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# Evaluation of anxiety-like behaviors following ethanol withdrawal in mice: effects of cannabidiol

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**ABSTRACT.** The abrupt interruption of ethanol consumption increases anxiety-like behaviors in rodents and may reflect different aspects of ethanol dependence in humans. Measuring emotional behaviors resulting from ethanol withdrawal may aid in testing potential pharmacological agents for the treatment of ethanol dependence. In the present study, we used forced expositon to ethanol 20% during 10 days to mice, followed by abrupt withdrawal of the substance. The animals were evaluated 7, 24 and 35 h after ethanol withdrawal in three different behavioral paradigms, i.e., the open field (OF), light dark (LD) transition and elevated plus maze (EPM), tests usually used to measure anxiety-like behaviors. This was done with the aim of identifying the best interval as well as the most appropriate behavioral test to detect the effects of drugs that can relieve anxiety induced by ethanol withdrawal in mice. We also evaluated the effect of cannabidiol (CBD 10, 30 and 60 mg $kg^{-1}$) in ethanol withdrawn mice because it has been shown to alliviate drug addicton and present anti-anxiety effects. Our results show significant behavioral changes at 24 h following ethanol withdrawal. Diazepam (4 mg $kg^{-1}$), used as a positive control, counteracted the effects of ethanol withdrawal in OF, LD box and EPM. Cannabidiol attenuated anxiety-like behavior produced at 24 h after abstinence from ethanol exposure only in the EPM test.

**Keywords:** ethanol withdrawal; anxiety; cannabidiol; mice.

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## Introduction

Withdrawal symptoms in response to cessation of ethanol repeated exposure has been considered as the core feature of ethanol dependence. Negative affect produced by ethanol withdrawal, such as depressed mood and elevated state of anxiety, represent important motivating factors for excessive ethanol consumption and account to the relapse of ethanol use and abuse (Koob & Volkow, 2010). As in humans, rodents exhibit depressive- and anxiety-like behaviors following ethanol withdrawal, indicating dysphoric emotions related to ethanol abstinence (Perez & De Biasi, 2015; Gong et al., 2017; Sidhu, Kreifeldt, & Contet, 2018). Measuring emotional behaviors resulting from ethanol withdrawal may aid in testing potential pharmacological agents for the treatment of ethanol dependence.

Although consistently demonstrated in rats, behavioral changes following ethanol withdrawal has been difficult to obtain and interpret in mice (Kliethermes, 2005; Sidhu et al., 2018). This occurs mainly because measurements of anxiety- and depressive-like behaviors differ among mice strains and the endpoints of the behavioural analysis (McCool & Chappel, 2015; Sidhu et al., 2018). For example, after 4 to 6 weeks of ethanol withdrawal from chronic intermitent ethanol (CIE) exposure, there was a reduction in social interaction in DBA/2J (DBA) but not C57BL/6J (C57) mice. In contrast, withdrawal from CIE, increased hyponeofagia in C57 but not in DBA mice. Both DBA and C57 mice showed increase digging and irritability-like behaviors, which are indicative of affective dysfunction associated with ethanol withdrawal (Sidhu et al., 2018). Otherwise, after 4 days of CIE, ethanol-withdrawn DBA but not C57 mice, exhibited increase anxiety-like behavior in the light dark (LD) transition test compared to their ethanol-naïve controls (McCool & Chappell, 2015). NMRI mice exhibited anxiety-like behaviors in the LD test following 8 h of ethanol abstinence following 8 days of 4% of ethanol liquid diet (Verleye, Heulard, & Gillardin, 2009). One study has failed for detecting anxiety in C57 or DBA mouse after a 72 h exposure to ethanol vapor (Finn, Gallaher, & Crabbe, 2000). Therefore, the use of different mouse strains, different behavioral tests and times of ethanol exposure and withdrawal may influence the outcome and interpretation of the results of ethanol withdrawal.

Information regarding the time course of behavioral changes in mice following ethanol withdrawal is scarce. In this study, we aimed to identify the most appropriate behavioral test and time interval to detect the effects of ethanol withdrawal in Swiss mice. The animlas were evaluated in the open field (OF), LD and elevated plus maze (EPM) behavioral tests to measure anxiety-related behaviors induced by ethanol withdrawal. Diazepam (DZ) was used as a positive control, since benzodiazepines have been considered a mainstay of therapy for the treatment of ethanol withdrawal syndrome. Because cannabidol (CBD) has been shown to alliviate drug addiciton and present anti-anxiety effects, we also evaluated the effects of CBD in ethanol withdrawn mice.

## Material and methods

### Animals

Adult male Swiss mice (25-30 gr) were used. The animals were obtained from the central *vivarium* of the State University of Maringá and were allowed to habituate in a local room with standard housing conditions, i.e., 12 h light cycle (lights on 7 a.m.), temperature controlled (22 ± 1°C), and supply of water and food *ad libitum*. The experimental procedures followed the "Basic Principles for Animal Use" and were approved by the Ethics Committee on Animal Experimentation of the State University of Maringá (CEUA # 4053230316). Every effort has been made to minimize the suffering of animals.

### Drugs

All drugs were administered *i.p.* in a volume of 10 mL $kg^{-1}$. Ethanol (Labsynth, Diadema, SP, Brazil) was diluted to 20% (w/v) in sterile saline solution 0.9%. Diazepam (DZ, Laboratório Teuto S.A, São Paulo, SP, Brazil) was diluted in sterile saline 0.9%. Cannabidiol (CBD, THC Pharma, Frankfurt, Germany) was diluted in 1% Tween 80 in sterile saline 0.9%. The doses were chosen based in revious studies showing anxiolytic-like effects for the compounds as follows: Ethanol 2 g $kg^{-1}$ (20% w/v) and DZ 1.0 and 4.0 mg $kg^{-1}$ (Botia, Legastelois, Houchi, & Naassila, 2015); CBD 10, 30 and 60 mg $kg^{-1}$ (Schiavon et al., 2016).

### Experimental design

The mice received vehicle (veh) or 2 g $kg^{-1}$ of 20% (w/v) ethanol *i.p.*, once a day (7:00 a.m.) during 10 days. Seven, 24 or 35 h after the last dose of ethanol, mice were tested for withdrawal behavioral symptoms in the OF, LD and EPM, with a 30 min interval between the tests (Figure 1A).

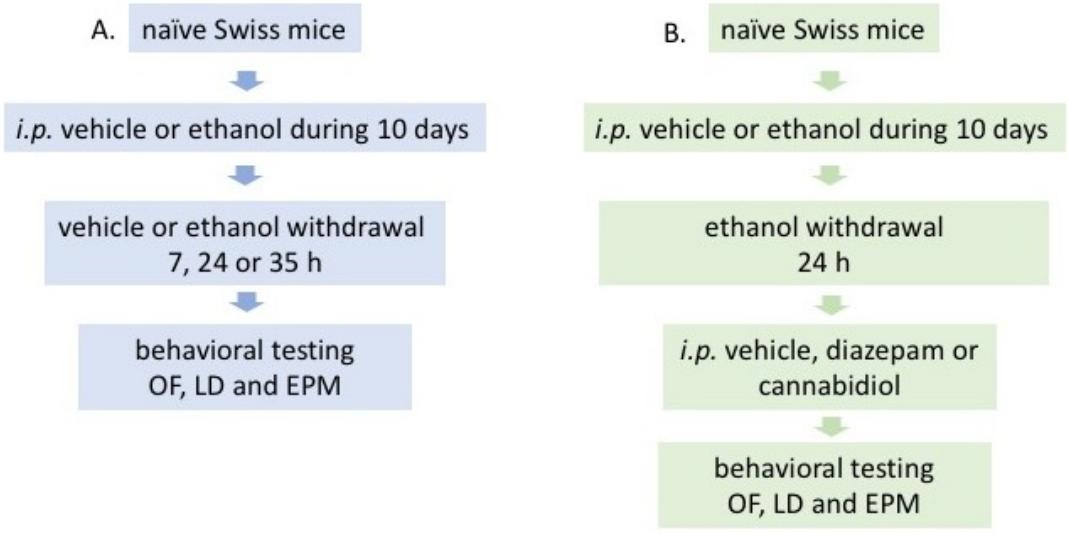


**Figure 1.** Experimental design.

After defining the best time interval to detect behavioral changes induced by ethanol withdrawal, other groups of mice were intoxicated with ethanol during 10 days. Therefore, 24 h after the ethanol withdrawal and 30 minutes before the first behavioral test, vehicle, DZ (1 and 4 mg $kg^{-1}$) or CBD (10, 30 and 60 mg $kg^{-1}$) were *i.p*. administered to the animals (Figure 1B).

Before the behavioral testing the animals were transported to a dimly iluminated (40 lux), sound attenuated and temperature controlled (23 ± 1°C) room and remained undisturbed at least 1 h prior to testing. All testing sessions were conducted during the daytime period (8:00 a.m. to 11 a.m.) and recorded for further analysis using ANY-maze software version 1.9 (Stoelting, Wood Dale, USA). The behavioral tests were performed from the least aversive test to the most aversive, i.e., OF, LD and EPM (Perez & De Biasi, 2015) with 30 min interval among them. Between one animal and another, the equipments were cleaned with 70% ethanol solution.

Mice received vehicle (veh) or 2 mg $kg^{-1}$ of 20% (w/v) ethanol *i.p.*, once a day (7:00 a.m.) during 10 days. Seven, 24 or 35 h after the last dose of ethanol, mice were tested for withdrawal behavioral symptoms in the open field (OF), light dark transition (LD) and elevated plus maze (EPM) (Figure 1A). Twenty four h after the ethanol withdrawal and 30 minutes before the first behavioral test, vehicle, DZ (1 and 4 mg $kg^{-1}$) or CBD (10, 30 and 60 mg $kg^{-1}$) were *i.p*. administered to the animals (Figure 1B). All testing sessions were conducted during the daytime period (between 7:00 and 12:00 a.m.) and recorded for further analysis using ANY-maze software version 1.9 (Stoelting, Wood Dale, USA). The behavioral tests were performed from the least aversive test to the most aversive, i.e., OF, LD and EPM (Perez & De Biasi, 2015) with 30 min interval among them.

## Blood ethanol concentrations

Blood ethanol concentrations were determined using separate groups of animals in order to verify if the *i.p.* ethanol administration would result in reliable BEC and to confirm ethanol clearance during the abstinence periods. Blood was collected at 1, 7, 24 and 35 hours after ethanol *i.p.* injections. Blood samples were collected from the inferior vena cava of animals anesthetized with sodium thiopental using heparinized syringes. The BEC measurements were performed on a Varian CP3380 gas chromatograph equipped with a flame ionization detector (Varian 1177, Palo Alto, CA, USA) and a carbowax fused silica capillary column (30 m, 0.25 mm id, thickness, 0.25 mm; Chrompack, Middelburg, The Netherlands). Sample injections were performed using an automated sampler (CombiPAL, CTC Analytics, Zwingen, Switzerland). Thus, 1 mL of the blood sample containing sodium fluoride as a preservative was added in a headspace compartment with 1 mL of distilled water, 1 g NaCl and isobutane (100 mg $dL^{-1}$) as the internal standard. The compartment was closed with silicone polytetrafluoroethylene and a steel cover. The samples were incubated at 80°C for 10 min before being injected into the capillary system of the gaseous chromatograph. The results were presented as the mean ± standard error of the mean (S.E.M) of the EBC in mg%.

## Behavioral tests

### Open field

The apparatus consisted of a wood squared arena and measured 40 x 40 x 40 cm. For analysis purposes, the OF was divided into two areas, the center (20 x 20 cm) and the outer peripheral area. At the beggining, the mice were placed in the center of the OF and left to explore the apparatus during 5 min. The distance traveled was used as a measurement of locomotor activity and the number of entries and time spent in the central area as a measurement of anxiety-related behavior.

### Light dark box

The apparatus used was a wooden box (40 x 20 x 20 cm) divided into two equal squares (20 x 20 cm) separated by a barrier that has an opening (8 x 12 cm), where the animals could transit between the two sides of the box. The dark compartment was painted black and the light compartment was white and brightly iluminated. The animals were placed in the middle of the light compartment, facing the dark side. The latency (sec) to enter the dark compartment with all four legs, and the percentage of time spent on the light side were used as measures of anxiety-like behaviors. The number of crossings was evaluated as a measure of general locomotion.

### Elevated plus maze

The EPM consisted of a Plexiglas with four arms apparatus with two closed arms in opposition to two open arms, which cross perpendicularly above ground level (30 cm), with a central platform (6 x 6 cm). Each arm measured 30 x 6 cm. The height of the closed arm walls was 15 cm. The open arms were surrounded by a

raised edge of 0.5 x 0.5 cm to prevent mice from falling off the arms. In the begning of the test, the mice were placed in the central platform facing a closed arm. The % of open arms entries and % of time spent in the open arms were used as a measure of anxiety-like behavior. The number of closed arm entries was used as a measure of locomotion.

### Statistical analysis

Data are expressed as mean ± standard error of the mean (SEM) of the experimental groups. Data were analysed by two-tailed Student *t*-test or oneway ANOVA when apropropriate. The Tukey *post-hoc* test was used for specific comparisons. Values of $p < 0.05$ were assumed as statistically significant.

## Results

### Blood ethanol concentrations

The BECs of the animals were 185 ± 104.1 mg% after 1 h of ethanol exposure, indicating ethanol intoxication. Very low amount of ethanol was detected 7, 24 and 35 h following ethanol withdrawal, where the BECs were 0.060 ± 2.5 mg%, 0.062 ± 1.8 mg% and 0.060 ± 2.0 mg%, respectively.

### Behavioral effects of ethanol withdrawal

As shown in Figure 2A, Student *t*-test detected significant differences between vehicle (veh) and ethanol withdrawn groups in all parameters evaluated in the OF, i.e., traveled distance (t=2.53, df=25, p=0.018), time in central area (t=3.43, df=25, p=0.002) and number of entries in the central area (t=2.87, df=25, p=0.008). No significant effects were observed in the OF behavioral parameters when the mice were tested at 7 or 35 hours after ethanol withdrawal. In the LD test (Figure 2B), significant effects of ethanol withdrawal were detected in the number of crossings at 24 hours (t=2.44, df=25, p=0.02) and 35 hours (t=3.34, df=22, p=0.003) when compared to vehicle treated group. Ethanol withdrawal did not impact the other measures in the LD test such as latency and time spent in the light compartment. Figure 3C shows the effects of ethanol withdrawal in mice evaluated in the EPM. Only at 24 hours, the number of closed arm entries was significativelly decreased by ethanol withdrawal when compared to control group. No other significant difference was detected in the other parameters evaluated in the EPM.

Figure 3 shows the effects of diazepam 24 hours after ethanol withdrawal in mice. While ANOVA did not detect any significant effect in the number of entries in the central zone of OF ($F_{2,41} = 3.07$, $p = 0.06$) there were effects in the distance traveled ($F_{2,41} = 1.78$, $p = 0.04$) and in the time spent in the central area of the OF ($F_{2,41} = 5.07$, $p = 0.06$). Diazepam 4 mg $kg^{-1}$ increased the time spent in the central area of the OF when compared to controls ($p = 0.01$; Figure 3A). In the LD test (Figure 3B), there were significant differences in the latency ($F_{2,41} = 14.77$, $p < 0.001$), % of time in the light compartment ($F_{2,41} = 14.90$, $p < 0.001$) and in the number of crossings ($F_{2,41} = 11.06$, $p = 0.002$). Diazepam 4 mg $kg^{-1}$ increased the latency ($p < 0.001$) and the time spent in the light compartment latency as compared to controls. Diazepam 1 mg $kg^{-1}$, in turn, increase the number of crossings in the LD test when compared to vehicle treated group ($p < 0.001$). In the EPM, there was significant differences between the experimental groups concerning % time spent ($F_{2,41} = 6.03$, $p = 0.005$) and entries in the open arms ($F_{2,41} = 4.86$, $p = 0.01$). Diazepam increased both parametes when compared to controls ($p < 0.05$). No significant effect was oberved concerning the number of entries in the closed arms of the EPM ($F_{2,41} = 2,35$, $p = 0.11$).

The effects of CBD in the ethanol-induced anxiety are shown in Figure 4. We observed significant effects in the traveled distance in mice evaluated in the OF ($F_{3,55} = 10.95$, $p < 0.0001$; Figure 4A). Ethanol withdrawn mice that received CBD presented a significant decrease in the traveled distance when compared to controls ($p < 0.05$). No differences were detected in the time spent ($F_{3,55} = 2.16$, $p = 0.10$) or in number of the entries ($F_{3,55} = 3,55$, $p = 0.40$) in the central area of the OF (Figure 4B). Again, no effects of CBD were observed in the withdrawn mice when they were evaluated in the LD test. However, when these animals were tested in the EPM, ANOVA detected significant differences in the % time spent ($F_{3,55} = 10.94$, $p < 0.001$) and in the % of entries ($F_{3,55} = 9.76$, $p < 0.001$) in the open arms and, in the number of closed arm entries ($F_{3,55} = 5.04$, $p = 0.004$) of the EPM. Animals that received CBD at 10 and 60 mg $kg^{-1}$ presented an increase in both parameters, i.e., the % of time ($p < 0.05$) and in the % of open arm entries ($p < 0.05$).

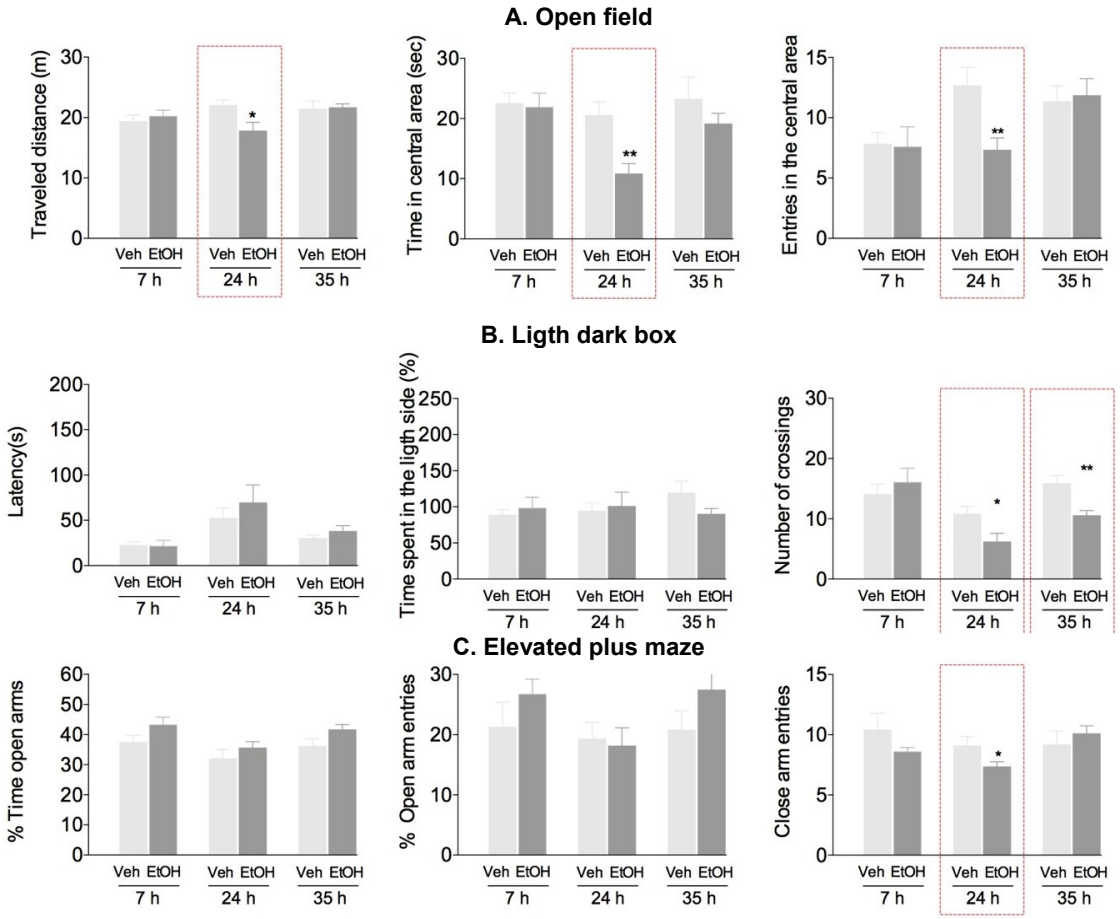


**Figure 2.** Time course of anxiety-related behavior expression in ethanol (etOH) withdrawn mice. The animals were tested 7, 24 or 35 hours following vehicle or ethanol withdrawal in the OF, LD and EPM. Columns represent the means and bars the standard error of the means (SEM). *p < 0.05 and **p < 0.001 compared to Veh (Student *t* test).

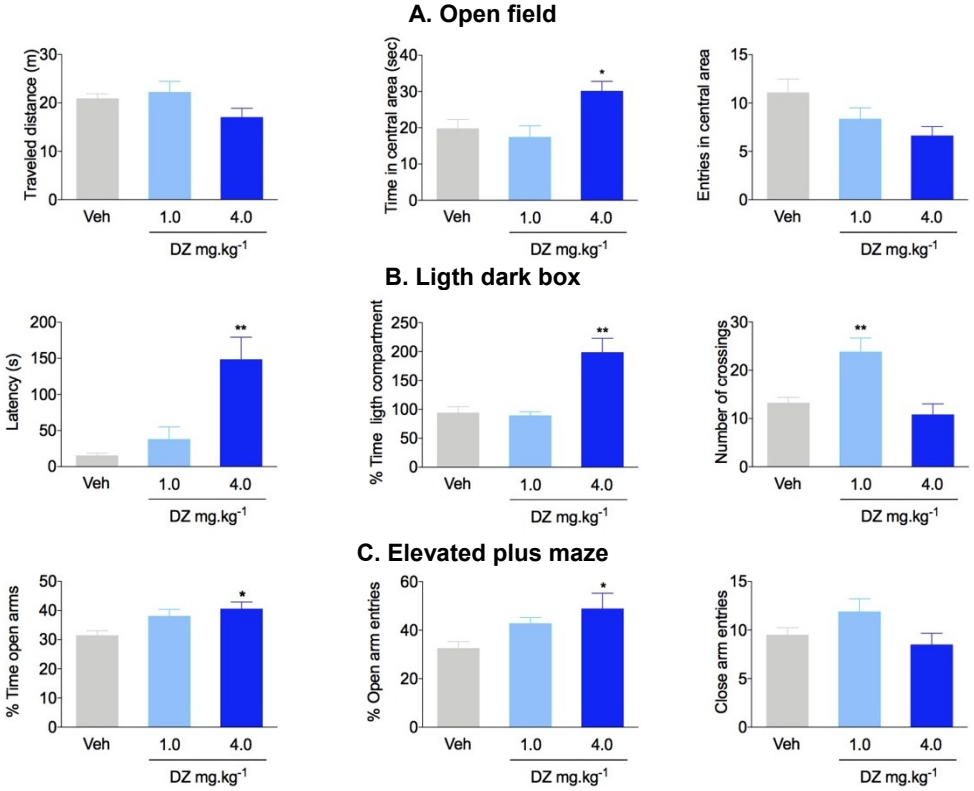


**Figure 3.** Effect of diazepam (DZ) 1 and 4 mg $kg^{-1}$ in anxiety-related behaviors induced by ethanol withdrawal in mice. Columns represent the means and bars the standard error of the means (SEM). *p < 0.05 and **p < 0.001 compared to Veh (ANOVA followed by the Tukey test).

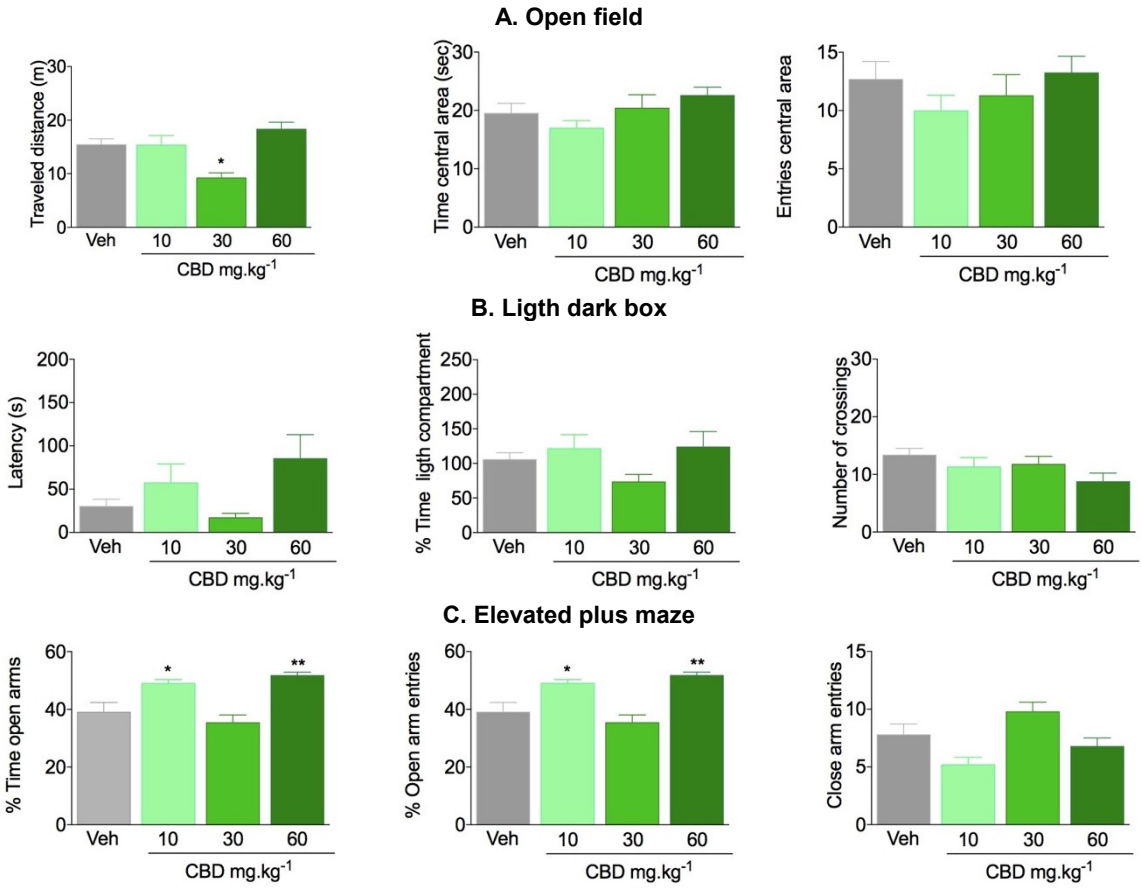


**Figure 4.** Effect of cannabidiol (CBD) 10, 30 and 60 mg $kg^{-1}$ in anxiety-related behaviors induced by ethanol withdrawal in mice. Columns represent the means and bars the standard error of the means (SEM). *p < 0.05 and **p < 0.001 compared to Veh (ANOVA followed by the Tukey test).

## Discussion

The abrupt interruption of ethanol consumption increases anxiety-like behaviors in rodents and may reflect different aspects of ethanol dependence in humans (Kliethermes, 2005). In the present study, we used forced expositon to ethanol 20% during 10 days to mice, followed by abrupt withdrawal of the substance. The animals were evaluated 7, 24 and 35 hours after ethanol withdrawal in three different behavioral paradigms usually used to measure anxiety-like behaviors. This was done with the aim of identifying the best interval as well as the most appropriate behavioral test to detect the effects of drugs that can relieve anxiety induced by ethanol withdrawal in mice. Our results show that OF, LD and EPM tests were sensitive to detect behavioral changes at 24 hours after ethanol withdrawal. The mice presented a significant reduction in the distance traveled and in the number of entries and % of time in the central zone of the OF test, a reduction in the number of crossings in the LD box and a decrease in the EPM closed arm entries. These results indicate an expression of anxiogenic-like effects due to ethanol withdrawal. Diazepam (4 mg $kg^{-1}$), used as a positive control, counteracted the effects of ethanol withdrawal in the OF, LD box and EPM. CBD attenuated anxiety-like behavior produced at 24 hours after abstinence from ethanol exposure only in the EPM test.

Liquid diet containing ethanol, exposure to vaporized ethanol, and ethanol gavage or *i.p.* injection, sustained high blood ethanol concentration in rodents (Becker & Ron, 2014; Botia, Legastelois, Houchi, & Naassila, 2015). Here in, 20% v/v ethanol administration during 10 days was used to establish a pattern of chronic ethanol exposure and subsequent ethanol withdrawal (Botia et al., 2015). After 7, 24 and 35 hours following ethanol withdrawal, the BEC were indetectable in the blood of mice, indicating a state of ethanol abstinence. The OF, LD and EPM, were used to measure the expression of anxiety-like behaviors produced by ethanol withdrawal.

The OF, LD and EPM behavioral tests are exploration-driven tests that rely on the voluntary locomotor activity of rodents. In general, rodents present a tendency to stay in a relatively safe area (the peripheral

zone of OF, the dark side of the LD box and the closed arms of the EPM) versus a more aversive area (the center of the OF, the light compartment of LD box and the open arms of the EPM) of the apparatus (Perez & De Biasi, 2015). Ethanol withdrawan mice showed decreased exploration (time and number of entries) in the central area of the OF at 24 hours after ethanol administration, indicating expression of anxiogenic-like behavior. These results are consistent with others showing that after 2 weeks of 14 days ethanol binge-drinking was suficient to increase anxiety-like behavior in C57 adult mice at 24 hours following ethanol withdrawal across various parafigms including the forced swim test, LD transition and marble burying tests (Lee, Coelho, Solton, & Szumlinski, 2017; Lee, Coelho, Class, & Szumlinski, 2018). However, the ethanol withdrawn mice also presented decrease in the total distance traveled in the OF, decrease in the number of crossings in the LD and in the closed arms of the EPM when compared to controls. These parameters usually indicate decreased locomotor activity. Accordingly, withdrawal decreased locomotion has been shown from 7 to 24 hours in genetically heterogeneous mice following ethanol vapor inhalation (Kliethermes, Cronise, & Crabbe, 2004). A decrease in the general locomotor activity makes complicate the interpretation of anxiogenic-like effects observed with ethanol withdrawal, since it may mask possible effects on anxiety parameters measured in the behavioral tests. However, rodents may express normal or even enhanced locomotor activity when measuring ethanol withdrawal in their homecage (Spanagel et al., 1996; Holter, Linthorst, Reul, & Spanagel, 2000; Kliethermes et al., 2004). Although there is no consensus, Kliethermes (2005) defends that decreased locomotor activity induced by ethanol withdrawal may be considered as an expression, and not a confound of an enhanced anxiety-like state.

One important aspect in the evaluation of ethanol withdrawal in rodents, is that the anxiety-related behaviors have to be affected by standard reference anxiolytic drugs commonly used in the withdrawal phase of ethanol dependence treatment, such as the benzodiazepines (Kliethermes, 2005). Here in, administration of DZ 4 mg $kg^{-1}$ in ethanol withdrawn mice resulted in anxiolytic-like effects in the OF, LD and EPM tests. These results indicated that the behavioral tests used in the present work were adequate to study the effects of potential anxiolytic compounds on ethanol withdrawn mice.

Cannabidiol, one of the main constutuints of *Cannabis sativa*, presents anxiolytic, anti-stress and anti-compulsive effects. CBD has been shown to interfere with brain circuits that mediate drug craving and seeking elicited by drug-related enviromental contexts (Koob & Volkow, 2010; Gonzalez-Cuevas et al., 2018). Moreover, CBD has presented therapeutic potential in treating drug and ethanol addiction in patients (Crippa et al., 2013; Hurd, 2015; Sloan, Gowin, Ramchandani, Hurd, & Le Foll, 2017; Batalla, Janssen, Gangadin, & Bossong, 2019). Cannabidiol also reduced the reinforcement properties and motivation and, prevented ethanol-induced relapse in C57 mice (Viudez-Martinez et al., 2018). Recently, it has shown that CBD attenuated context- and stress-induced ethanol seeking and reduced experimental anxiety of withdrawn rats in the EPM (Gonzalez-Cuevas et al., 2018). In the present study, CBD at 10 and 60 mgkg, but not at 30 mg $kg^{-1}$, decrease the anxiety-like behaviors of withdrawan mice. These findings corraborate previous studies showing that acute CBD administration may produce anxiolytic-like effects according to an inverted U-shaped dose–response curve in several anxiety paradigms (Campos, Moreira, Gomes, Del Bel, & Guimarães, 2012). Curiously, different from DZ, CBD induced anxiolytic-like effects only in animals tested in the EPM.

The reason for the lack of CBD anxiolytic-like effects in the ethanol withdrawn mice in the OF and LD tests is unknown. In fact, it is widely recognised that the OF, LD and EPM do not measure exactly the same type of anxiety-related behavior, althought it is not clear what relationship the inter-test differences have with the various forms of human anxiety (Cryan & Holmes, 2005). Moreover, performance of rodents in tasks measuring anxiety-like behaviors may be influenced by prior handling, lightening conditions and, age and strain of the animals among other influences (Kliethermes, 2005). Also, repeated *i.p.* injections and manipulation may have interfered with the expression of anxiety-like behaviors. (personal appointments).

Increased anxiety-related behavior during withdrawal is likely a reflection of the diret effects of ethanol exposure on the neuronal functioning (Kliethermes, 2005). While BZD are known to positively modulate the function of GABAa receptors (Kumar et al., 2009), different molecular mechanisms may underlie the behavioral effects of single or repeated CBD administration in mice (Schiavon, Bonato, Milani, Guimarães, & Weffort de Oliveira, 2016). CBD has been proposed to activate or modify the function of several receptors in the brain including the CB1, CB2, GPR55, TRPV, 5-HT1a and opioid receptors (Bisogno et al., 2001; Campos, Fogaça, Sonego, & Guimarães, 2016). Facilitation of serotonin 5-HT1A receptor-mediated neurotransmission, for example, have been reported to be involved in the anxiolytic-like effects of CBD (Campos et al., 2012). In addition, antioxidant properties of CBD has been implicated in neuroprotective

mecanisms against ethanol induced neurotoxicity in a model of binge ethanol consumption (Hamelink, Hampson, Wink, Eiden, & Eskay, 2005). However, it is nuclear whether CBD's anti-anxiety effects observed in the present study might be explained by direct interaction of CBD with different receptors or by its antioxidant and/or neuroprotective effects.

## Conclusion

In summary, the present results show that OF, LD and EPM tests were sensitive to detect anxiogenic-like behavior at 24 hours after ethanol withdrawal. Diazepam, used as a positive control, counteracted the effects of ethanol withdrawal in the OF LD box and EPM. Cannabidiol attenuated anxiety-like behavior produced at 24 h after abstinence from ethanol exposure only in the EPM. Further studies are needed to clear the mechanisms underlying the CBD effects following ethanol withdrawal.

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