the VTA upon self-administration (between 17 and 66 mM) (Rodd et al., 2005; Bassareo et al., 2021). Therefore, the data indicate that adolescent AMED suppresses the ability of VTA DA neurons to respond, in adulthood, to subsequent stimuli. We recognize that one limitation of the present work, which may deserve further and more detailed investigation, is the lack of a precise identification of DA neurons of the VTA projecting to the mPFC and or to the nucleus accumbens, which can be solved by the use of retrograde tracers into the mPFC and subsequent recordings of the firing rate from selectively stained cell bodies in the VTA.

The molecular mechanism underlying the acute alcohol effect exerted by its association with the ED in the present study was not addressed in this work and will undoubtedly be the subject of future and more detailed investigations. However, it is essential to mention that caffeine, a main psychostimulant component of EDs, can exert a direct negative control over the firing of DA neurons in the VTA at concentrations ranging from 5 to 20 μM (Stoner et al., 1988), an effect that may be mediated through an antagonistic action on A2A adenosine receptors (Valenti et al., 2021; our unpublished observations). In addition, taurine, also a common component of EDs, at concentrations ranging from 0.01 to 30 mM can markedly reduce the spontaneous firing of VTA DA neurons via activation of extrasynaptic strychnine-sensitive glycine receptors, which, in turn, leads to membrane hyperpolarization (Wang et al., 2005). Thus, as our recordings were done in slices taken from adult rats that were exposed during adolescence to a binge-like protocol of AMED administration, our results may suggest that similarly to acutely administered or applied individual components of EDs, i.e., caffeine and taurine, AMED exposure during adolescence may induce long-lasting adaptive synaptic plasticity by acting at distinct receptor targets on VTA DA neurons, resulting in the sustained suppression of the responsivity of these nerve cells to acute alcohol that we detected in our study.

Altogether, our results show that exposure to alcohol, ED, or AMED, during the critical period of adolescence induces different adaptive changes in the function of mesocortical dopaminergic neurons and indicate that exposure of adolescent rats to AMED decreases their sensitivity to external stimuli. This finding might lay the foundation for an increased vulnerability to alcohol addiction.

The mPFC is a key structure for higher level cognitive functions like decision-making, behavioral control and flexibility, and goal-directed behavior planning (Goto and Grace, 2005). Binge-like AMED administration, involving early exposure to alcohol and ED components, could disrupt the mPFC's neuroplastic processes during adolescence. Alcohol negatively affects brain development, especially the mPFC, leading to neurotransmitter and neuroplasticity alterations, inflammation, myelin integrity issues, and impaired glial function (Seemiller and Gould, 2020; Tetteh-Quarshie and Risher, 2023). Our findings suggest that alcohol and ED constituents during adolescence may induce long-lasting modifications in the mPFC, resulting in increased alcohol intake, decreased sensitivity of dopaminergic neurons, and disrupted PPI in adulthood. Studies show that chronic caffeine exposure during adolescence can also affect the mPFC's function related to the DA system (Boeck et al., 2009), potentially altering dendritic plasticity and natural pruning processes (Christensen et al., 2020; Juárez-Méndez et al., 2006).

This study has been focused on male rats as several pieces of evidence suggest a significant influence of hormones on the effect of alcohol on mPFC DA-projecting neurons (Dazzi et al., 2002, 2007). Moreover, the current knowledge of DA developmental processes during adolescence is largely derived from studies on male subjects (Reynolds and Flores, 2021). The results of this study can be used to pursue further insight into possible sex differences in the sensitivity of mesocortical dopaminergic neurons induced by early exposure to AMED.

5. Conclusion

In conclusion, to the best of our knowledge, the present study provides the first evidence for a comprehensive and coherent set of behavioral, neurochemical and electrophysiological data on the plastic changes of the mesocortical DA function of adult rats early exposed, in adolescence, to a protocol that closely mimics the intermittent, binge-like, simultaneous consumption of highly alcoholic beverages and EDs. Moreover, the present data strongly point out that future studies aimed at further characterizing the neurobiological bases and consequences of AMED consumption should consider using EDs as a whole and not their

individual main components or their partial combinations. This notwithstanding, we acknowledge that the present results suffer from some main limitations that refer to the issue of sex (only males were used in the study) as well as to the lack of voluntary control over the binge-like exposure to alcohol or ED or AMED during adolescence. Overall, the present results are in agreement with the finding that addicted subjects show hypofrontality (Wallace et al., 1996; Sun and Rebec, 2006) which was suggested to be the mechanism responsible for the decreased ability to inhibit both the compulsive search for the substance as well as the increase of its assumption (Jentsch and Taylor, 1999).

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CRediT authorship contribution statement

Laura Dazzi: Conceptualization, Methodology, Validation, Investigation, Formal analysis, Visualization, Funding acquisition, Data curation, Writing – original draft, Writing – review & editing. **Fabrizio Sanna:** Conceptualization, Methodology, Validation, Investigation, Formal analysis, Visualization, Writing – original draft, Writing – review & editing, Funding acquisition, Data curation. **Giuseppe Talani:** Conceptualization, Methodology, Validation, Investigation, Formal analysis, Visualization, Writing – original draft, Writing – review & editing, Funding acquisition, Data curation. **Valentina Bassareo:** Visualization, Writing – review & editing. **Francesca Biggio:** Visualization, Writing – review & editing. **Paolo Follesa:** Visualization, Writing – review & editing. **Maria Giuseppina Pisu:** Visualization, Writing – review & editing. **Patrizia Porcu:** Visualization, Writing – review & editing. **Roberta Puliga:** Visualization, Writing – review & editing, Data curation, Formal analysis. **Marina Quartu:** Visualization, Writing – review & editing, Funding acquisition. **Mariangela Serra:** Visualization, Writing – review & editing, Funding acquisition. **Maria Pina Serra:** Visualization, Writing – review & editing. **Enrico Sanna:** Conceptualization, Methodology, Validation, Investigation, Formal analysis, Visualization, Writing – original draft, Writing – review & editing, Funding acquisition, Data curation. **Elio Acquas:** Conceptualization, Methodology, Project administration, Supervision, Writing – review & editing, Funding acquisition.

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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