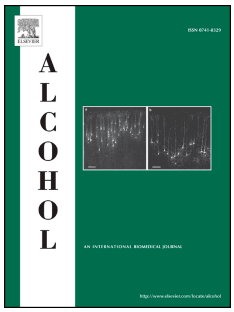

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Effects of the hallucinogenic beverage ayahuasca on voluntary ethanol intake by rats and on cFos expression in brain areas relevant to drug addiction

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Abstract

Ayahuasca is a hallucinogenic infusion used in religious ritualsthat 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 pc ip) or ayahuasca (Aya) at 0.5, 1 or 2X the ritual dose in the final 5 days. A naïve group had only access to water. Ethanol intake was estimated throughoutthe experimentand cFos expression was evaluated in medial orbital cortex (MO), ventral orbital (VO), lateral orbital (LO), nucleus accumbens (NAc) and striatum. Treatment with either NTX or Aya (oral) did not decrease ethanol intake compared to the baseline level (5^{th} to 7^{th} week), but the NTX group intake was significantly lower than control ($p< 0.05$). 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 ($p <0.05$), 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 control 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 humann. 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 needs further investigation.

Key words: Alcohol; ayahuasca; behavior; cFos protein; rats

1. 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 (WHO, 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) (NIH, 2016). There are various pharmacological options to treat AUD, including naltrexone, acamprosate, baclofen and topiramate, although most treatments show low to medium efficacy (Papacuer et al., 2018).

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

Changes in serotonin (5-HT) transmission and reuptake are associated with alcohol addiction, and it has been shown that increasedserotonergic activity decreases ethanol intake, while decreased serotonergic functioning increases ethanol intake as well as aggressive behavior (LeMarquand et al., 1994; Heinz et al., 2011). Decreased serotonin neurotransmission in dependent animals may be associated with relapse drinking (Lê et al. 2008; Clapp et al., 2008), and administration of fluoxetine, a 5-HT reuptake inhibitor, and of 5-HT_{1A} and 5-HT_{1B} receptoragonists 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 a reduced serotonin availability in the brain (Johnson et al., 2008;Thompson and Kenna, 2016).

Transcribed *c-fos*mRNA and the translated protein product cFos can be used as a marker of strongly activated neurons. Cruz et al. (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 (Li et al; 2010; Jaramillo et al. 2016; Sharma et al., 2016).

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

2. Material and methods

2.1. 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 showed to contain 0.12 mg/mL DMT, 1.19 mg/mL harmine, 0.08 mg/mL harmaline, and 0.15 mg/mL tetrahydroharmine.

2.2. 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 beddingand kept under controlled environmental conditions (12h-12h, dark-light; 22-25˚C; 45-60% humidity). Filtered water and a commercial laboratory rat feed (Labina, Purina®, Brazil) were provided *ad libitum.* This study was conducted according to the Guide for the Care and Use of Laboratory Animals and The Guideline for the Testing of Chemicals (OECD, 2003) and approved by the University of Brasilia Ethics Committee on Animal Use (License N° 73276/2014).

2.3. IA2BC (intermittent access to 2-bottle choice) protocol

Sixty animals were exposed to 20% solution of ethanol (Dinâmica®) for 8 weeks according to the IA2BC protocol (Carnicela*et al.*, 2014). Every Monday, Wednesday and Friday at 05:00 pm (beginning of the dark period), the animals were weighed and put individually in a cage with simultaneous access to a bottle of filtered water and a bottle of ethanol solution (250 mL acrylic or plastic bottle). The bottles were weighed before being made available to the animals, and re-weighed 24 hours later, at the end of each exposure day. The ethanol bottle was then replaced by another water bottle for the 24-hour period of non-ethanol exposure, except for the weekend (48-hour 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 in 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, 4 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 in 5 groups: water (control, gavage), naltrexone (NTX, 2 mg/kg pc ip) 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 (Santos et al., 2017; Motta et al., 2018).

On Saturday morning, 18 to 20 hours after the beginning of the last ethanol session and treatment (Friday, 05:00 PM), the animals were euthanized for biological samples collection..

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The animals were euthanized with a thiopental overdose (100 mg/kg bwi.p). The thorax was opened, and 5 mL blood collected by cardiac puncture for hemogram analysis: leucocytes, erythrocytes, hemoglobin, hematocrit, mean corpuscular volume, mean corpuscular hemoglobin, platelet, 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 minutes and 4% formaldehyde for 7 minutes (Gage, et al., 2012). The head was separated from the body, the dura-mater carefully removed, the brain weighed, transferred to a falcon tube containing 4% formaldehyde in PBS for 24 hours, and then maintained in 30% sucrose solution in a refrigerator for at least 72 hours before processing. Additionally, the heart, lungs, liver, kidney and stomach were removed, washed in saline, weighed and submitted to macroscopic evaluation.

2.5. cFos immunohistochemistry

Brain sections of 30 µm were obtained in an electric vibratome (KD-400; 0.15 mm/min; 100Hz); 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 hours at 4ºC, incubation with anti-Rabbit IgG (B8895; 1:800 dilution) for 2h, incubation with peroxidase anti-peroxidase soluble complex rabbit antibody (PAP, 1:500 dilution) for 1.5 hours, and staining for 10

minutes in 3,3' - diaminobenzidine (DAB) 0.06% in PBS followed by 10 minutes 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 cover lid. 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 brain side of MO, VO, LO, NAc and CPu regions were evaluated for cFos expression (total of 8 readings per animal in MO and 2 readings per animal inothers regions), as shown in Fig. 1. The identity of the sample was blind to the evaluator.

2.6. 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 $p \leq 0.05$.

3. Results

3.1. 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 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 et al. (2014), when the IA2BC protocol is used to estimate the decrease in ethanol intake due to a drug treatment, only excessive drinkersshould be included. In this study, we considered heavy drinkers those animals showing a mean ethanol intake of at least 3 g/kg bw/24h 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 8^{th} 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 5^{th} week, remaining constant until the 7^{th} week, with a mean intake of 6.0 ± 0.13 g/kg bw/24 h (5-7^{th} weeks), which was considered the baseline level. Fig. 2B shows the ethanol intake during the 8^{th} week of exposure and 5 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 control (6.7 ± 0.57 g/kg bw/24 h; $p< 0.05$).

3.2. Macroscopy, body and organ weights and hemogram

No animal died during the experiment, but 5 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 control (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).

3.3. 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, and furthermore, cFos was measured in brain 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 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 Aya0.5 group and $p< 0.005$ for the other groups); NTX and ayahuasca groups also had higher

(but not significant) levels of cFos compared to control (Fig. 3E). Fig. 4 illustrates the immunohistochemistry labeling of the various groups in the MO area.

4. Discussion

Alcohol use disorder (AUD) is characterized by a progressive escalation from low or moderate to excessive alcohol consumption (Vilpoux et al., 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, Aya2). The treatment with naltrexone did decrease significantly 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 (Grob et al., 1996; Halpern et al., 2008; Fabregas et al., 2010; Lawn et al., 2017). Thomas et al. (2013) showed that ayahuasca-assisted therapy was associated with statistically significant improvements in factors related to drug abuse among a rural aboriginal 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 (0.5, 1 and 2X) had no significant impact on ethanol intake by male Wistar rats using the IA2BC protocol. Although this result does not corroborate the humans studies, it

should be repeated using other rat strains, including alcohol preferring rats, or other exposure protocols (Boerngen-Lacerda et al., 2013; Carnicella et al., 2014; Goltseker et al., 2019).

lw)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 et al., 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 (Buckholtz and Boggan, 1977; Airaksinen et al., 1980).

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 (Clapp et al., 2008). Indeed, 5-HT_2 receptors modulate dopamine release in the NAc (Deurwaerdère et al., 2004; Navailles et al., 2004;Boerngen-Lacerda et al., 2013), although there are conflicting evidences on how this modulation affects drug use (Boerngen-Lacerda et al., 2013). Deurwaerdère et al. (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} receptorantagonists (SB 206553 and SB 242084), while theagonist Ro 60-0175 failed to exert this effect but reduced the increase in dopamine outflow induced by haloperidol (Navaille et al., 2004). Canal and Murnane (2017) proposed that activation of 5-HT_{2C} receptors on 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& Prickett (2012) hypothesized that the positive ayahuasca effect observed by some authors on drug abuse and addiction involves the reduction of dopamine levels in the mesolimbic system as 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 et al. (2013) showed that mianserin, an antagonist/inverse agonist of 5-HT_2 receptor, and ketanserin, an antagonist of 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 Aya2 Wistar rat group compared to control. 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.

Chronic use of ethanol and other drugs leads to a series of adaptive responses in the mesolimbic dopaminergic system, including a change in transcript factors and gene expression (Clapp et al., 2008), such as those coding for Fos proteins (Cruz et al., 2015; Nestler, 2012; Perroti 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 and Crews, 2015; Vilpoux et al., 2009). Jupp et al. (2011a,b) showed that cFos was elevated during reinstatement of alcohol seeking in the OFC, and both reinstatement and cFos expression were reduced by treatment of SB-334860, an antagonist of the orexin receptor-1, a neuropeptide that can selectively increase ethanol consumption (Schneider et al., 2007). Sharma et al. (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 et al. (2016) found that alcohol (1 g/kg, intragastric) decreased cFos expression in the median prefrontal cortex (mPFC) and NAc. Using the IA2BC protocol, Li et al. (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 increased significantly 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 nuclei, amygdaloid nucleus, and hippocampal formation brain areas of rats treated once at 30X ayahuasca dose. All together, these results confirm the need to investigate different activated brain areas involved in drug addiction to build upa body of mechanistic information involved in AUD and other drug desorders 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 hours. 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 was mentioned previously, daily doses at 4X or higher are lethal do 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 safeto the animals (Santos et al., 2018). Furthermore, water and total fluid intake should alsobe measured to evaluate alcohol preference during the IA2BC protocol.

5. 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 intakeby 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 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 needs further investigation, with the goal of understanding the mechanisms involved in the effects observed in human studies.

Contributors

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

Conflict of interest

None

Acknowledgments

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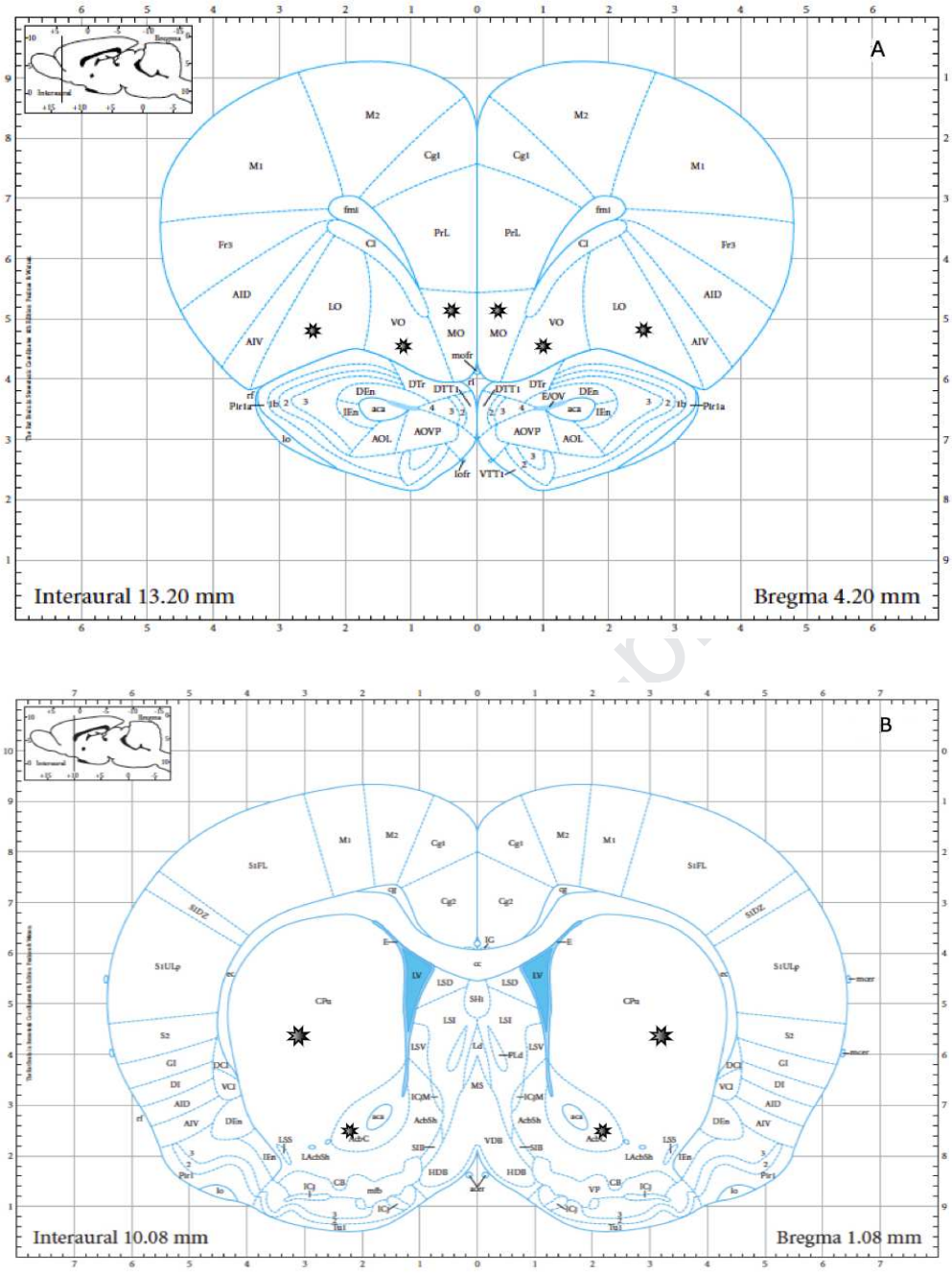

Fig. 1. Brain areas evaluated. A: MO:medial orbital cortex; LO: lateral orbital cortex; VO: ventral orbital cortex; B: AcbC accumbens nu (nucleus accumbens, NAc), core; CPu: caudate putamen (striatum). Adapted from Paxinos & Watson (6th ed.; 2007).

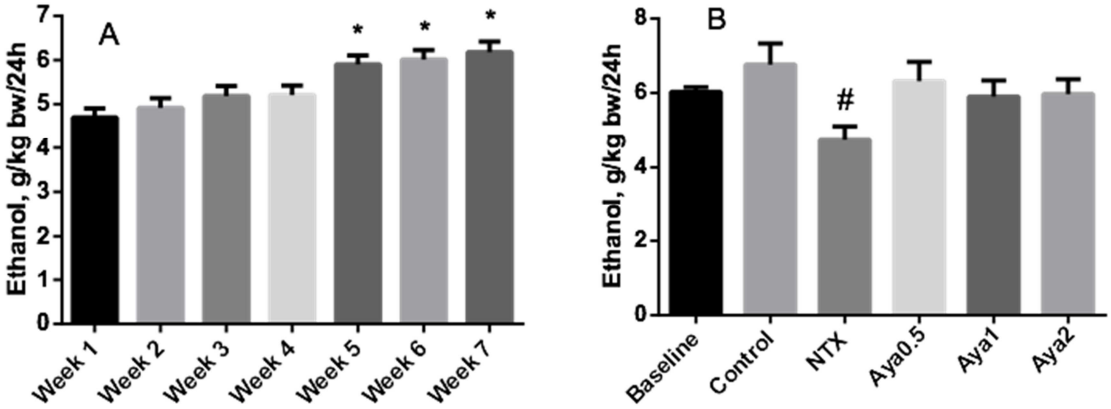

Fig. 2. (A) Ethanol intake during the first 7 weeks by the 52 heavy-drinker rats**.** (B) Baseline ethanol intake (weeks 5 to 7) and intake during the 8th week and treatment. Control, n= 10; NTX, n= 11; Aya0.5, n= 10; Aya1, n= 11; Aya2, n= 10. Mean± SEM, one-way ANOVA followed by Turkey. * significant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

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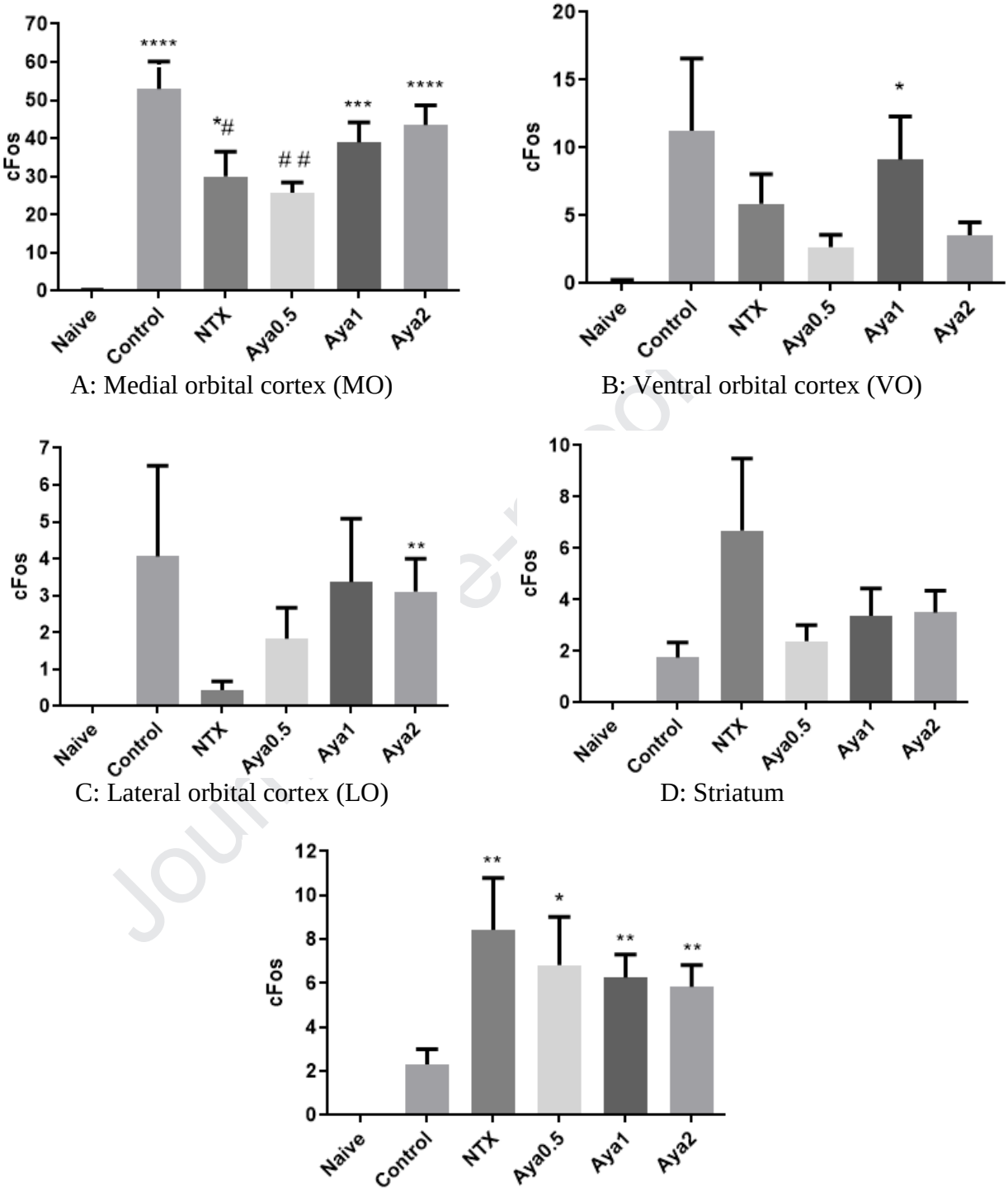

A: Medial orbital cortex (MO)

B: Ventral orbital cortex (VO)

C: Lateral orbital cortex (LO)

D: Striatum

E: Nucleus accumbens (NAc)

574 **Fig. 3.** cFos expression in the brain regions (n/0.1 mm^2). Naïve, n=4; Control, n=8; NTX, n=8;
575 Aya0.5, n=9; Ay1, n=8; Aya2, n=9. Mean± SEM, A: One-way ANOVA followed by Turkey
576 or Holm-Sidak test. B-E: Kruskal-Wallis test; * Significant compared to naïve: *p<0.05, **
577 p<0.01, *** p<0.001, **** p<0.0001; # significant compared to control: # p<0.05, ## p< 0.01

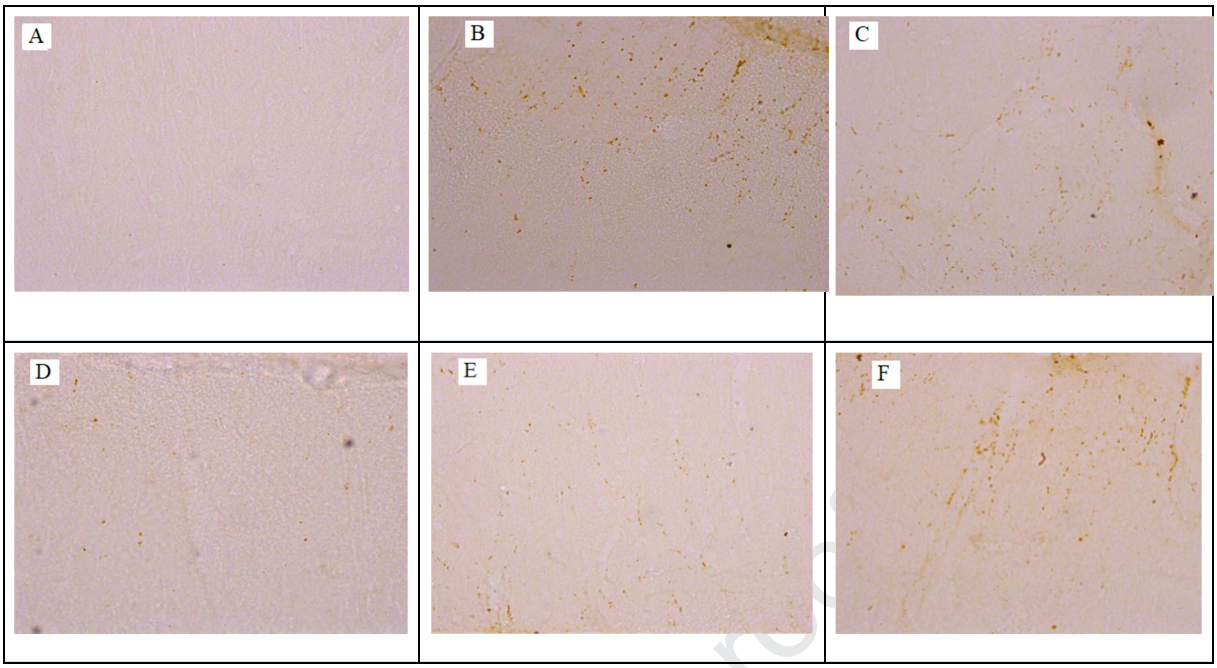

Fig. 4. Photobiography of cFos immunohistochemistry in the medial orbital cortex. A: naïve, B: control, C: NTX, D: Aya0.5, E: Aya1, F: Aya2.

Highlights:

- Ayahuasca treatment did not affect ethanol intake by rats
- Ethanol intake significantly increased cFos in the MO and nucleus accumbens
- NTX and Aya0.5 treatment decreased cFos expression compared to control in the MO
- Only the Aya group reached the levels of the naïve group