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# Effects of the hallucinogenic beverage ayahuasca on voluntary ethanol intake by rats and on cFos expression in brain areas relevant to drug addiction

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ABSTRACT

Ayahuasca is a hallucinogenic infusion used in religious rituals that has serotoninergic properties and may be a potential therapeutic option for drug addiction. In this study, Wistar rats had intermittent access to ethanol for 8 weeks, receiving water (control), naltrexone (NTX, 2 mg/kg body weight [bw] intraperitoneally [i.p.]) or ayahuasca (Aya) at 0.5x, 1x, or 2x the ritual dose in the final 5 days. A naïve group had access only to water. Ethanol intake was estimated throughout the experiment, and cFos expression was evaluated in medial orbital cortex (MO), ventral orbital cortex (VO), lateral orbital cortex (LO), nucleus accumbens (NAc), and striatum. Treatment with either NTX or Aya (oral) did not decrease ethanol intake compared to the baseline level (5th to 7th week), but the NTX group intake was significantly lower than controls ($p < 0.05$). Ethanol significantly increased cFos expression in the MO region for control ($p < 0.0001$), NTX ($p < 0.05$), Aya1 ($p < 0.001$), and Aya2 ($p < 0.0001$) groups. This increase was also observed in the VO for the Aya1 group ($p = 0.035$), in the LO for the Aya2 group ($p < 0.01$), and in NAc for NTX and ayahuasca groups ($p < 0.005$). Furthermore, NTX and Aya0.5 treatment decreased cFos expression compared to controls in the MO region ($p < 0.05$ and $p < 0.01$, respectively), but only the ayahuasca group reached levels not significantly different from the naïve group. Studies using other protocols and dose regime are necessary to better investigate the impact of ayahuasca on alcohol intake by rats to support the observations in humans. Additionally, the role of ayahuasca in mediating cFos expression in other selected brain regions and its relationship with the serotoninergic/dopaminergic systems and drug addiction need further investigation.



## Introduction

Alcohol abuse is one of the five major risk factors for disease worldwide, linked to over 200 health conditions, including cancer, liver and cardiovascular diseases, road injuries, violence, and suicides. In 2016, the harmful use of alcohol resulted in about 3 million deaths worldwide, higher than that caused by tuberculosis, HIV/AIDS, and diabetes, representing 5.3% of all deaths (World Health Organization, 2018). Alcohol, like other drugs of abuse, is consumed for its positive reinforcing effect, and chronic exposure leads to changes in brain chemistry, withdrawal symptoms, and behaviors characteristic of alcohol use disorder (AUD), as described in the most recent Diagnostic and Statistical Manual of Mental Disorders (DSM5) (National Institutes of Health, 2016). There are various pharmacological options to treat AUD, including naltrexone, acamprosate, baclofen, and topiramate, although most treatments show low to medium efficacy (Palpacuer et al., 2018).

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The therapeutic potential of ayahuasca for various conditions and diseases has been investigated, including for drug addiction (Domínguez-Clavé et al., 2016; Frecska, Bokor, & Winkelman, 2016; Hamill, Hallak, Dursun, & Baker, 2019; Nunes et al., 2016). Ayahuasca is a psychoactive beverage used in spiritual rituals and for healing since ancient times by native groups from the Amazon region (Labate & Cavnar, 2014; Luna, 2011). The beverage was introduced to non-indigenous groups in the 1930s in Brazil, where it has been legally used in the religious context since 1984 (CONAD, 2010). In recent decades, this use has spread to other South American countries, the United States, Canada, and some European countries (Health Canada, 2017; Labate & Jungaberle, 2011). Ayahuasca infusion is generally prepared by the decoction of the leaves of *Psychotria viridis*, which contain N,N-dimethyltryptamine (DMT), a non-selective 5-HT receptor agonist, and vines of *Banisteriopsis caapi*, which contain β-carboline alkaloids (mainly harmine, harmaline, and tetrahydroharmine), which are monoamine oxidase (MAO) inhibitors (Domínguez-Clavé et al., 2016; Smith, Canton, Barret, & Sanders-Bush, 1998).

Changes in serotonin (5-HT) transmission and reuptake are associated with alcohol addiction, and it has been shown that increased serotonergic activity decreases ethanol intake, while decreased serotonergic function increases ethanol intake as well as increasing aggressive behavior (Heinz, Beck, Meyer-Lindenberg, Sterzer, & Heinz, 2011; LeMarquand, Pihl, & Benkelfat, 1994). Decreased serotonin neurotransmission in dependent animals may be associated with relapse drinking (Clapp, Bhave, & Hoffman, 2008; Lê et al., 1999), and administration of fluoxetine, a 5-HT reuptake inhibitor, and of 5-$HT_{1A}$ and 5-$HT_{1B}$ receptor agonists significantly reduced ethanol intake by rats (Murphy et al., 2002). The serotonin transporter gene has been linked to excessive drinking, early-onset problem drinking, alcohol dependence, anxiety, and impulsiveness, probably due to reduced serotonin availability in the brain (Johnson et al., 2008; Thompson & Kenna, 2016).

Transcribed *c-fos* mRNA and the translated protein product cFos can be used as a marker of strongly activated neurons. Cruz and coauthors (Cruz, Javier Rubio, & Hope, 2015) have hypothesized that Fos is expressed in the small number of neurons that received the highest levels of cue-induced glutamatergic excitatory input during conditioned drug behavior. Various studies have investigated the expression of Fos proteins, including cFos, in brain regions of rats exposed to alcohol, such as the prefrontal cortex, nucleus accumbens, and striatum (Jaramillo, Randall, Frisbee, & Besheer, 2016; Li et al., 2010; Sharma, Dumontier, DeRoode, Sahota, & Thakkar, 2014).

The objectives of this study were to evaluate whether ayahuasca treatment can decrease ethanol intake in rats exposed to intermittent access to the beverage, and to investigate the effects of ayahuasca in the neural activity (through cFos expression) in brain areas relevant to drug addiction of ethanol-exposed animals.

## Materials and methods

### *Ayahuasca*

The ayahuasca infusion was prepared by a religious group (*União do Vegetal*, UDV) using *B. caapi* and *P. viridis* collected in the Federal District of Brazil (Pic-Taylor et al., 2015). Soon after preparation, the beverage was frozen and stored at −20 °C for lyophilization (Liotop L101), with calculated dry matter content of 16% (w/v). Harmine and harmaline analytical standards were obtained from Sigma–Aldrich Co., DMT was synthesized as described by Qu et al. (2011), and tetrahydroharmine was synthesized from harmaline according to Callaway et al. (1996). The identity and purity of the synthesized compounds were determined by LC-MS/MS (Shimadzu LC system coupled to a mass spectrometer 4000 QTRAP, Applied Biosystem), 1H and 13C NMR (Varian Mercury Plus spectrometer 7.05 operating at 300 MHz for 1H and at 75.46 MHz for 13C) and LC-MSD-TOF (Agilent 1100 Series). The ayahuasca material was analyzed prior to the experiment using GC–MS/MS (Trace Ultra coupled with TSQ Quantum XLS Triple Quadrupole; Thermo Scientific), and was shown to contain 0.12 mg/mL DMT, 1.19 mg/mL harmine, 0.08 mg/mL harmaline, and 0.15 mg/mL tetrahydroharmine.

### *Animals*

A total of 64 male *Wistar* rats (about 7 weeks old and weighing ~ 250 g, with a maximum weight variation of 20%) were obtained from the University of São Paulo (Brazil) and kept for 7 days at the animal facility of the Faculty of Medicine of the University of Brasilia for acclimation prior to starting the study. Rats were housed individually in standard polypropylene cages with stainless steel coverlids and pinewood shavings as bedding and kept under controlled environmental conditions (12 h/12 h, dark/light; 22–25 °C; 45–60% humidity). Filtered water and a commercial laboratory rat chow (Labina, Purina®, Brazil) were provided *ad libitum*. This study was conducted according to the Guide for the Care and Use of Laboratory Animals (National Research Council, 2011) and approved by the University of Brasilia Ethics Committee on Animal Use (License No. 73276/2014).

### *IA2BC (intermittent access to 2-bottle choice) protocol*

Sixty animals were exposed to a 20% solution of ethanol (Dinâmica®) for 8 weeks according to the IA2BC protocol (Carnicella, Ron, & Barak, 2014). Every Monday, Wednesday, and Friday at 5:00 PM (beginning of the dark period), the animals were weighed and put individually into a cage with simultaneous access to a bottle of filtered water and a bottle of 20% ethanol solution (250-mL acrylic or plastic bottle). The bottles were weighed before being made available to the animals, and re-weighed 24 h later, at the end of each exposure day. The ethanol bottle was then replaced by another water bottle for the 24-h period of non-ethanol exposure, except for the weekend (48-h non-exposure period). In each exposure session, the position of the ethanol bottle in the cage was switched to avoid any side preference by the animal. To test for ethanol leakage during the experiment, an ethanol bottle was put into an empty cage and weighed in each session, showing a loss of lower than 1 mL, which is considered acceptable (Li et al., 2010). In addition to the 60 animals submitted to the IA2BC protocol, four animals (naïve, not exposed to ethanol) received only water *ad libitum* for 7 weeks, when they were euthanized for biological sample collection.

The treatments started on the Monday of the 8th week of ethanol exposure, just before the ethanol session, and continued daily until Friday. The animals were divided into five groups: water (control, gavage), naltrexone (NTX, 2 mg/kg bw i.p.), and ayahuasca (Aya, gavage) groups at 0.5x, 1x, and 2x the ritual dose. NTX was prepared from the medicament Uninaltrex® (tablet, GENOM®), which was solubilized in NaCl 0.9% in a sonicator to prepare a final solution of 1 mg/mL. A 1x dose corresponds to 150 mL of ayahuasca taken by a 70-kg person, and to 0.26 mg/kg bw DMT, 2.58 mg/kg bw harmine, 0.171 mg/kg bw harmaline, and 0.33 mg/kg bw of tetrahydroharmine. The doses used were selected based on previous studies conducted by our research group with the same material, showing that ayahuasca intake on consecutive days at doses 4x or higher leads to death of male and female Wistar rats (da Motta et al., 2018; Santos, Vieira, Pic-Taylor, & Caldas, 2017).

On Saturday morning, 18–20 h after the beginning of the last ethanol session and treatment (Friday, 5:00 PM), the animals were euthanized for biological samples collection.

### *Euthanasia, blood collection, and perfusion*

The animals were euthanized with a thiopental overdose (100 mg/kg bw i.p.). The thorax was opened, and 5 mL of blood was collected by cardiac puncture for hemogram analysis: leukocytes, erythrocytes, hemoglobin, hematocrit, mean corpuscular volume, mean corpuscular hemoglobin, platelet count, mean platelet volume, blood cell distribution width, lymphocytes, monocytes, and granulocytes. The animals were then submitted to a cardiac perfusion with phosphate-buffered saline (PBS, pH 7.4) for 10 min and 4% formaldehyde for 7 min (Gage, Kipke, & Shain, 2012). The head was separated from the body, the dura mater was carefully removed, the brain was weighed, transferred to a falcon tube containing 4% formaldehyde in PBS for 24 h, and then maintained in 30% sucrose solution in a refrigerator for at least 72 h before processing. Additionally, the heart, lungs, liver, kidney, and stomach were removed, washed in saline, weighed, and submitted to macroscopic evaluation.

### *cFos immunohistochemistry*

Brain sections of 30 μm were obtained in an electric vibratome (KD-400; 0.15 mm/min; 100 Hz); the sections were placed in an antifreeze solution (glycerol, ethylene glycol, and $H_2O$) and kept in the refrigerator for cFos counting. Three subsequent sections were obtained from each brain region evaluated in this study, which are relevant to drug addiction: medial orbital cortex (MO), lateral orbital cortex (LO) and ventral orbital cortex (VO) (bregma 4.2 mm; Paxinos & Watson, 2007), nucleus accumbens (NAc), and caudate putamen (CPu, striatum) (bregma 1.08 mm; Paxinos & Watson, 2007) (Fig. 1).

The brain sections were prepared for cFos counting according to the manufacturer's instructions (Sigma–Aldrich). In summary, the sections were first blocked with 3% $H_2O_2$ in methanol, washed in PBS with 0.3% Triton X-100 (PBS-T), and blocked with normal goat serum 0.01% diluted in PBS-T. Washing with PBS occurred between the following steps: incubation with rabbit cFos antibody (F7799; 1:5000 dilution) for 48 h at 4 °C, incubation with anti-rabbit IgG (B8895; 1:800 dilution) for 2 h, incubation with peroxidase anti-peroxidase soluble complex rabbit antibody (PAP, 1:500 dilution) for 1.5 h, and staining for 10 min in 3,3′-diaminobenzidine (DAB) 0.06% in PBS followed by 10 min in DAB 0.3% in $H_2O_2$. The prepared brain sections were washed again with PBS, placed on a slide, dehydrated in graded ethanol, cleaned with xylene, mounted with Entellan® (Merck) and protected with a coverlid. cFos positive neurons were counted in a Leica DM 2000 microscope (40x) with a Leica Application Suite (LAS) V4.1 Core. Areas (0.1 $mm^2$) from each side of the brain of MO, VO, LO, NAc, and CPu regions were evaluated for cFos expression (total of eight readings per animal in MO and two readings per animal in other regions), as shown Fig. 1. The identity of the sample was blind to the evaluator.

### *Statistical analysis*

Data were analyzed using GraphPad Prism 6.01 (September 21, 2012) by one-way analysis of variance (ANOVA) followed by Tukey or Holm–Sidak test, or by Kruskal–Wallis test and Dunn's multi-comparison test (non-parametric). Results are given as mean ± standard error (SEM). In all cases, a difference was significant when $p \leq 0.05$.

## Results

### *IA2BC protocol for voluntary chronic ethanol consumption*

During the study, many rats with high ethanol intake showed signs of stress and aggressiveness during handling, and some rats chewed the base of the bottle, leading to leakage of the content and loss of the ethanol intake data. This occurred with 15 measurements, corresponding to less than 1% of all expected measurements during the experiment (60 animals, 3 measurements/week, for 8 weeks).

According to Carnicella and co-authors (2014), when the IA2BC protocol is used to estimate the decrease in ethanol intake due to a drug treatment, only excessive drinkers should be included. In this study, we considered heavy drinkers to be those animals showing a mean ethanol intake of at least 3 g/kg bw/24 h during the first 7 weeks of exposure. Eight of the 60 animals that underwent the protocol did not reach this threshold and were excluded. The remaining 52 animals were submitted to treatment at the 8th week, while continuing the exposure protocol. The animals were randomly distributed among the treatment groups: control (n = 10), NTX (n = 11), Aya0.5 (n = 10), Aya1 (n = 11), and Aya2 (n = 10).

Fig. 2A shows the ethanol intake data of the 52 rats during the first 7 weeks of exposure. The intake increased slowly during the first 4 weeks, became significantly higher in the 5th week, remaining constant until the 7th week, with a mean intake of 6.0 ± 0.13 g/kg bw/24 h (5th–7th weeks), which was considered the baseline level. Fig. 2B shows the ethanol intake during the 8th week of exposure and five consecutive days of treatment. No significant differences were found between the ethanol intake of treatment groups and the baseline level, but the naltrexone group intake (4.7 ± 0.35 g/kg bw/24 h) was significantly lower than the control group intake (6.7 ± 0.57 g/kg bw/24 h; $p < 0.05$).

### *Macroscopy, body and organ weights, and hemogram*

No animal died during the experiment, but five animals exposed to ethanol showed important liver lesions (two from each control and NTX groups and one from the Aya2 group). No macroscopic alterations were observed in the other organs of any animal. There was no difference among the body weights and organ weights of naïve and ethanol-exposed animals, except for the absolute brain weight, which was significantly higher than naïve (1.98 ± 0.10 g) in the control ($p = 0.006$), NTX ($p = 0.037$), Aya0.5 ($p = 0.017$), and Aya2 ($p = 0.0084$) groups. However, no differences were found when the brain weight was expressed relative to body weight (data not shown). Hemogram results showed a significant decrease in hemoglobin levels in the NTX group (13.6 ± 0.89 g/dL) compared with the control group (16.6 ± 0.59 g/dL; $p = 0.022$), and a significant increase of the mean corpuscular hemoglobin of the control group (32.7 ± 0.54 g/dL) compared to naïve (30.3 ± 0.22 g/dL; $p = 0.0074$). No other significant changes in the hemogram parameters were found (data not shown).

### *cFos expression*

Fig. 3 shows the cFos counts of labeling nuclei in the five brain regions investigated in this study. Slides of six animals were lost during the mounting procedure; cFos was measured in brain

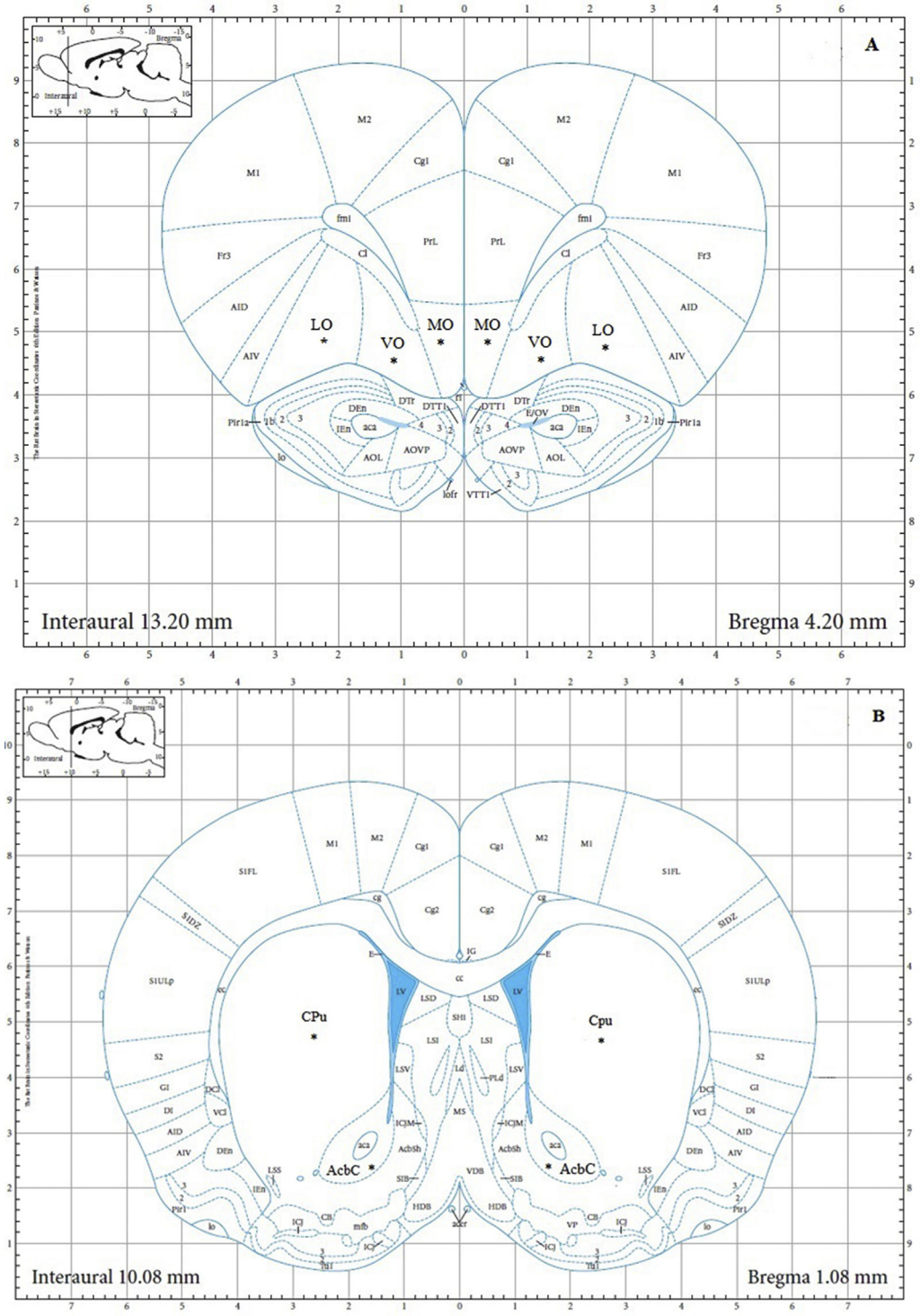


**Fig. 1.** Brain areas evaluated. (**A**) MO: medial orbital cortex; LO: lateral orbital cortex; VO: ventral orbital cortex; (**B**) AcbC accumbens nu (nucleus accumbens, NAc), core; CPu: caudate putamen (striatum). Adapted from Paxinos and Watson, 2007.

regions of 46 animals. Naïve animals, not exposed to ethanol, showed no or very little cFos expression (maximum of 4 counts in the MO), but ethanol intake induced expression in all regions, although statistical significance was not observed in all cases.

In the MO (Fig. 3A), cFos expression was significantly higher than naïve in the control ($p < 0.0001$), NTX ($p < 0.05$), Aya1 ($p < 0.001$), and Aya2 groups ($p < 0.0001$) groups; expression was significantly lower than control for the NTX ($p < 0.05$) and the Aya0.5 groups ($p < 0.01$), but only the ayahuasca group achieved the levels of the naïve group. Ethanol intake significantly increased cFos expression in the VO for the Aya1 group ($p = 0.035$; Fig. 3B), and in the LO for the Aya2 group ($p < 0.01$; Fig. 3C), but it did not significantly affect the expression for any group in the striatum (Fig. 3D). In the NAc, treatment with naltrexone or ayahuasca significantly increased cFos expression compared to the naïve group ($p = 0.022$ for the Aya0.5 group and $p < 0.005$ for the other

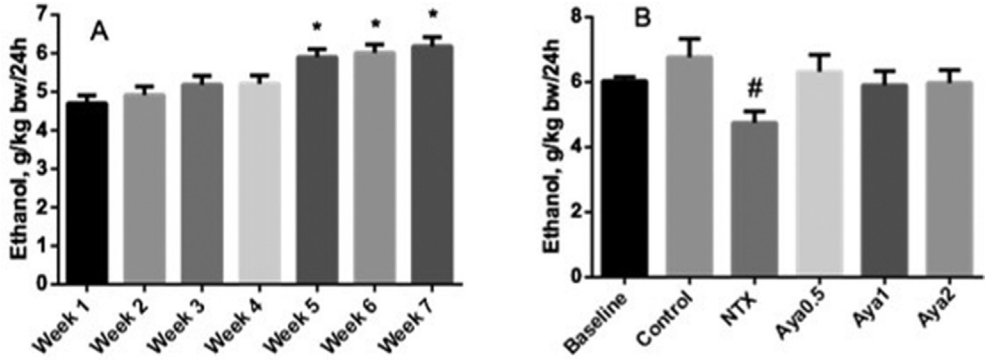


**Fig. 2.** (**A**) Ethanol intake during the first 7 weeks by the 52 heavy-drinker rats. (**B**) Baseline ethanol intake (weeks 5–7) and intake during the 8th week and treatment. Control, n = 10; NTX, n = 11; Aya0.5, n = 10; Aya1, n = 11; Aya2, n = 10. Mean ± SEM, one-way ANOVA followed by Tukey. * significant from weeks 1–4; # significant compared to control.

groups); the NTX and the ayahuasca groups also had higher (but not significant) levels of cFos compared to controls (Fig. 3E). Fig. 4 illustrates the immunohistochemistry labeling of the various groups in the MO area.

## Discussion

Alcohol use disorder (AUD) is characterized by a progressive escalation from low or moderate to excessive alcohol consumption (Vilpoux, Warnault, Pierrefiche, Daoust, & Naassila, 2009). The intermittent access to 20% ethanol in a 2-bottle choice procedure (IA2BC) has been shown to induce a gradual escalation of voluntary ethanol intake by rats and is a useful approach for preclinical evaluation of potential therapeutic options against AUD (Carnicella et al., 2014). In this protocol, the ethanol intake by rats increases gradually, eventually reaching a significantly higher level after the 4th week (5–6 g/kg bw/24 h), a level that is enough to induce pharmacologically relevant blood ethanol concentrations (Carnicella et al., 2014; Li et al., 2010). Indeed, in the present study, a significantly higher ethanol intake level was reached at the 5th week of exposure, remaining constant up to the 7th week.

In the 8th week of exposure, the animals were treated for five consecutive days either with water (control group), naltrexone (an opioid receptor antagonist widely used for treating AUD; Anton, 2008), or ayahuasca at doses related to the doses used in a UDV religious ritual (Aya0.5, Aya1, and Aya2). The treatment with naltrexone did significantly decrease the ethanol intake compared to the control group, but not compared to the baseline level reached at 5–7 weeks of exposure. Li et al. (2010) did find a decrease in ethanol intake by rats submitted to the IA2BC protocol and treated with naltrexone when compared to both baseline and control groups.

Ayahuasca used under religious/ritual context has been shown to have a positive effect on individuals with AUD and other drug problems (Fábregas et al., 2010; Grob et al., 1996; Halpern, Sherwood, Passie, Blackwell, & Ruttenber, 2008; Lawn et al., 2017). Thomas and co-workers (Thomas, Lucas, Capler, Tupper, & Martin, 2013) showed that ayahuasca-assisted therapy was associated with statistically significant improvements in factors related to drug abuse among a rural native population in Canada. Oliveira-Lima et al. (2015) showed that ayahuasca (i.p. injection) inhibits early behaviors of mice associated with initiation and development of alcohol addiction, measured by the locomotor activity in an open field apparatus. Preclinical studies with ayahuasca using laboratory animals, however, are limited in the literature, but are essential to investigate the mechanisms underlying the effects without the religious/ritualistic aspects that may influence the outcome beyond the biological response. In the present study, ayahuasca exposure for five consecutive days at doses around the ritual dose (0.5x, 1x, and 2x) had no significant impact on ethanol intake by male Wistar rats using the IA2BC protocol. Although this result does not corroborate the human studies, it should be repeated using other rat strains, including alcohol-preferring rats, or other exposure protocols (Boerngen-Lacerda, Jamal, Correia, & Goeldner, 2013; Carnicella et al., 2014; Goltseker, Hopf, & Barak, 2019).

The psychoactive and potential therapeutic properties of ayahuasca are mainly due to the compounds present in the plants used to prepare the beverage. DMT, present in *P. viridis*, is an agonist of 5-HT receptors, with the highest affinity for 5-$HT_{2A}$ (39 nM) and 5-$HT_{2C}$ (127 nM) (Keiser et al., 2009), although 5-$HT_{2C}$ showed an important desensitization to DMT over time (Smith et al., 1998). After ayahuasca consumption, the degradation of the orally inactive DMT in the gastrointestinal tract is prevented by the β-carbolines present in the *B. caapi* (harmine and harmaline), which are MAO A inhibitors (Santillo, Liu, Ferguson, Vohra, & Wiesenfeld, 2014). Tetrahydroharmine is not a strong MAO inhibitor, but possibly contributes to the neuroactivity of the infusion by inhibiting the uptake of serotonin at presynaptic sites, like other 1-methyl-tetrahydro-β-carbolines (Airaksinen, Svensk, Tuomisto, & Komulainen, 1980; Buckholtz & Boggan, 1977).

Like other drugs of abuse, alcohol increases firing of the ventral tegmental area projecting to the NAc, which increases dopamine release, a process that is mediated by other signaling systems, including the 5-HT system (Clapp et al., 2008). Indeed, 5-$HT_2$ receptors modulate dopamine release in the NAc (Boerngen-Lacerda et al., 2013; De Deurwaerdère, Navailles, Berg, Clarke, & Spampinato, 2004; Navailles, De Deurwaerdère, Porras, & Spampinato, 2004), although there is conflicting evidence regarding how this modulation affects drug use (Boerngen-Lacerda et al., 2013). De Deurwaerdère and co-authors (2004) showed that constitutively active 5-$HT_{2C}$ receptors are responsible for a tonic inhibitory control on nigrostriatal and mesolimbic dopamine neuronal pathways. Cocaine increased dopamine levels in the NAc and striatum, being boosted by the 5-$HT_{2C}$ receptor antagonists (SB 206553 and SB 242084), while the agonist Ro 60–0175 failed to exert this effect but reduced the increase in dopamine outflow induced by haloperidol (Navailles et al., 2004). Canal and Murnane (2017) proposed that activation of 5-$HT_{2C}$ receptors on the NAc shell could inhibit potassium Kv1.x channels, enhancing the anti-cocaine addiction mechanism, which may explain the non-addictive nature of classic 5-$HT_2$ receptor agonist hallucinogens, such as LSD. Indeed, Liester and Prickett (2012) hypothesized that

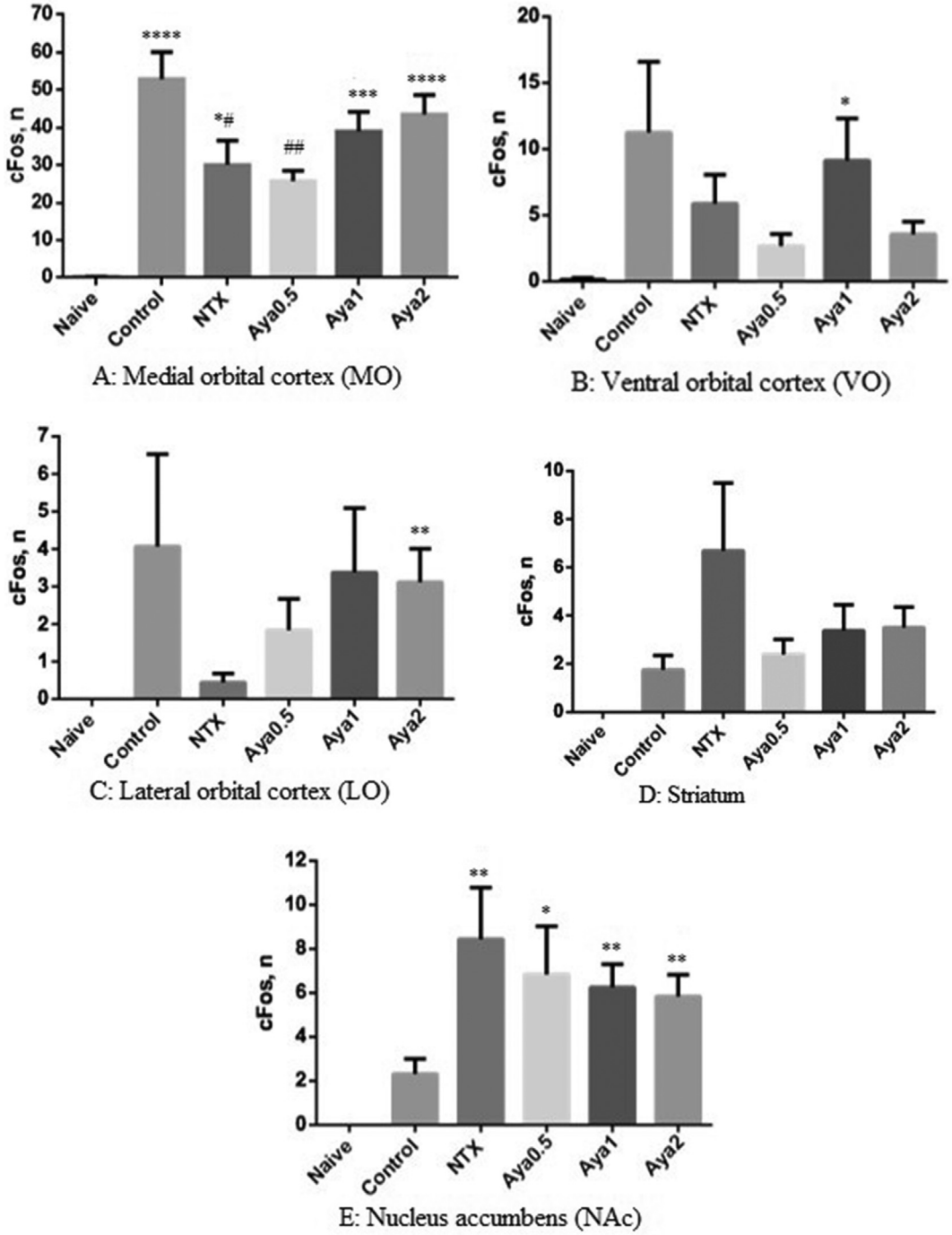


**Fig. 3.** cFos expression in the brain regions. Naïve, n = 4; Control, n = 8; NTX, n = 8; Aya0.5, n = 9; Ay1, n = 8; Aya2, n = 9. Mean ± SEM. (**A**) One-way ANOVA followed by Tukey or Holm–Sidak test. (**B**–**E**) Kruskal–Wallis test; * significant compared to naïve; # significant compared to controls.

the positive ayahuasca effect observed by some authors on drug abuse and addiction involves the reduction of dopamine levels in the mesolimbic system as a result of agonism on the 5-$HT_2$ receptors by the DMT present in the infusion.

On the other hand, Lankford and Myers (1996) showed that amperozide, a 5-$HT_{2A}$ antagonist, reduced ethanol consumption by rats. Furthermore, Boerngen-Lacerda and co-authors (2013) showed that mianserin, an antagonist/inverse agonist of the 5-$HT_2$ receptor, and ketanserin, an antagonist of the 5-$HT_{2A}$ receptor, blocked the development of ethanol-induced sensitization in mice, while daily co-administration of fluoxetine and paroxetine (selective serotonin reuptake inhibitors) during 28 days of ethanol treatment potentiated this effect. A yet non-published study conducted by our research group has shown that chronic exposure to ayahuasca for 28 days did increase serotonin levels in the brain (without the hippocampus) of the Aya2 Wistar rat group compared to controls. Ayahuasca intake did not affect dopamine levels, but significantly increased the levels of its main metabolite DOPAC (3,4 dihydroxyphenyl acetic acid) at the Aya1 and Aya2 doses, compared with controls. The impact of ayahuasca exposure on dopamine/serotonin levels of specific brain areas and its role in drug addiction needs further investigation.

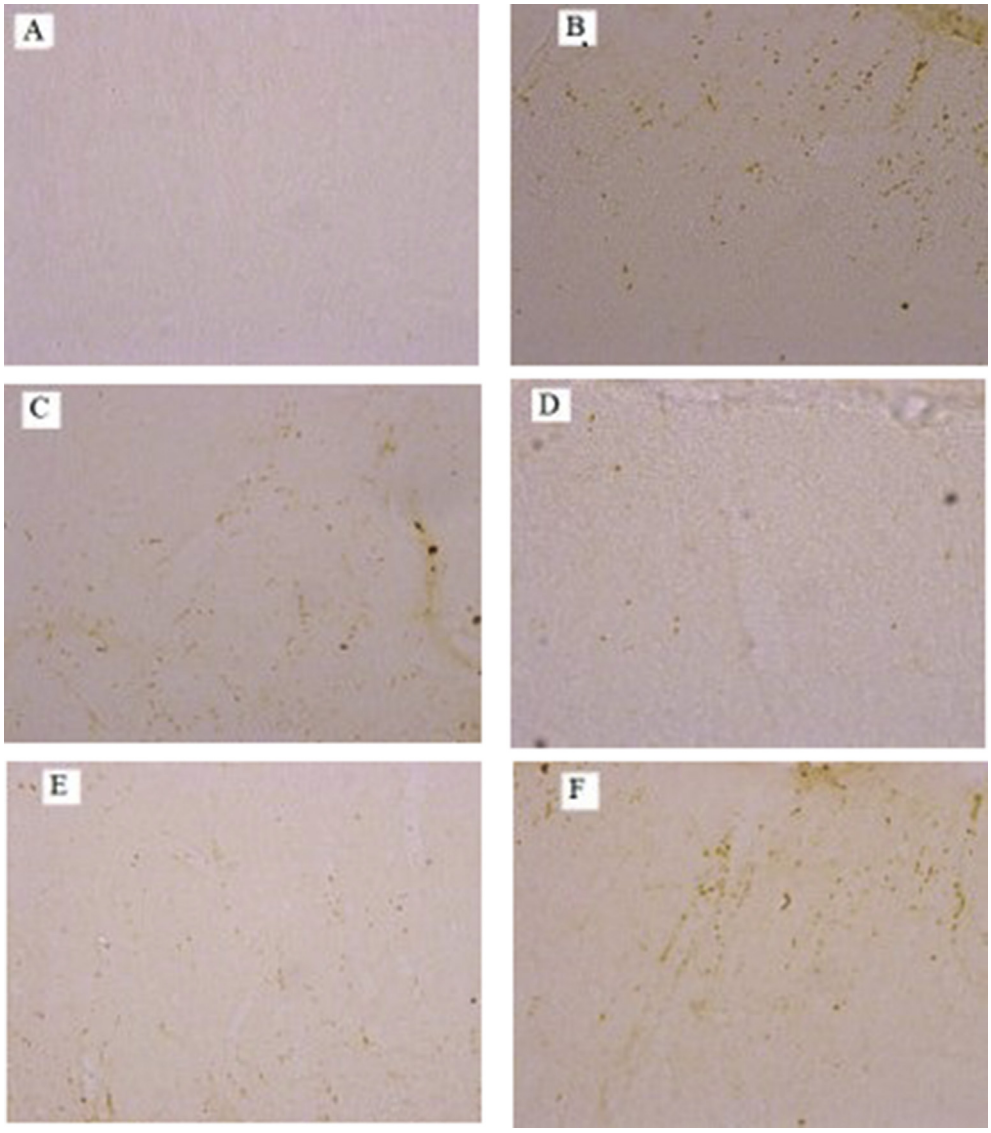


**Fig. 4.** Photobiography of cFos immunohistochemistry in the medial orbital cortex. (**A**) naïve, (**B**) control, (**C**) NTX, (**D**) Aya0.5, (**E**) Aya1, (**F**) Aya2.

Chronic use of ethanol and other drugs leads to a series of adaptive responses in the mesolimbic dopaminergic system, including a change in transcription factors and gene expression (Clapp et al., 2008), such as those coding for Fos proteins (Cruz et al., 2015; Nestler, 2012; Perrotti et al., 2008). George et al. (2012) found a significant increase in Fos protein expression in the medial prefrontal cortex and the central nucleus of the amygdala in rats trained in the IA2BC protocol. Increased cFos expression was also found in the orbitofrontal cortex (OFC) during alcohol seeking in animals (Liu & Crews, 2015; Vilpoux et al., 2009). Jupp and co-authors (Jupp, Krivdic, Krstew, & Lawrence, 2011; Jupp, Krstew, Dezsi, & Lawrence, 2011) showed that cFos was elevated during reinstatement of alcohol seeking in the OFC, and both reinstatement and cFos expression were reduced by treatment with SB-334860, an antagonist of the orexin receptor-1, a neuropeptide that can selectively increase ethanol consumption (Schneider, Rada, Darby, Leibowitz, & Hoebel, 2007). Sharma and co-authors (2014) also showed a significant cFos increase in the nucleus accumbens of rats exposed to alcohol after direct infusion in the anterior basal brain region, although Jaramillo and co-authors (2016) found that alcohol (1 g/kg, intragastric) decreased cFos expression in the median prefrontal cortex (mPFC) and NAc. Using the IA2BC protocol, Li and co-authors (2010) showed that alcohol intake by Wistar rats significantly increased ΔFosB (a FosB truncated spliced variant) expression in the NAc core, dorsolateral striatum, and LO, but not in the NAc shell, dorsomedial striatum, and mPFC. This effect was reversed by naltrexone treatment in all regions, except in the mPFC. In the present study, ethanol exposure using the IA2BC protocol increased cFos expression in all investigated brain sections, but significance was mainly found in the MO and NAc. Naltrexone and ayahuasca at the lower dose (Aya0.5) significantly decreased cFos expression compared to controls in the MO region, although this decrease was enough to reach cFos levels not significantly different from naïve animals only for the Aya0.5 group. Higher ayahuasca treatment doses did not show any impact on cFos levels caused by alcohol exposure. These results seem to indicate a protective effect of ayahuasca at low levels in this region. On the other hand, treatment with either naltrexone or ayahuasca significantly increased cFos expression in the NAc compared to naïve animals, an increase that was not found in controls. Indeed, neuronal activation indicated by c-Fos was also observed by Pic-Taylor et al. (2015) in the dorsal raphe nucleus, amygdaloid nucleus, and hippocampal

formation brain areas of rats treated once at the 30xayahuasca dose. All together, these results confirm the need to investigate different activated brain areas involved in drug addiction to construct a body of mechanistic information of the factors involved in AUD and other drug disorders and the role that ayahuasca may play to treat them.

This is the first study that investigated the effects of ayahuasca on ethanol intake of alcohol-addicted rats. However, the study has some limitations that should be discussed. The ethanol intake was not measured at an earlier stage (30–60 min after exposure), which could have captured an early intake change due to the ayahuasca treatment, which was not possible after 24 h. It is also possible that no impact on ethanol intake was observed because the ayahuasca doses tested were too low (up to 2x the ritual dose). However, as it were mentioned previously, daily doses at 4x or higher are lethal to the animals and could not be tested. One option for future studies is to use intermittent exposure at higher doses, which was shown to be safe to the animals (Santos et al., 2017). Furthermore, water and total fluid intake should also be measured to evaluate alcohol preference during the IA2BC protocol.

## Conclusions

Human studies have shown a potential use of ayahuasca to treat drug addiction. In this study, ayahuasca daily exposure for 5 days at doses up to 2x the ritual dose did not affect chronic intermittent voluntary ethanol intake by rats. However, cFos expression due to ethanol intake was partially reversed in the medial orbital cortex brain region by naltrexone, a medicament used to alcohol use disorder, and it was reversed to levels not significantly different from the naïve group by ayahuasca treatment at the lowest dose tested (0.5x the ritual dose). The potential role and pathways for mediating cFos expression by ayahuasca in selected brain regions and its relationship with the serotoninergic/dopaminergic systems and drug addiction need further investigation, with the goal of understanding the mechanisms involved in the effects observed in human studies.

## Contributors

The last and corresponding authors conceptualized the study and prepared the first draft of the manuscript. The first author conducted most of the experiments, with the contributions of the second and third authors. All contributors to this manuscript reviewed the manuscript drafts and approved the submission.

## Declaration of Competing Interest

None.

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