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Immersive virtual plus-maze to examine behavior and psychophysiological-related variables in young people with problematic alcohol and cannabis consumption

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ABSTRACT

Stressful events appear to be risky situations that can precipitate the consumption of drugs. One way to recreate stressful contexts, in an ecological and controlled method, is through immersive virtual reality (VR). In our study, we designed the scenario of an elevated plus-maze (EPM) using VR, which is widely used in animal models to assess unconditioned anxiety. This task allowed us to analyze the behavioral, psychophysiological (heart rate and electrodermal activity), and hormonal response (salivary cortisol and Alpha-amylase) to this stressful situation in different moments (before VR task (anticipation), at the end of the task and 10 minutes later) in young people with problematic alcohol use (AU, n = 27), alcohol combined with cannabis consumption (AU + C, n = 10), as well as in a control group (CO, n = 33). Behavioral analysis revealed that the AU group displayed fewer entries into open arms than the CO group, whereas both experimental groups spent less time at the end of the open arms, as well as lower *time by look down index* compared to the CO group. Moreover, our VR EPM induced different psychophysiological responses in the different moments measured. In general, electrodermal activity seemed to be a good biomarker of recovery from a stressful situation, as once the exposure to the stressful situation ended, the AU + C group took longer to recover compared to the CO group. Regarding hormonal analyses, we observed a similar response pattern in all groups suggesting that our VR task was able to activate both stress systems. The alpha-amylase to cortisol ratio, proposed as a biomarker of stress systems dysregulation, was higher in the group of young participants with alcohol abuse. Interestingly, our VR EPM was able to induce a slight alcohol craving in both experimental groups. In conclusion, our results suggest certain subtle behavioral and physiological differences that could be used to detect young individuals at risk of future severe addictions or other stress-related comorbidities. Moreover, it could help us to develop prevention strategies focused on emotional, cognitive, and psychophysiological aspects.

1. Introduction

Alcohol and cannabis use peaks during young adulthood (ages 18–24) (Messina et al., 2021). These drugs are commonly used together despite inducing several health and social problems (Thomasius et al., 2022). Furthermore, the involvement in substance use during early or late young adulthood has long been a significant health concern due to its predictive association with subsequent drug use behaviors in adulthood (Mooney-Leber and Gould, 2018; Patton et al., 2007).

Recent studies have found that university students consume more alcohol than individuals of the same age who do not attend university (Verhooga et al., 2020). Some factors that can explain, in part, this finding are the greater autonomy and economic independence, making them more likely to have access to alcohol and other substances (García-Carretero et al., 2019; Llamosas-Falcón, et al., 2022), the social pressure along with the fact that alcohol consumption is normalized and associated with fun and good times, eventually leading to high consumption at university parties, particularly on weekends

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(Teixidó-Compañó et al., 2022). On the other hand, the university stage entails a greater number of responsibilities, resulting in less free time for leisure activities, as well as stress derived from academic activities, which can negatively impact their mental health and serve as a potential risk factor for consumption. Finally, another associated risk factor is the absence of supervision and control by parents due to the increased independence that characterizes this stage, often accompanied by a distance from the family home (Canova-Barrios, et al., 2018).

Young adulthood is also characterized by a high vulnerability to stress (Tschetter et al., 2022). In this regard, both clinical and preclinical studies have reported that an increase in alcohol consumption is associated with stress (Kirsch and Lippard, 2022). Other studies in rodent models have also shown that early alcohol consumption induces a long-term enhancement of anxiety-like behaviors (Sampedro-Piquero et al., 2022; Sánchez-Marín et al., 2022). Considering these findings, early life stress is a recognized risk factor for developing common psychiatric disorders, such as depression and anxiety, as well as a higher vulnerability to suffer substance use disorders (Cabanis et al., 2021). Thus, the combination of stress and substance consumption, especially alcohol and cannabis, during young adulthood might promote vulnerability to mental disorders (Evans et al., 2016; Frobel et al., 2022). However, this association is complex, and a better understanding of the neurobiological underlying mechanisms involved is needed.

The connections between brain stress systems and substance use disorders are predominantly corroborated by studies conducted on individuals with alcohol and substance use disorders (Koob et al., 2014). As we mentioned before, the onset of alcohol and cannabis misuse often occurs during youth, and variations in how young adults respond to stress may serve as a potential biomarker for predicting the risk of a future addiction (Sinha, 2009; Sinha et al., 2016). Thus, although age differences in reactivity could depend upon stressor type, several studies have suggested that effects of stress on the young brain may be longer lasting when compared to the adult (Romeo, 2013). Besides, research suggests that, compared to older or adult people, young participants experience more interpersonal stressors, and they are more reactive to them (Birditt et al., 2005; Neupert et al., 2007). These age differences may reflect hypothesized improvements in emotion regulation (Carstensen et al., 2000) or a higher perceived control over the management of the stressful events (Neupert et al., 2007). Even more, studies in animal models have found that in both male and female rats, for instance, indicate that young animals show significantly protracted ACTH and corticosterone responses compared to adults following a brief exposure to a variety of stressors (e.g., foot shock, hypoxia, restraint) (Goldman et al., 1973; Romeo and McEwen, 2006). Nevertheless, despite this evidence, previous research has been limited to adult populations and studies are scarce in young individuals with risky alcohol consumption, and there is insufficient evidence in those who also display problematic cannabis use (Croissant et al., 2006; Zhang et al., 2020).

In the pursuit of ecological contexts and more reliable measures of stress reactivity, virtual reality (VR) has been increasingly applied because it is able to provoke stress reactivity despite the environment being virtual (Dammen et al., 2022; Martens et al., 2019). To date, there is a scarcity of studies that have examined stress reactivity and anxiety-related behaviors in young individuals with substance use through exposure to virtual scenarios. On the one hand, most studies utilizing VR have focused on the potential of this technology as a treatment (Monarque et al., 2023), or as a method to assess variables related to consumption, such as craving (Kim and Lee, 2015) or cognitive state (Montgomery et al., 2011, 2012). On the other hand, there is a lack of standardized laboratory tests to study human anxiety-related behaviors. Hence, considering that approach-avoidance conflicts are a hallmark of anxiety-related behaviors and that the Elevated Plus-maze (EPM) is the gold standard task to assess this behavior in rodents (Biedermann et al., 2022), we aimed to develop a human version of this test using a VR setup. We have selected this methodology because it provides a first-person perspective that enables participants to experience a direct influence from the virtual environment leading to genuine physiological and behavioral responses (Schöne et al., 2021). Besides, VR scenarios along with the measurement of different biomarkers might provide an answer regarding identification of at-risk patients (Mazza et al., 2021).

Finally, while many VR simulations used for research purposes focus on a limited set of biomarkers, a careful examination of patterns of response across multiple domains requires the measurement of several indexes to avoid ambiguity (Stritzke et al., 1996). Thus, our study sought to explore a broader array of physiological stress indicators. Specifically, we aimed to analyze the impact of the VR environment on key biomarkers, including heart rate (HR), electrodermal activity (EDA), salivary Alpha-amylase (sAA) and salivary cortisol (sCORT) levels. These biomarkers collectively provide insights into the complex interplay between the Hypothalamic Pituitary Adrenal (HPA) axis and the autonomic nervous system (ANS) during stress responses. Several studies have described that chronic drug use alters peripheral and central brain stress pathways, but there is no conclusive data about young people with risky alcohol and cannabis consumption. Interestingly, growing evidence suggests that these alterations may play a role in compulsive motivation and drug craving and in predicting relapse and treatment outcome, rather than in chronic drug abuse (Milivojevic and Sinha, 2018).

Overall, we aimed to analyze the behavioral and psychophysiological response of young individuals with problematic alcohol consumption, with or without cannabis use, in a human VR EPM. Hence, it could be used as a predictor of vulnerability to substance consumption, as well as relapse.

2. Material and method

2.1. Participants

This study is part of the cross-sectional study "Impact of an aerobic exercise program to reduce the cognitive, neurophysiological, and psychophysiological alterations related to the consumption of alcohol and cannabis in young people" (Identifier 2022I004) funded by Government Delegation for the National Plan on Drugs. The study was carried out from December 2022 to May 2023. Undergraduate students aged between 18 and 25 years were recruited through verbal disclosure, e-mails, and notice boards at Autonomous University of Madrid (Caucasian, 21 male and 49 female, age 19.2 ± 0.10). All volunteers included in the study signed the informed consent, accompanied by an informative note, and they created an alphanumeric code based on the first letter of their first name, the last letter of your first surname followed by your date of birth (e.g., PO1304) to guarantee privacy during the data processing and analysis phases. To collect information about alcohol and cannabis use and anxiety trait on-line standardized questionnaires were administered (Alcohol use disorders identification test (AUDIT, Spanish adaptation: Contel Guillamon et al., 1999); Cannabis Abuse Screening Test (CAST, Legleye et al., 2007); State-Trait Anxiety Inventory (STAI-t, Spielberger et al., 1970). The risky consumption of both drugs was determined using the AUDIT (girls $\geq$6; boys $\geq$8 points) and CAST (>4 point for both sexes) questionnaires (AU and AU + C groups respectively) (EDADES, 2022). Based on these scores, we divided the sample into 3 groups: control group (CO, n = 33 (12 boys)); alcohol use group (AU, n = 27 (4 boys)); alcohol use and cannabis group (AU + C, n = 10 (5 boys)). Inclusion criteria were: 1) aged 18–25 years; 2) an absence of diagnosis of substance use disorder; 3) an absence of comorbid disease, such as anxiety-related disorders, depression, psychotic disorder, or Attention-Deficit/Hyperactivity-Disorder (ADHD). Moreover, healthy controls should not present a history of drug abuse, including nicotine and alcohol. All volunteers were excluded if they presented severe difficulties in understanding the test instructions, altered consciousness or agitation, a score in the AUDIT above 19 points and if they consumed prescription drugs affecting the central nervous system (mainly anxiolytics and antidepressants). Additionally, during the initial interview,

we asked them about possible phobias and individuals with fear of heights or claustrophobia were excluded from the study.

The study received approval from the Ethics Committee of the Autonomous University of Madrid (Code: CEI-122- 2490) in accordance with the Ethical Principles for Medical Research Involving Human Subjects adopted in the Declaration of Helsinki by the World Medical Association (64th WMA General Assembly, Fortaleza, Brazil, October 2013), Recommendation No. R (97) 5 of the Committee of Ministers to Member States on the Protection of Medical Data (1997), and the Spanish Data Protection Act (Ley Orgánica 15/1999 de Protección de Datos, LOPD). All participants were informed about the study prior to their inclusion in the study and then provided their written informed consent.

2.2. EPM – VR setup

The human VR EPM used consisted in a metallic elevated platform on which the participant appeared when pressed a button and remained in that area 5 minutes. With the aim of increasing the augmented reality perception of height and instability, the shoes of the participants were attached and secured to a 10 cm platform (Fig. 1). Participants received a VR glass (Meta Quest 2, 128 Gb, Meta Platforms Technologies Ireland Ltd.) and after checking the vision of participants, our VR EPM started. The participant could freely move along the four arms of the cross, two closed (5 m in total) and two open (5 m in total) in an originally created city of skyscrapers with cars moving below. To reduce the artificiality of the virtual environment, we included visual and acoustic enrichment. The behavioral variables registered were the following: time spent in the different parts of the maze (closed and open arms and the end of the open areas), frequency of visits, as well as the time and frequency looking down in the edge of the open arms and the index *time by look down* (ratio between the time spent looking down in the open section and the square-root of the number of looks down). Behavioral variables registered were analysed manually by *Raton Time* program. After the EPM experience (5 minutes), there was an additional task for the participants that consisted of three trials in which they had to find a box, walk towards it in the EPM and then, touch box (2 minutes approximately, 40 seconds each trial). When the subjects found the box in the first trial, they appeared in the distal part of an open arm and the new box was placed in the distal part of the opposite open arm. The third situation was the same as the first one, except snakes appeared in the open arms (Fig. 1 shows the timeline of our VR EPM). Participants read the instructions by a written message presented before starting each part of the test and the researcher made sure to clarify any doubts: first part (5 minutes of free exploration): *You will now find yourself in a virtual environment. Your task is to freely explore the environment for 5 minutes*. Press any button on the joystick to begin; Second part (3 trials, 2 minutes to find and touch the boxes): *Next, you will see a box in the environment, and you should move to touch it with your hand. Press any button on the joystick to begin*. In the following link, a video extract of our VR EPM can be observed: https://drive.google.com/file/d/13ASiZJCTtOxxfEclcBMK6gijO9Zqd2Kn/view?usp=drive_link When the task finished, participants fulfilled different questionnaires on-line through Qualtrics platform (Scale subjectively rating anxiety provoked by the VR environment (How stressful has the virtual reality situation been for you? Nothing/ Some/Quite a lot/Much); STAI state (STAI-s) and Multidimensional Alcoholism Craving Scale (MACS) (Guardia-Serecigni et al., 2004, 2006).

2.3. Psychophysiological measurements

To estimate ANS reactivity during the study, EDA and HR (electrocardiogram ECG) were recorded using Versatile Bio (Bitbrain Technologies 2018; Zaragoza, Spain). This equipment is a mobile real-time biosignal amplifier which allows physiological measurements with a total freedom of movement for the user (sampling rate and data resolution: 256 Hz at 24 bits). The ultralight biosignal amplifier (172 g) was placed on the waist of the participants and the two EDA response electrodes were placed on the fingers of the non-dominant hand, making sure that the sensor is in contact with the inner part of the participant's finger. ECG was made up by 2 sensors which were connected to the sticker and then, placed in the lower nape of the participant and the other sensor on the left arm (heart must be between the sensors). Finally, the ECG sensor was connected to an ExG connector (CMRR/impedance: >100 dB @50Hz > 50 GΩ). Data transmission was performed by Bluetooth 2.1. and Bitbrain Software Kit was used for data acquisition and programming.

Saliva samples were also recollected through salivettes (Salivette®, Sarstedt, S.A., Barcelona, Spain). Saliva samples were coded and stored in the refrigerator at −20 °C. Then, they were centrifuged at 1500 rpm for 15 min, resulting in a clear supernatant of low viscosity that was stored at −80 °C until the analyses of the sCORT and sAA levels. Both, sCORT and sAA have individually been identified as meaningful and reliable markers of the stress systems (Pitman and Orr, 1990; Granger et al., 2007). sCORT and sAA concentrations were measured using a commercially available enzyme-linked immunosorbent assay (Human Alpha-amylase Kinetic reaction for saliva samples (1-1902-1); Human Cortisol (expanded range) Kit for saliva samples (1-3002-1, Salimetrics®, BioNOVA científica S.L., Madrid, Spain). sCORT and sAA levels were expressed in nmol/L and U/mL, respectively (Ali and Pruessner, 2012).

All these physiological measurements were analysed before the start

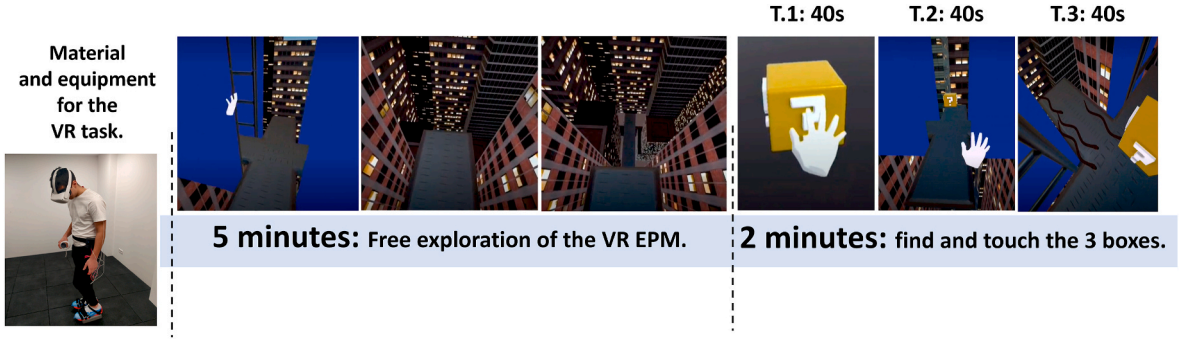

Fig. 1. Timeline of the virtual Elevated Plus-Maze. The center area constituted the starting point of the exploration phase lasting for 5 min. The first image also shows the VR equipment, such as the glasses and the joystick, as well as the EDA and HR sensors in one participant. After the EPM experience, there was an additional task for the participants that consisted of three trials in which they had to find and touch a box (2 minutes approximately, 40 seconds each trial). When the subjects found the box in the first trial, they appeared in the distal part of an open arm and the new box was placed in the distal part of the opposite open arm. The third situation was the same as the first one, except snakes appeared in the open arms. In addition, to increase the perception of height and instability, the shoes of the participants were attached and secured to a 10 cm platform.

of the VR task (t1, *anticipatory stress*), immediately after the end (t2) and 10 minutes later (t3, *stress recuperation*) (12 pm–18 pm). Moreover, as an index of *uncertainty stress*, we also studied HR and EDA in the three additional trials in which participants had to find a box and touch them without knowing what occurred.

2.4. Statistical analysis

All statistical analyses were performed using SPSS 25 (IBM SPSS Statistics, Corporate headquarters, New Orchard Road, Armonk, New York 10504-1722, USA) and all p values were two-tailed, and the level of significance was taken as $p \leq 0.05$. Descriptive analyses were performed on the demographic variables (sex, years, education, etc.), the information related to drug use (age of onset, days since the last consumption, etc.) and the AUDIT and CAST questionnaires scores (Table 1). We reported mean ± SEM. Statistical differences between groups in the different questionnaires and the behavioral variables registered in the virtual EPM were analysed by the non-parametric Kruskal Wallis test ($p \leq 0.05$) (Table 2). Physiological measurements (HR, EDA, as well as sAA and sCORT levels) were analysed by the non-parametric Friedmann Test for repeated measures to investigate whether our VR EPM produced significant stress responses at different moments in all groups, and Kruskal-Wallis's test to explore the differences among groups. Besides, area under the curve, with respect to ground (AUCg), was first calculated for the raw, untransformed values of both sAA and cortisol using the trapezoid formula described previously (Pruessner et al., 2003). This score incorporates information regarding both baseline and reactivity individually for Alpha-amylase and cortisol, within one score. We then divided the AUCg of Alpha-amylase by the AUCg of cortisol to derive an overall ratio variable of Alpha-amylase over cortisol based on the individual AUCg variables named AOCg (Ali and Pruessner, 2012). Multiple linear regression analysis was performed including the physiological parameters to search predictors of drug consumption (AUDIT, CAST and craving). Finally, correlations were also conducted to examine the associations between behavioral and psychophysiological variables. In supplementary information, a correlation matrix for each group between behavioral and psychophysiological measurements can be consulted (Fig. S1).

Table 1
Demographic, alcohol, and cannabis use characteristics of participants.

	CO	AU	AU + C
DEMOGRAPHICS			
Age ± SEM	18.87 ± 0.26	19.11 ± 0.27	18.5 ± 0.17
Sex male	12 (12/33)	4 (4/27)	5 (5/10)
Marital status (single) - %	100%	100%	100%
SUBSTANCE US			
Onset age	16.3 ± 0.4	14.7 ± 0.8	15.1 ± 0.3
Days since last used drug (alcohol)	61.4 ± 0,6	3.2 ± 0.7	3.9 ± 0.5
Days since last used drug (cannabis)			2.1 ± 0.3
AUDIT	3.15 ± 0.33	10.71 ± 0.81	10.70 ± 1.12
Frequency of alcohol consumption (AUDIT item 1) [0 = Never, 1 = Monthly or less, 2 = 2–4/month, 3 = 2–3/week, 4 = ≥ 4/week]	1.57 ± 0.15	2.11 ± 0.17	1.80 ± 0.20
Quantity of alcohol use in a day (AUDIT item 2) [0 = 1 or 2, 1 = 3 or 4, 2 = 5 or 6, 3 = 7, 8 or 9, 4 = 10 or more]	0.45 ± 0.12	0.59 ± 0.13	0.30 ± 0.15
CAST	- -	7.3 ± 1.3	

Values are means ± SEM or percentages.

Table 2
Questionnaires and behavioral-related variables of participants.

	CO	AU	AU + C	p	η_p^2
QUESTIONNAIRES					
STAI-t	24.30 ± 1.75	32.81 ± 1.84	22.1 ± 3.24	0.02*; 0.01*	0.18
STAI-s	21.5 ± 3.10	22.96 ± 1.83	15.4 ± 1.57	0.12	
MACS	12.27 ± 0.47	17.40 ± 1.13	20.4 ± 2.44	0.002*	0.23
Subjective anxiety scale	1.60 ± 0.35	1.15 ± 0.16	0.90 ± 0.23	0.22	
BEHAVIORAL VARIABLES					
Frequency open arms	9.79 ± 0.87	6.25 ± 0.70	9.00 ± 1.23	0.02*	0.13
Frequency closed arms	3.24 ± 0.46	2.85 ± 0.34	4.10 ± 0.60	0.25	
Frequency end of open arms	4.42 ± 0.72	2.37 ± 0.49	3.00 ± 0.47	0.08	
Time in open arms	105.76 ± 6.25	97.84 ± 11.13	106.39 ± 13.65	0.86	
Time in closed arms	25.80 ± 3.25	31.68 ± 4.54	53.17 ± 20.46	0.35	
Time end of open arms	48.33 ± 7.96	18.44 ± 4.77	27.40 ± 6.62	0.02*	0.14
Latency open arms	13.10 ± 1.61	25.17 ± 5.02	9.03 ± 1.72	0.13	
Latency closed arms	34.56 ± 6	33.41 ± 7.93	24.31 ± 4.03	0.98	
Latency end open arms	45.70 ± 8.64	33.78 ± 8.69	31.88 ± 8.43	0.35	
Time by entry index	37.88 ± 4.46	38.51 ± 4.89	34.10 ± 4.30	0.83	
Time by look down index	20.36 ± 1.69	8.00 ± 1.06	13.15 ± 1.24	0.001*	0.35
Latency look down	24.05 ± 3.76	29.61 ± 6.88	26.01 ± 9.44	0.88	

Values are means ± SEM. *Asterisks represent significant differences between groups ($p \leq 0.05$).

3. Results

3.1. Questionnaires and behavioral data in the VR EPM

STAI-t revealed significant differences among groups ($H = 12.42$, $p = 0.002$). Post hoc analysis showed that the AU group presented higher trait anxiety compared to the CO group ($p = 0.01$) and the AU + C group ($p = 0.02$) (Fig. 2a). Regarding the behavioral pattern of exploration, significant differences among groups were found in the frequency of entries into the open arms ($H = 8.40$, $p = 0.02$), time spent in the edge of the open arms ($H = 7.86$, $p = 0.02$), and in the *time by look down* index ($H = 24.09$, $p = 0.001$). Specifically, the AU group showed fewer number of entries into the open arms compared to the CO group ($p = 0.02$). Moreover, both experimental groups (AU and AU + C) spent less time in the edge of the open arms ($p = 0.016$, $p = 0.02$, respectively) and displayed a reduced *time by look down* index ($p = 0.001$, $p = 0.03$, respectively) compared to the CO group (Fig. 2b, c, and 2d). Finally, differences among groups were also displayed in alcohol craving levels ($H = 17.40$, $p = 0.001$) with both experimental groups showing higher craving after the VR task (AU: $p = 0.002$, AU + C: $p = 0.002$, respectively) compared to the CO group (Fig. 2e). Negative correlations between the variables *Time at the end of the open arms* and STAI-t score was observed in the AU group ($r = -0.49$, $p = 0.003$). STAI-s score and the rest of behavioral variables registered in the VR EPM did not show significant differences among groups ($p > 0.05$). More details about these results are available in Table 2. Besides, we have performed the statistical analysis using sex as a covariate and the results were the same that the obtained before (more information is displayed in Supplementary Results).

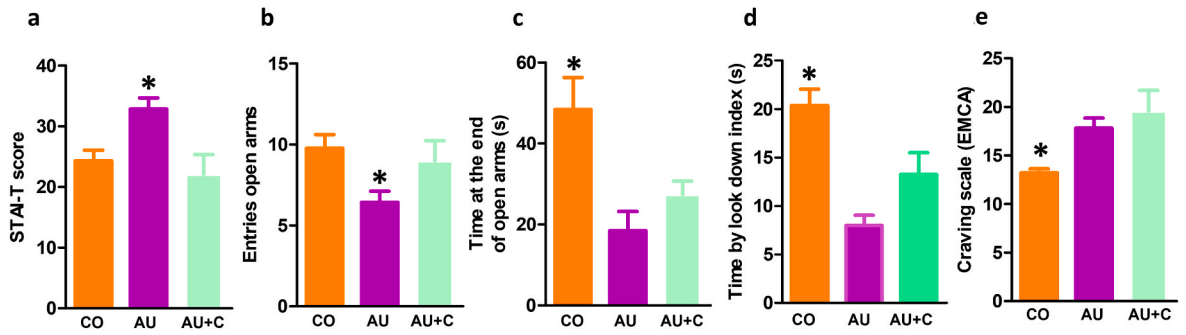

Fig. 2. Exploration behavior and associated traits and emotional variables. AU group presented higher trait anxiety compared to the CO group ($p = 0.01$) and the AU + C group ($p = 0.02$) (2a). Regarding the behavioral pattern of exploration, AU group showed fewer number of entries into the open arms compared to the CO group ($p = 0.02$) (2b). Moreover, both experimental groups spent lower time in the edge of the open arms (AU: $p = 0.016$, AU + C: $p = 0.02$, respectively (2c)) and displayed a reduced *time by look down* index ($p = 0.001$, $p = 0.03$, respectively (2d)) compared to the CO group. Finally, both experimental groups showed a higher score in MACS scale after the VR task (AU: $p = 0.002$, AU + C: $p = 0.002$, respectively) compared to CO group, suggesting the presence of slight alcohol craving (2e). All data are mean ± S.E.M. and statistically significant differences were considered when $p \leq 0.05$ (*).

3.2. Psychophysiological measurements

Regarding HR, the statistical analysis revealed a significant effect on time (Fr = 94.48; $p = 0.001$) (Fig. 3a). No difference was observed among groups within the temporal dynamics of HR before, during and after, nor the accumulated measure (AUC) (Fig. 3b). With concern to EDA, we also found a significant effect on time (Fr = 44.57; $p = 0.001$) and comparison among groups revealed that the AU + C group exhibited higher EDA values during (t2) and after (t3) the VR EPM compared to the CO group (t2: H = 7.36; $p = 0.02$; t3: H = 7.18; $p = 0.02$) group (Fig. 3c). sAA showed a significant effect of time on the different measures (t1, t2 and t3), suggesting that the VR task activated the SNS (Fr = 15.88; $p = 0.0003$; Fig. 4a). This time effect was also statistically significant for the cortisol levels measured (Fr = 23.46; $p = 0.00001$; Fig. 4b). The ratio of sAA over sCORT (AOCg) was higher in the AU group compared to the CO group (H = 5.77, $p = 0.05$) (Fig. 4c). Interestingly, after multiple linear regression analysis with all the physiological parameters as independent variables and craving as dependent variable, the predictor which reached the statistical significance was precisely AOCg index ($\beta = 1{,}01$; $p < 0.02$; $R^2 = 0.419$; $F_{(14,32)} = 1.184$, $p < 0.05$).

4. Discussion

In the current study, we investigated the behavioral and psychophysiological response of young individuals with problematic alcohol consumption, with or without cannabis use, in a human EPM performed in a VR environment. Our stress-related measurements, registered at three moments of the VR task (behavioral pattern of ambulation, HR, and EDA response, as well as sCORT and sAA levels), could be used as a predictor of vulnerability to substance consumption, severity, as well as anxiety-related disorders. As expected, our results showed several behavioral and physiological differences in young people with problematic alcohol, with or without cannabis abuse, compared to a group of healthy participants matched by age and educative level.

Young participants with risky substance consumption (alcohol/cannabis) exhibited more anxious-related behaviors in our VR EPM task and showed slight alcohol craving after the end of the stress.

To our knowledge, this study is the first experiment to analyze human approach-avoidance behavior, as a hallmark of anxiety-related

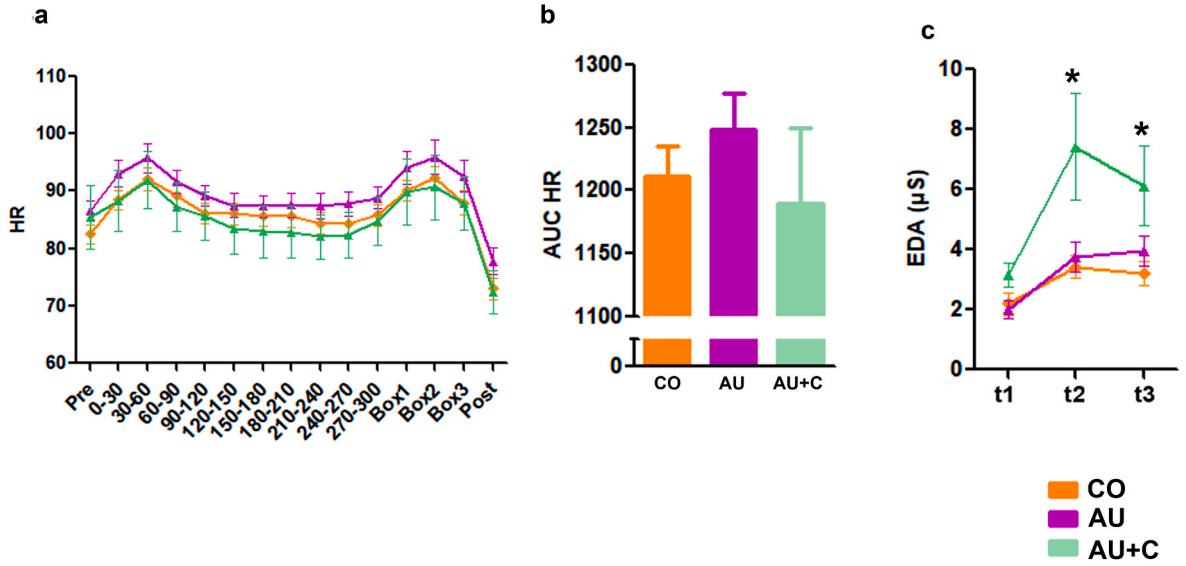

Fig. 3. HR and EDA responses to VR EPM. HR activity is displayed at baseline, 0–30s over the task, during boxes condition and 10 minutes after the end of the EPM (3a). As it can be observed, this autonomic response showed a similar pattern of response in the three groups reflecting two peaks of higher activity, at the onset of the task and during the uncertainty stress condition (Boxes) ($p = 0.001$). AUC HR did not show significant differences between groups (3b). On the other hand, EDA analysis also showed a significant effect on time ($p = 0.001$) and comparison among groups revealed that AU + C group exhibited higher EDA values during and after the VR EPM compared to CO ($p = 0.02$) group. All data are mean ± S.E.M. and statistically significant differences were considered when $p \leq 0.05$ (*).

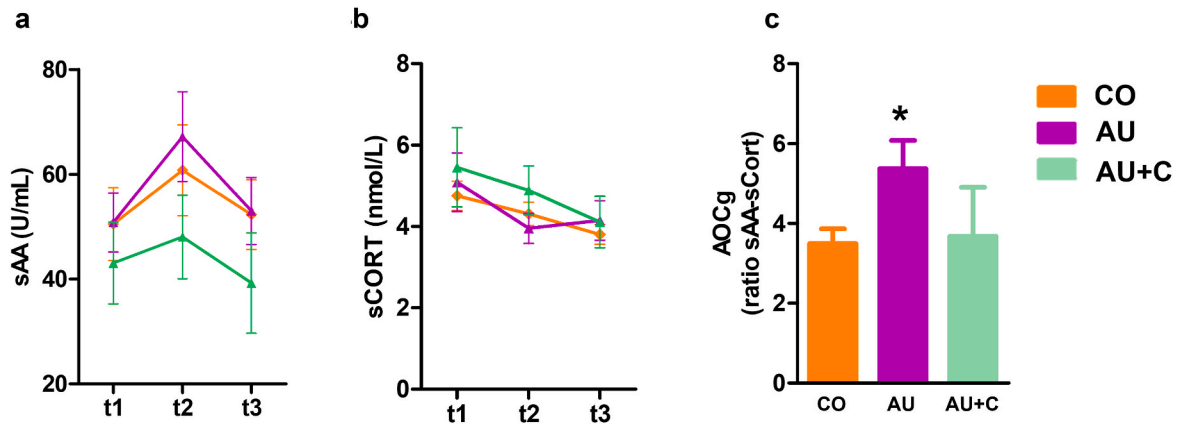

Fig. 4. Comparisons among groups in sAA and sCORT levels during the three moments of study of the VR EPM. As it can be appreciated, the levels of these biomarkers were not significantly different among groups (4a and 4b). The sAA to sCORT ratio (AOCg), calculated from the AUCg results, was increased in the AU group ($p = 0.05$) suggesting a possible dysregulation between SNS and HPA stress systems (Ali and Pruessner, 2012). All data are mean ± S.E.M. and statistically significant differences were considered when $p \leq 0.05$ (*).

behaviors, in a VR EPM in a sample of young participants with problematic substance consumption. In rodents, avoidance of the open arms is linked to heightened anxiety, while approach is associated with a desire for novelty and curiosity so, in humans, it is possible that avoidance is also connected to fear of heights (acrophobia), whereas approach is associated with sensation seeking (Biedermann et al., 2017). Considering these aspects, our AU group made fewer entries into the open arms compared to the CO group and both experimental groups (AU and AU + C) spent fewer time at the end of the open arms and displayed a lower *time by look* index suggesting a more anxious profile of exploration. These results suggest a cross-species similarity between animal models and humans because rodents with ethanol consumption during their young adulthood usually also show anxiety-like behaviors in the EPM (Hefner and Holmes, 2007; Sampedro-Piquero et al., 2022). Besides, and according to the study of Madeira et al. (2021), the open arm avoidance observed in our experimental groups could be related to acrophobia, as these authors proposed, or trait anxiety owing to the negative correlation observed between the behavioral variables and the score in this questionnaire (STAI-t) in the AU group. Nevertheless, although the trait anxiety showed by our participants was low (20–30 points), it was not close to zero, which could be related to certain discomfort experienced by them when they were exposed to a height situation with a risk of death by falling (Coelho et al., 2009; Öhman and Mineka, 2001). Moreover, and as we mentioned in the Methodology section, an exclusion criterion in our study is the presence of fear of heights or claustrophobia, so we could discard this explanation in our sample.

Interestingly, we also found that our VR EPM was able to trigger a slight level of alcohol craving in both experimental groups, as MACS score revealed. While we did not observe high levels of craving, it is noteworthy that our VR environment, validated in animal research, was capable of eliciting alcohol desire, even at mild levels, in a non-clinical young sample (Guardia-Serecigni et al., 2004). Other studies performed in clinical samples have found that other stress paradigms such as the Trier Social Stress Test (TSST) was able to increase alcohol craving and the motivation to drink (McCaul et al., 2018). The role of stress in alcohol-seeking is well-established in the animal literature. Hence, physical, social, and emotional stress have demonstrated to facilitate acquisition or increase alcohol self-administration in rodents and nonhuman primates (Sinha et al., 2003). Nevertheless, in general, more invasive stress methods are applied to rodents to induce craving, foot-shock being the most predominant (Lê and Shaham, 2002).

Therefore, in this initial approach focused on young participants with problematic drug consumption, it appears that a VR environment was able to induce anxious behaviors during the exploration of the maze and, in turn, triggered some mild anxiety and craving as shown by the administered questionnaires and behavioral pattern of ambulation. However, further research on this matter is necessary.

Young participants with problematic substance consumption presented higher EDA response after the end of the VR task, as well as an increased ratio sAA over sCORT levels.

Our VR EPM stimulated the ANS, as demonstrated by changes in HR and EDA, as well sAA and sCORT levels in the three testing conditions (t1, t2 and t3) in all our groups (Fig. 3). Regarding EDA, we observed that the AU + C group exhibited higher reactivity during and after the VR EPM compared to the CO group. Several studies have shown that this physiological response during emotional processing is correlated with activity in the amygdala (Phelps et al., 2001; Williams et al., 2001). It is unclear whether amygdala hypersensitivity preceded cannabis use or was a consequence of use, but it is possible that young people may use cannabis owing to its acute anxiolytic effects (Phan et al., 2008). Hence, cannabinoids could potentially weaken the primary neural inhibitory process in the amygdala, reducing the threshold for activation, particularly in response to threatening/stressful stimuli, such as our VR EPM (Spechler et al., 2015). Regarding the AU group, it showed a similar pattern of HR and EDA as the CO group. Some studies have suggested that blunted reactivity is a general characteristic of alcohol dependency, particularly in adults with a long-standing history of consumption, rather than in individuals with time-limited excessive alcohol use (Bibbey et al., 2015). Moreover, there is preliminary evidence that those young adults with abuse of different substances are more likely to show blunted physiological responses to an acute stress task than those with abuse of only one drug (Panknin et al., 2002). In our study, and as we mentioned before, our participants did not have a diagnosed addiction and were not receiving treatment.

sCORT has been widely acknowledged as a reliable biological marker for assessing the stress response of the HPA axis for many decades, whereas recently, more attention is given to the SNS activity, especially to sAA as a valid biological index of stress research reflecting central noradrenergic activation (Ali and Nater, 2020; Warren et al., 2017). To date, no study has examined the sCORT and sAA levels to a mildly virtual stressful situation in young individuals with substance risk consumption, especially alcohol and cannabis. Moreover, we have studied the levels of both biomarkers at three different moments (before the VR EMP as index of anticipatory stress (t1), after the end of the task (t2) and after 10 minutes as an index of stress recuperation (t3)). Our results reflected that our VR EPM was able to trigger the activity of the SNS in the three groups, above all after the end of the task. Specifically, we found a rapid response in sAA displaying a peak level immediately after the VR EPM and recovering to pre-task levels 10 min after the end of the

EPM. This result agrees with the data found by Biedermann et al.'s study (2022) in which sAA levels were also increased at first exposure to the VR EPM (Fig. 4a and b). Similar pattern of response of these salivary biomarkers were observed by Maruyama et al. (2012) after the TSST in healthy adult participants. Despite the fact that we did not observe significant differences between groups, it is noteworthy that the AU + C group showed the lowest levels of sAA in all three measurements during the VR test. In relation to this finding, previous studies have also observed that cannabis consumption led to reduced reactivity to a stressful situation, as well as significantly lower levels sAA after the TSST (Simon et al., 2023). This blunted physiological response has been also associated in some studies with a blunted emotional regulation during stressful events possibly suggesting the development of tolerance to the neuroendocrine effects of cannabinoids (Cuttler et al., 2017; Rubino et al., 2008). Even more, it has been suggested that the capacity of stress systems to respond to stressful stimuli/negative emotions seems to be only partially recovered after 6 months of cannabis abstinence in dependent subjects (Somaini et al., 2012). In line with this evidence, a possible alteration of safety system sensitivity, particularly of the reactions to threat, dangerous situations, negative and unpleasant emotions, has been previously reported in cannabis users (Boyd and Gumley, 2007). Moreover, a reduced response to negative emotions in terms of anxiety scores at STAI-t was evidenced in our experiment in the AU + C group, suggesting a possible lack of reactivity to stressful stimuli in these subjects (Table 2).

Regarding sCORT (Fig. 4b), we observed a downward trend of cortisol levels across the three time points examined. A study of Herbison et al.'s group (2016) defined these subjects with this type of cortisol pattern in response to the TSST as *Anticipatory Responders (AR)*. Interestingly, there is some prior indication that AR are more prevalent among young people (13–20 years) than other samples (Evans et al., 2013). The occurrence of an anticipatory cortisol stress response could constitute a manifestation of increased subjective stress sensitivity related to altered psychological wellbeing or chronic stress, among other factors (Engert et al., 2013). Besides, according to our results, it seems that sAA is insensitive to anticipatory stress (Fig. 4a) (Engert et al., 2013). Concerning the time course of these hormones, in the case of sAA, the levels of this hormone usually show an immediate increase with the presentation of a stressor (1–5 minutes) (Takai et al., 2004), peaking around 10 min (Granger et al., 2007; Nater et al., 2005) and they quickly return to baseline within 20–30 minutes after the stressor (Maruyama et al., 2012; Petrakova et al., 2015). With a higher threshold for activation and on a longer timescale involving sustained stress, changes in salivary cortisol are initiated 5–10 min after stressor onset, peak between 20 and 30 min, and can continue to be elevated for an hour or more (Kudielka et al., 2009; Joseph et al., 2021). In a comparison of different time points after a similar stress protocol (being T1: directly after, T2: 15 min, T3: 30 min, T4: 45 min, T5: 60 min), Biedermann et al. (2017) found that the peak of sAA was at T1 (directly after) and sCORT at T2 (15 minutes) following a decrease in the expression of both measures, especially sCORT.

On the other hand, only a limited number of studies have investigated the links between chronic stress and the regulation of both the SNS and the HPA axis (Rotenberg and McGrath, 2016; Sajaniemi et al., 2011; Vigil et al., 2010). Imbalances or disconnections between the SNS and HPA axis can have negative consequences and may be associated with a higher prevalence of health issues or behavioral complications. Some recent literature suggests that the sAA over sCORT ratio could serve as a more accurate indicator of dysregulation within the stress systems, particularly when correlated with self-reported levels of social stress, anxiety, as well as depressive symptoms (Ali and Pruessner, 2012; Engert et al., 2011). Thus, in our study we have calculated the index AOCg which was higher in the AU group compared to the CO group (Fig. 4c) suggesting a possible blunted HPA axis activity with a complimentary increase in SNS stress response. Our regression analysis reflected that AOCg index was the better predictor variable of alcohol craving level measured by MACS questionnaire. This result could be understood whether we considered the existence of a HPA axis dysregulation along with a sympathetic dominance toward stressful events in our AU group. Some studies have related similar results with the future development of an alcohol use disorder or with heavy alcohol use owing to the influence of elevated craving levels (Blaine et al., 2016; Boschloo et al., 2011; Schepis et al., 2011). Finally, because this ratio has been shown to be a sensitive marker of stress system dysregulation in adults and, given that the patterns of biological stress responsivity differ significantly between youth and adults, it is mandatory to understand stress responsivity during these early and critical periods of life (Allwood et al., 2011; Jopling et al., 2021).

Overall, this research field will enable us to develop preventive strategies that can provide support to young individuals who engage in substance use buffering the negative impacts of stress on their well-being. Mood and the emotional valence of a given situation may elicit alcohol craving and intake (Ghiţă et al., 2019; Wardell and Read, 2013). Thus, subjects with problematic alcohol consumption or alcohol use disorder are at great risk for relapse because they have increased vulnerability and fewer coping skills in stressful environments (Sinha, 2008). Hence, certain negative moods should be treated as triggering factors for alcohol use, focusing on boredom, stress, anxiety or tension, irritability, frustration, sadness, and anger (Pla-Sanjuanelo et al., 2015). Similarly, Wiers et al. (2003) suggested that subjective cognitive states such as fatigue, disgust, or distress were highly associated with alcohol misuse patterns. In this context, VR environments could help us to detect greater anxiety and/or altered physiological responses when people cope with more realistic and challenging stressful scenarios that are fully controlled by the researcher or clinician (Tsamitros et al., 2021). In this regard, the recent review by Dammen et al. (2022) has described how VR stress tasks elicited a varied magnitude of physiological stress reactivity constituting an effective tool in stress research. Specifically, the meta-analysis supported the idea that laboratory stress tasks administered in VR induce HPA and ANS reactivity in healthy adults across broad populations. Recently, the study of Rodrigues et al. (2021) has described the development of an interesting VR task, IMVEST, in which participants are simultaneously exposed to mental -arithmetic calculations- and environmental challenges, along with intense visual and auditory stimulation. It contains critical elements of stress elicitation, such as perceived threat to physical self, social-evaluative threat and negative feedback, uncontrollability, and unpredictability. Authors proposed that this type of stress-eliciting VR task could help us to assess whether an individual exhibits abnormal stress responses when faced with stressful challenges, as well as to evaluate the effectivity of specific treatments (e.g., mindfulness or psychotherapy) in mitigating heightened stress responses individuals or groups of developing at treatment. Meanwhile, individuals undergoing treatment could appreciate the opportunity to recreate and utilize a genuine experiential world within the confines of their therapists' clinical offices. Nonetheless, despite existing research in both physiological stress response and VR, more studies about this topic are necessary.

5. Conclusion and limitations

In summary, the present study focused on analyzing behavioral manifestations of anxiety by a VR EPM along with their underlying neural or psychophysiological pathways. The study of different biomarkers leads to important insights between the interplay of body and mind and VR seem to be a very useful tool to investigate anxiety in humans along with its attractiveness for young people. Nevertheless, further investigations with larger sample sizes and incorporating additional behavioral and physiological assessments are necessary. These studies could also examine potential associations between these patterns and other phenotypes, such as personality traits and social anxiety, as well as their potential links to psychosomatic issues. Finally, we must consider several limitations in our study. First, we used a relatively small

sample size (n = 70) and somewhat unbalanced groups (33 CO, 27 AU and 10 AU + C). Thus, testing more participants would increase the statistical power needed to draw stronger conclusions from our findings, and thus we need to consider these findings preliminary. Moreover, it should be noted that the ratio of men to women was not homogenous in each group and therefore, the findings of this study could be attributed to differential sex ratios in the three groups. Due to the larger representation of women in our sample, we should have considered their menstrual phase as a variable that influences in stress response. On the other hand, we have not analysed the influence of drug-related variables, such as the amount of alcohol/cannabis consumed by the participants which could impact in the heterogeneity of the individuals assigned to the experimental groups. Besides, the inclusion of other behavioral variables, such as the analyses of walking distances could give us more valuable information as an indicator of general activity. Finally, this study is observational and, therefore, conclusions regarding causality cannot be addressed.

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CRediT authorship contribution statement

R.D. Moreno-Fernández: Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Writing – original draft. **D. García-León:** Resources, Software. **G. Peñas:** Resources, Software. **R. Martín-Romero:** Investigation. **F. Buades-Sitjar:** Data curation, Formal analysis, Investigation. **P. Sampedro-Piquero:** Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Supervision, Writing – original draft, Writing – review & editing.

Declaration of competing interest

Authors declare no conflicts of interest.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

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