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ORIGINAL ARTICLE

Toxicity of ayahuasca after 28 days daily exposure and effects on monoamines and brain-derived neurotrophic factor (BDNF) in brain of Wistar rats

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Abstract

Ayahuasca is a hallucinogenic beverage that affects the serotonergic system and have therapeutic potential for many diseases and disorders, including depression and drug addiction. The objectives of this study were to evaluate the potential toxic effects of ayahuasca on rats after chronic exposure, and the levels of monoamines, their metabolites and the brain-derived neurotrophic factor (BDNF) in the brain. Female and male rats were treated orally for 28 days with H_2O (control), fluoxetine (FLX), a selective serotonin reuptake inhibitor antidepressant, or ayahuasca (Aya) at doses of 0.5X, 1X and 2X the ritualistic dose (7 to 10 animals/group). Clinical, hematological and macroscopic results showed that ayahuasca was safe to the rats. Behavior tests conducted one hour after the last treatment showed that male rats from the Aya1 group explored the open field central area less than the control group, and the number of entries in the central area compared to total locomotion was also significantly lower in this group and in the FLX group. The hippocampus was removed for BDNF analysis and the remaining brain was used for monoamine analysis by HPLC-FL. Serotonin levels were significantly higher than control only in the Aya2 female group, while a significant reduction of its metabolite 5-HIAA was observed in the FLX group. Dopamine levels were similar among the experimental groups, but the levels of its metabolite DOPAC increased significantly in the Aya1 and Aya2 groups compared to controls, especially in females, and the DOPAC/dopamine turnover was significantly higher in Aya2 group. The levels of HVA, another dopamine metabolite, did not change with the treatments compared to controls, but HVA/DOPAC ratio was significantly lower in all ayahuasca male groups. Norepinephrine was not detected in any brain sample, and the levels of its metabolite MHPG did not change significantly among the groups. BDNF levels in the hippocampus were significantly higher in the FLX and Aya2 female groups compared to controls when expressed in relation to the total brain weight. The mechanisms involved in the increase in serotonin, dopamine turnover and BDNF levels observed in ayahuasca treated animals should be further investigated in specific brain areas.

Keywords Ayahuasca · Serotonin · Dopamine · BDNF · Brain · Depression

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Introduction

Ayahuasca has been used for millennia as a sacred drink in religious and healing rituals by traditional communities in the Amazon region, a use that has expanded to Christian religions in Brazil since 1930, including the *Santo Daime* and the *União do Vegetal* (Frecska et al. 2016). In the last 20 years, the Brazilian ayahuasca religions have established congregations in various countries, including the USA, Canada, some European countries and elsewhere in Latin America (Labate and Feeney 2012; Health Canada 2017). The use of ayahuasca under a ritual/religious context is safe and brings many benefits to the users (Bouso et al. 2012; Domínguez-Clavé et al.

2019). However, the recreational use of this beverage has increased over the last decades, which has raised concerns regarding its safety when it is used at very high doses and frequency (dos Santos et al. 2017; da Motta et al. 2018).

Ayahuasca is usually prepared from *Banisteriopsis caapi* vine, which contains the β-carboline alkaloids harmine, tetrahydroharmine and harmaline, which are monoamine oxidase (MAO) inhibitors, and *Psychotria viridis* leaves, which contain the psychoactive agent N,N-dimethyltryptamine (DMT), a potent serotonin receptor agonist (Riba 2003; Carbonaro and Gatch 2016; Cameron et al. 2018). DMT is not orally active as it is degraded by MAO in the gastrointestinal tract, but the presence of β-carboline prevents its degradation and allows it to reach the CNS, an interaction that is the basis of the psychotropic action of the beverage (Mckenna 2004; Domínguez-Clavé et al. 2016).

A number of studies have investigated the potential of ayahuasca and its components on the treatment of various nervous system disorders, either in animal models or in humans, including depression (Ahlem et al. 2015; Osoŕio et al. 2015; Sanches et al. 2016; Palhano-Fontes et al. 2018; Cameron et al. 2019) and drug addiction (Oliveira-Lima et al. 2015; Frecska et al. 2016; Domínguez-Clavé et al. 2016; Hamill et al. 2019). Although the etiology, neurobiological processes and molecular mechanisms of depression are not completely understood (Manji et al. 2001; Peng et al. 2015), many studies have associated this disorder with dysfunction, functional imbalance or deficiency of neurotransmitters, including dopamine, serotonin and norepinephrine (Krishnan and Nestler 2008; Moret and Briley 2011; Haenisch and Bönisch 2011; Catena-Dell'Osso et al. 2013; Duman et al. 2016). Furthermore, the brain-derived neurotrophic factor (BDNF), the most abundant neurotrophin in the central nervous system (CNS), is decreased in the hippocampus of depressed individuals (Huang and Reichardt 2001; Krishnan and Nestler 2008). More recently, de Almeida et al. (2019) have suggested a potential link between the observed antidepressant effects of ayahuasca and increased serum BDNF levels in humans. The role of the serotonergic system in the establishment of drug use-associated behaviors and the transition and maintenance of addiction have also been widely investigated (Müller and Homberg 2015). Changes in serotonin transmission and reuptake are associated with alcohol addiction, with increased serotonergic activity decreasing ethanol intake, while decreased serotonergic functioning increases the intake (LeMarquand et al. 1994; Heinz et al. 2011). Furthermore, the β-carbolines and harmol, the main harmine metabolite in humans, have been shown to stimulate neurogenesis in vitro (Morales-García et al., 2017), and the potential neuroprotective and neuroplasticity-enhancing effects of DMT and 5-MeO-DMT have been described (Szabo et al. 2016; Dakic et al. 2017).

Most studies using animal models to investigate the effects of ayahuasca intake on the neural system were conducted with single and/or high dose levels (Castro-Neto et al. 2013; Pic-Taylor et al. 2015; Oliveira-Lima et al. 2015), and the extrapolation to a therapeutic regime in humans maybe limited. More recently, Cameron et al. (2019) showed a potential antidepressant effect of DMT on male rats after chronic, intermittent microdose exposure.

The objectives of this study were to investigate the effects of the chronic intake of ayahuasca to Wistar rats at doses approximating those used in a religious ritual and the levels of serotonin, dopamine, norepinephrine and their metabolites and of BDNF in the brain of exposed rats.

Materials and methods

Animals

A total of 85 Wistar rats (43 males and 42 females) were obtained from the Biology Institute of the University of São Paulo, Brazil. The rats were 5 weeks old, and uniform in weight (180 ± 15 g males and 140 ± 15 g females). The animals were kept in the animal facility of the Medical School of University of Brasília (UnB) in galvanized polypropylene cages on a ventilated shelf (Alesco®) under controlled conditions of temperature (23 ± 2 °C), humidity (45–60%) and light/dark cycle of 12 h/12 h; they received commercial rodent feed (Purina®) and filtered water ad libitum. Before study initiation, the animals underwent a 10-day acclimatization period, with each box accommodating only one animal. The project was approved by the Committee for Ethics in Animal Use (CEUA) of the Institute of Biological Sciences of the University of Brasília (No. 66693/2016). Environmental conditions, animal handling and care followed the recommendations of The Guide for the Care and Use of Laboratory Animals (National Research Council 2011). Animal handling also followed the ethical principles from the National Council for the Control of Animal Experimentation (CONCEA) and the Brazilian Arouca Law (Number 11794/2008), and every effort was made to minimize the potential suffering of experimental rats.

Ayahuasca material

The ayahuasca used in this study is the same used in previous studies conducted by our research group (Pic-Taylor et al. 2015; Melo Junior et al. 2016; Santos et al. 2017; Andrade et al. 2018; da Motta et al. 2018), and was kept at −20 °C in the freezer until lyophilized (K105, LIOBRAS) for the conduction of the study. Prior to the experiment, the material was submitted to chemical analysis by GC-MS/MS (Trace Ultra coupled with TSQ Quantum XLS Triple Quadrupole; Thermo Scientific). The harmaline and harmine standards, 99.2% and

98% purity, respectively, were obtained from Sigma Aldrich. 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). Quantitative analysis of the ayahuasca used in this study showed 0.12 mg/mL DMT, 1.19 mg/mL harmine, 0.08 mg/mL harmaline, and 0.15 mg/mL tetrahydroharmine. The structures are shown in Fig. 1.

Study design

The study protocol followed the general parameters recommended by the OECD Guidelines for the Testing of Chemicals No. 407/95 (Repeated Dose 28-day Oral Toxicity Study in Rodents). The animals were randomly distributed into five groups, with 7 to 10 animals of each sex per group: a control group (distilled water), a fluoxetine group (10 mg/kg bw - manipulated by Farmacotécnica®) and three groups treated with ayahuasca at doses of 0.5X (Aya0.5), 1X (Aya1) and 2X (Aya2) the dose used in a religious ritual of *União do Vegetal*. These doses were selected based on our previous studies showing that daily ayahuasca intake at doses 4X or

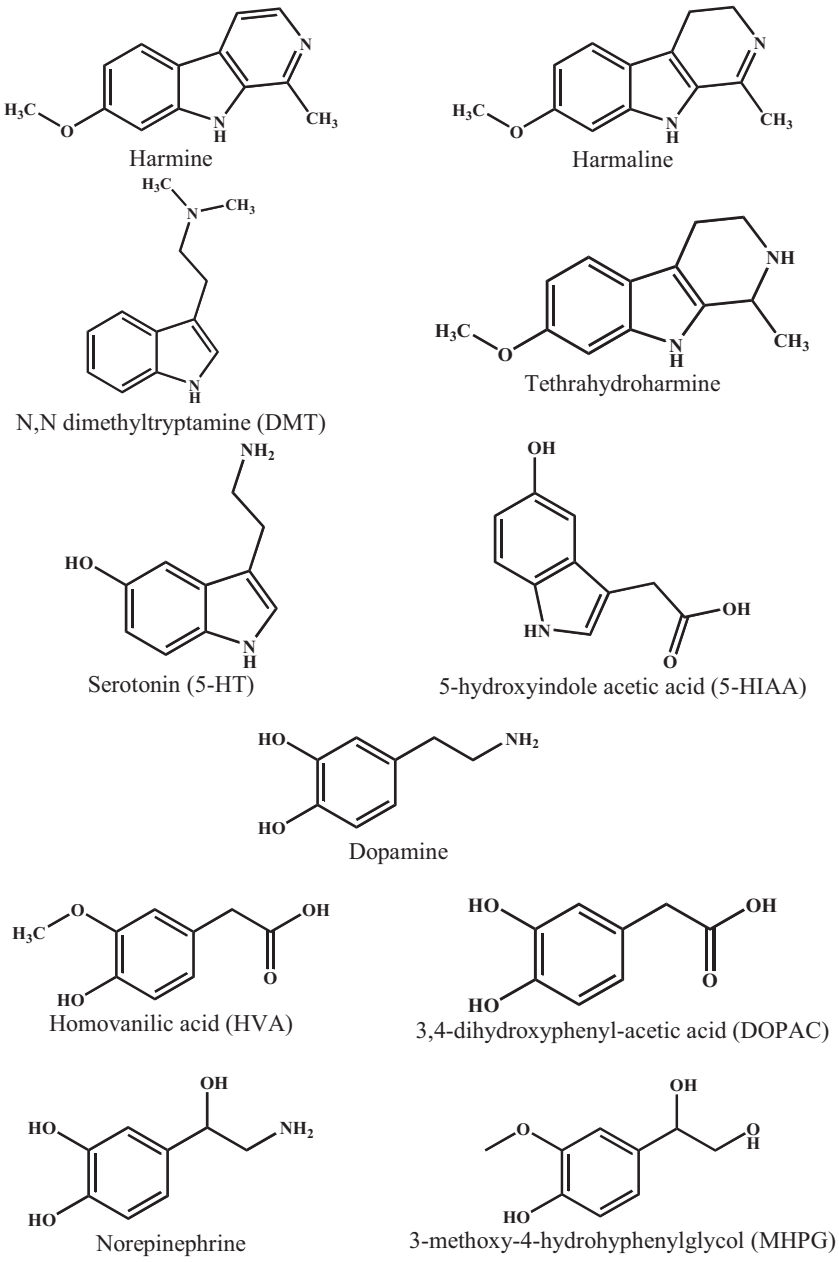

Fig. 1 Chemical structures of the ayahuasca components (harmine, harmaline, tetrahydroharmine and DMT, and the monoamines and their main metabolites

higher is fatal to male and female Wistar rats (Santos et al. 2017; da Motta et al. 2018). One ritual dose (1X, Aya1) corresponds to 150 mL for a 70 kg (1X) individual, by body weight (bw), 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. Animals were treated daily in the morning by gavage for 28 days, maintaining a final volume of 2 mL. To ensure that all females were in the same phase during the experiment (estrus), vaginal secretion was collected and observed fresh under an optical microscope to determine the presence of specific cells. Animals were clinically evaluated daily and body weight was checked every three days in a calibrated balance (Camry Mark, model EK9333).

Animals were euthanized by decapitation using a rat guillotine 3 h after the last treatment. Blood was immediately collected by cardiac puncture and stored at −20 °C. The brain was removed, washed with phosphate-saline buffer (PBS), weighed and dissected to isolate the hippocampus from both hemispheres. Both hippocampus and remaining brain tissue were flash frozen with liquid nitrogen and stored separately in microcentrifuge (Eppendorf) tubes at −80 °C. BDNF plays a crucial role in depression as a central regulator of neuronal plasticity in the hippocampus (von Bohlen Und Halbach and von Bohlen Und Halbach 2018), justifying the analysis of this neurotrophin in this specific brain region. The remaining brain tissue was used for monoamines analysis.

Behavior tests

The behavior tests were conducted one hour after the last treatment. First, the animal was placed individually in the central quadrant of the open field (OF) arena and observed for 5 min for locomotion (number of quadrants covered) and number of entries in the central quadrant (Prut and Belzung 2003). Then, the animal was placed in the central open cross area of the elevated-plus-maze (EPM) apparatus and evaluated for 5 min for the number of entries in the closed and open arms (including the central) and the time spent in each arm (Pellow et al. 1985). In both tests, rearing, grooming, urination and defecation were also evaluated. The dimensions of each apparatus were described previously (Pic-Taylor et al. 2015).

Hematological and biochemical evaluation

Blood samples were analyzed about five hours after collection on a Sysmex Poch 100iVDiff ™ automated hematometer calibrated for rats. The following parameters were analyzed: hemoglobin, total hematocrit, erythrogram, leukogram, corpuscular volumes and their levels of variation. Due to the time elapsed between blood collection and analysis, platelets were not measured. Approximately 1.5 mL of serum from each animal was obtained by centrifugation and stored at −20 °C for biochemical analysis on a Chemwell-t Labtest automatic biochemical analyzer: aspartate transaminase; alanine transaminase and alkaline phosphatase for hepatic function; urea and serum creatinine for renal function, and lactate dehydrogenase for tissue damage.

Analysis of monoamines by HPLC-FL

The analytical standards of serotonin (5-HT, 5-hydroxytryptamine; 99% purity), 5-hydroxyindole-3-acetic acid (5-HIAA; 98.2% purity), dopamine (DA, purity ≥98%), 3,4 dihydroxyphenyl acetic acid (DOPAC; purity 99.5%), homovalinic acid (HVA; purity 99.5%), and 3-methoxy 4-hydroxyphenylglycol sulfate (MHPG; purity 98.4%) were purchased from Sigma-Aldrich®. Norepinephrine (NE; purity 99.8%) was purchased from USP (American Pharmacopoeia). Stock solutions of each analytical standard were prepared in ultrapure water (Millipore®) at 1 mg/mL and kept at −20 °C until used. Standard curves were prepared from working mix solutions at 0.01; 0.02; 0.05; 0.1 and 0.2 ng/μL for NE; 0.05; 0.2; 1; 2 and 4 ng/μL for DA; 0.01; 0.02; 0.05; 0.1 and 0.2 ng/μL for 5-HT; 0.02; 0.05; 0.1; 0.2 and 1 ng/μL for 5-HIAA; 0.1; 0.2; 0.5; 1 and 2 ng/μL for DOPAC; 0.02; 0.05; 0.1; 0.2 and 1 ng/μL for HVA and 2; 4; 8; 10 and 15 ng/μL for MHPG. The structures of the monoamines and their metabolites are shown in Fig. 1.

The HPLC-FL system used was from Shimadzu® (Japan), model LC20AT, with automatic injector (SIL-20SA), quaternary LC pump system (20AT), and fluorescence detector (10AXL) connected to LC Solution® software. The fluorescence of the monoamines was monitored at excitation and emission wavelengths of 279 nm and 320 nm, respectively. Gemini C18 analytical column (150 × 4.6 mm, 5 μm) was preceded by a pre-safety column C18 (4.0 × 3.0 mm, 5 μm), both from Phenomenex® (Torrance, CA, USA). The aqueous mobile phase contained Na_2EDTA (Vetec®) at 0.26 mM, with the pH adjusted (UJ Micronal®, model AJX-512) to approximately 3.2 with acetic acid. HPLC grade methanol (purity 98%; Merck®) was used as the organic phase. The mobile phases were filtered under vacuum through a 0.45 μm PTFE membrane (Millipore®) and ultrasonicated for 10 min (Unique®, Model USC-3300) before use. The optimized mobile phase conditions are: flow rate of 0.5 mL/min and methanol concentration at 5% for 7.5 min.; from 7.5 to 9 min. Methanol increases to 16%, with flow rate at 1 mL/min; from 9 to 20 min. Methanol rises to 25%, from 20 to 25 min. to 30%, from 25 to 35 min. to 80%, returning to the initial conditions, and kept for 10 min. Total running time was 50 min. and injection volume 20 μL.

Sample preparation: Approximately 50 mg of brain (without the hippocampus, weighed quantitatively) was transferred to a microcentrifuge tube, 100 μL of a 0.2 M solution of perchloric acid (IMPEX®) and 3 mM cysteine (SIGMA®) were added, the tube was vortexed for 20 s and centrifuged

for 20 min (5000 RPM, 4 °C). The supernatant was injected in the HPLC-FL. For each sample, 2–3 subsamples were weighed and submitted to extraction and analysis, and the mean of the replicates used in the statistical analysis.

BDNF quantification

BDNF levels in the hippocampus were determined using the ChemiKine ™ BDNF sandwich-ELISA kit, according to the manufacturer's instructions (Milipore, USA). The BDNF standard curve showed satisfactory linearity (coefficient of determination of 0.99). Two replicate samples were analyzed and the mean concentration was expressed in terms of total proteins, weight of total brain and weight of hippocampus. Total proteins were quantified by a modified Lowry validated method using bovine serum (Sigma-Aldrich®) to prepare a seven-point standard curve.

Statistical analysis

Data were analyzed using GraphPad Prism 6.01 (September 21, 2012) by one-way analysis of variance (ANOVA) followed by Tukey test, or by Kruskal-Wallis test and Dunn's multicomparison test (non-parametric). Values are means ± standard error (SEM). In any case, a difference was significant when $p \leq 0.05$.

Results

No clinical signs of toxicity were observed and all animals survived through the 28 days of the experiment. There were no significant differences in body and organ weights between the experimental groups, but the weight gain over the treatment of the fluoxetine female group was significantly lower than controls (Table S1; Supplemental Material). No macroscopic alterations were observed in the organs of the animals, except for one lung with black spots in a female of the control group. Blood analysis data showed that the percentage of lymphocytes of the Aya 0.5 female group was lower than controls and aspartate transaminase activity in the Aya1 male group was higher than controls (Table S2 – S4; Supplemental Material).

Behavior tests

No significant differences in locomotion in the OF test (number of quadrants covered) were observed among the experimental groups (data not shown). Male rats from the Aya1 group explored the central quadrant less than control animals, and the ratio between the number of entries in the central area and number of quadrants covered (% of central entries) was significantly lower in male rats of Aya1 and FLX groups; this decrease was also significant when considering both genders in the FLX group (Fig. 2). Treatment with ayahuasca (and fluoxetine) did not affect the other parameters evaluated in the OF test, and all parameters evaluated in the EPM (data not shown).

Monoamine and metabolite levels in brain samples (without hippocampus) by HPLC-FL

Selectivity of the HPLC-FL method was shown by comparing the extract of a brain fortified with the analytes of interest and a brain extract without fortification. The matrix effects were evaluated by comparing the angular coefficients of a NE calibration curve in water and a standard curve prepared in brain extract. There was a significant NE signal decrease (area in the chromatogram) of 17–40% when present in brain compared with water. This result shows that, in principle, quantification of the analytes should be done against a standard curve in brain matrix. However, as the matrix effect will occur in all cases and the objective of the study was to compare the monoamine and metabolite concentrations among the groups and not to quantify the absolute concentrations, the quantification of the analytes was done with an analytical curve prepared in water. This approach is similar to that taken by other authors (Fonseca et al. 2017, Lakshmana and Raju 1997; Castro-Neto et al. 2013). The linearity of the standard curves showed correlation coefficients (r) ≥ 0.99 for all analytes. Instrumental precision, assessed though standard curves prepared on 5 different days, was satisfactory, with relative standard deviation ranging from 1.7 to 19.2% for all analytes at all levels, except for 5-HT at 0.01 ng/μL level (22.5%).

In this study, monoamines and their metabolites were analyzed in the brain tissues without the hippocampus, and the results were expressed in relation to the total brain weight. Serotonin (5-HT) levels were significantly higher than controls only for the Aya2 female group (Fig. 3A), and there was a significant reduction of 5-HIAA metabolite levels in the brains of the fluoxetine-treated rats when compared to controls (Fig. 3B). The 5-HIAA/5-HT ratio was decreased compared to controls in fluoxetine and Aya treated female rats, but with no significance (Fig. 3C).

Figure 4 shows the levels of dopamine, its metabolites DOPAC and HVA, and the turnover ratios for the control and treated groups. DA levels did not change significantly when rats were exposed to fluoxetine or ayahuasca (Fig. 4A), but the DOPAC levels increased gradually with ayahuasca treatment, being significantly higher than controls for Aya1 and Aya2 female groups, and for Aya2 male group ($p < 0.0001$ for Aya2 both sexes group). Furthermore, the Aya2 group had significantly higher DOPAC levels than FLX and Aya0.5 groups (Fig. 4B), and higher DOPAC/DA turnover than controls and the fluoxetine group (Fig. 4C).

There was no significant difference for HVA levels and for HVA/DA turnover among the groups (data not shown), but the HVA/DOPAC turnover was significantly lower than control for male ayahuasca-treated groups and for Aya1 and Aya2 groups when considering both sexes (Fig. 4D).

Norepinephrine was not detected in any brain sample analyzed, and no significant difference was found in the levels of its metabolite MHPG among the groups (Fig. 5). In the control group, a significantly higher level of this metabolite was observed in females compared to males (13.58 ± 2.55 and 8.09 ± 1.67, respectively).

BDNF in the hippocampus

No significant difference was observed in the ratio between the weight of the hippocampus and the total weight of each brain between the study groups (data not shown). BDNF levels in the rat hippocampus were estimated in relation to total proteins, to brain weight and to hippocampus weight (Fig. 6). BDNF levels were significantly higher than control for FLX group (both sexes) when expressed in relation to total proteins and to brain weight (Fig. 6A and B), and for the Aya2 female group when expressed in relation to brain weight (Fig.

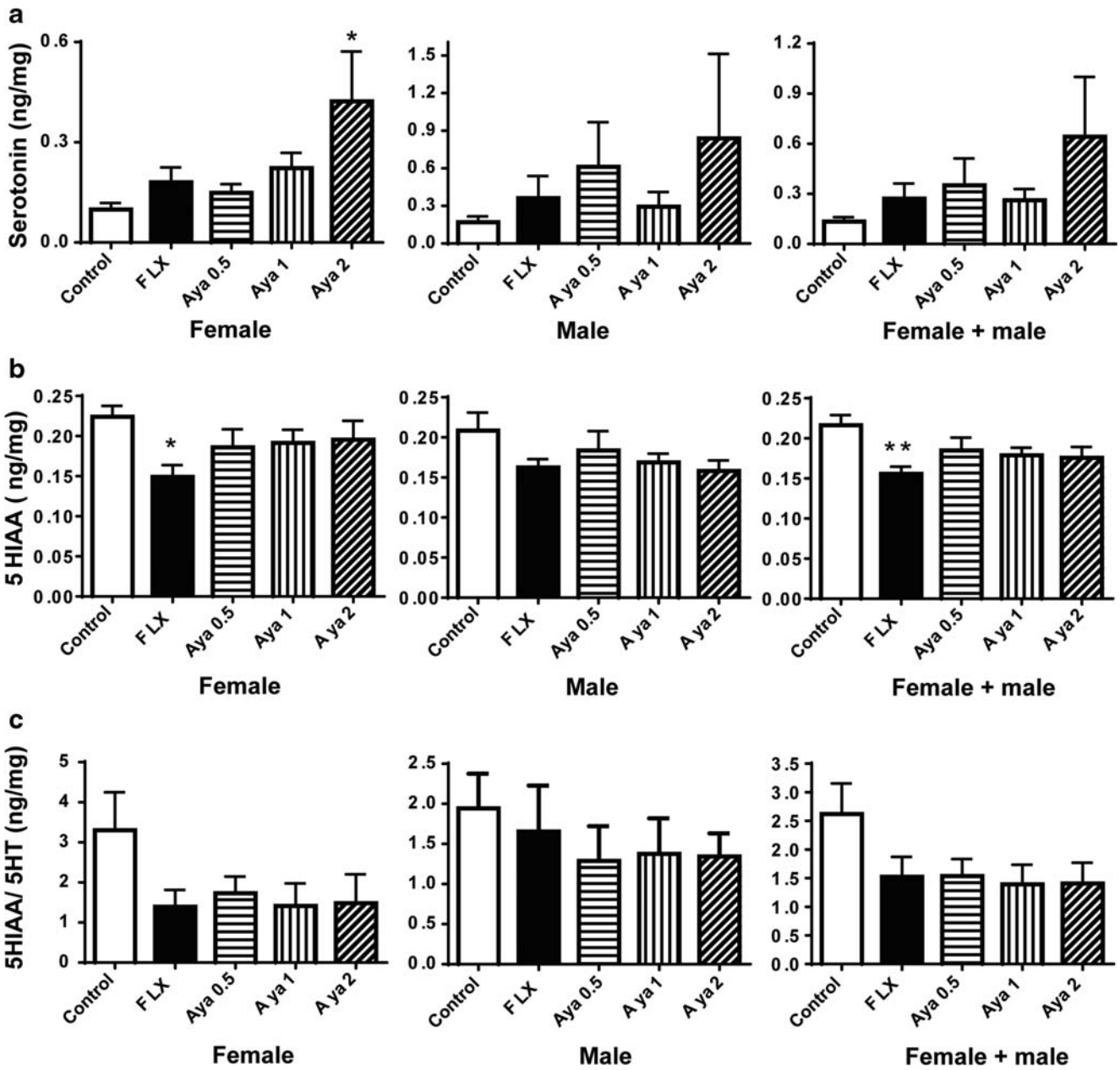

Fig. 2 A: Exploration of the central area of the open field (number of entries) and B: % of central entries

5B). No significant differences were observed among the groups when the BDNF was related to hippocampus weight (Fig. 6C).

Discussion

Chronic treatment with ayahuasca for 28 days at doses 0.5 to 2X the ritual dose did not affect body weight, weight gain or organ weights, nor showed any effect on organs that could be observed in a macroscopic evaluation. The hematologic (lymphocyte) and biochemical (aspartate transaminase) alterations found in the lower doses have, alone, no clinical significance. All together, these results indicate that chronic exposure to ayahuasca for 28 days up to 2X the ritual dose is safe for Wistar rats, which is the estimated no observed adverse effect level (NOAEL).

Using the same ayahuasca material of the present study, Santos et al. (2017) found no variation in the biochemical results of treated male rats exposed every two days for 72 days to ayahuasca at 1X to 8X the ritual dose. However, this dose regime increased the relative stomach weight at all doses tested, and decreased feed consumption and weight gain at the 4X and 8X doses, with also a significant effect in the body weight observed in the highest dose group. Indeed, daily exposure to ayahuasca at 4 and 8X for 14 days reduced food consumption of pregnant rats (da Motta et al. 2018). On the other hand, Cameron et al. (2019) did find that 7 days intermittent DMT intake at 1 mg/kg (which correspond to ~ 4X the ritual dose of the material used in the present study) increased the body weight of male rats (but not of females). These results indicated that in addition to the dose, the DMT present in ayahuasca is probably involved in the modulation of weight gain after chronic exposure.

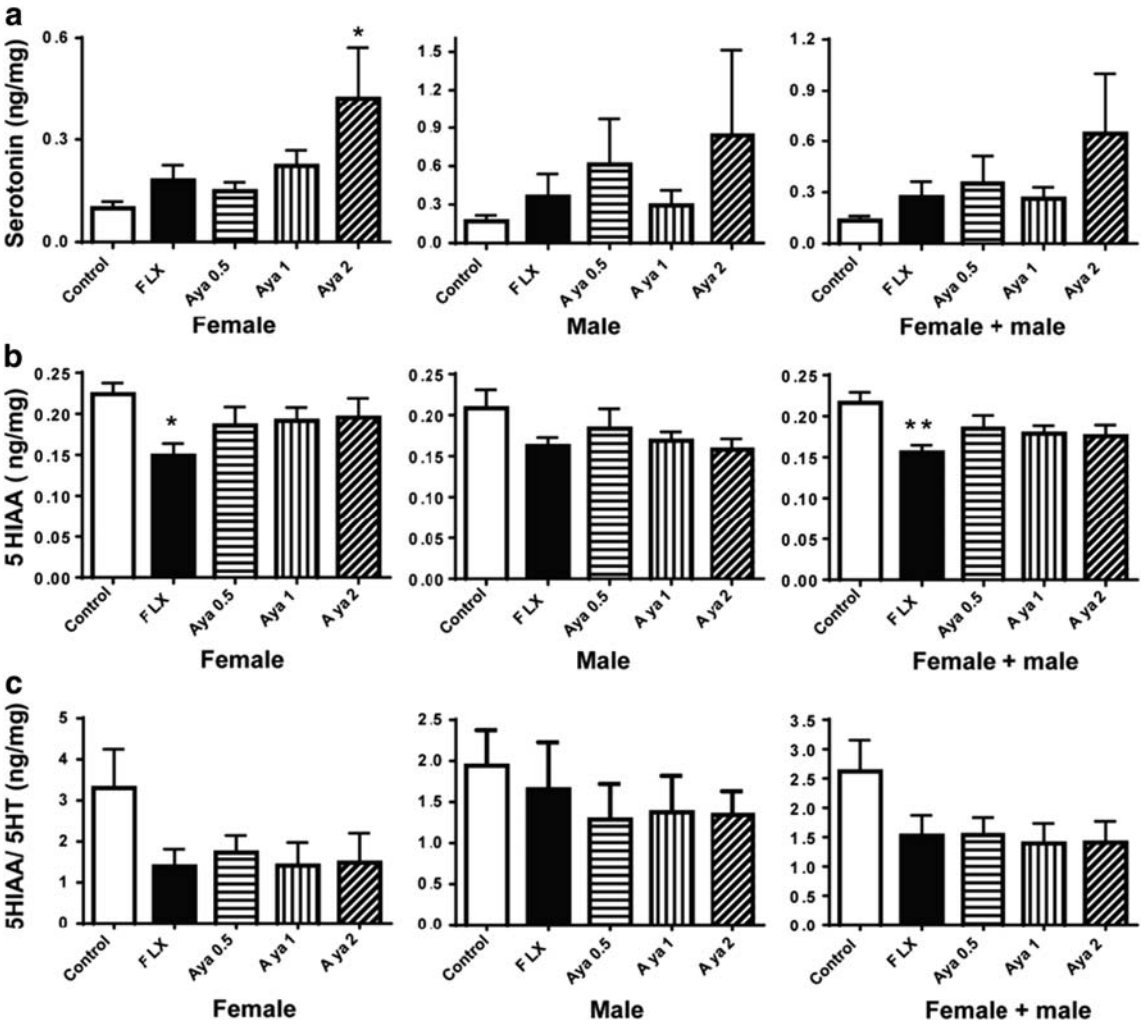

Fig. 3 A: Levels of serotonin (5-HT) and B: levels of 5-HIAA, in ng/mg of brain; C: 5-HIAA/5HT. Mean ± SEM, Control: $n = 8$ females and 8 males, FLX: $n = 9$ females and 9 males, Aya0.5: n = 9 females and 7 males, Aya1: n = 8 females and 10 males, and Aya2: n = 8 females and 9 males. * $p < 0.05$ and ** $p < 0.001$ relative to control

The results of this chronic study add to the ayahuasca toxicological profile on Wistar rats obtained from previous studies. Ayahuasca has a low acute toxicity (LD_{50} higher than 50X the ritual dose; Pic-Taylor et al. 2015), a low acute genotoxicity, with a NOAEL of 5X the dose (Melo Junior et al. 2016), a potential toxic effect in male rats at 4X the ritual dose after 72 days of intermittent exposure (Santos et al. 2017) and an important developmental toxicity, with a NOAEL of 1X the ritual dose (da Motta et al. 2018).

The EPM and the OF tests are a widely used animal model to investigate anxiety-like behavior, based on the spontaneous exploratory behavior of rodents and their natural aversion to open areas caused by fear and anxiety (Pellow et al. 1985; Walf and Frye 2007). The OF test was pharmacologically validated with classical benzodiazepines, and results with serotonin-like acting drugs are contradictory. Chronic administration of SSRI, such as fluoxetine, normally do not elicited anxiolytic-like effects, and non-specific 5-HT receptor

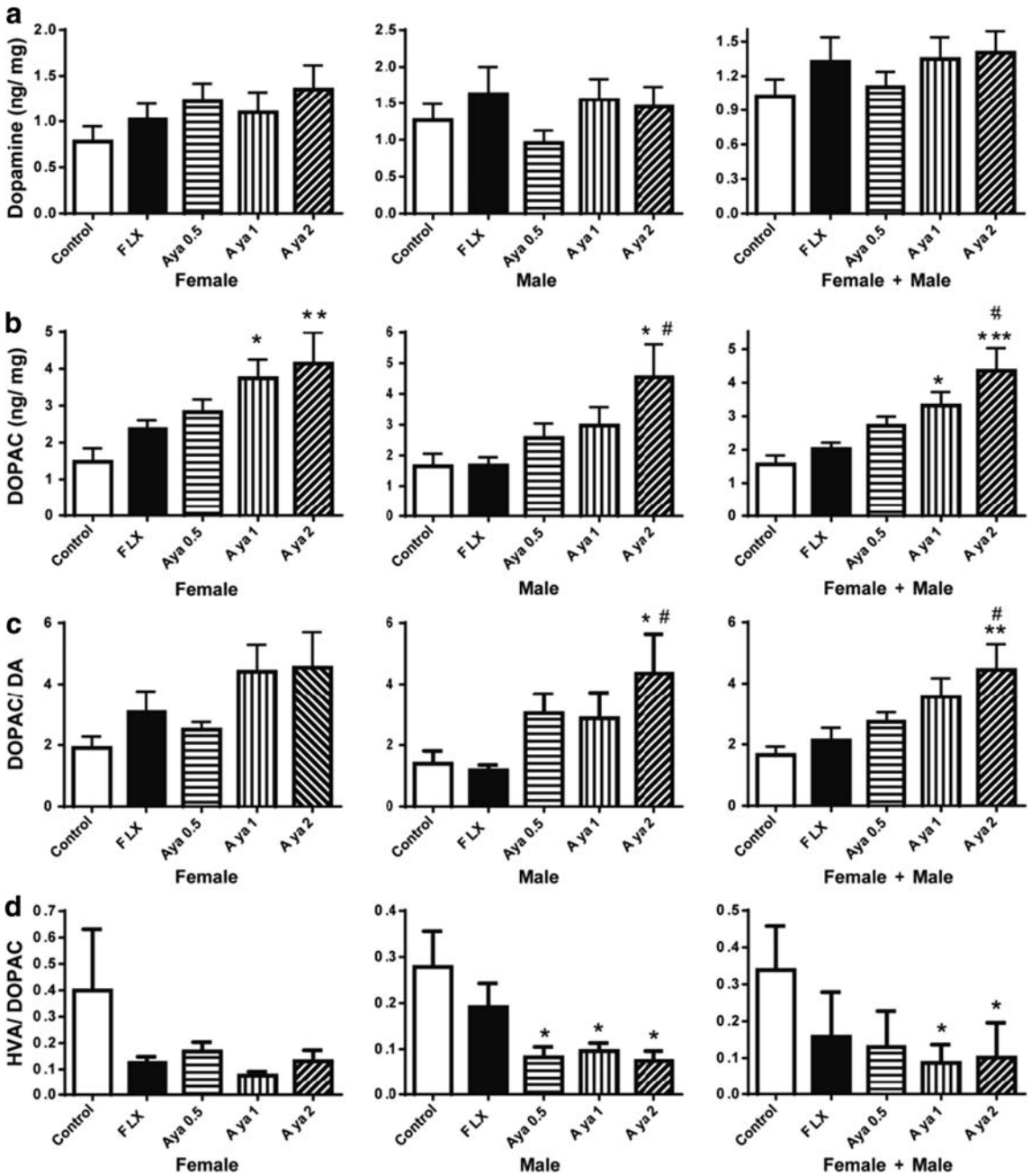

Fig. 4 A: Levels of dopamine and B: levels of DOPAC, in ng/mg of brain, C: DOPAC/dopamine and D: HVA/DOPAC. Results in, expressed as mean ± SEM, Control: n = 8 females and 8 males, FLX: n = 9 females and 9 males, Aya0.5: n = 9 females and 7 males, Aya1: n = 8 females and 10 males, and Aya2: n = 8 females and 9 males. * significant in relation to control, $p < 0.05$, ** significant in relation to control, $p < 0.001$, *** $p < 0.0001$ in relation to control, # significant relative to fluoxetine, $p < 0.05$

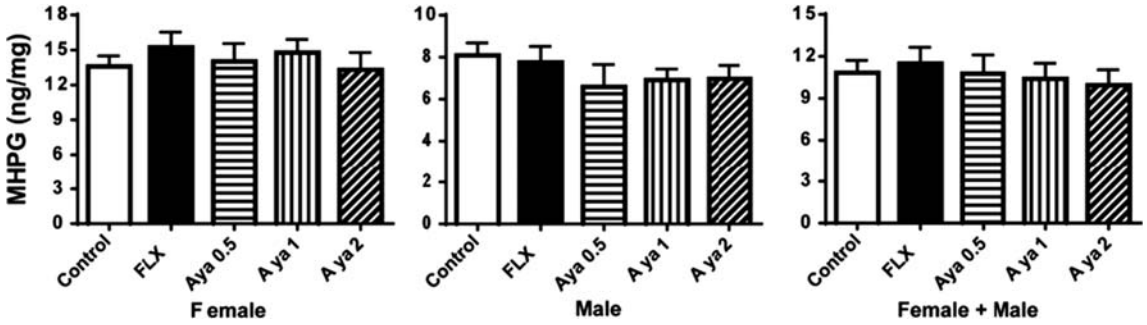

Fig. 5 Levels of MHPG in rat tissue, in ng/mg of brain, expressed as mean ± SEM, Control: n = 8 females and 8 males, FLX: n = 9 females and 9 males, Aya0.5: n = 9 females and 7 males, Aya1: n = 8 females and 10 males, and Aya2: n = 8 females and 9 males

agonists (such as DMT) are normally anxiogenic (Prut and Belzung 2003). In the present study, chronic treatment with ayahuasca (and fluoxetine) did not affect locomotion of the rats in the OF arena, but male animals from the Aya1 group explored the central area less and showed a lower % of central entries, an anxiogenic behavior that was also observed in the FLX male and both gender groups. It is important, however, to emphasize that although the rats were exposed for 28 days, the tests were conducted one hour after the last exposure and can reflect also an acute response. Previous studies showed that single high ayahuasca dose (15-30X the ritual dose) and fluoxetine significantly decreased rat locomotion and rearing in the OF and in the EPM, and also increased rat activity in the forced swimming test (indicating a potential antidepressant

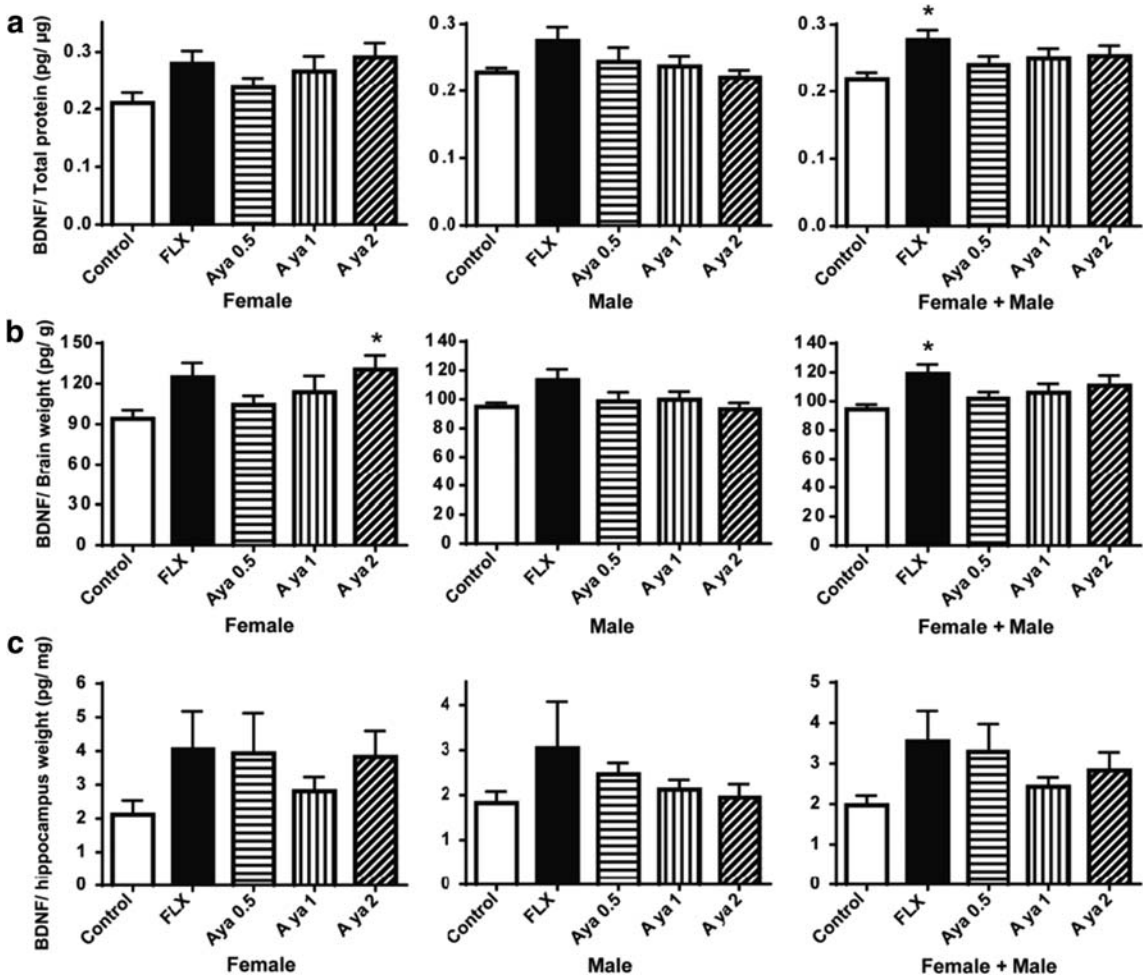

Fig. 6 BDNF levels in rats' hippocampus, expressed in A: pg/µg total proteins, B: pg/g brain and C: pg/mg hippocampus. Mean ± SEM, Control: n = 8 females and 8 males, FLX: n = 9 females and 9 males, Aya0.5: n = 9 females and 7 males, Aya1: n = 8 females and 10 males, and Aya2: n = 8 females and 9 males. * $p < 0.05$ relative to control

effect after acute exposure (Pic-Taylor et al. 2015; Melo Junior et al. 2016). This antidepressant effect in the FST was also shown after acute exposure to DMT (Cameron et al. 2018) and harmine (Farzin & Mansure 2006; Fortunato et al. 2009).

According to the monoamine theory, depression can be attributed to functional imbalance or deficiency of neurotransmitters, a hypothesis that is supported by the therapeutic effects of tricyclic antidepressants, monoamine oxidase inhibitors (MAOIs), serotonin and norepinephrine receptor inhibitors (SNRIs), and selective serotonin reuptake inhibitors (SSRIs) (Krishnan and Nestler 2008; Domínguez-Clavé et al. 2016). Ayahuasca contains DMT, a 5-HT_2 agonist, and β-carbolines, which are MAOIs (Riba 2003). Hence, it is reasonable to hypothesize that the ayahuasca may have an antidepressant action through the serotoninergic system. To investigate this hypothesis, Mckenna (2004) looked at serotonin transporter sites in blood platelets of 15 healthy male ayahuasca users, using [3H]-citalopram to label the transporters in binding assays, and found a significant up-regulation in the density of the citalopram binding sites compared with control subjects. Although the ayahuasca drinkers had a higher density of transporters, there was no change in their affinity for the labeled citalopram binding site. A recent study with humans showed evidence of a rapid antidepressant effect after a single dosing session with ayahuasca when compared with placebo, with a decrease in depression severity and improvements in the psychiatric scales (Palhano-Fontes et al. 2018).

In the present study, serotonin levels were significantly increased in brain tissue without the hippocampus of Aya2 female group compared to controls, while there was an important, but non-significant, reduction in serotonin turnover (5-HIAA/5-HT) in all ayahuasca-treated groups. The turnover can be used as an index of neurotransmitter metabolism: the reduction of turnover indicates a deceleration, and its increase an acceleration of the metabolism (Donato et al. 2013). Castro-Neto et al. (2013) found a significant increase in serotonin levels and turnover in the hippocampus and the amygdala of rats treated with ayahuasca at single oral doses of 250 to 800 mg/kg bw. These doses correspond to ~ 6 to 20 mg/kg bw of harmine, higher than the highest correspondent harmine dose used in the present study (5.2 mg/kg bw). The increased serotonin levels in the brain of rats treated with ayahuasca is expected, due to the action of β-carbolines (MAO inhibitors) present in the beverage. On the other hand, treatment with fluoxetine, a known SSRI, did not significantly increase serotonin levels, although it significantly decreased the 5-HIAA metabolite levels.

Castro-Neto et al. (2013) observed a significant increase in dopamine levels in the amygdala of the ayahuasca-treated animals and Iurlo et al. (2001) reported an increase in dopamine in the striatum of mice treated with a single dose of harmine (0.5 to 10 mg/kg bw, ip) and decreased DOPAC and HVA metabolites levels. Brierley and Davidson (2013) suggested that harmine increases dopamine efflux via a presynaptic mechanism specific for the 5-HT2A receptor, independent of its MAO inhibitory activity. In the present work, no significant differences were found in dopamine levels in the brain of ayahuasca-treated animals, but ayahuasca increased the metabolism of dopamine to DOPAC, its main metabolite in rats, and slows the methylation of DOPAC to HVA (Fig. 1). These impacts were not observed with fluoxetine. Although there is a tendency for dopamine turnover to be lower in depressive states, the results from various studies are not consistent due to the complexity of dopamine neuronal activity, which depends on their release, metabolism, sensitivity of different receptors, and balance with other neurotransmitters (Ackenheil 2001).

In the present study, norepinephrine was not detected in brain tissue without the hippocampus of any animal used in the study. Other studies, however, reported concentrations between 0.15 and 1.86 ng/mg of this monoamine in various regions of the brain, with the highest concentration found in the hypothalamus (Fonseca et al. 2017; Lakshmana and Raju 1997; Castro-Neto et al. 2013). MHPG levels, a useful marker of the usage rate of central norepinephrine (Glavin 1985), were not affected by the treatment with ayahuasca (or fluoxetine), and the levels found in control animals (~ 8 ng/mg in males) were much higher than those reported in the literature in several brain regions (0.05–0.16 ng/mg; male Wistar rats; Martínez et al. 2018). It seems that the Wistar rats used in the experiment have a very high NE turnover rate, leading to non-detected levels of the neurotransmitter and to very high levels of its metabolite, mainly in females.

BDNF plays a crucial role in depression as a central regulator of neuronal plasticity in the hippocampus (von Bohlen Und Halbach and von Bohlen Und Halbach 2018). BDNF levels are decreased in bipolar mania and bipolar depression in human plasma/serum (Fernandes et al. 2011), and post-mortem data from depressed individuals show decreased levels of BDNF in the hippocampus (Krishnan and Nestler 2008). Chronic treatment with antidepressants increases BDNF expression in the hippocampus of adult rats (Malberg et al. 2000), and it is believed that the antidepressant action of fluoxetine, an SSRI, is a result of the synergism between an increase in serotonin receptor activation and BDNF levels in the brain (Li et al. 2017). In the present study, the levels of BDNF in the hippocampus were significantly higher in the fluoxetine and Aya2 (only female) groups compared to controls. A significant increase in BDNF levels was also observed in the hippocampus of rats treated once with 15 mg/kg, i.p harmine (Fortunato et al. 2009) and for 14 days at 10 and 15 mg/kg, i.p. (Fortunato et al. 2010a, b). Furthermore, Vaidya et al. (1997) have shown that 5-HT2A/2C receptor agonist 4-iodo-2,5-dimethoxyphenylisopropylamine (DOI) significantly decreased BDNF mRNA expression in the dentate gyrus granule cell layer of the hippocampus but had no

effect on the CA1 and CA3 regions. Hence, although fluoxetine also induced BDNF in the hippocampus, the effect observed in the ayahuasca treated animals in this study is most likely due to the presence of the β-carbolines, with no involvement of the 5-HT2A agonist DMT, a hypothesis that should be further investigated.

The results of this study show that chronic oral use of ayahuasca affects male and female rats differently. Males showed higher sensitivity than females in monoamine turnovers, while females showed more sensitivity in the serotonin and BDNF levels. Carlsson and Carlsson (1988) also found an increase in serotonin activity, synthesis, and metabolite levels in the brain of female Sprague-Dawley rats compared to males treated with NSD 1034 (N-(3-hydroxybenzyl) N-methyl hydrazine dihydrogen phosphate), an inhibitor of L-amino acid decarboxylase. Saylor et al. (2019) suggested that serotonin sensitivity differences may be due to the presence of auto receptor effects in female mice, emphasizing the importance of conducting pre-clinical studies with both sexes.

The results of this study contribute to the scientific literature about the toxicological profile of ayahuasca and the investigation of the mechanisms in the nervous system involved in the effects of ayahuasca observed in human studies. However, the study has some limitations that should be discussed. A major limitation was that the monoamines and their metabolites were analyzed in a large portion of brain (total brain after removing the hippocampus), leading to dilution factors that decreased the possibility of detecting changes due to ayahuasca treatment. This is very relevant as the doses used were relatively low, close to the ritualistic doses. Furthermore, in addition to the hippocampus, BDNF levels should also be investigated in other brain regions. Other relevant areas that should be investigated in future studies include the prefrontal cortex, nucleus accumbens and striatum, which are important for the serotoninergic and dopaminergic systems (Clapp et al. 2008).

Conclusion

This study showed that the chronic use of ayahuasca for 28 days at doses of 0.5 to 2X the ritual dose was safe to Wistar rats, with a NOAEL of 2X the ritual dose, corresponding to 0.52 mg/kg bw DMT, 5.2 mg/kg bw harmine, 0.34 mg/kg bw harmaline and 0.66 mg/kg bw tetrahydroharmine. Treatment with ayahuasca led to increased levels of serotonin and increased dopamine turnover in the brain (without the hippocampus) and BDNF levels in the hippocampus, although did not affect dopamine levels. This is a preliminary study and the results should be further investigated in specific brain regions and with different dosing regime, including higher, intermittent ayahuasca doses. Additionally, animal models of depression should be used in future studies when investigating the mechanisms of ayahuasca as antidepressant.

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Compliance with ethical standards

Conflict of interest The authors have no conflict of interest to declare.

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