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Effects of ayahuasca on the development of ethanol-induced behavioral sensitization and on a post-sensitization treatment in mice

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HIGHLIGHTS

- Ayahuasca (Aya) did not exert effects on the spontaneous locomotor activity of mice.
- Aya prevented the development of ethanol(Eth)-induced behavioral sensitization (BS).
- At high doses, Aya also inhibited acute Eth-induced hyperlocomotion.
- An 8-day treatment with Aya in the open-field did not induce BS to this drug.
- Counter-sensitization with Aya blocked the reinstatement of Eth-induced BS.

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ABSTRACT

Background: Hallucinogenic drugs were used to treat alcoholic patients in the past, and recent developments in the study of hallucinogens led to a renewal of interest regarding the application of these drugs in the treatment of addiction. In this scenario, accumulating evidence suggests that the hallucinogenic brew ayahuasca (Aya) may have therapeutic effects on substance abuse problems.
Methods: We investigated the effects of Aya on spontaneous locomotor activity and ethanol(Eth)-induced hyperlocomotion and subsequent locomotor sensitization by a two-injection protocol. Additionally, we tested the effect of Aya on an 8-day counter-sensitization protocol to modify sensitized responses induced by a repeated treatment with Eth (1.8 g/kg) for 8 alternate days.
Results: Aya showed high sensitivity in preventing the development of Eth-induced behavioral sensitization, attenuating it at all doses (30, 100, 200, 300 or 500 mg/kg) without modifying spontaneous locomotor activity. At the highest doses (300 and 500 mg/kg), Aya also showed selectivity to both acute and sensitized Eth responses. Finally, a counter-sensitization strategy with 100 or 300 mg/kg of Aya for 8 consecutive days after the establishment of Eth-induced behavioral sensitization was effective in blocking its subsequent expression on an Eth challenge.
Conclusions: We demonstrated that Aya not only inhibits early behaviors associated with the initiation and development of Eth addiction, but also showed effectiveness in reversing long-term drug effects expression, inhibiting the reinstatement of Eth-induced behavioral sensitization when administered in the Eth-associated environment.

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[1] This paper is in memory of Dr. Roberto Frussa-Filho, who dedicated his entire life to Science, because a man is alive while his name is still spoken.

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1. Introduction

Alcohol (ethanol) abuse is a major contributor to more than 60 types of diseases and injuries and accounts for approximately 2.5 million deaths each year [38]. Ethanol addiction is a chronic and often progressive and fatal disease with genetic, psychosocial, and environmental factors influencing its development and manifestations [28]. Currently available psychological and pharmacological treatments are only partially effective [3] and further research on new intervention approaches remain necessary.

Hallucinogenic drugs were used to treat alcoholic patients during the decades of 1960 and early seventies. These studies came prematurely to a halt due to the classification of hallucinogens into Schedule I class, i.e., drugs with high abuse potential, no accepted therapeutic use and lack of accepted level of safety for use under medical supervision. After four decades banned from human psychiatric research, hallucinogen research has resumed by using psilocybin, a serotonergic hallucinogen, to treat alcoholism and nicotine dependence [4].

Accumulating evidence from observational epidemiological studies suggests that the hallucinogenic brew ayahuasca may have therapeutic effects on substance related problems. This brew is produced from the decoction of N,N-dimethyltryptamine (DMT) and harmala alkaloid-containing plants, such as harmine, tetrahydroharmine (THH) and harmaline [26], and is used in syncretic religions in major cities of Brazil and parts of Europe, Japan, Canada, and the USA [40]. Case–control, longitudinal and cross-sectional studies showed that ritual and religious ayahuasca users present fewer alcohol-related problems than control groups and that drug use diminished after joining ayahuasca churches [13,20,22,39]. It is an open question whether ayahuasca has anti-addictive properties per se or if the social factors (e.g. religious social reinforcement) play a major role in these results [2]. By ruling out the ceremonial religious aspects of the aforementioned studies, pharmacological studies using rodent models can contribute to elucidate the role of the brew per se into the neurobiological mechanisms of ayahuasca on alcohol-related behavior.

In the current paper we used the behavioral sensitization model to investigate the effects of ayahuasca on alcohol-related behavior in mice. Alcohol increases dopamine levels in the nucleus accumbens, which elicits locomotor stimulation in rodents, and repetitive administration intensifies this response [37]. This phenomenon called behavioral sensitization is thought to be an underlying adaptation responsible for addiction to drugs of abuse and to share neuronal mechanisms with craving [33]. Behavioral sensitization depends on the temporal pattern of drug exposure. Repeated intermittent treatment regimens are usually more effective to induce sensitization than continuous exposure to high or escalating drug doses [32,37,44]. However, single dose drug abuse exposure has also been reported to induce long-term behavioral sensitization [42,43].

Additionally, an important aspect concerning both drug craving in humans and behavioral sensitization in rodents is the potentiating effect of environmental cues previously paired with drug effects on their development [7,9,15,29]. Therefore, recent efforts to develop effective treatments for addiction have focused on manipulations of learning and memory processes involved in encoding drug-cue associations. In this scenario, it has been suggested that reconsolidation and/or counter-sensitization procedures permit the therapeutic drug treatment to become linked to the contextual stimuli and in effect form a new and different drug association with the contextual cues.

This paper reports two experiments designed to evaluate the effects of ayahuasca on ethanol-related behaviors. In the first experiment, we evaluated the effects of ayahuasca on mice spontaneous locomotion in the open-field apparatus, hyperlocomotion induced by ethanol and ethanol-induced behavioral sensitization in a single injection protocol. The second experiment was designed to test the effect of ayahuasca on a counter-sensitization protocol to modify sensitized responses induced by a repeated treatment with ethanol.

2. Material and methods

2.1. Animals

Male 3-month-old Swiss EPM-M2 mice (30–35 g) were obtained from the Centre for Development of Experimental Models in Medicine and Biology of Braz Cubas University. Animals were housed in groups of 12 in polypropylene cages (32 cm × 42 cm × 18 cm) under controlled temperature (22–23 °C) and lighting (12/12 h light/dark; lights on at 6 h 45 a.m.) conditions. Food and water were available ad libitum throughout the experiments. The experiments were performed in accordance with the National Institute of Health Guide for the care and use of laboratory animals (NIH Publications No. 80–23, revised 1996), and animals were maintained in accordance with the Brazilian Law for Procedures for Animal Scientific Use (#11794/2008). The experimental procedures were approved by the Institutional Ethical Committee of Braz Cubas University under the protocol #176/2008.

2.2. Drugs

One liter batch of ayahuasca was obtained by a member of the Santo Daime church. The liquid was lyophilized and rendered 88 g of freeze dried material. The ratio of dry tea/volume of liquid tea was calculated to establish the doses to be administered in the experiments.

Ethanol (Merck®) and ayahuasca were diluted in saline 0.9% solution. All solutions were given intraperitoneally (i.p.) at a volume of 10 ml/kg of body weight. Ethanol was administered at the dose of 1.8 mg/kg. The dose of ethanol was chosen based on previous studies showing that if is effective in inducing both acute and sensitized locomotor responses in mice [10,17,24].

2.3. Ayahuasca compounds analysis

In order to quantify the amount of the main compounds of ayahuasca (DMT, tetrahydroharmine, harmine and harmaline) in our preparation, the sample of ayahuasca was analyzed by liquid chromatography-tandem mass spectrometry (LC-MS/MS) conducted on a high performance liquid chromatography equipment Prominence system (Shimadzu, Kyoto, Japan). The analysis was conducted by the Criminalistics Institute of São Paulo.

Harmine hydrochloride and harmaline hydrochloride were purchased from Sigma®. The synthesis of tetrahydroharmine was performed according to previously published procedure (Callaway et al., 1996) and DMT was synthesized according to a modified procedure based on the selective dimethylation method (Giumanini et al., 1980; Pires et al., 2009). The stock solutions (1.0 mg/ml) of DMT, harmine, harmaline and tetrahydroharmine were prepared in methanol and stored at −20 °C until the performance of the LC-MS/MS.

2.4. Open-field evaluation

Locomotor activity was measured in an open field apparatus previously described by [9]. The apparatus is a circular wooden arena (40 cm in diameter and 50 cm high) with an open top and a floor divided into 19 squares. Hand-operated counters were used to score the locomotion frequency (total number of any square entered) during 10-min sessions by an observer, who was blind to the treatment allocation. Ten-minute sessions were proposed because it has been shown that even shorter periods are effective in reliably evaluating the effects of drugs acting on dopaminergic systems [8,16].

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2.5. Experimental procedure

2.5.1. Experiment 1. Effects of ayahuasca on spontaneous locomotor activity, acute ethanol-induced hyperlocomotion and ethanol-induced behavioral sensitization

Eighty mice were given a 10-min habituation period in the open-field on 2 consecutive days after a saline i.p. injection. Basal locomotor activity was measured on day 2. Six groups of animals were formed (n = 10 or 30), which were statistically equivalent with respect to the basal levels of locomotor activity. Previous habituation sessions are important to ensure the accuracy of the data due to the effect that environmental novelty exerts on spontaneous [21], ethanol- [17] and hallucinogenic drugs-induced locomotor activity [21].

On the third day, animals were i.p. acutely treated with saline (Sal, n = 30) or ayahuasca at the doses of 30, 100, 200, 300 or 500 mg/kg (Aya, n = 10 for each group) followed by initial exposure to the open-field environment 30 min after treatment to quantify their locomotor activities. During the interval between the treatment and the open-field exposure, animals were returned to their home-cages (animals under the same treatment housed together). A 30-min interval between the injection of ayahuasca and the open-field exposure was determined based on previous studies showing that hallucinogenic drugs might show a biphasic locomotor profile, with drug-induced hyperlocomotion only being observed after longer post-treatment periods [21,27]. The following groups were compared in the first open-field exposure: Sal, Aya30, Aya100, Aya200, Aya300 and Aya500. Immediately after the first behavioral evaluation, or 40 min after the saline/ayahuasca injection, 20 animals from the Sal group received a saline i.p. injection, and the remaining 10 mice were treated with 1.8 g/kg i.p. ethanol (Eth). All animals pretreated with ayahuasca also received 1.8 g/kg i.p. ethanol. After the second treatment, animals were placed in a clean cage until the subsequent exposure to the open-field apparatus. Five minutes after administration of either saline or ethanol, animals were returned to the open-field and for locomotion quantification. Thus, the following groups were formed: Sal–Sal, Sal–Eth, Aya30–Eth, Aya100–Eth, Aya200–Eth, Aya300–Eth and Aya500–Eth.

Seven days later, 10 out of 20 animals that were treated twice with saline on the previous week (Sal–Sal group) received a saline i.p. injection again (forming the Sal–Sal–Sal group) and the remaining 10 mice were treated with 1.8 g/kg i.p. ethanol for the first time (forming the Sal–Sal–Eth group). Ethanol (1.8 g/kg) was also administered to all the other animals for the second time, forming the Sal–Eth–Eth, Aya30–Eth–Eth, Aya100–Eth–Eth, Aya200–Eth–Eth, Aya300–Eth–Eth and Aya500–Eth–Eth groups. After treatment, animals were placed in a clean cage until their behavioral evaluations. Five minutes after the injections, mice were placed in the open-field for locomotor activity quantification. The experimental design of Experiment 1 is summarized in Fig. 1.

2.5.2. Experiment 2. Effects of ayahuasca on a counter-sensitization protocol to modify sensitized responses induced by a repeated treatment with ethanol

Sixty-six mice were given a 10-min habituation period in the open-field on 2 consecutive days after a saline i.p. injection. Basal locomotor activity was measured on day 2. Six groups of animals were formed (n = 11 for each group), which were statistically equivalent with respect to the basal levels of locomotor activity. Twenty-four hours after the second habituation day, the behavioral sensitization procedure began. Three groups of animals received an i.p. injection of saline (Sal groups) and the other 3 groups were treated with 1.8 g/kg ethanol (Eth groups) 5 min prior to being placed in the open-field apparatus every other day for 15 days from the 3th to 17th days (ethanol-induced behavioral sensitization, sensitization phase). After treatments, animals were placed in a clean cage until their behavioral evaluations. During the alternate non-sensitization days, mice were left undisturbed in their home-cages. On days 3 and 17 animals were observed for the quantification of their locomotion frequency.

Forty-eight hours after the last injection of the sensitization phase (19th day), the counter-sensitization protocol began. For 8 consecutive days (19th to 26th days) 11 animals from the Sal group received daily saline i.p. injections (Sal–Sal group) and the remaining mice received daily i.p. injections of ayahuasca (Aya) at the doses of 100 (Sal–Aya100, n = 11) or 300 (Sal–Aya300, n = 11) mg/kg. Those doses were chosen because in the first experiment 100 mg/kg of ayahuasca was the highest dose that specifically prevented ethanol-induced behavioral sensitization and 300 mg/kg was the lower dose that inhibited both ethanol-induced hyperlocomotion and behavioral sensitization. The ethanol-sensitized groups underwent the same procedure. Eleven animals from the Eth group received daily saline i.p. injections (Eth–Sal group) and the remaining mice received daily i.p. injections of ayahuasca at the doses of 100 (Eth–Aya100, n = 11) or 300 (Eth–Aya300, n = 11) mg/kg. Therefore, the following groups were formed: Sal–Sal, Sal–Aya100, Sal–Aya300, Eth–Sal, Eth–Aya100 and Eth–Aya300. During the interval between the treatment and the open-field exposure, animals were returned to their home-cages (animals under the same treatment housed together). Thirty minutes after each administration of saline or ayahuasca, animals were individually exposed to the open-field arena for 10-min sessions (counter-sensitization phase).

Four days after the last counter-sensitization day (30th day), all animals received an i.p. saline injection and were placed, 5 min later, in the open-field apparatus for quantification of their locomotion frequency. Two days after the Saline challenge, animals were tested for drug-induced reinstatement of ethanol-induced behavioral sensitization (day 32). All animals received an i.p. injection of 1.8 g/kg ethanol and were placed, 5 min later, in the open-field apparatus for quantification of their locomotion frequency. In both saline and ethanol challenge sessions, animals were placed in a clean cage during the interval between the treatment and the behavioral evaluation. The experimental design of Experiment 2 is summarized in Fig. 2.

2.6. Statistical analysis

Before conducting the statistical analysis, all variables were checked for normality (Shapiro–Wilk test) and homogeneity (Levene's test), which validated the use of the parametric tests. Data were analyzed by 1 or 2-way ANOVA, and multiple comparisons were performed using the Tukey's *post hoc* test when necessary or the paired Student *t*-test. A p value less than 0.05 was considered as a statistically significant difference.

Fig. 1. Design of experiment 1. OFQ: Open-field quantification; Sal: saline i.p. injection; Aya: ayahuasca (30, 100, 200, 300 or 500 mg/kg) i.p. injection; and Eth: ethanol 1.8 g/kg i.p. injection.

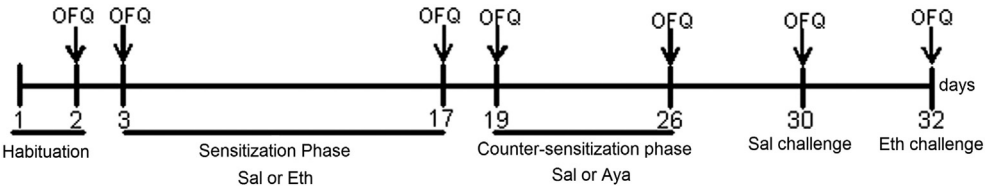

Fig. 2. Design of experiment 2. OFQ: Open-field quantification; Sal: saline i.p. injection; Aya: ayahuasca (100 or 300 mg/kg) i.p. injection; and Eth: ethanol 1.8 g/kg i.p. injection.

3. Results

3.1. Ayahuasca compound analysis

LC-MS/MS analysis indicated the following active constituents in our sample of ayahuasca:

- DMT: 0.4 mg/100 mg (35 mg/ml of initial batch)
- Tetrahydroharmine: 3.07 mg/100 mg (2.70 mg/ml of initial batch)
- Harmine: 3.85 mg/100 mg (3.39 mg/ml of initial batch)
- Harmaline: 0.17 mg/100 mg (0.15 mg/ml of initial batch).

3.2. Experiment 1. Effects of ayahuasca on spontaneous locomotor activity, acute ethanol-induced hyperlocomotion and ethanol-induced behavioral sensitization

Analysis of the second habituation session using 1-way ANOVA revealed no significant difference between groups [$F(5,74) = 0.09$; $p = 0.99$] (data not shown). In the first behavioral evaluation after saline or ayahuasca administration (spontaneous locomotor activity), ANOVA did not reveal significant differences between groups [$F(5,74) = 0.41$; $p = 0.83$], demonstrating that, at all doses, ayahuasca did not modify spontaneous locomotor activity per se (Fig. 3a).

In the evaluation of acute ethanol-induced hyperlocomotion after ayahuasca treatment, statistically significant differences were observed between groups [$F(6,73) = 11.74$; $p < 0.0001$]. An acute ethanol effect was observed based on the significantly higher locomotion frequency of the Sal–Eth group compared to the Sal–Sal group (Tukey's test, $p < 0.001$). Ayahuasca at the doses of 30, 100 and 200 mg/kg did not affect acute ethanol-induced hyperlocomotion. However, at the doses of 300 and 500 mg/kg, ayahuasca prevented the acute stimulating effect of ethanol (Tukey's test, $p < 0.05$) (Fig. 3b).

Mice were previously exposed/habituated to the open-field during the spontaneous locomotion evaluation for the subsequent within-day session on the first ethanol challenge and were re-exposed to the open-field on the test session only 7 days after the first ethanol injection. These different conditions could affect the locomotor activity of mice per se. Thus, to avoid an effect of these habituation factor between-sessions, the locomotor frequencies of mice were evaluated within-session, compared to the respective control groups. After one week, ethanol-induced locomotor sensitization was evaluated, and statistically significant differences were observed [$F(7,72) = 7.87$; $p < 0.0001$]. As shown in Fig. 3c, an acute ethanol injection for the first time induced enhanced locomotion frequency (Sal–Sal–Eth > Sal–Sal–Sal), which was potentiated in the Sal–Eth–Eth group (Sal–Eth–Eth > Sal–Sal–Eth) (Tukey's test, $p < 0.05$), indicating the development of behavioral sensitization. Treatment with ayahuasca at all doses before the first ethanol administration prevented the development of ethanol-induced sensitization, as shown by a significant decrease in the locomotor activity of these groups compared to the Sal–Eth–Eth group (Tukey's test, $p < 0.05$). These data together indicate that ayahuasca prevented the development of single dose ethanol-induced behavioral sensitization even at doses that did not inhibit acute ethanol-induced hyperlocomotion.

3.3. Experiment 2. Effects of ayahuasca on a counter-sensitization protocol to modify sensitized responses induced by a repeated treatment with ethanol

Analysis of the second habituation session using Student *t*-test revealed no significant difference between groups [$t(64) = 0.0085$; $p = 0.99$] (data not shown).

For the ethanol-induced behavioral sensitization analysis (sensitization phase), 2-way ANOVA with repeated measures showed a significant interaction effect between time (Day 3 vs Day 17) and treatment (ethanol vs saline) [$F(5,60) = 2.70$; $p < 0.05$]. As illustrated in Fig. 4a, Tukey's *post hoc* test showed that the acute ethanol injection (first day of sensitization phase) induced a significant increase in the locomotor activity of mice (Eth groups > Sal groups), thereby revealing the locomotor-stimulating effect of ethanol. In addition, paired *t*-test demonstrated that repeated treatment with ethanol increased the locomotor activity of the animals, as demonstrated by an increased locomotion of ethanol-treated groups on Day 17 compared with Day 3, thereby revealing the development of behavioral sensitization.

For the analysis of the counter-sensitization phase with ayahuasca, 2-way ANOVA with repeated measures revealed no significant effect of pre-treatment (ethanol vs saline) [$F(1,60) = 0.370$; $p = 0.54$], counter-sensitization treatment (ayahuasca vs saline) [$F(1,60) = 0.282$; $p = 0.75$] and time (Day 19 vs Day 26) [$F(1,60) = 1.57$; $p = 0.21$] or interaction between these factors [$F(1,60) = 0.66$; $p = 0.93$]. This result suggests that animals pre-treated with ethanol did not differ from the Sal group, and that, again, ayahuasca per se did not modify locomotor activity, even after a treatment for 8 consecutive days (Fig. 4b).

Four days after the last counter-sensitization phase (day 30), 2-way ANOVA revealed no significant effect of pre-treatment (ethanol vs saline) [$F(1,60) = 2.43$; $p = 0.12$] and counter-sensitization treatment (ayahuasca vs saline) [$F(1,60) = 0.12$; $p = 0.88$] or interaction between these factors [$F(1,60) = 0.81$; $p = 0.45$] during the saline challenge (Fig. 4c).

However, during the Ethanol challenge, 2-way ANOVA revealed a significant interaction effect between pre- (ethanol vs saline) and counter-sensitization (ayahuasca vs saline) treatments [$F(2,60) = 4.95$; $p < 0.01$]. As illustrated in Fig. 4c, paired *t*-test showed that an acute ethanol injection promoted an enhanced locomotion frequency in the group that was experiencing ethanol for the first time, as shown by a higher locomotion frequency of Sal–Sal group on the ethanol challenge compared to itself on the saline challenge. Of note, previous treatment with ayahuasca for 8 consecutive days did not inhibit the acute ethanol-induced hyperlocomotion phenomenon, because Sal–Aya100 and Sal–Aya300 groups did not differ from Sal–Sal group on the ethanol challenge day.

Fig. 3. Locomotor activity quantification in the open-field apparatus demonstrating the behavioral effects of i.p. treatment with either ayahuasca (Aya, 30, 100, 200, 300 or 500 mg/kg) or saline on (a) spontaneous locomotor activity and its subsequent effects on (b) acute hyperlocomotion induced by ethanol (Eth, 1.8 g/kg) and (c) ethanol-induced behavioral sensitization after a 7-day interval. Data are reported as mean ± S.E.M. ★ $p < 0.05$ compared with Sal–Sal (b) or Sal–Sal–Sal (c); ♦ $p < 0.05$ compared with Sal–Eth (b) or Sal–Eth–Eth (c); and • $p < 0.05$ compared with Sal–Sal–Eth (c). One- or two-way analysis of variance (ANOVA) followed by Tukey's test.

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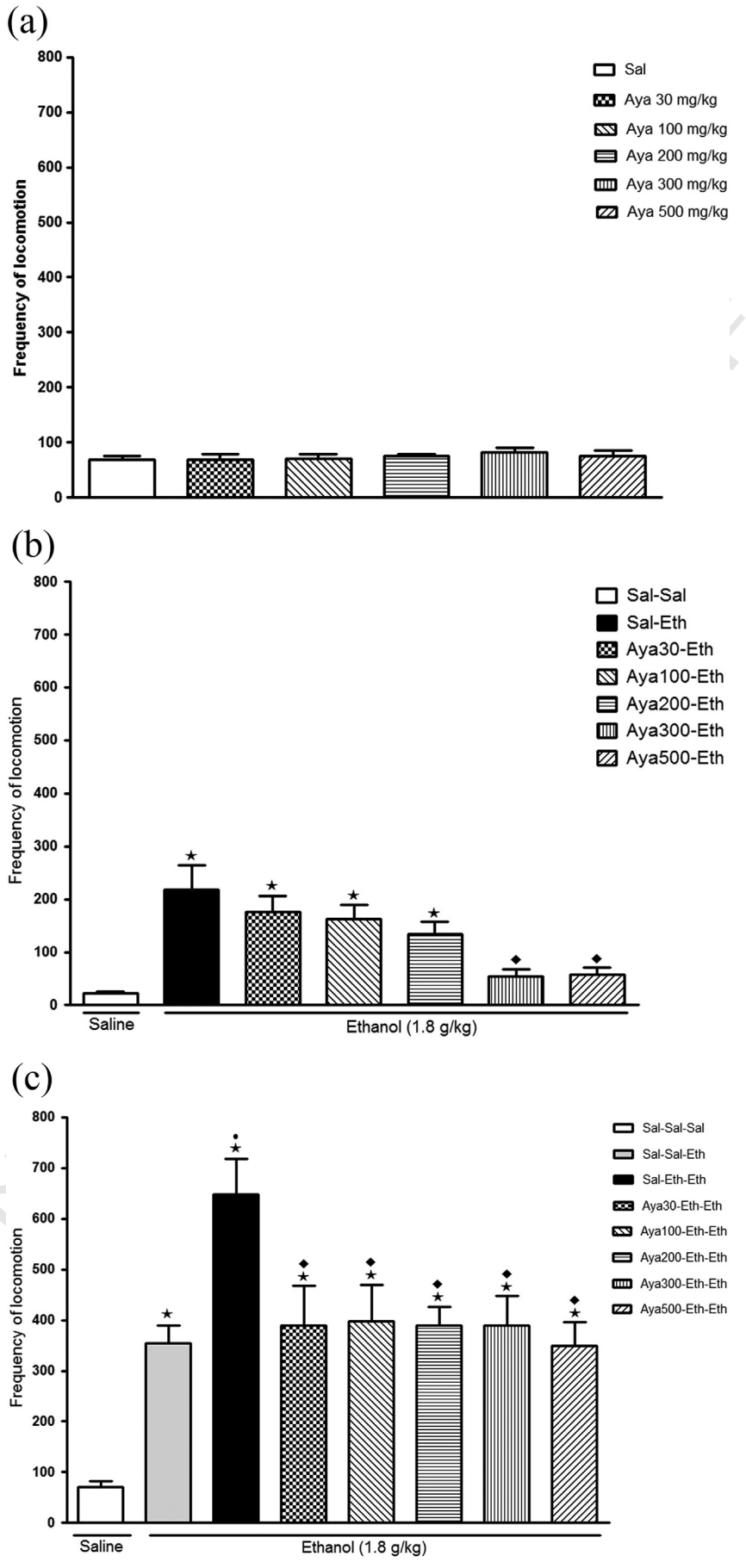

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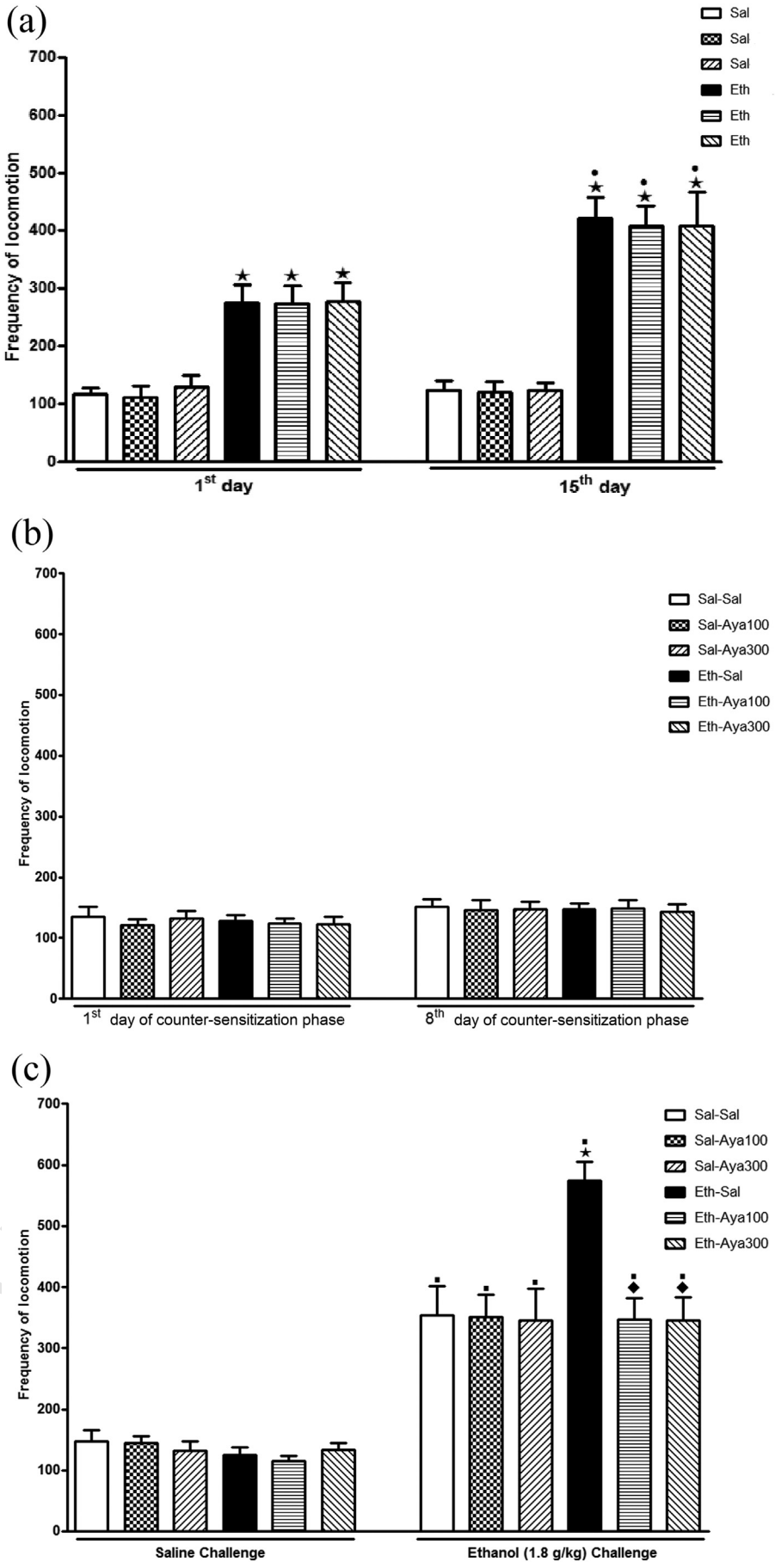

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Additionally, the ethanol-induced hyperlocomotion of the Sal–Sal group was potentiated in Eth–Sal group (Tukey's test, $p < 0.05$), indicating the expression of behavioral sensitization reinstatement with a new ethanol challenge in the previous group and repeatedly sensitized with ethanol that received saline during the counter-sensitization phase even after 15 days of drug withdrawal. However, Tukey's test indicated that the groups previously sensitized with ethanol and treated with 100 or 300 mg/kg of ayahuasca in the counter-sensitization phase (Eth–Aya100 and Eth–Aya300 groups), showed a lower locomotor activity compared to the group pretreated with ethanol in the sensitization phase but treated with saline in the counter-sensitization phase (Eth–Sal > Eth–Aya100 and Eth–Aya300). Moreover, the locomotor activity of both groups pre-treated with ethanol in the sensitization phase and treated with ayahuasca in the counter-sensitization phase (Eth–Aya100 and Eth–Aya300 groups) did not differ from that showed by the group pretreated with saline which received ethanol for the first time in the Ethanol challenge (Sal–Sal group). Taken together, these results indicate that the counter-sensitization with ayahuasca at both doses was effective in blocking the expression of the reinstatement of ethanol-induced behavioral sensitization.

4. Discussion

The most important findings of the present study were the following: (1) ayahuasca showed high sensitivity in preventing the development of ethanol-induced behavioral sensitization because it was attenuated by all tested doses, even lower doses than those required to reduce acute ethanol response, without modifying spontaneous locomotor activity; (2) at the highest doses (300 and 500 mg/kg), ayahuasca showed selectivity to both acute and sensitized ethanol responses, blocking these phenomena without affecting spontaneous locomotor activity; (3) a prolonged 8-day treatment with 100 or 300 mg/kg of ayahuasca in the open-field apparatus did not implicate in the development of behavioral sensitization to this substance; and (4) counter-sensitization with 100 or 300 mg/kg of ayahuasca in the open-field for 8 consecutive days after the establishment of behavioral sensitization to ethanol was effective in blocking the expression of the reinstatement of ethanol-induced behavioral sensitization.

The presumed biochemical mechanism of action for ayahuasca brews includes the presence of beta-carboline monoamine oxidase inhibitors (harmala alkaloids) coupled with dimethyltryptamine, a compound that acts on specific serotonin receptors, particularly 5-HT2$_A$ receptors [5]. Evidence of 5-HT receptor agonist activity has been reported in a drug-discriminant animal model study [36]. However 5-HT2 receptor antagonist activity of DMT reported in a previous in vitro study [11] suggests that the purported agonist or antagonist properties of this compound deserve further investigation. Regarding the inhibitory effects of ayahuasca on ethanol-induced hyperlocomotion showed in the present study (Fig. 3b), it has been demonstrated that treatment with ritanserin, a 5HT2$_A$/2$_C$ receptor antagonist, caused a dose-dependent reduction of ethanol-induced auto-administration and locomotor activity [19]. In addition, a recent study from our group demonstrated that pre-treatment with ziprasidone, an antipsychotic drug with high affinity for both dopamine D$_2$ and 5-HT receptors that acts as a potent 5-HT2$_A$ receptor antagonist [35], inhibited not only acute cocaine-induced hyperlocomotion, but also cocaine-induced behavioral sensitization [25].

Within this context, there is extensive experimental evidence demonstrating that in addition to dopaminergic transmission, serotonergic transmission is necessary for the development of ethanol-induced behavioral sensitization. Treatment with the serotonergic antagonist ondansetron blocks the development and expression of ethanol-induced locomotor sensitization [41]. Indeed, simultaneous treatment with a serotonin 5-HT2 receptor antagonist exerts the same effects, preventing the induction and expression of ethanol-induced behavioral sensitization [14]. Additionally, the administration of the 5-HT2$_C$ receptor antagonist SB-242084 directly into the nucleus accumbens blocked the expression of ethanol-induced behavioral sensitization in mice [1]. Taken together, these findings are in line with the high selectivity of ayahuasca in inhibiting both ethanol-induced hyperlocomotion and behavioral sensitization (Fig. 3b and c).

Importantly, despite an altered state of consciousness linked to the use of ayahuasca [31], the ritual use of this substance does not typically produce health or psychosocial problems such as addiction [12,13]. Indeed, a review of the literature on ayahuasca suggests that consumption of traditional preparations in social settings carries a minimal risk of abuse potential or dependence formation [18]. Within this context, our results are among the first to demonstrate that acute (Figs. 3a and 4b) or repeated (Fig. 4b) treatments with ayahuasca do not lead to enhanced locomotor activity in mice, a well-established parameter as an animal model of addiction that shares neuronal mechanisms with craving in humans [33].

Rather, ceremonial ayahuasca drinking has been correlated with lower amounts or severities of substance dependence. Importantly, clinical studies carried with members from Brazilian ayahuasca churches demonstrated that these ayahuasca users show less substance abuse disorders despite prior histories of moderate to severe problems with alcohol or other drugs and higher lifetime illicit drug use [13,20]. However, all these studies involve subjects who are regular and committed members of religious communities, so it remained unclear whether fewer reported substance use problems could be attributed to the ayahuasca drinking rather than being a church member. By ruling out the ceremonial religious aspects of the aforementioned studies, pharmacological studies using rodent models can contribute to elucidate the role of the brew per se into the neurobiological mechanisms of ayahuasca on alcohol-related behavior.

As far as we know, this is the first study showing that a counter-sensitization strategy with ayahuasca inhibits the expression of a pre-established ethanol-induced behavioral sensitization (Fig. 4c). Usually, as showed in the present study (Fig. 4b), ethanol-treated animals do not express a conditioned locomotion in the environment previously associated with this drug (the open-field apparatus, in the present study) in a free-drug session. Instead, ethanol exerts its memory effects through a phenomenon called state-dependency [30], which is reversible by pre-test ethanol administration [34]. Thus, ethanol-induced conditioning remains silent but present, and is expressed in a subsequent ethanol challenge, which has difficult extinction strategies. Indeed, this difficulty was shown by the persistent expression of ethanol-induced behavioral sensitization in the ethanol control group of Experiment 2 even after a 15-day withdrawal period with re-exposure to the open-field apparatus for 8 consecutive days (group Eth–Sal, Fig. 4c).

Therefore, recent efforts to develop effective treatments for addiction have focused on manipulations of learning and memory processes involved in encoding drug-cue associations. Among them, the re-consolidation phenomenon has been extensively used [6]. However, it requires a brief re-exposure to the test environment cues before the pharmacological intervention, while in the strategy proposed in the present study (counter-sensitization) animals are re-exposed to the drug-associated context only and right after the pharmacological therapy intervention. Thus, re-consolidation strategies could be dangerous

Fig. 4. Locomotor activity quantification in the open-field apparatus demonstrating acute hyperlocomotion induced by ethanol (Eth, 1.8 g/kg) (Day 1) and ethanol-induced behavioral sensitization (Day 15) after a 15-day intermittent treatment (8 ethanol injections) (a) and the behavioral effects of i.p. treatment with either ayahuasca (Aya, 100 or 300 mg/kg) or saline on the counter-sensitization phase for 8 consecutive days (Day 19 to Day 26) (b) and on subsequent saline (Day 30) and ethanol (Day 32) challenges. Data are reported as mean ± S.E.M. • $p < 0.05$ compared with itself on the first ethanol treatment day (Day 1) (a); ★ $p < 0.05$ compared with Sal (a) or Sal–Sal (c) on the same experimental day; ♦ $p < 0.05$ compared with Eth–Sal (c); and ■ $p < 0.05$ compared with itself on the saline challenge. Two-way analysis of variance (ANOVA) followed by Tukey's test or paired Student's *t*-test.

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regarding relapse and perhaps not feasible in the clinic. The tactic proposed herein would not present this risk.

In this scenario, the clinical implications of the present findings might be far reaching. Although some programs for addiction recovery claim improved health outcomes for patients who combine ayahuasca during treatment [23,45], neither has been evaluated with sufficient scientific rigor to provide definitive evidence of the success of their approaches [39]. In the present study, we demonstrated that ayahuasca not only inhibits early behaviors associated with initiation and development of drug addiction, but also showed effectiveness in reversing long-term drug effect expression, inhibiting the reinstatement of ethanol-induced behavioral sensitization when administered in the ethanol-associated environment without exerting addictive potential.

5. Conclusions

Ayahuasca inhibited the initiation and development of ethanol-induced behavioral sensitization, also showing effectiveness in preventing its reinstatement when administered in the ethanol-associated environment without exerting addictive potential.

Conflict of interest

The authors disclose any actual or potential conflict of interest including any financial, personal or other relationships with other people or organizations within three years of beginning the present work that could inappropriately influence, or be perceived to influence it.

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