EFFECT OF AYAHUASCA ON EXPERIMENTAL MODELS OF ETHANOL ADDICTION AND IMMUNOHISTOCHEMISTRY ANALYSIS IN RODENTS

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Conflict of interest

All the authors have nothing to disclose.

Abstract

Ayahuasca, a psychoactive brew originally used ritually and therapeutically by various traditional peoples in the Amazon, is receiving special attention for its potential therapeutic effects on addiction. This study investigates the effects of lyophilized ayahuasca in alcohol-induced animal model, examining behavioral, pharmacological, and neurobiological aspects. Phytochemical analysis confirmed the presence of β-carbolines and dimethyltryptamine, with all compound's levels within literature ranges. Pharmacological screening revealed dose-dependent behavioral changes, with the highest dose inducing transient effects. Motor coordination deficits were observed at the highest dose, consistent with pharmacological screening. Pretreatment with ayahuasca prevented ethanol-induced conditioned place preference, suggesting anti-addictive potential. Immunoreactivity analysis revealed no significant changes in DeltaFosB and c-Fos expression in the nucleus accumbens core and shell subsections. This study suggests low acute toxicity of ayahuasca, motor coordination impairment at high doses, and potential anti-addictive effects in preventing ethanol-induced conditioned place preference. These findings contribute to the understanding of the pharmacological and behavioral effects of ayahuasca and highlight the need for further research and clinical studies to elucidate its therapeutic potential in substance use disorders.

1. Introduction

Ayahuasca (from Quechua "vine of souls") is a psychedelic botanical brew used by various traditional peoples in the Amazon for ritual and therapeutic purposes (McKenna & Riba, 2016). It is usually prepared by boiling the bark and stems of the vine *Banisteriopsis caapi* with the leaves of the shrub *Psychotria viridis*. The vine contains β-carboline alkaloids such as harmine, tetrahydroharmine (THH), and harmaline, while the shrub leaves contain N, N-dimethyltryptamine (DMT) (McKenna et al., 1984). Depending on the group of users and where the brew is prepared, other plants can be used or added to the traditional cooking process (dos Santos et al., 2011; Konrath et al., 2021; Schultes et al., 2001).

The β-carbolines inhibit the monoamine oxidase A (MAO-A), resulting in a synergistic effect that allows DMT to enter the bloodstream and cross the blood-brain barrier, triggering the psychoactive effect (McKenna & Riba, 2016). DMT is a 5-HT_{2A}, 5-HT_{2C}, 5-HT_{1A}, $_{1B}$, $_{1D,}$ and 5-HT_7 serotoninergic receptor agonist (Callaway et al., 1996; Grob et al., 1996; Nichols, 2016; Speranza et al., 2017). These receptors may be involved in the modulation of synaptogenesis and neuritogenesis (David & Gardier, 2016). Some studies have noted the interaction between DMT and sigma-1 receptors, which has been associated with potential neuroprotective effects (Bouso et al., 2012; dos Santos & Hallak, 2020; Rossi et al., 2022). In addition, DMT has been shown to interact with the endocannabinoid system as well as the vesicular monoamine transporter and trace amine-associated receptor 1 (TAAR1). TAAR1 has been shown as a promising therapeutic target for conditions such as schizophrenia, psychosis in Parkinson's disease, and substance use disorders (Dodd et al., 2021). Other studies suggest that β-carbolines, particularly harmine, may cross the blood-brain barrier and inhibits central nervous system MAO (He et al., 2015; Meinguet et al., 2015)). There is also evidence that tetrahydroharmine (THH), one of the most common β-carboline alkaloids found in ayahuasca brew, besides its action on MAO-A, acts as a serotonin reuptake inhibitor, resulting in increased concentrations of serotonin and DMT in the synaptic cleft (Callaway et al., 1999; McKenna, 2004).

The therapeutic potential of ayahuasca, although observed for centuries by traditional communities, has only emerged in the scientific literature in recent decades. Studies suggest that ayahuasca has therapeutic potential for psychiatric disorders such as anxiety, depression, and substance use disorder (SUD) (dos Santos & Hallak, 2020; Mendes et al., 2022; Wiltenburg et al., 2021). Qualitative and quantitative observational studies support the therapeutic potential of ayahuasca on SUD treatment (Barbosa et al., 2018; Doering-Silveira et al., 2005; Jiménez-Garrido et al., 2020; Mendes et al., 2022; Thomas et al., 2013). Additionally, preclinical studies seem to confirm the potential of ayahuasca in drug addiction treatment (Daldegan-Bueno et al., 2023; Mendes et al., 2022). Ayahuasca administration has been shown to reduce voluntary amphetamine consumption, amphetamine-induced locomotor activity and anxiety in rats (Godinho et al., 2017), and attenuated naloxone-induced withdrawal syndrome in morphine-dependent rats (Aricioglu-Kartal et al., 2003). The treatment with harmaline reduced self-administration of morphine and cocaine (Glick et al., 1994), as well as β-carbolines altered patterns of Fos expression in brain regions associated with addictive behavior (Daldegan-Bueno, Favaro, Morais, et al., 2022; Nolli et al., 2020; Reis et al., 2020).

Animal studies have also shown the potential therapeutic use of ayahuasca in alcohol use disorders (AUD) (Mendes et al., 2022). Preclinical studies showed that ayahuasca inhibited alcohol-induced sensitization and reduced the alcohol withdrawal symptoms in mice (Almeida et al., 2021; Oliveira-Lima et al., 2015). It was also observed that ayahuasca inhibited ethanol-induced conditioned place preference (CPP) expression (Cata-Preta et al., 2018; Gianfratti et al., 2022) and blocked the reinstatement of methylphenidate CPP induced by priming-injection (Reis et al., 2020).

Despite its burden on public health, pharmacological treatments for SUD, especially AUD, are ineffective and limited (Bozkurt, 2022). Also, in the context of the neurobiology of craving in AUD, the changes in the reward system involve central relay stations such as the ventral tegmental area (VTA), nucleus accumbens (NAc), and orbitofrontal cortex (Bracht et al., 2021). Given the therapeutic potential observed in previous studies, in this study we evaluated the effect of lyophilized ayahuasca pretreatment on alcohol-induced behavioral sensitization and CPP, along with the expression of c-Fos and DeltaFosB in the NAc.

2. Materials and Methods

2.1. Botanic material

The Scientific Commission of the União do Vegetal (UDV) provided the ayahuasca brew. Samples of the plants used to produce the brew were collected in the city of Mogi das Cruzes, São Paulo State, Brazil, and identified as *Banisteriopsis caapi* (Spruce ex Griseb.) Morton (Malpighiaceae) and *Psychotria viridis* Ruiz & Pav (Rubiaceae). The voucher species were deposited in the Herbarium of the Federal University of ABC (UFABC) under registration numbers F.R.Mendes 18 and F.R.Mendes 19, respectively. This study follows the Brazilian legislation on access to the biodiversity, and it is registered on the SISGEN platform (#AF827A4).

2.2. Ayahuasca preparation and lyophilization

The ayahuasca brew was initially concentrated in a rotavapor to a final volume of 1/10 of the initial volume and then frozen and lyophilized (Liotop k105) to produce a dry extract. The brew

concentration was measured as 356 mg/mL. The lyophilized was stored and resuspended in drinking water as needed. This method has several advantages, including preservation of all physical and nutritional properties of the product, storage capabilities, ease quantification, measurement of appropriate doses for the study, and allows for the use of more concentrated samples (Daldegan-Bueno, Favaro, Tófoli, et al., 2022).

2.3. Phytochemical analysis (quantification of compound alkaloids)

Phytochemical analysis was performed on lyophilized ayahuasca sample resuspended in distilled water at the original concentration (356 mg/mL). DMT and the β-carbolines harmine, harmaline, and THH were quantified by high-performance liquid chromatography (HPLC) coupled with a mass spectrometer (LC-MS /MS), according to the method described in Silveira et al. (2020).

The sample was initially diluted to a final ratio of 1:5000 by stepwise serial dilution (1:10 x 1:10 x 1:50). First, 100 µL of the aliquots, in triplicates, were diluted in 900 µL of mobile phase A (ammonium formate buffer with 0.1% formic acid). From this phase, another 100 µL was taken and diluted in 900 µL of mobile phase A, and finally, 100 µL of the final solution was diluted in 4900 µL of mobile phase A. The solution was shaken with a vortex at each dilution step for better homogenization. Ten microliters of the internal standard (DMT-d6 1 µg/mL) were added to 90 µL of the final solution to reach a final 100 ng/mL concentration. Finally, 5 µL of this solution were injected into the ultra-efficient liquid chromatography system (model Acquity System), coupled to a triple quadrupole mass spectrophotometer in tandem mode (model Quattro Premier) with electrospray ionization (UPLC-ESI-MS/MS). After quantification, the value obtained with the equation of the straight line of measurement was multiplied by five thousand to correct for dilution.

2.4. Animals

Male Swiss mice (2 to 5 months old) were obtained from the Center for the Development of Experimental Models in Medicine and Biology at Universidade Federal de São Paulo (CEDEME/UNIFESP) and the animal facility of the Federal University of ABC (UFABC Bioterium) for the toxicological and behavioral testing. The mice were housed and maintained in micro-insulated polypropylene cages (maximum of 5 animals per cage, without enriched environment) under a 12h/12h light/dark cycle (lights off at 7:00 pm) and controlled temperature (21 ± 4 ◦C). Water and food (Nuvilab®) were provided *ad libitum*. The mice were habituated for 15 days prior to the start of the experiments. The experiments were conducted during the light phase. All experimental procedures were approved by the Research Ethics Committee of the UFABC under registration number 2502090217 and the guidelines of the "principles of Care of Laboratory Animals" were followed.

2.5. Drugs and treatments

Analytical reagents were purchased from Sigma/Merck laboratories or equivalent. Antibodies and specific reagents used for immunohistochemistry are described in the respective topic. For intraperitoneal (i.p.) administration, absolute ethyl alcohol (Synth) was diluted in saline solution (0.9% sodium chloride) at a concentration of 20% and injected in a volume of 0.14 ml/10 g to achieve a dose of 2 mg/kg. Lyophilized ayahuasca (dry extract) was diluted in filtered water and administered by gavage (orally, v.o.) at a volume of 0.1mL/10g weight. The doses used for all behavioral and pharmacological tests were calculated based on a human adult (~70kg) average consumption of ayahuasca at UDV members during a routine religious ceremony (128ml), which is equivalent to a dose of approximately 650 mg/kg (mean unitary dose in humans). Doses of 130 (0.2X unitary), 650 (1X unitary), 1950 (3X unitary), 3560 (equivalent to 10X the initial concentration), and 5000 mg/kg

(maximum recommended dose in toxicological studies) were employed on the animal studies, as described in the following sessions. For the rest of the article, lyophilized ayahuasca is referred to as ayahuasca.

2.6. Initial pharmacological screening

The mice were divided into four groups (n = 7/group) and received an oral dose of water (control) or increasing doses of ayahuasca (650, 3560, and 5000 mg/kg). The animals were placed in wire cages immediately after administration and observed at intervals of 0 to 15 minutes, 15 to 30 minutes, 30 to 60 minutes, 1 to 2 hours, 2 to 4 hours, and after 24 hours (Carlini, 2011). The presence or absence of several signs and behaviors (such as signs of stereotypy, Straub's tail, in addition to trembling and ruffled fur) were registered, according to (Carlini, 2011).

2.7. Rota-rod test

Motor coordination was measured using a Rota-Rod apparatus (Panlab® model LE8500), and the mice received the same doses as described in the initial pharmacological screening (n = 7 /group). The objective was to certify that the ayahuasca doses employed in this study did not interfere with the animals' coordination. The rotarod consisted of a swivel bar of 5 cm in diameter, suitably machined to provide grip, divided into four equal compartments. Each animal underwent a training session and a test session 24h later. The training session consisted of placing the animal's four feet on the bar while serially increasing the rotations speed. During the test, the animals were placed back in the apparatus at a constant speed of 12 revolutions per minute and left for a maximum of three consecutive minutes. The test phase consisted of evaluations at 0 (basal measurement), 45, 90, and 180 minutes after vehicle or drug administration (Carlini, 2011). The latency and number of falls on the rotarod were measured. Each animal had to make three consecutive attempts to complete the task and the average time spent in the bar was calculated (each attempt was limited to a maximum of 60 seconds).

2.8. Conditioned place preference (CPP)

The CPP test evaluated the effect of ayahuasca pretreatment on alcohol-induced place preference. The CPP apparatus consisted of wooden boxes with two chambers (20x20x40cm) connected by sliding doors and a central passageway (12x12x40cm). The chambers had unique features such as wall colors (one was white, the other black), floor texture, and visual stimuli (different geometric symbols were placed on the walls). The test chambers were placed in a room with 40 lx illuminance. The time the mice spent in each chamber was recorded with a camera (Go Pro Hero 4) for later analysis with ToxTrac software (https://toxtrac.sourceforge.io). The CPP protocol was adapted from Prus et al. (2009) and consisted of two habituation sessions (one session/day), one preconditioning test, eight consecutive conditioning sessions (one conditioning session/day, with a total of four alternated conditioning sessions for ethanol and saline), and a post-conditioning test, 24 h after the last conditioning session, without any treatment, as described below.

2.8.1. Habituation and preconditioning test

The mice (not previously exposed to experimental procedures or treatments) were habituated for three days by placing them in the central passage with both sliding doors open and free access to both chambers for 15 minutes (habituation phase). On the third day, the time the animals spent in each chamber was measured to determine the animals' baseline preference (preconditioning test). No injections were administered on either the habituation days or the preconditioning test.

2.8.2. Conditioning

After the preconditioning test, animals were evenly distributed among four experimental groups (n = 9/10): Control water (CTR); ayahuasca 130 mg/kg (AYA130), 650 mg/kg (AYA650), and 1950 mg/kg (AYA1950). To reduce possible bias, half of the mice were paired with ethanol in the less preferred chamber, and the other half in the more preferred chamber in the preconditioning test (Prus et al., 2009). The conditioning sessions were performed over the following 8 days, in which animals received ethanol (2.0 g/kg i.p. at a concentration of 20%) or saline (0.9% i.p.), every other day, five minutes before being confined to one of the chambers. The first paring session was randomized. The ayahuasca or water was administered orally (v.gavage) 30min before each conditioning/pairing session with the animals in their home cages. All conditioning sessions lasted 5 min (Figure 1).

2.8.3. Postconditioning test

Twenty-four hours after the last conditioning session, the animals were placed again in the center of the CPP apparatus to explore the apparatus freely for 15 min. The time spent in each chamber was recorded using the same system from the preconditioning test. No injections were administered during the postconditioning test. Twenty-four hours after the test, the animals were euthanized and perfused for tissue collection and subsequent DeltaFosB immunohistochemistry (see Section 2.11). Figure 1 shows the illustrative scheme of the conditioned place preference protocol used and the scheme of treatment.

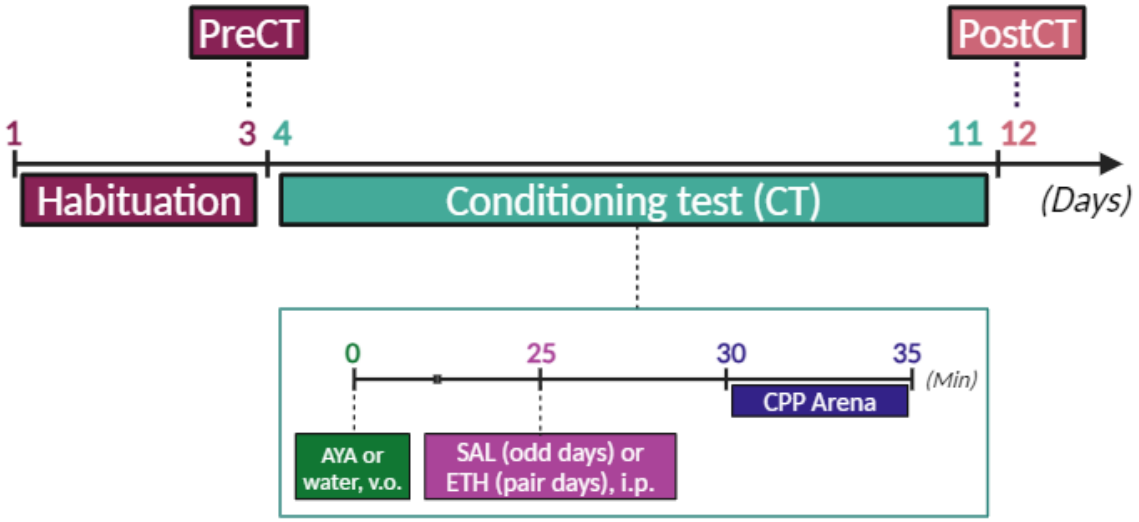

Figure 1. Design of the conditioned place preference (CPP) protocol and treatment schedule to investigate the effects of ayahuasca pre-treatment on alcohol-induced CPP. The CPP apparatus consisted of two chambers with different characteristics, such as colour, floor texture and visual stimuli, and a corridor connecting the two chambers. Habituation - mice had free access to both chambers for 15 minutes (for three days); PreCT, drug-free preconditioning test (mice preference was recorded); Conditioning, mice were divided into four experimental groups: Water, Ayahuasca (AYA 130, 650 or 1950 mg/kg). The conditioning sessions were carried out over 8 days. Ayahuasca or water was administered orally 30 minutes before each session (time = 0). Ethanol (ETH, 2.0 g/kg) or saline (SAL) was administered 5 minutes before the animals were confined to a chamber (time = 25) for 5 minutes (four days each); PostCT, drug-free postconditioning test. The post-conditioning test, conducted 24 hours after the last session, assessed the time spent in each chamber. No injections were administered during this test.

2.9. Behavioral sensitization

This protocol was based on a study that investigated the effect of ayahuasca on ethanol-induced sensitization (Oliveira-Lima et al., 2015) and was modified, as described below.

Animals were evaluated on the open-field apparatus, which consisted of an opaque white polyethylene arena with a cylindrical shape. The cylinder body was 50 cm high, and its base was a wooden circular disk 40 cm in diameter and 2 cm high. The behavioral sensitization procedure consisted of the following phases: habituation, sensitization, and ethanol challenge (Figure 2). Mice behavior was recorded with a camera (Go Pro Hero 4) and analyzed with the ToxTrac program (https://toxtrac.sourceforge.io). The behavioral parameters evaluated were locomotor activity (total distance traveled) and exploration rate (percentage of the number of zones crossed and the total number of zones generated by the ToxTrac software).

In the habituation phase all animals were placed in the center of the open field apparatus, and they could freely explore the arena for 30 min. After the habituation, the animals were randomly distributed among six experimental groups (n = 7-8/group): water x saline (WATER-SAL); water x ethanol (WATER-ETH); ayahuasca 650mg/kg x saline (AYA650-SAL); ayahuasca 650mg/kg x ethanol (AYA650-ETH); ayahuasca 1950mg/kg x saline (AYA1950-SAL); ayahuasca 1950mg/kg x ethanol (AYA1950-ETH).

The sensitization phase lasted 14 days. The animals received daily doses of water or ayahuasca by gavage in their home cages followed by an intraperitoneal injection of ethanol (2.0 g/kg at 20% concentration) or saline (0.9%) 30 min later. Twenty minutes after gavage, they were placed in the open-field apparatus for 10 minutes to acclimate, on days 1, 5, 9, and 14. The animals then received an intraperitoneal injection of ethanol (2.0 g/kg at 20% concentration) or saline (0.9%) and their behavioral parameters were recorded from minute 1 to minute 11 (10 minutes total).

Ninety minutes after the last sensitization session, the mice were deeply anesthetized and perfused, and the brains were collected and processed for immunohistochemistry.

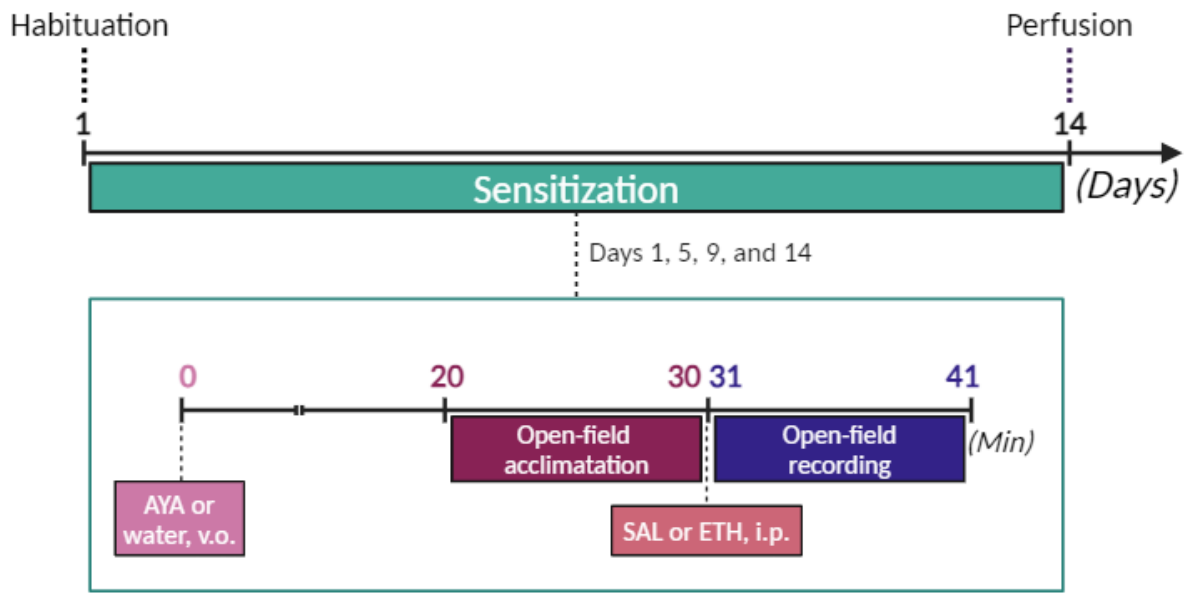

Figure 2. Design of the behavioral sensitization protocol and treatment schedule to study the effects of ayahuasca pretreatment on locomotor activity and open-field exploration rate in mice. Habituation phase, mice freely explored the open field for 30 min; sensitization phase, mice were randomly assigned to six experimental groups (n = 7-8/group): water, ayahuasca (AYA 650 or 1950 mg/kg) and saline (SAL) or ethanol (ETH, 2.0 g/kg). The sensitization phase lasted 14 days and consisted of daily gavage of water or AYA followed by i.p. injections of ETH or SAL 30 min later. On days 1, 5, 9, and 14, animals were exposed to the open field, allowed to acclimate for 10 min after gavage, followed by i.p. injections of ETH or SAL 30 min after gavage. Recording was performed from minute 1 to minute 11 (10 min total); perfusion was performed ninety minutes after the last sensitization session.

2.10. Tissue Preparation

The mice submitted to the CPP protocol were anesthetized twenty-four hours after the postconditioning test, for DeltaFosB immunohistochemistry, while the animals from sensitization test were euthanized ninety minutes after the last exposure in the open field, for c-Fos immunohistochemistry. The mice were profoundly anesthetized with 30% urethane (Sigma-Aldrich, St. Louis, MO, USA) and transcardiacally perfused. The brains were pre and post fixed in 4% paraformaldehyde (Sigma-Aldrich, St. Louis, MO, USA) in a 0.1 M phosphate buffer (pH 7.4) and then cryoprotected in a 30% sucrose/phosphate buffer for 48 h (4ºC). After that, brains were quickly frozen in cold isopentane (-40ºC; Sigma-Aldrich, St. Louis, MO, USA) and stored at -80ºC for further analyses.

The brains were cut in slices of 30 µm in a cryostat (Leica CM1860 UV Cryostat; Leica Microsystems Inc, IL, USA) at -15° to -20°C. Serial sections taken through the striatum (containing the NAc) were collected in ethylene glycolanti-freezing solution and stored at -20°C until the day of the immunohistochemistry assay (Prieto et al., 2023).

2.11. Free-floating immunohistochemistry

Coronal sections containing nucleus accumbens were washed in a 0.1 M phosphate buffer solution (PBS) and incubated with 10 mM sodium citrate buffer at 70 ºC for 20-30 minutes. Then, the sections rested at room temperature for 30 minutes and were then washed with 0.1M PBS with Triton X-100 (PBST), and blocked with 2% bovine serum albumin in PBST (for DeltaFosB) or 3% normal goat serum in PBST (for c-Fos). The tissues were then incubated overnight at 4°C with anti-DeltaFosB primary antibody (DeltaFOSB; rabbit; Cell Signaling Technology, ref. 22509; 1:1000 dilution) or anti-Fos antibody (c-Fos; rabbit; Cell Signaling Technology, ref. 22509; 1:1000 dilution) diluted in blocking solution. After, sections were washed in PBST, and incubated for 2-3 hours with biotinylated anti-rabbit secondary antibody (1:450 for DeltaFosB and 1:600 for c-Fos, Vector Laboratories, Burlingame, CA, USA). Finally, the sections were washed in PBST and incubated for 90-120 min in the avidin-biotin-peroxidase complex (ABC Elite kit, PK-610, Vector Laboratories) in PBST, washed and then incubated with 3,3′-diaminobenzidine for approximately 5 min and washed 4-5 times. The sections were transferred into 0.1M PBS and mounted onto chrome alum-gelatin-coated slides. Once dry, the sections were dehydrated with a graded series of alcohol (distilled water; alcohol 30%, 60%, 90%, 95%, and 100%) and xylol before coverslipping with Entellan (Merck, Darmstadt, Germany). A sample of negative control with absence of primary antibody was always carried out.

2.12. Image analysis

The NAc core and NAc shell regions were identified using Paxinos Atlas (Franklin & Paxinos, 2013). Images of c-Fos and DeltaFosB immunoreactivity were acquired with a camera (DFC295) connected to a microscope (Leika DM5500) with a 1.25x/0.0375 and 5x/0.12 objective. Labeling was visualized by a higher brown reaction product. Images of both hemispheres were acquired for each animal (3-6 per animal). C-Fos and DeltaFosB positive cells were quantified using ImageJ software (https://imagej.nih.gov/). Quantification of immunoreactivity was measured by the density of c-Fos and DeltaFosB cells per 0.1 mm^2. The sections representing the entire length of the NAc were analyzed, and the mean was used for further statistical analysis (See Sections 3.6 and 3.7).

2.13. Statistical analysis

The data was analyzed using JAMOVI (https://www.jamovi.org/) and GraphPad Prism (https://www.graphpad.com/) software. The Student's t-test, ANOVA, and repeated measures ANOVA (RM ANOVA) were used to analyze parametric behavioral data over time. The Shapiro-Wilk test and

Q-Q plot were used to analyze the normality of the data. The homogeneity of the data was also evaluated using Levene's test. For immunohistochemistry, ANOVA was used to compare the different experimental groups. The Tukey test was used for multiple comparison procedures between the treatment conditions and their respective control group. Statistically significant differences were considered if $p<0.05$.

3. Results

3.1. Phytochemical analysis (quantification of compound alkaloids)

The concentrations of DMT and the beta-carbolines harmine, harmaline, and THH are shown in **Table 1** together with the values described by Callaway et al. (2005). The DMT concentration was relatively low but within to those found in the literature.

Table 1. Quantification of compound alkaloids in comparison with data described by Callaway et al., (2005).

	Concentration (mg/ml)			
Sample:	DMT	THH	Harmaline	Harmine
Ayahuasca	0.08	1.86	0.18	1.4
Callaway, 2005	0.00-14.15	0.48-23.80	0.01-0.90	0.45-22.85

DMT - dimethyltryptamine; THH - tetrahydroharmine

3.2. Pharmacological screening

The qualitative analysis showed that the group treated with the highest dose of ayahuasca (5000 mg/kg) showed signs of stereotypy, Straub's tail, trembling and ruffled fur in the first hour. However, these behaviors decreased in the last hours, and after 24 hours they no longer showed atypical behaviors. The other groups showed no changes in behavior compared to the control group.

3.3. Motor coordination measurement (Rota-rod test)

The RM ANOVA revealed a statistical difference between and within groups in the Rota-rot performance over time [$F(12,72) = 4.11$, $p<0.001$]. Tukey`s post hoc found that only the group treated with the highest dose of ayahuasca (5000 mg/kg) showed a significant deficit in motor coordination in the 45-min trial ($p = 0.008$ relative to control, and $p < 0.001$ relative to its baseline time 0 min), supporting the pharmacological screening data (Figure 3). Subsequent trials, however, showed that the animals recovered their normal performance.

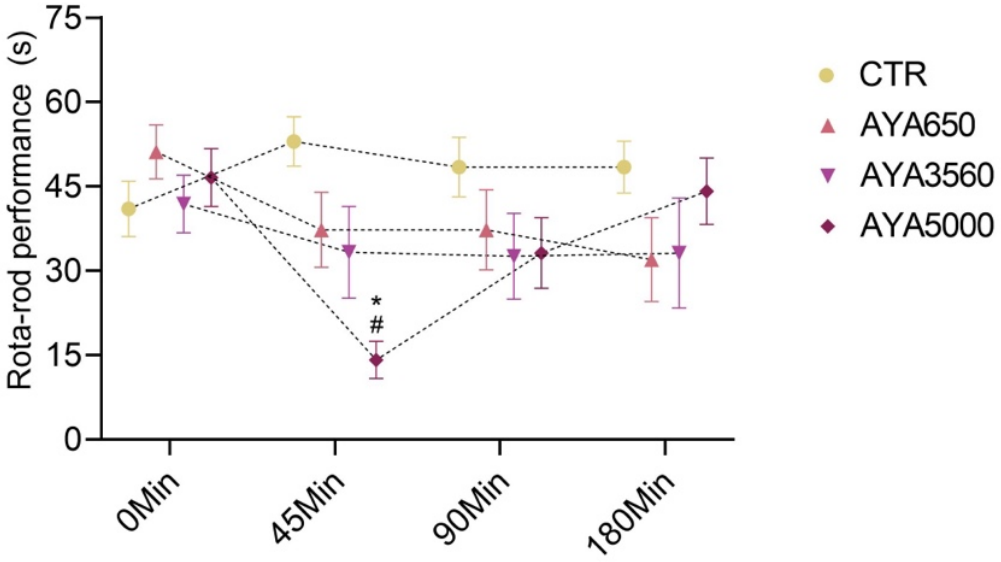

Figure 3. Spent time (seconds) on rota-rod (12 RPM) of mice orally administered water (CTR), ayahuasca (AYA) 650, 3560, and 5000 mg/kg. Data are reported as means ± SEM. * $p < 0.05$ - statistical difference from control; # $p < 0.05$ - statistical difference from baseline (0 min). Repeated ANOVA followed by Tukey's posthoc test (n = 7/group).

3.4. Conditioned place preference

The RM ANOVA revealed no statistically significant difference between the groups with respect to the time the animals spent in the two chambers in the preconditioning phase [$F(3,35) = 0.73$, $p = 0.55$] and in the postconditioning phase [$F(3,35) = 0.30$, $p = 0.831$] (Figure 4A). On the other hand, the Student's t-test revealed a statistically significant difference between time spent in the two chambers in the postconditioning phase for the control group [$p = 0.021$], while no differences were observed for the other groups (Figure 4B).

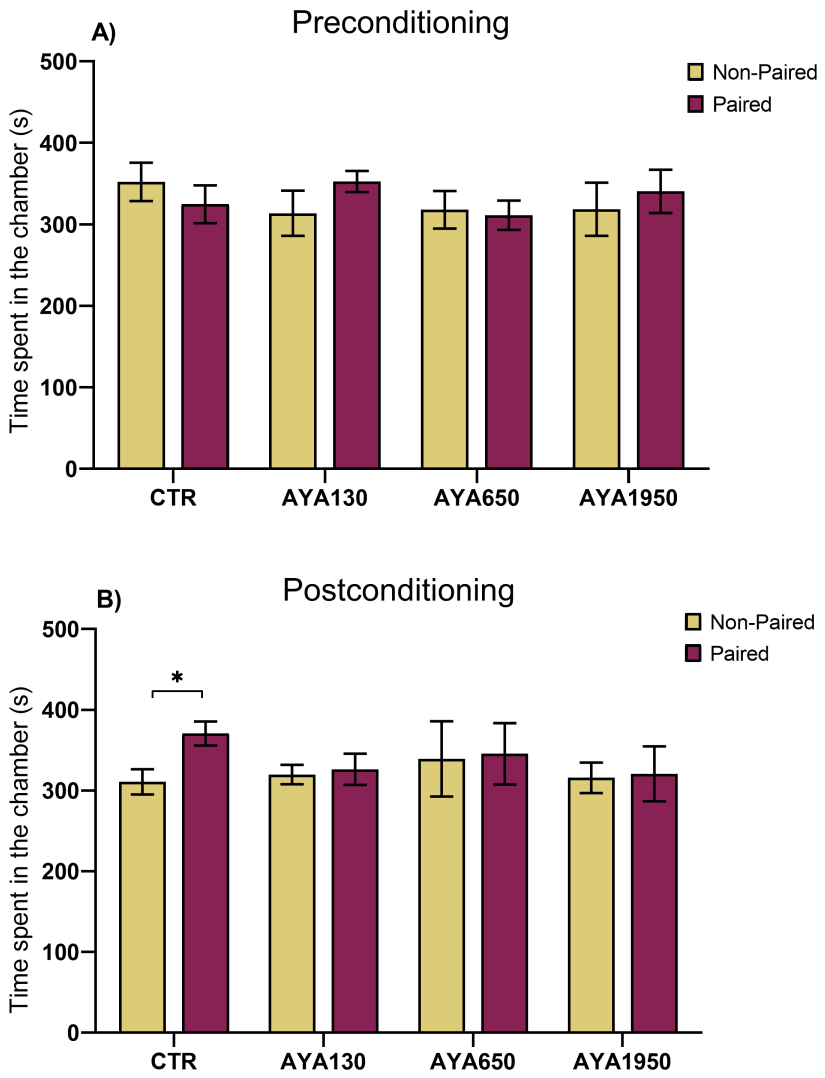

Figure 4. Time spent (seconds) in unpaired and paired chambers of mice administered orally with water (CTR), or ayahuasca (AYA) 130, 650, and 1950 mg/kg and i.p. with 0.9% saline (unpaired) and 2.0 g/kg ethanol (paired). Data reported as means ± SEM. A) Before the conditioning phase (pairing with ethanol and saline), ANOVA = n.s. and t-student = n.s. (n = 9 10/group). B) After the conditioning phase (pairing with ethanol and saline), t-student * $p < 0.05$ - statistically different on within-group comparison (n = 9 10/group).

3.5. Behavioral sensitization

The mice ambulation (distance traveled) and exploration rate were analyzed on open field before sensitization (baseline) and on days 1, 5, 9, and 14. The RM ANOVA followed by Tukey's test was used to evaluate between and within-group over time. The RM ANOVA did not show a statistically significant interaction between and within-groups over time for ambulation [$F(20,144) = 0.931$, $p = 0.550$ (Figure 5A). An analysis of the ambulation growth rate was also performed, but no statistically significant interaction was found among the groups at different assessment times.

In the open field exploration rate, the RM ANOVA showed a statistically significant interaction within groups over time [$F(20,132) = 1.76$, $p = 0.031$]. Tukey's post hoc analysis showed statistically significant interaction for AYA1950-ETH ($p < 0.001$; Figure 5B) and AYA1950-SAL ($p = 0.005$) groups comparing day 1 and their baseline. No other differences were observed among groups or over time.

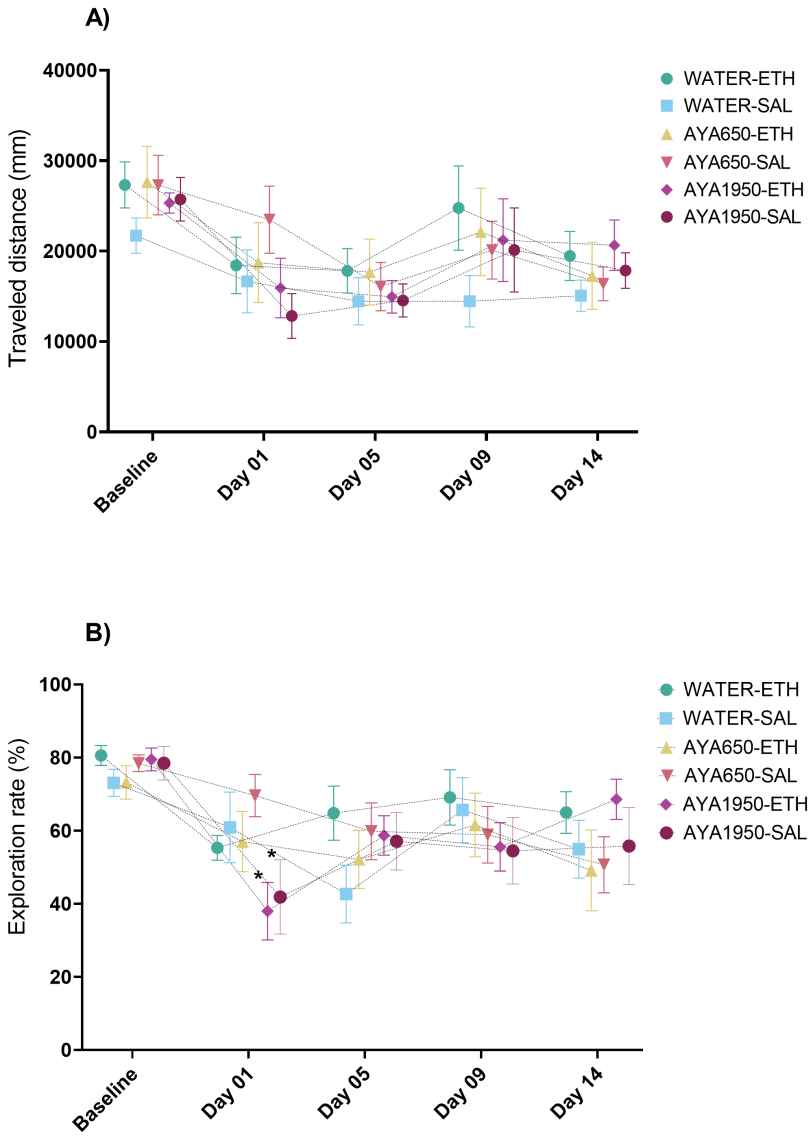

Figure 5. Mice exposed to the open-field arena administered orally with water and ayahuasca (AYA) 650 and 1950 mg/kg and i.p. with 0.9% saline (SAL) or 2.0 g/kg ethanol (ETH). Animals were evaluated for 10 min on different days, from baseline (no treatment) and from day 01 to day 14. A) Traveled distance (millimeters), data reported as means ± SEM, RM ANOVA followed by Tukey (n = 6-8/group), $p > 0.05$, n = 6-8/group. B) Exploration rate (percentage), data reported as means ± SEM. RM ANOVA followed by Tukey (n = 6 8/group), * $p < 0.05$ - statistical difference from baseline.

3.6. DeltaFosB protein immunoreactivity

Quantification of immunoreactivity was measured by the density of c-Fos and DeltaFosB cells in shell and core regions of the NAc (Figure 6A-B). The one-way ANOVA of DeltaFosB protein immunoreactivity of mice from alcohol-induced CPP revealed no statistically significant interaction between the groups, both in the core [$F(3,3.69) = 2.17$, $p = 0.243$] and in the shell region [$F(3,5.94) = 3.83$, $p = 0.077$] of nucleus accumbens (Figure 6, pannels C and E).

3.7. c-Fos protein immunoreactivity

The ANOVA of c-Fos protein immunoreactivity of mice from behavioral sensitization revealed no statistically significant difference between the groups, both in the core [F (5,34) = 2.006, p = 0.103] and in the shell region [F (5,32) = 0.987, p = 0.441] of nucleus accumbens (Figure 6, pannels D and F).

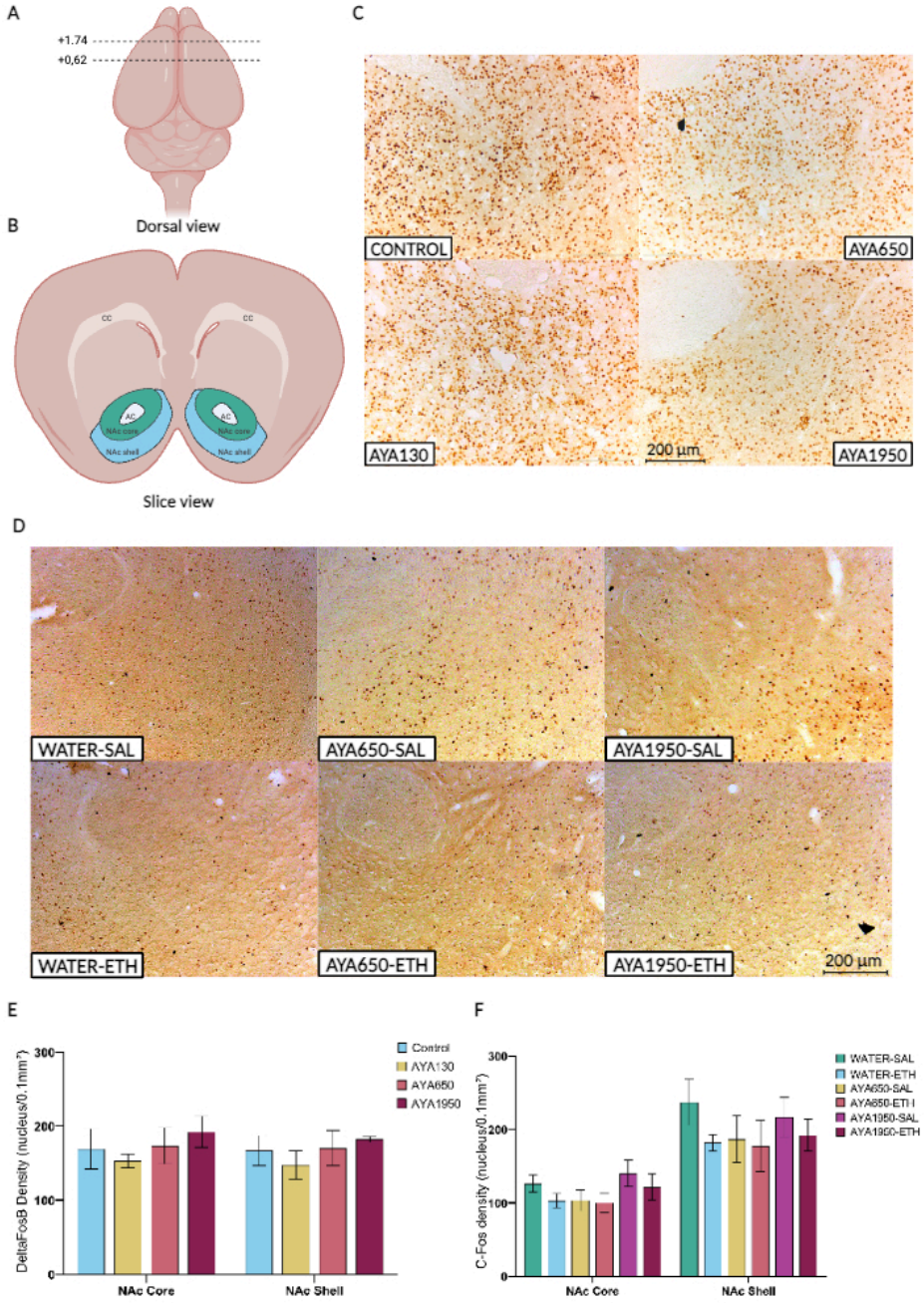

Figure 6. A) Range of sections analyzed by immunohistochemistry (dorsal view). B) Slice view of the regions of NAc analyzed (NAc core and NAc shell) and anatomical references (AC – Anterior commissure; CC – Corpus callosum). C) Representative photomicrographs showing DeltaFosB-ir cells. D) Representative pictures of c-fos staining. E) DeltaFosB quantification (nuclei/0.1mm²) in the NAc regions of mice receiving orally water (Control) and ayahuasca (AYA) 130, 650 and 1950 mg/kg and i.p. with 0.9% saline and 2.0 g/kg ethanol and subjected to the CPP protocol. Data reported as means ± SEM (ANOVA, p > 0.05, n = 4-5/group. F) c-Fos quantification (nuclei/0.1mm²) in the NAc regions of mice receiving orally water and ayahuasca (AYA) 650 and 1950 mg/kg and i.p. with 0.9% saline (SAL) and 2.0 g/kg ethanol (ETH) and subjected to the behavioral sensitization protocol. Data reported as means ± SEM (ANOVA, p > 0.05, n = 6-8/group).

4. Discussion

Several observational studies suggest a beneficial effect of ayahuasca on drug addiction and other psychiatric conditions (Mendes et al., 2022; Wiltenburg et al., 2021). Previous preclinical studies support the potential anti-alcohol effect of ayahuasca (Daldegan-Bueno et al., 2023; Gianfratti et al., 2022; Nunes et al., 2016). However, these studies are still sparse, lack reproducibility, especially because of the high heterogeneity associated with the brew, and few have investigated behavior and protein expression together. Preclinical studies with ayahuasca are an important source of new insights and knowledge and are in line with the current increase in observational and clinical studies. This study aimed to examine the effects of lyophilized ayahuasca pretreatment in two alcohol-induced animal models and its effects on FOS expression in the nucleus accumbens, an area that plays an important role in substance use disorders.

The phytochemical analysis of the ayahuasca sample used in the current study revealed the presence of β-carbolines in concentrations compatible to those found in the literature. Particularly, DMT levels were below the averages commonly reported in other experimental studies, but within the range reported in the literature (Callaway, 2005; Souza et al., 2019). It is worth citing that the ayahuasca sample employed in this study induced the desired subjective effects when consumed as part of the group's ritual, according to members of UDV (personal communication). Also, the doses used in animal's treatment were based on the average ritualistic unitary dose calculated for an individual of 70 kg, that is 651 mg/kg (as shown on session 2.5).

The concentration of alkaloids contained in ayahuasca can vary depending on the sample, even when prepared by the same group. Factors such as the brew's cooking time, harvest time, and even the timing of plant collection can affect the amount of these alkaloids (Callaway, 2005). In this study the amount of harmaline was slightly higher in the sample than that observed in other studies, suggesting that the higher concentration of harmaline might had helped compensate for the lower level of DMT, in terms of the subjective effects on the participants in the ritual.

In the pharmacological screening, the highest dose tested (5000 mg/kg) elicited behavioral alteration in the animals, including Straub tail, tremor, and increased sensitivity. These behaviors can be characterized by the so-called "serotonergic syndrome" (Carlini, 2011). Although ayahuasca induced some toxic effects at the highest dose, no animal died and behavioral changes decreased over time, with no apparent toxic behavioral features 24 hours later. Additionally, this dose of ayahuasca decreased the animal performance on the rota-rod, suggesting that ayahuasca at high doses may impair behavioral performance in tests requiring preserved motor coordination. However, all the other doses did not cause motor impairment. These results are consistent with data from the literature and confirm the low toxicity of ayahuasca brew when administered orally and in doses equivalent to those used in ayahuasca ceremonies (Morais, 2014; Pires et al., 2010).

The possible anti-addiction effect of ayahuasca was evaluated in two animal models: conditioning place preference and behavioral sensitization test. The conditioning protocol was suitable to induce an increase in time spent in the chamber paired with alcohol in the control group and the pretreatment with ayahuasca was sufficient to block the development of the alcohol-induced CPP in all tested doses. However, no difference among groups was observed, likely because the alcohol-induced CPP in the control group was moderate. The blocking of alcohol-induced CPP by ayahuasca has already been described. Cata-Preta et al. (2018) and Gianfratti et al. (2022) observed that ayahuasca blocked alcohol-induced CPP at different doses and that ayahuasca might induces CPP by itself. On the other hand, Cata-Preta et al. (2018) showed that the extracts of *Psychotria viridis* and *Banisteriopsis caapi* did not induce CPP *per se*, nor blocked ethanol-induced CPP, highlighting the importance of the synergistic effects of ayahuasca constituents.

Locomotor hyperactivity is one of the factors most affected by repeated administration of drugs that can increase dopaminergic function (Fish et al., 2002; Lessov & Phillips, 1998). Oliveira-Lima et al. (2015) showed that ayahuasca at doses between 30 and 500 mg/kg blocked ethanol-induced sensitization in mice. In this study, however, there was no development of ethanol-induced locomotor sensitization; therefore, the effect of ayahuasca pretreatment could not be evaluated as planned. The absence of alcohol-induced behavioral sensitization may be due to several factors. Some authors suggest that behavioral sensitization may depend on the predisposition of some animals to alcohol self-administration (Grahame et al., 2000; Lessov & Phillips, 1998). Outbred mice usually exhibit high variability (observed in our work with the high within-group variance and standard deviation) and may not exhibit behavioral sensitization (De Pauli et al., 2014; Quadros et al., 2002). The environment of the treatment, housing conditions, the number of sessions and amount of time the animals are exposed to the open field also seems to be crucial for the development of behavioral sensitization (Araujo et al., 2006; Badiani et al., 1995; Crombag & Robinson, 2004). Our protocol was based on the study by Oliveira et al. (2015), but unfortunately it was not able to produce behavioral sensitization.

In addition to evaluating the animal's ambulation in the open field, we also evaluated the exploration rate of the animals, as well as the growth rate of the evaluated parameters over time. Again, the control group showed no difference over time, and there was no substantial difference between groups. On the other hand, the animals receiving the highest dose of ayahuasca decreased their exploration rate on day 1 compared to baseline measurement, both in the group treated with ethanol and treated with saline. This effect might be related to a possible sedative or anxiogenic effect induced by the highest ayahuasca dose, but such effect was not sustained on subsequent measurement days, suggesting a habituation to the dose employed. Additionally to behavioral analysis, we assessed the weight of the animals, but no animal or experimental group showed significant differences in weight (data not shown).

Even though the behavioral effects of ayahuasca in the animal models was limited, the repeated treatment with alcohol and ayahuasca could have modulated the expression of early genes. Quantification of positive DeltaFosB and C-Fos cells was performed in the CPP and sensitization protocol, respectively, to evaluate possible intracellular changes in the NAc core and shell after alcohol and ayahuasca administration.

DeltaFosB is known to be a transcription factor that accumulates in the brain after any drug exposure. It is a stable molecule that can remain present for long periods of time (Damez-Werno et al., 2012; Nestler et al., 2001). DeltaFosB is also known to increase the sensitivity of the reward circuit to a drug and may be one of the mechanisms behind drug-induced craving and euphoria (Nestler et al., 2001). Interestingly, structural changes in the NAc caused by DeltaFosB are also considered to cause drug relapse, and the overexpression of the protein is known to occur in D1 neurons of the NAc of people suffering from AUD (MacNicol, 2017; Olsen, 2011). This mechanism through DeltaFosB may produce sensitivity in former addicts when they are reintroduced to drugs, leading to rapidly escalating relapse (MacNicol, 2017). To our knowledge, this is the first work evaluating DeltaFosB-positive cells after ayahuasca treatment. However, our analysis revealed no difference in the number of DeltaFosB positive cells between and within experimental groups, both in core and shell subregions of the NAc. These results suggest that the moderate alcohol-induced CPP in the control group and the absence of CPP in the other groups did not induce significant intracellular changes in these markers in NAc. However, another control group that did not receive ayahuasca nor alcohol would be important to understand whether these changes would be comparable to those in a naïve group.

C-Fos is a gene with immediate expression, and the c-Fos antibody is usually used in acute studies to demonstrate the effect of short-term manipulations or the effect on the last day of chronic or sub-chronic procedures. In our study, the results correspond to the last day of sensitization. Our analysis revealed no difference in the number of c-Fos positive cells between the experimental groups, both in

core and shell subregions of the NAc. There are contradictory results in the literature as the effect of ayahuasca on c-Fos expression, which may be related to the fact that chronic procedures with discreet or no contextual changes do not increase c-Fos expression, possibly due to the stabilization of neural adaptation mechanisms (Daldegan-Bueno, Favaro, Morais, et al., 2022; Faria et al., 2008; Nolli et al., 2020; Walter et al., 2017). For instance, Faria et al. (2008) observed that acute administration of ethanol increased the expression of Egr-1 and c-Fos in regions such as the prefrontal cortex, motor cortex, amygdala, hippocampus, and NAc of mice, while chronic administration resulted in the opposite effect. On the other hand, Walter et al. (2017) did not observe an alteration in c-Fos levels in the NAc core and shell after acute or chronic alcohol administration.

Few studies have examined the levels of c-Fos positive cells in brain of mice treated with ayahuasca and ethanol. In this sense, Daldegan-Bueno et al. (2022) showed that rats treated with low and intermediate doses (120 to 480 mg/kg) of ayahuasca (comparable to those used in this study) did not show difference in c-Fos positive cells between the ayahuasca-treated and control groups in the hippocampus, amygdala, pre-frontal and barrel field cortex. Nolli et al. (2020) also observed a similar effect, in which mice treated with ayahuasca did not show difference in c-Fos positive cells in the NAc region compared with the control group.

Although studies are inconsistent regarding the specific NAc subregion associated with the development of addiction and behavioral sensitization, current evidence suggests that the NAc shell region might play a predominant role (Brenhouse & Stellar, 2006; Cruz et al., 2015; McReynolds et al., 2018). In our study, the absence of differences in c-Fos positive cells density between NAc subregions reinforces that behavioral sensitization and ayahuasca administration did not elicit any significant effects on c-Fos positive cells density.

Recent studies suggest the benefits of ayahuasca brew in the treatment of substance use disorder, particularly alcohol (dos Santos & Hallak, 2020; Mendes et al., 2022; Wiltenburg et al., 2021). There are a variety of physiological and psychological mechanisms by which ayahuasca can effectively impact addiction. The fact that the effect of ayahuasca results from the synergistic action of two complex plants, each containing its alkaloids, provides a variety of direct and indirect mechanisms of action. Since DMT belongs to the class of tryptamines, ayahuasca therapeutic role seems to be shared with the general actions of this class, combined with some unique properties beyond tryptamines, such as ayahuasca affinity to sigma-1 receptors (Quadir et al., 2019; Robson et al., 2012). On the other hand, β-carboline alkaloids exhibit special properties and should also be studied separately (Politi et al., 2021; Wiltenburg et al., 2021).

To understand the hypothesis of how ayahuasca can be used therapeutically for AUD, it is important to emphasise that addiction is known to be a chronic disease that can impair the ability of the brain's reward circuitry to respond to reward, as well as the modulation of stress and self-regulation (Volkow et al., 2019). Addictive drugs have the potential to increase dopamine release in the mesolimbic reward pathway, either directly by acting on the dopaminergic system or indirectly by affecting the modulatory activity of other neurotransmitters, such as serotonin (Tomkins & Sellers, 2001; Volkow et al., 2019).

Ayahuasca may modulate brain dopamine levels primarily through its effects on serotoninergic pathways (Liester & Prickett, 2012). There is evidence that stimulation of 5-HT_{2A} and 5-HT_{2C} receptors by agonists directly modulates dopamine release in dopaminergic pathways (mesolimbic, nigrostriatal, and mesocortical) (Howell & Cunningham, 2015; Sari et al., 2011; Sullivan et al., 2015). Moreover, stimulation of postsynaptic serotonergic receptors appears to excite GABAergic interneurons, and consequently, these neurons inhibit dopamine release (Howell & Cunningham, 2015; Sari et al., 2011). In addition, high doses of DMT could also activate the 5-HT_{1B} receptor, which is important for presynaptic inhibition (Guo & Rainnie, 2010; Sari et al., 2011). The effect of ayahuasca on dopaminergic neurons suggests that it reduces the activation of reward circuits in the brain, thereby

reducing the effects associated with substances with addictive potential (Liester & Prickett, 2012; Rossi et al., 2022). In our ayahuasca sample, the amount of DMT was low compared to other studies (Callaway, 2005), as described previously; therefore, it is possible that this low amount was not sufficient to act efficiently on all of the aforementioned serotonin receptors. In general, the anti-addictive effects of ayahuasca, at least from a pharmacological perspective, are associated with the dopaminergic and serotonergic systems.

In addition to pharmacological mechanisms, non-pharmacological mechanisms also appear to be essential for the anti-addictive effects of ayahuasca. Several observational studies suggest the importance of the context (often referred to as *set* and *settings*) as part of the treatment (Liester & Prickett, 2012; Mendes et al., 2022). Therefore, animal models may not be the best tool to investigate the potential effect of ayahuasca in drug addiction. Controlled clinical studies, including therapeutic setting and support as in psychedelic-assisted therapy are necessary for a better understanding of ayahuasca therapeutic effect on SUD.

To summarize, the results of this study suggest that ayahuasca administered in doses comparable to those typically used in religious contexts carries a minimal risk of acute toxicity, does not affect general motor coordination and activity, and prevents ethanol-induced conditioned place preference in mice. These findings may indicate that ayahuasca's pharmacological properties could potentially stabilize serotonergic neurotransmission and suppress the dopamine-mediated reward reaction to ethanol. However, due to the limited number of studies conducted on animal models thus far, additional research is necessary to enhance our understanding of these mechanisms and further validate our results concerning ayahuasca's impact on ethanol's rewarding properties. In addition, metabolomic analysis of the brew should be performed to verify and identify other target and non-target components of the beverage and to allow investigation of the role of these components in the psychopharmacological and behavioral effects observed.

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