



Research report

Disulfiram impairs the development of behavioural sensitization to the stimulant effect of ethanol

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ARTICLE INFO

Article history:

Received 28 May 2009

Received in revised form 22 October 2009

Accepted 24 October 2009

Available online 3 November 2009

Keywords:

Ethanol

Disulfiram

Behavioural sensitization

Locomotor activity

Mice

ABSTRACT

Background: Although disulfiram has been used in the treatment of alcoholism due to the unpleasant sensations its concomitant ingestion with ethanol provokes, some patients reported stimulant effects after its ingestion. This issue has not been addressed in studies with animals. In mice, the stimulant effect of ethanol has been associated with increased locomotor activity and behavioural sensitization. This study sought to analyze the influence of disulfiram on the development of behavioural sensitization to the stimulant effect of ethanol.

Methods: Male Swiss mice pre-treated with vehicle or disulfiram (15 mg/kg) received saline or ethanol (2.0 g/kg) every other day, for 5 days. Forty-eight hours afterwards mice were challenged with Saline, and 48 h later they received Disulfiram, or Disulfiram + Ethanol or Ethanol.

Results: The co-administration of disulfiram (15 mg/kg) blocked the development of behavioural sensitization induced by ethanol (2.0 g/kg). Although the acute administration of disulfiram did not alter the locomotor activity, its acute administration-induced higher levels of locomotor activity in mice previously sensitized to ethanol than in controls which received saline.

Conclusions: Our data suggest that besides the known psychological effects (fear of aversive effects) disulfiram efficacy on alcohol dependency treatment could also be due to its pharmacological interference in the brain neurotransmission.

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1. Introduction

Disulfiram is a potent inhibitor of the mitochondrial aldehyde dehydrogenase (ALDH), the main enzyme involved in the hepatic metabolism of ethanol. Disulfiram has been traditionally used in the treatment of alcoholism. If a patient ingests alcohol and disulfiram together, a severe discomfort can be experienced, including facial flushing, alteration in blood pressure, palpitations, sympathetic overactivity, headache, dyspnea, nausea and vomiting [15,16]. Patients are advised that drinking while on disulfiram can cause severe illness and eventually death [30,31]. These effects are supposed to discourage the patient from drinking while taking the medicine and have been attributed to the increase in the levels of acetaldehyde, the main ethanol metabolite, after the ingestion of alcoholic beverages [4,13,16,19].

Recently, disulfiram administration has been evaluated in the treatment of cocaine dependence. Carroll et al. [3] conducted a randomized, placebo-controlled, double-blind study examining the efficacy of disulfiram and cognitive behavioural therapy among cocaine-dependent patients, providing strong evidence for the

effectiveness of disulfiram in cocaine treatment. However, since disulfiram does not cause classical aversive reactions if administered with cocaine, as seen when disulfiram is co-administered with alcoholic beverages, the mechanism underlying disulfiram effectiveness on cocaine dependence is unclear.

There are some reports according to which disulfiram increases the levels of dopamine in rodents [1,18] and in humans [35]. Disulfiram also reduces norepinephrine synthesis and increases dopamine levels due to its inhibitory effect of dopamine beta-hydroxylase activity, an enzyme which catalyzes the conversion of dopamine to norepinephrine in peripheral and central noradrenergic-containing neurons [44]. The effectiveness of disulfiram on cocaine dependence could be related to its action on the catecholaminergic system [7,18,44]. Theoretically, it would be possible that in increasing dopamine levels, disulfiram could act as a “substitution treatment” for both cocaine and alcohol. Besides, if repeatedly administered, disulfiram could affect catecholaminergic transmission and neuroadaptive changes in dopaminergic system usually observed after chronic consumption of alcohol or cocaine.

Cocaine and low doses of alcohol shared similar behavioural stimulant characteristics. The rewarding and powerful addictive effects of stimulants have been attributed to their capacity to increase dopamine levels in the nucleus accumbens, an important component of mesocorticolimbic dopaminergic pathway [20,42].

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Table 1
Schedule of the experimental protocol. Pre-treatment drug administration (i.p.) was given 4 h before treatment.

Group	Treatment phase		Challenge phase			
	Days 1, 3, 5, 7 and 9		Day 11 (saline challenge)		Day 13 (drug challenge)	
	Pre-treatment	Treatment	Pre-treatment	Treatment	Pre-treatment	Treatment
V+S	Vehicle	Saline	Vehicle	Saline	Vehicle Disulfiram Disulfiram	Ethanol Saline Ethanol
D+S	Disulfiram	Saline	Vehicle	Saline	Vehicle Disulfiram Disulfiram	Ethanol Saline Ethanol
V+E	Vehicle	Ethanol	Vehicle	Saline	Vehicle Disulfiram Disulfiram	Ethanol Saline Ethanol
D+E	Disulfiram	Ethanol	Vehicle	Saline	Vehicle Disulfiram Disulfiram	Ethanol Saline Ethanol

Both alcohol and cocaine, when repeatedly administered can induce behavioural sensitization to the stimulant effect on locomotion. Behavioural sensitization, also known as “reverse tolerance” [37], is a phenomenon characterized by a progressive increase in the locomotor response to a same dose of the drug [28] after repeated administration. Mesocorticolimbic dopaminergic projections seem to mediate the behavioural sensitization phenomenon. Although some authors dissociate the neural mechanisms underlying sensitization from those underlying addiction [8,34], many others suggest that the sensitizing effects of several drugs of abuse could be related to their reinforcing/positive effects [5,9,17,33]. Although behavioural sensitization to ethanol has not been studied as an animal model of addiction, there is some evidence (in human and animal studies) showing that this phenomenon may have clinical relevance in the understanding of alcoholism development and in the knowledge of the underlying neurobiological processes involved [11,14,26].

Hypersensitive and hyper responsive mesolimbic dopaminergic pathways are typically associated with behavioural sensitization, leading to sensitized increases of dopamine concentrations in the nucleus accumbens after a challenge with psychostimulants [28]. Besides the dopaminergic system, other neurotransmitter systems are involved in behavioural sensitization, among them the glutamatergic [2,21,29], GABAergic [28] and cholinergic systems [25].

There are few controlled studies in rodents examining the effects of disulfiram associated with ethanol [24] and cocaine [22]. Haile et al. [12] observed that disulfiram facilitated the development of locomotor sensitization to cocaine, although disulfiram alone had minimal effect on activity levels. Furthermore, the expression of sensitization was higher in rats previously administered with disulfiram [12]. However, we found no studies on the influence of disulfiram on sensitization to ethanol.

If alterations of the dopaminergic system induced by disulfiram could explain its efficacy on cocaine dependence treatment, it would be possible that a similar mechanism could provide an additional explanation about its efficacy on alcoholism treatment. Based on this hypothesis, the first goal of the present study was to test the effects of low doses of ethanol and disulfiram on the locomotor activity of mice. The second goal was to evaluate the effects of disulfiram on the behavioural sensitization to the stimulant effect of ethanol.

2. Materials and methods

2.1. Animals

Male albino Swiss mice 3 months old, weighing 30–45 g (colony of the Departamento de Psicobiologia - Universidade Federal de Sao Paulo), housed under standard

laboratory conditions of 12 h light/dark cycle (lights on from 7:00 to 19:00), 22–26 °C and 40–70% humidity, were used in all experiments. They were provided food and water *ad libitum*. All studies were conducted in accordance with the strictest ethical principles of the National Institute of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 80-23) revised 1996. All efforts were made to minimize animals' suffering and to keep the number of animals used to a minimum. This project was approved by the Committee of Ethics in Research of Universidade Federal de Sao Paulo (CEP 0165/05 and 0078/06).

2.2. Drugs

Ethanol (Synth[®], Diadema, Brazil) solution was prepared with saline (15%, w/v in 0.9% NaCl). Tetraethylthiuram disulfide (disulfiram; Sigma–Aldrich[®], Saint Louis, MO) solution was prepared with saline (2%, w/v in 0.9% NaCl) dissolved in Tween 80 (Synth[®], Diadema, Brazil). The vehicle solution given as control of disulfiram was saline with Tween 80.

2.3. Apparatus

All locomotor activity tests were carried out in Opto-Varimex activity cages [model Opto-M3, with acrylic boxes (47.5–25.7–20.5 cm); Columbus Instruments, Columbus, OH] which detect locomotion by the interruption of photoelectrical beams.

2.4. Experimental procedures

2.4.1. Dose–effect curve with four doses of ethanol

A dose–effect curve was constructed with four doses of ethanol (EtOH: 1.8, 2.0, 2.2 and 2.4 g/kg) in order for us to determine their acute effects on locomotor activity. Sixty-four Swiss male albino mice (naïve) were habituated to the activity chamber for 30 min. Twenty-four hours later, they received ethanol or saline i.p. and their locomotor activity was measured for 30 min.

2.4.2. Dose–effect curve with four doses of disulfiram

Another dose–effect curve was constructed with four doses of disulfiram (Dis: 15, 25, 50 and 100 mg/kg) in order for us to detect the acute effects on locomotor activity. Sixty-four Swiss male albino mice (naïve) were habituated to the activity chamber for 30 min and 24 h later received disulfiram or vehicle (i.p.). After 4 h they received saline and their locomotor activity was measured for 30 min.

2.4.3. Treatment and challenge with ethanol, disulfiram or disulfiram + ethanol

In order to evaluate the effect of disulfiram on the development and on the expression of sensitization to ethanol, we utilized protocols (for treatment and challenge test) based on previous studies [23,27,38]. The effects *per se* of disulfiram on locomotor activity and its influence on the development of sensitization to the stimulant effect of ethanol were evaluated in the “treatment” phase of the protocol (10-day treatment). In the second phase of the protocol (challenges) the same drugs above mentioned were administered in order for us to evaluate the influence of disulfiram on the expression of sensitization and of contextual conditioning.

2.4.3.1. Procedures. Table 1 summarizes the procedures. Initially, all mice were placed in activity cages for us to measure their baseline activity for 15 min, in drug-free situation. This procedure was repeated three times within a 24-h interval. Forty-eight hours after the third baseline test, the mice were allocated to one out of four experimental groups (equated on basis of their locomotor activity in baseline sessions). The mice received disulfiram (15 mg/kg) or vehicle i.p. 4 h before ethanol (2.0 g/kg) or saline administration, composing four groups of

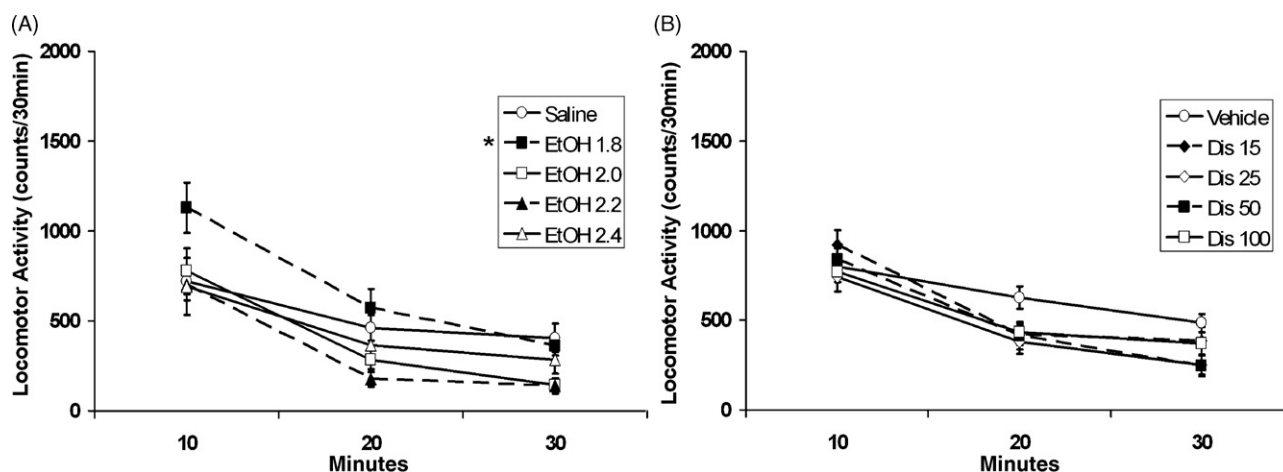


Fig. 1. Locomotor activity (mean \pm S.E.M., counts in 30 min) after different doses of ethanol (EtOH; part A) or disulfiram (Dis; part B) administration. (A) Mice were tested for 30 min immediately after ethanol 1.8, 2.0, 2.2 or 2.4 g/kg i.p. (EtOH 1.8, $n=13$; EtOH 2.0, $n=13$; EtOH 2.2, $n=13$ or EtOH 2.4, $n=13$) or saline ($n=12$) administration. *Higher activity levels than the EtOH 2.0 ($p<0.05$), EtOH 2.2 ($p<0.05$) and the EtOH 2.4 ($p<0.05$) groups. (B) Mice were tested for 30 min immediately after saline administration, 4 h after pre-treatment with disulfiram 15, 25, 50 or 100 mg/kg i.p. (Dis 15, $n=13$; Dis 25, $n=13$; Dis 50, $n=13$ or Dis 100, $n=13$) or vehicle ($n=12$) administration.

treatment: vehicle + saline (V+S; $n=57$), disulfiram 15 mg/kg + saline (D+S; $n=62$), vehicle + ethanol 2.0 g/kg (V+E; $n=59$) and disulfiram 15 mg/kg + ethanol 2.0 g/kg (D+E; $n=61$). The animals were tested in the locomotor activity cages for 15 min, immediately after ethanol (or saline) administration. This procedure was repeated every other day, during a 10-day period (five tests). Forty-eight hours after the end of this treatment, the challenge phase started.

In the challenge phase, each of the four experimental groups of mice (V+S, D+S, V+E and D+E) was assigned to one out of three different subgroups, similar regarding their locomotor activity in the 5th locomotor activity test. The subgroups were submitted to two challenges. In the first, called "Saline challenge", all animals received vehicle (administered 4 h before) and saline. Immediately after saline administration the mice were tested in the activity cages for 15 min. Forty hours later, in the "drug challenge", mice received disulfiram (15 mg/kg) or vehicle 4 h before ethanol (2.0 g/kg) or saline administration, composing three subgroups: disulfiram 15 mg/kg + saline (Disulfiram; $n=20$); vehicle + ethanol 2.0 g/kg (Ethanol; $n=19$) and disulfiram 15 mg/kg + ethanol 2.0 g/kg (Disulfiram + Ethanol; $n=20$). The animals were tested in the locomotor activity cages for 15 min immediately after ethanol (or saline) administration.

2.5. Statistical analysis

The analysis comparing the groups regarding locomotor activity was carried out by the Statistica software (Statsoft Inc., 2004). All variables are expressed as mean and S.E.M. unless otherwise stated. After we tested distributions for normality and variance for homogeneity, the mean locomotor activity was compared by ANOVA for repeated measures followed by Newman–Keuls test. ANCOVA was used in the drug challenge data analysis, with saline challenge data as a covariate. The significance level was set at 5%.

3. Results

3.1. Dose–effect curve with four doses of ethanol

A two-way ANOVA revealed a significant effect of doses of ethanol [$F(4, 59)=4.02$; $p<0.05$] and time [$F(2, 118)=80.35$; $p<0.01$] but no doses \times time interaction [$F(8, 118)=1.79$; $p=0.08$]. A post hoc Newman–Keuls test detected that the EtOH 1.8 group presented higher locomotor activity than the EtOH 2.0 ($p<0.05$), EtOH 2.2 ($p<0.05$) and the EtOH 2.4 ($p<0.05$) groups (Fig. 1A). The main significant effect of time is explained by a progressive decline in the locomotor activity of all groups over the test session ($p<0.05$).

3.2. Dose–effect curve with four doses of disulfiram

A two-way ANOVA showed no significant effect of disulfiram at any doses [$F(4, 59)=1.62$; $p>0.05$], but a significant time [$F(2, 118)=148.33$; $p<0.01$] and dose \times time interaction [$F(8, 118)=2.43$; $p<0.05$] effects. According to the Newman–Keuls test,

all groups presented lower levels of locomotor activity at the 30 min than at the 10 min measure (Fig. 1B).

3.3. Repeated administration of ethanol, disulfiram or disulfiram + ethanol

Fig. 2 shows the locomotor activity of mice in five tests performed during the 10-day treatment with disulfiram 15 mg/kg or saline associated with ethanol 2.0 g/kg or saline. A $2 \times 2 \times 5$ ANOVA detected significant effects of pre-treatment [$F(1, 235)=9.59$; $p<0.01$]; treatment [$F(1, 235)=51.17$; $p<0.01$]; time [$F(4, 940)=5.37$; $p<0.01$]; and of the interactions pre-treatment \times treatment [$F(1, 235)=25.54$; $p<0.01$], pre-treatment \times time [$F(4, 940)=8.48$; $p<0.01$] treatment \times time [$F(4, 940)=31.66$; $p<0.01$] and pre-treatment \times treatment \times time [$F(4, 940)=4.17$; $p<0.01$]. The Newman–Keuls test detected signifi-

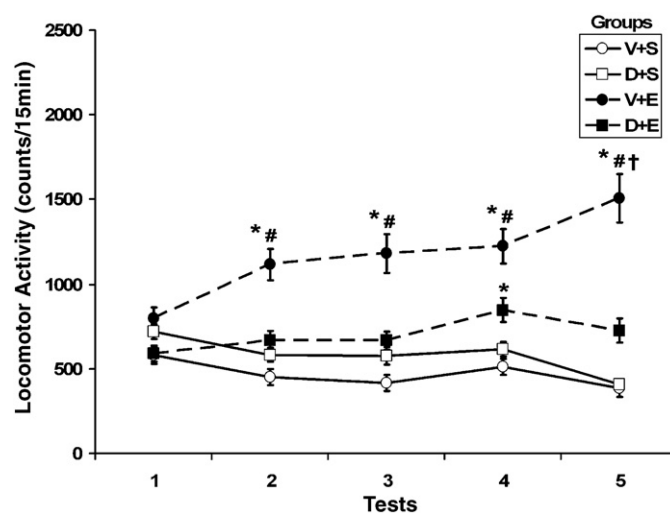


Fig. 2. Locomotor activity (mean \pm S.E.M., counts in 15 min) in the 5 tests performed every other day during the 10-day period of treatment, immediately after ethanol (2.0 g/kg) or saline administration. Mice were pre-treated i.p. with disulfiram (15 mg/kg) or vehicle 4 h before the test. Groups: vehicle + saline (V+S, $n=57$), disulfiram 15 mg/kg + saline (D+S, $n=62$), vehicle + ethanol 2.0 g/kg (V+E, $n=59$) and disulfiram 15 mg/kg + ethanol 2.0 g/kg (D+E, $n=61$). *Higher activity levels than in test 1 ($p<0.01$). #Higher levels than S+S group in tests 2, 3, 4 and 5 ($p<0.05$). †Higher levels than D+E group in test 5 ($p<0.01$).

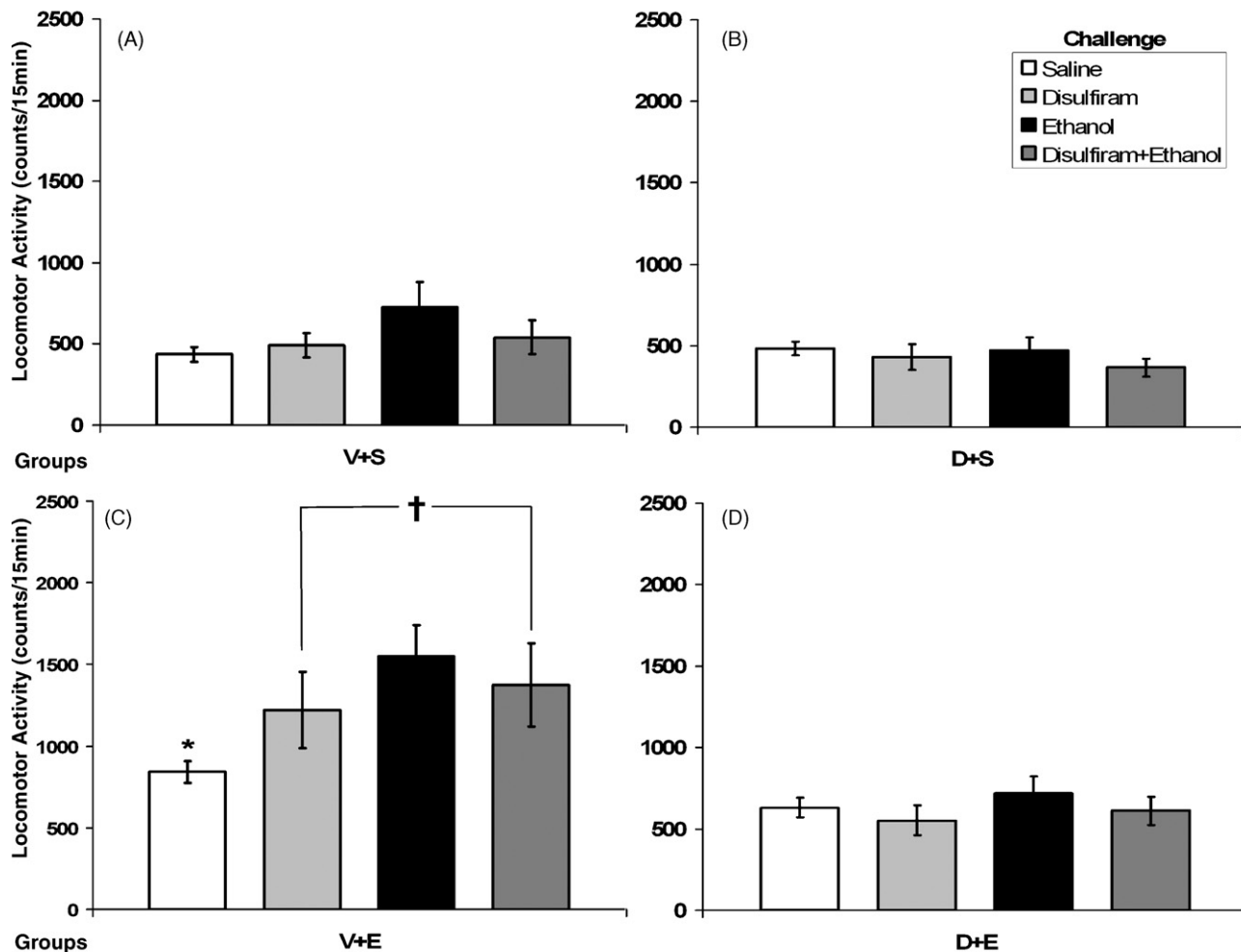


Fig. 3. Locomotor activity (mean \pm S.E.M., counts in 15 min) in challenge tests of mice previously treated with (A) vehicle + saline (V+S); (C) vehicle + 2.0 g/kg of ethanol (V+E); (B) 15 mg/kg of disulfiram + saline (D+S) or (D) 15 mg/kg of disulfiram + 2.0 g/kg of ethanol (D+E). All animals were tested under vehicle + saline ("saline challenge") with 4 h of interval between vehicle and saline administration. "Drug challenge" was assigned 48 h after "saline challenge". Different animals were used in each "drug challenge": Disulfiram 15 mg/kg + saline (Disulfiram; $n = 20$); vehicle + ethanol 2.0 g/kg (Ethanol; $n = 19$) and disulfiram 15 mg/kg + ethanol 2.0 g/kg (Disulfiram + Ethanol; $n = 20$). The animals were tested in the locomotor activity cages for 15 min immediately after ethanol (or saline) administration. *Higher activity levels than those from all other treatment groups in the saline challenge ($p < 0.05$). †Higher locomotor activity levels than all the other groups, in all drug challenges ($p < 0.01$).

cantly higher locomotor activity levels in the V+E-treated group than in the V+S-treated group, in tests 2, 3, 4 and 5 ($p < 0.01$). In the 5th test, the V+E-treated group presented higher activity than the D+E-treated group ($p < 0.01$). The Newman-Keuls post hoc test also revealed that in the 5th test the V+E-treated group presented higher levels of locomotor activity than in previous tests ($p < 0.01$). Similarly, in the 4th test, the D+E-treated group presented higher levels of activity ($p < 0.05$) than in the previous tests.

A one-way ANOVA yielded significant effect of experimental groups during saline challenge [$F(3, 235) = 11.18$; $p < 0.01$]. The Newman-Keuls test detected higher locomotor activity levels in the V+E-treated group than in all others ($p < 0.01$). In Fig. 3 we can observe the locomotor activity of the previously treated group (V+S, V+E, D+S, D+E) in the four challenges (Saline, Disulfiram, Ethanol, Disulfiram + Ethanol).

An ANCOVA ($4 \times 3 \times 1$), considering the factors treatment (four levels: V+S, V+E, D+S and D+E) and drug challenge (three levels: Disulfiram, Ethanol and Disulfiram + Ethanol), with saline challenge as covariate, detected a significant effect of treatment [$F(3, 226) = 17.52$; $p < 0.01$]. The V+E group presented significantly higher locomotor activity levels than all the other groups, independently of the drug challenge ($p < 0.01$).

4. Discussion

The main and original finding of the present study is that concurrent administration of disulfiram prevents the development of sensitization to the stimulant effect of ethanol. The theoretical basis for the use of disulfiram usually emphasizes its "aversive effect", i.e. the patient who ingests disulfiram does not intake alcohol due to fear of the adverse reactions (as flushing, nausea, etc.) induced by its combined use with alcohol. Although some authors consider this is as a "learned fear" or "psychological effect" [40], our data suggest that a neuropharmacological explanation to this phenomenon cannot be discharged.

In the present study, mice previously treated with disulfiram + ethanol when challenged with ethanol alone did not present sensitization (increased locomotion after repeated administration), while those treated with the same dose of ethanol without disulfiram when challenged with ethanol (with or without disulfiram) presented a clear sensitized locomotor response. These data indicate that the co-administration of disulfiram and ethanol impaired the development but not the expression of behavioural sensitization to the stimulant effect of ethanol. Some authors consider that the experience of the stimulant effect is essential to the development

of sensitization [33,45]. We could hypothesize that, in the present study, an aversive effect, induced by the simultaneous administration of disulfiram, could have masked the stimulant effect of ethanol, impairing a possible reinforcement sensation that could be essential to the development of sensitization. On the other hand, the mice treated with ethanol alone experienced the stimulant/reinforcing effect of ethanol during the treatment, which might have allowed them to develop sensitization and express it in the challenge.

We could also consider the possibility that ethanol-treated mice developed tolerance to the peripheral aversive effects, induced by low levels of acetaldehyde, produced during treatment as a consequence of ethanol metabolism, being “tolerant” to its aversive effects in the challenge test with disulfiram + ethanol. This hypothesis is supported by Tampier et al. [41], who reported no effect of disulfiram or cyanamide in inhibiting ethanol intake on alcohol-preferring rats (UChB rats) that had consumed ethanol for 30 days. They suggested that the animals had developed tolerance to the aversive effects of acetaldehyde.

Another possible hypothesis to explain the impairment of behavioural sensitization development is the distinct effect of acetaldehyde formed “peripherally” or “centrally” [10,32]. According to some theories, a stimulant effect of acetaldehyde could be observed when it passes the blood brain barrier, or when it is formed in the CNS. In rats, Correa et al. [6] observed that intraventricular (i.c.v.) infusion of acetaldehyde showed an inverted U-shaped dose–response curve, with moderate doses increasing motor activity. They showed that central administration of low doses of ethanol can increase locomotor activity, and suggested that acetaldehyde may be an active metabolite of ethanol that can also facilitate locomotor activity. On the other hand, in mice, Tambour et al. [39] observed an increase in locomotion after low doses of ethanol (1.0 or 2.0 g/kg i.p., or 6.87 μ mol i.c.v.). They also observed a reduction in locomotor activity, after high doses of acetaldehyde (i.p. or i.c.v. administration). Although in our study we could not distinguish whether a putative “central” or “peripheral” (or both) acetaldehyde accumulation contributed to the impairment of sensitization development, we suppose that those effects were related to the “peripheral acetaldehyde” accumulation.

In this study we observed that none of doses of disulfiram administered acutely affected the locomotor activity of mice. The selection of the dose (15 mg/kg) was based on studies reported in the literature [43] and on a pilot study in which it had no toxic effects nor affected the locomotion per se. Based on the results of the dose–effect curve, we have chosen 2.0 g/kg of ethanol for repeated ethanol administration because it was the lowest dose tested that acutely did not induce a stimulant effect, and the purpose of this experiment was to observe disulfiram effects on behavioural sensitization.

The fact that mice pre-treated with ethanol presented stimulant effect on locomotor activity, even when tested under disulfiram alone, suggests the interaction of effects between ethanol and disulfiram. This phenomenon could not be justified as a conditioned response, since mice treated with ethanol presented lower locomotor activity levels in the *saline challenge* than in the other challenges. We could hypothesize that since disulfiram also inhibits dopamine beta-hydroxylase, it could interfere in the regular balance monoamines metabolism. As previously described, the inhibition of dopamine beta-hydroxylase reduces the norepinephrine synthesis and increases dopamine levels, in rodents [1,18] and in humans [35]. Haile et al. [12] showed that the development of cocaine locomotor sensitization was facilitated by disulfiram and the expression of sensitization was greater in rats previously treated with disulfiram. Although this effect is usually observed at doses higher than those used in the present study [1], the fact that disulfiram induced a stimulant response *only* in mice

already sensitized to ethanol suggests the occurrence of previous neuroadaptations due to the repetitive administration of ethanol.

A study from our laboratory showed increased D2-receptor binding in caudate–putamen anterior and ventrolateral in mice sensitized to ethanol [36]. A possible explanation for the interaction of effects between ethanol and disulfiram could be the increased levels of dopamine in sensitized mesolimbic and mesocortical dopaminergic pathways or hypersensitized D2 receptors. This could have facilitated the expression of the stimulant effect of an originally non-stimulant dose of disulfiram. However, other studies are needed to explore the exact mechanisms underlying cross-sensitization between ethanol and disulfiram.

In summary, we showed in this paper that repeated administration of disulfiram and ethanol impaired the development of behavioural sensitization to the stimulant effects of ethanol, but did not block these effects in animals previously sensitized to ethanol. If a similar process occurs in humans, this information should be taken into account by those who prescribe disulfiram to alcohol dependent patients. It would also be possible to consider that disulfiram efficacy could be attributable to a “substitute therapy”, replacing the ethanol stimulant effect. However, this hypothesis still has to be pre-clinically tested.

Acknowledgements

This work was supported by FAPESP (fellowship grant 04/12448-7 and 05/57295-6), AFIP and CNPq (Brazil). The authors thank Drs. Ana Regina Noto, Débora C. Hipólido and Deborah Suchecki for their suggestions to the manuscript and for their helpful comments.

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