Impact of caffeine on ethanol induced sensitization: relation to dopamine metabotropic changes in ERK and DARPP-32.

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ABSTRACT

In recreational contexts, caffeine is frequently consumed with ethanol to reduce psychomotor slowing or incoordiantion induced by ethanol. To determine if caffeine can reverse these ethanol-induced impairments we evaluated different types of voluntary locomotion in an open field in adult male CD-1 mice. Both drugs modulate dopamine (DA) transmission in an indirect and complex way. Accumbens (Acb) DA has been involved in behavioral activation, voluntary locomotion and locomotor sensitization. Thus, we also assessed the impact of caffeine on repeated ethanol-induced locomotor sensitization. Acute effects of caffeine were evaluated on measures of exploration (horizontal locomotion and supported rearing) as well as incoordination (rearing not supported by the wall) modulated by acute or repeated administration of ethanol. Acutely, ethanol decreased both types of rearing. Caffeine increased supported rearing but did not block ethanol-induced incoordination. As for horizontal locomotion, both substances increased it in a biphasic manner, and caffeine even potentiated ethanol-induced locomotion. Although ethanol administered repeatedly induced sensitization of both types of exploration, sensitized mice that were administered caffeine acutely in an ethanol-free state showed blunted stimulant effects compared to ethanol naïve mice. Phosphorylation of markers dependent on dopamine D_1 and D_2 receptor activation were evaluated (pERK and pDARPP-32(Thr75) respectively) in Acb core (AcbC) and Acb shell (AcbSh). Ethanol increased pERK immunoreactivity in both subregions of the Acb, consistent with an increase in DA, but co-administration with caffeine blunted that increase. There were no effects on pDARPP-32(Thr75) suggesting a role for D_1 but not D_2 receptor modulation in the interaction.

Keywords: alcohol, caffeine, dopamine, stimulation, incoordination, sensitization.

INTRODUCTION

Caffeine is a substance commonly used for its known psychostimulant properties (Temple *et al.* 2017). Preferentially in form of beverages, contained in tea, coffee or more recently in popular caffeine-rich beverages called "energy drinks", it is taken daily with the aim of improving cognitive and physical performance. A large number of studies in humans have highlighted its beneficial effects in terms of fatigue reduction, increase alertness and energy (Astorino & Roberson 2010; Duncan *et al.* 2012, 2013; Smirmaul *et al.* 2017). Moreover, its use in association with ethanol has, in the last decades, become widespread in order to counteract the sedative effects and the locomotor impairment of high, intoxicating, doses of ethanol (Hasenfratz *et al.* 1993; Drake *et al.* 2003; Attwood *et al.* 2012). While under laboratory conditions participants sometimes report feeling less intoxicated and impaired when alcohol is consumed with caffeine (Ferreira et al. 2016; Marczinski and Fillmore 2006), others do not report feeling less sedated or incoordinated (Arria et al. 2011; Peacock et al. 2013). Moreover, objective measures of motor function have been demonstrated to be largely unaffected by co-administration of caffeine with ethanol (Weldy 2010; Ulbrich et al. 2013), although some mild stimulating effects have also been observed such as reductions in reaction time (Howland et al. 2010) after consuming ethanol plus caffeine.

In animal studies, it has been shown that when acutely administered, both ethanol and caffeine are able to stimulate or suppress locomotor activity in a dose dependent manner, typically with bell-shaped (or inverted-U) dose-response functions (Correa *et al.* 2001a; Arizzi-LaFrance et al. 2006; Hilbert *et al.* 2013; López-Cruz *et al.* 2013). Low doses of caffeine seem to potentiate psychomotor stimulation induced by ethanol (Waldek 1974; López-Cruz et al. 2011). However, the impact of higher doses such as the ones found in energy drinks are much less known. Moreover, the repeated administration of a stimulant substance may produce sensitization, measured as a potentiation of locomotion (Steketee & Kalivas 2011; Kawa & Robinson 2019). Cross-sensitization also has been describe, which is said to occur when a new drug shows potentiated stimulating effects in subjects that show sensitization to another drug, for example among ethanol and cocaine in rats (Xu & Kang 2017). Caffeine induces locomotor sensitization (Ulenius et al., 2019) as well as cross-sensitization with other substances such as amphetamine and nicotine (Celik *et al.* 2006). Recently it has been reported that repeated intragastric coadministration of ethanol and caffeine induced significantly greater locomotor sensitization than the drugs alone (May *et al.* 2015). However, cross-sensitization between both drugs remains largely unexplored.

Sensitization in locomotor exploration is related to the motivational properties of drugs of abuse (Robinson & Berridge 2000), such as the regulation of behavioral activation, and processes such as incentive salience and invigoration of goal directed responses, which are functions regulated by the mesolimbic dopamine system (Robinson & Berridge 2000; Salamone *et al.* 2016). Ethanol and caffeine act on the dopamine systems via different mechanisms: ethanol preferentially increases the firing of dopaminergic neurons in the ventral tegmental area leading to an increase in dopamine (DA) transmission in nucleus accumbens (Acb) (Gessa *et al.* 1985; Di Chiara & Imperato 1988; Carboni *et al.* 2000; Vena *et al.* 2016) while caffeine causes an increase of extracellular DA in prefrontal cortex, but not in Acb (Acquas *et al.* 2002, 2010). The Extracellular signal-Regulated Kinase (ERK) plays a critical role in signal transduction and neuroplasticity induced by substances of abuse (Valjent et al., 2005). In particular, phosphorylated ERK (pERK) in the core (AcbC) and shell (AcbSh) subregions of the Acb, increases after acute ethanol administration (Ibba et al., 2009; Spanos et al., 2012). In addition, pERK seems also to increase, at least in prefrontal and cingulate cortices, after caffeine administration, and this effect is mediated by D_1 receptors (Acquas et al. 2010).

These two drugs also act on the adenosinergic system; ethanol increases endogenous adenosine (López-Cruz *et al.* 2014), while caffeine is an antagonist of adenosine receptors (Ferré 2008). Adenosinergic and dopaminergic receptors converge on common mechanisms, showing opposite effects on metabotropic intracellular cascades (Agnati *et al.* 2003; Fuxe *et al.* 2003; Ferré 2008), such as Dopamine- and cAMP-regulated phosphoprotein Mr 32 kDa phosphorylated at the Threonine75 (Thr-75) site (pDARPP-32-Thr75), which is associated with activation of DA D_2 receptors (Svenningsson *et al.* 1999; Nunes *et al.* 2013).

The present experiments were developed to determine the effects of acute administration of caffeine (7.5, 15 or 30 mg/kg), ethanol (1.5, 2.5 or 3.5 g/kg) and their interaction on different indicators of novelty induced behavioral activation and exploration measured in an open field apparatus. In addition, we also studied if an acute administration of caffeine at different doses (15 and 30 mg/kg) can reverse the motor sensitization elicited by repeated administration of ethanol (1.5 g/kg). Furthermore, we assessed the effects of an acute challenge with caffeine on ethanol-elicited pERK and pDARPP-32(Thr75) immunoreactivity in AcbC and AcbSh), as a measure of neuronal markers of DA receptor activation.

MATERIALS AND METHODS

Animals

Adult male CD-1 mice (N=277) (30-40 g, Janvier, France S.A.) were 8-10 weeks old at the beginning of the study. Mice were housed in groups of three or four per cage, with standard laboratory rodent chow and tap water available *ad libitum*. The colony was kept at a temperature of 22± 2 ºC with lights on from 08:00 to 20:00 h. All animals were under a protocol approved by the Institutional Animal Care and Use committee of Universitat Jaume I. All experimental procedures complied with directive 2010/63/EU of the European Parliament and of the Council, and with the "Guidelines for the Care and Use of Mammals in Neuroscience and Behavioral Research", National Research Council 2003, USA. All efforts were made to minimize animal suffering and to reduce the number of animals used.

Drugs

Ethanol (Panreac Quimica S.A., Spain) 20% (v/v) in isotonic saline (0.9 % w/v) was administered intraperitoneally (IP) 10 minutes (min) before testing. Caffeine (Sigma-Aldrich, Spain) was dissolved in 0.9% w/v saline and administered IP 30 min before testing. Saline solution was used as vehicle. Doses and time leads were chosen based on previous studies (Correa *et al.* 2004; López-Cruz *et al.* 2016).

Apparatus and testing procedures

Open Field (OF)

The OF apparatus consists of a clear glass cylinder 25 cm in diameter and 30 cm high previously used to observe the effects of ethanol on spontaneous locomotion and locomotor sensitization (Correa *et al.* 2004). The floor of the cylinder was divided into four equal quadrants by two intersecting lines drawn on the floor. Animals were placed in the center of the cylinder and immediately observed for 10 min. The behavioral test room was illuminated with a soft light, and external noise was attenuated. Horizontal and vertical locomotion in the OF were simultaneously recorded and registered manually. For horizontal locomotion, an activity count was registered each time the animal crossed from one quadrant to another with all four legs. A count of vertical locomotion was registered each time the animal raised its forepaws in the air higher than its back (unsupported rearing) or rested them on the wall (supported rearing).

For the sensitization experiment (experiment 5), two groups of animals received either vehicle or ethanol and were introduced in the OF (session 1). This treatment was repated in alternating days until a total of 5 sessions were performed. In the cros-sensitization test (experiment 6), these same

animals were further divided in 3 groups and two days after session 5, they received a single dose of vehicle, caffeine 15 mg/kg or caffeine 30 mg/kg.

Blood ethanol determinations

Additional mice were used to determine whether caffeine influenced blood ethanol levels at the high doses and times used in the behavioral studies. For that purpose, animals were injected with caffeine (0 or 30 mg/kg) and with ethanol (1.5 or 2.5 g/kg). Trunk blood samples (20 µl) were collected 10 and 20 min after ethanol and caffeine administration respectively. Following Boehm et al. (2000), each blood sample was immediately placed in a microcentrifuge tube containing 50 µl of ice-cold 5% $ZnSO_4$ solution. A 50-µl aliquot of 0.3 N $Ba(OH)_2$ and 300 µl of deionized water was added. After centrifugation at 4°C (5 min, 12,000 rpm), the supernatant was removed and blood ethanol concentrations were determined by headspace gas chromatography with a flame-ionized detector (CE Instruments GC 8000, HS 850).

pERK immunohistochemistry

Mice were anesthetized with carbon dioxide for 30 s and perfused 15 min after the last treatment. The time interval between ethanol administration and anesthesia was selected on the basis of the time of the peak effect on DA transmission (Melis *et al.* 2007; Ibba *et al.* 2009). Brains were collected and stored in paraformaldehyde solution during 24 h and refrigerated in sucrose (30%), sodium azide (2%) and phosphate buffer PB (0.1M) solution prior to slicing. Free floating coronal sections (40 µm) were serially cut using a microtome cryostat (Weymouth, MA, USA) according to plates 21-23 (approximately from AP 1.18 to AP 0.98 mm from bregma for the AcbC and AcbSh) of the mouse brain atlas (Paxinos & Franklin 2001). After rinsing in 0.01 M 0.01 M phosphate buffer saline (PBS) (pH 7.4) (3 times for 10 min) and incubating for 30 min with 1% hydrogen peroxide and after three rinses of 30 min each one, the slices were incubated for 1 hr with 0.1% Triton X-100 (T.X) in TBS and 3% Bovine Albumin serum (BSA). The incubation with the primary anti-pERK antibody (phosphorylated ERK, Cell Signaling Technology, Beverly, MA, USA) at 1:350 was conducted overnight at 4°C. On the following day, after rinsing, the slices were incubated with the second antibody, the anti-rabbit HRP conjugate envision plus (DAKO) for 1.5 h on a rotating shaker at room temperature. Finally, sections were washed and rinsed for 1-3 min in 3,3-diaminobenzidine chromagen (DAKO).

pDARPP-32(Thr75) immunohistochemistry

Alternating brain slices obtained from the same animals and not used for pERK immunohistochemistry were processed for pDARPP-32(Thr75) immunoreaction. Brain slices were rinsed in 0.01 M PBS (pH 7.4)

and incubated in 1% hydrogen peroxide for 30 min to block endogenous staining. Sections were then rinsed in 0.01 M PBS (pH 7.4) (3 times for 5 min). To measure the immunoreactivity to pDARPP-32 nonspecific binding sites were blocked, and cells were permeabilized in a solution containing 0.1% T.X and 3% BSA in PBS for 1h at room temperature on a rotating platform before primary antibody incubation. pDARPP-32 immunoreactivity was visualized with a polyclonal rabbit antibody for pDARPP-32 phosphorylated at the threonine 75 residue (pDARPP32-Thr75, 1:500; Cell Signaling Technology, Beverly, MA, USA). The antibody was dissolved in solutions that also contained 3% BSA and 0.1% T.X in PBS for 24 h incubation at 4°C. After the primary antibody treatment, the sections were rinsed in PBS (3 times for 5 min) and incubated in the secondary antibody, anti-rabbit HRP conjugate envision plus (DAKO) for 1.5 h on a rotating shaker at room temperature. Finally, sections were washed and rinsed for 1-3 min in 3,3-diaminobenzidine chromagen (DAKO).

Image analysis

Processed brain sections were mounted to microscope slides (Menzel-Gläser, Superfrost® Plus, Thermo scientific), air dried, processed through alcohol-xylene and cover-slipped using Eukitt® (Sigma AldrichMerck KGaA) as a mounting medium. The sections were examined and photographed using a Nikon Eclipse E600 (Melville, NY, USA) upright microscope equipped with an Insight Spot digital camera (Diagnostic Instruments, Inc). Images of the regions of interest were magnified at 20X and captured digitally using Stereo Investigator software. Cells were quantified with ImageJ software (v. 1.42, National Institutes of Health sponsored image analysis program) in three sections per animal, and the average value per mm^2 was used for statistical analysis.

Statistical analysis

One-way analysis of variance (ANOVA) was used to analyze the effect of drug administration on the different dependent variables; horizontal and vertical supported and unsupported locomotion. Two-way factorial ANOVA was used for the interaction studies. When the overall ANOVA was significant, non-orthogonal planned comparisons using the overall error term were used to compare each treatment with the control group (Keppel 1991). For these comparisons, a level was kept at 0.05 alpha because the number of comparisons was restricted to the number of treatments minus one. A probability level of 0.05 or smaller was used to indicate statistical significance. Statistics were done using STATISTICA 8 (StatSoft Inc., Tulsa (OK), USA) software.

RESULTS

Experiment 1. Effects of acute administration of caffeine on several measures of locomotion.

Figure 1 shows the effects of caffeine (0.0, 7.5, 15.0 or 30.0 mg/kg) administered 30 min before the OF test (N=38). One way-ANOVA showed an overall effect of caffeine on horizontal crosses [$F_{3,30}$=4.06, p<0.05], as well as on supported rearing [$F_{3,30}$=3.48, p<0.05]. Planned comparisons revealed that caffeine at low and moderate doses (7.5 and 15 mg/kg) significantly increased horizontal locomotion (p<0.05 and p<0.01, respectively) (**Fig 1A**). These same doses of caffeine also produced significant increases in the number of supported rears (p<0.05) (**Fig 1B**). No significant effect of caffeine treatment on unsupported rearing was observed [$F_{3,30}$=0.45, n.s.] (**Fig 1C**).

Experiment 2. Effects of acute administration of ethanol on several measures of locomotion.

Figure 2 shows the effects of ethanol (0.0, 1.5, 2.5 or 3.5 g/kg) administered 10 min before the OF test (N=40). One-way ANOVA revealed an overall effect of ethanol treatment on horizontal crosses [$F_{3,39}$=3.75, p<0.05], supported rearing [$F_{3,39}$=24.11, p<0.01], and unsupported rearing [$F_{3,39}$=19.13, p<0.01]. Planned comparisons showed that ethanol significantly increased horizontal crosses at the dose of 2.5 g/kg (p<0.05) **(Fig 2A**). Supported rearing was decreased by the highest doses of ethanol (2.5 and 3.5 g/kg, p<0.01) (**Fig 2B**). All ethanol doses significantly decreased unsupported rearing (p<0.01) (**Fig 2C**).

Experiment 3. Effects of acute administration of caffeine and ethanol on several measures of locomotion.

Figure 3 shows the effects of caffeine (0, 15 or 30 mg/kg) and ethanol (0.0, 1.5, 2.5 or 3.5 g/kg) combination in mice (N=115) evaluated in the OF. Factorial ANOVA (Caffeine x Ethanol) showed an overall effect of caffeine [$F_{2,112}$=11.18, p<0.01], ethanol [$F_{3,112}$=59.35, p<0.01] and caffeine-ethanol interaction [$F_{6,112}$=6.64, p<0.01] on horizontal crosses in the OF. Planned comparisons revealed that in caffeine 30 mg/kg treated mice, ethanol 1.5 g/kg produced a significant increase in locomotion compared to caffeine (0 mg/kg) + ethanol (1.5 g/kg) treated mice (p<0.05). In addition, caffeine (15 and 30 mg/kg) + ethanol (2.5 g/kg) groups were significantly different from caffeine (0 mg/kg) + ethanol (2.5 g/kg) treated mice (p<0.01). Moreover, caffeine (0 mg/kg) + ethanol (2.5 g/kg) was significantly different compared to vehicle + vehicle group (p<0.01) (**Fig 3A**). The factorial ANOVA (Caffeine x Ethanol) for the variable supported rearing, as a measure of vertical locomotion, also showed an overall effect of caffeine [$F_{2,112}$=3.81, p<0.05], ethanol [$F_{3,112}$=62.26, p<0.01] and their interaction [$F_{6,112}$=2.29, p<0.05]. Planned comparisons showed that in the vehicle treated group, caffeine 15 mg/kg increased

supported rearing compared to control ($p<0.01$). Among ethanol 1.5 g/kg treated mice, both caffeine (15 and 30 mg/kg) treated groups increased supported rearing compared to caffeine (0 mg/kg) + ethanol (1.5 g/kg) group ($p<0.01$). In addition, caffeine (0 mg/kg) + ethanol (2.5 and 3.5 g/kg) groups were significantly different from vehicle control group ($p<0.01$) (**Fig 3B**). Finally, the factorial ANOVA (Caffeine x Ethanol) for unsupported rearing (**Fig 3C**) showed a significant effect of ethanol treatment [$F_{3,112}=66.89$, $p<0.01$]. However, there was no significant effect of caffeine [$F_{2,112}=0.94$, NS], and no significant interaction [$F_{6,112}=0.83$, n.s.].

Experiment 4. Effects of caffeine administration on blood ethanol levels.

Additional mice (N=24) were used to determine if caffeine influenced blood-ethanol levels after motor stimulating doses. Animals received caffeine (0 or 30 mg/kg) and 20 min later ethanol (1.5 or 2.5 g/kg) was administered. Two-way factorial ANOVA (Ethanol x Caffeine) showed a significant effect of ethanol [$F_{1,25}= 326.82$, $p<0.01$], but no significant effect of caffeine [$F_{1,25}=0.31$, n.s.], and no interaction [$F_{1,25}=3.39$, n.s.]. These data (table 1) suggest that the observed behavioral effects of ethanol co-administered with caffeine are not due to changes in blood ethanol concentration.

Experiment 5. Effects of repeated administration of ethanol on several measures of locomotion.

Figure 4 shows the effects of repeated ethanol (0 or 1.5 g/kg) administration in mice (N=32) exposed to the OF during 5 sessions in alternating days. Factorial ANOVA with a between factor (session: 1 and 5) and a within factor (ethanol dose: 0 or 1.5 g/kg ethanol) showed a significant effect of treatment [$F_{1,64}=30.87$, $p<0.01$], a significant effect of session [$F_{1,64}=18.15$, $p<0.01$] and treatment x session interaction [$F_{1,64}=8.24$, $p<0.01$] for the first dependent variable; horizontal crosses. Planned comparisons showed a locomotor stimulant effect of ethanol (1.5 g/kg) compared with vehicle treated mice in the first session ($p<0.01$), and the ethanol treated group in session 5 was also significantly different compared to the vehicle group in the same session ($p<0.01$). Moreover, ethanol in session 5 further increased locomotion compared to session 1 ($p<0.01$). This increase in locomotion over sessions was not observed in the vehicle treated groups, suggesting a sensitization of locomotion induced by ethanol (Fig 4A). The factorial ANOVA for the variable supported rearing showed a significant effect of ethanol dose [$F_{1,64}=6.42$, $p<0.01$], no significant effect of session [$F_{1,64}=3.32$, n.s.], but a significant effect of ethanol dose x session interaction [$F_{1,64}=4.61$, $p<0.05$] (Fig 4B). Planned comparisons showed that although ethanol did not change supported rearing in session 1 when compared to the vehicle group, it increased the number of supported rears when administered in session 5 compared to the vehicle group in the same session ($p<0.01$), and also compared to its

administration in session 1 ($p<0.05$). The ANOVA for the dependent variable unsupported rearing showed a significant effect of ethanol dose [$F_{1,64}=127.4$, $p<0.01$], a significant effect of session [$F_{1,64}=21.62$, $p<0.01$], but did not show a significant interaction [$F_{1,64}=2.60$, n.s.] (Fig 4C).

Experiment 6. Effects of acute administration of caffeine on ethanol-induced locomotor sensitization in several behavioral measures.

Figure 5 shows the effects of an acute administration of caffeine on ethanol-sensitized mice. Two days after the last drug administration, animals received an acute administration of caffeine (0, 15 or 30 mg/kg) in order to observe if there was a cross-sensitization effect. The factorial ANOVA; previous ethanol treatment (0 or 1.5 g/kg) x caffeine dose (0, 15 or 30 mg/kg) showed an overall effect of previous ethanol dose [$F_{1,65}=11.48$, $p<0.01$], an effect of caffeine dose [$F_{2,65}=25.45$, $p<0.01$] and also a significant interaction [$F_{1,65}=3.82$, $p<0.05$] on horizontal locomotion (Fig 5A). Planned comparison showed a stimulant effect of caffeine at both doses (15 and 30 mg/kg) in the vehicle pretreated group ($p<0.01$). However, only the dose of 15 mg/kg of caffeine induced locomotion in the ethanol (1.5 g/kg) pretreated group ($p<0.01$). Interestingly, caffeine at the highest dose (30 mg/kg) significantly decreased locomotion in animals that had received ethanol (1.5 g/kg) in previous sessions when compared to the effect of this dose of caffeine in the vehicle pretreated group ($p<0.01$) (Fig 5A).

The same pattern of results was observed on the other two variables; supported (Fig 5B) and unsupported rearing (Fig 5C). Thus, for supported rearing the results were: ethanol pretreatment [$F_{1,65}=9.66$, $p<0.01$], caffeine [$F_{2,65}=29.28$, $p<0.01$], and the interaction [$F_{1,65}=3.25$, $p<0.05$]. The effect of caffeine on supported rearing showed a similar pattern of effects. Caffeine increased supported rearing at both doses (15 and 30 mg/kg, $p<0.01$ and $p<0.05$, respectively) in the vehicle pretreated group. However, only the smaller dose of caffeine (15 mg/kg) significantly increased rearing in the ethanol pretreated group ($p<0.01$) compared to its vehicle group, and this dose produced a blunted induction of supported rearing that showed significant differences between the two caffeine 15 mg/kg groups ($p<0.05$). In addition, caffeine 30 mg/kg decreased supported rearing in the ethanol (1.5 g/kg) pretreated group compared to the same dose of caffeine in the ethanol (0 g/kg) pretreated group ($p<0.05$) (Fig 5B).

Finally, the same pattern of results was observed on unsupported rearing (Fig 5C) [$F_{1,65}=4.87$, $p<0.05$]; [$F_{2,65}=10.90$, $p<0.01$]; [$F_{1,65}=5.36$, $p<0.01$, respectively]. While caffeine at both doses increased unsupported rearing in the vehicle pretreated group ($p<0.01$ and $p<0.05$ respectively), only caffeine 15 mg/kg increased unsupported rearing in the ethanol (1.5 g/kg) pretreated group, and the highest dose of caffeine (30 mg/kg) significantly decreased this type of rearing ($p<0.05$) compared to its vehicle.

Moreover, the effect of caffeine 30 mg/kg in the ethanol (1.5 g/kg) pretreated group was significantly different from the effect observed in the vehicle pretreated group ($p<0.01$).

Experiment 7. Effects of caffeine on the expression of ethanol-elicited pERK and pDARPP32(Thr75) in AcbC and AcbSh.

Figures 6 and 7 show the effects of caffeine (0, 15 or 30 mg/kg) administered 20 min before of administration ethanol (0 or 1.5 g/kg) on the number of pERK- and pDARPP-32(Thr75)-positive neurons in the AcbC and AcbSh. Immunoreactivity levels were analyzed separately for Acb subregions. One-way ANOVA on the number of pERK-positive cells revealed an overall effect of treatment on AcbC [$F_{3,22}=10.18$; $p<0.01$] and AcbSh [$F_{3,22}=5.69$; $p<0.01$]. Planned comparison's analysis showed a significant increase on pERK expression after caffeine (0 mg/kg) + ethanol (1.5 g/kg) treatment relative to caffeine (0 mg/kg) + ethanol (0 g/kg) groups in the AcbC and AcbSh ($p<0.01$). Furthermore, the administration of caffeine (15 and 30 mg/kg) + ethanol (1.5 g/kg) was significantly different from caffeine (0 mg/kg) + ethanol (1.5 g/kg) in the AcbC and AcbSh ($p<0.01$, for both doses in both structures).

Conversely, one-way ANOVA did not revealed a significant effect of these treatments on the number of pDARPP-32(Thr75)–positive cells in the AcbC [$F_{3,24}=0.29$; n.s.] and AcbSh [$F_{3,24}=0.27$; n.s.].

DISCUSSION

In the present studies with male CD-1 mice, we investigated the acute interaction of caffeine and ethanol, two of the most widely used psychoactive drugs, on different measures of locomotor exploration in an OF: horizontal and vertical locomotion. Moreover, vertical locomotion was separated in two parametters; one more dependent on postural coordination (non-supported rearing), and another less so (wall-supported rearing).

The biphasic effects of ethanol on locomotion have been widely known, often in mouse studies (Phillips & Shen 1996; Correa *et al.* 2001a; Karlsson & Roman 2016), but also in rats after central administration (Correa *et al.* 2003a b). Thus, at low doses, ethanol has stimulatory effects, whereas at high doses prevails the suppressant effect on locomotion (Correa *et al.* 2001b; Chuck *et al.* 2006), and then ataxia, incoordination and sedation predominate (Correa *et al.* 2001b; Chuck *et al.* 2006). Furthermore, the biphasic nature of caffeine on locomotion has previously been described in mice (El Yacoubi *et al.* 2003; Zhang *et al.* 2011; López-Cruz *et al.* 2014), although only very high doses of caffeine (100 mg/kg), much higher than the ones used in the present study, suppress locomotion (Zhang *et al.* 2011). Accordingly,

in the present studies, acute intraperitoneal administration of caffeine or ethanol showed a dose dependent effect on locomotion, with low and moderate doses (caffeine 7.5 and 15 mg/kg, and ethanol 2.5 g/kg) stimulating, and high doses (caffeine 30 mg/kg and ethanol 3.5 g/kg) reducing horizontal locomotion compared to the vehicle group. Furthermore, caffeine showed a bell-shaped dose response curve, with an induction of stimulant effects on supported rearing at low and moderate doses (7.5 and 15 mg/kg), but not at the highest (30 mg/kg) dose. In contrast, ethanol dose dependently decreased both types of rearing. Interestingly, acute administration of both drugs revealed that stimulant (15 mg/kg) and non-stimulant (30 mg/kg) doses of caffeine enhanced locomotion in mice treated with low and moderate (1.5 and 2.5 g/kg, respectively) doses of ethanol. Importantly, caffeine (30 mg/kg) increased horizontal locomotion and supported rearing in combination with a dose of ethanol (1.5 g/kg) that administered alone was not stimulatory. However, at the highest dose of ethanol (3.5 g/kg) caffeine was not able to reverse ethanol's effects in any of the three locomotion parameters. Our results are similar to previous results, in terms of the effects of drug combinations; caffeine in combination with low doses of ethanol (1.75 g/kg) potentiated stimulation and at higher ethanol doses (2.5 and 3.25 g/kg) caffeine potentiated reductions in locomotion (Hilbert *et al.* 2013). Thus, the synergistic activity of the two substances is revealed at low doses, while if one of the two doses used for either drug is particularly high, the antagonistic effect prevails and at even higher doses there is a potentiation of the suppression of locomotion (Waldeck 1974; Hilbert *et al.* 2013). For example, 100 mg/kg of caffeine totally suppresses the locomotor activity induced by a low dose of ethanol (1 g/kg) (Waldeck 1974), and, in terms of coordination, the oral administration of low dose of caffeine (10 mg/kg) reduced ethanol induced-ataxia (Kuribara *et al.* 1992). Local intracerebroventricularly administration of caffeine at low doses (less than 25 micrograms) dose-dependently reduced ethanol-elicited incoordination in mice, while a high dose (75 micrograms) potentiated it (Dar 1988). In our study, a high dose of ethanol (3.5 g/kg) co-administered with moderate doses of caffeine (15 or 30 mg/kg) produced an even deeper suppression of locomotion.

In addition, we also evaluated the impact of acute administration of caffeine on ethanol-induced sensitization, which involves a progressive increase in the motor response resulting from repeated, intermitent ethanol exposure (Camarini & Pautassi 2016). There is very little evidence of cross-sensitization between ethanol and caffeine. Thus, repeated intragastric administration of both drugs induced significantly greater locomotor sensitization than either substances alone (May *et al.* 2015). In our experiment, repeated administration of a low dose of ethanol (1.5 g/kg) induced sensitization of

exploration both horizontal and on the walls of the OF. However, there was no sensitization of the measure that more closely reflected incoordination (unsupported rearing). This measure of incoordination however, showed a slight tendency to be reversed, indicating tolerance rather than sensitization induced by repeated ethanol exposure in this particular context. On the other hand, there was no cross-sensitization after acute administration of caffeine to ethanol-sensitized mice. Although both doses of caffeine (15 and 30 mg/kg) induced all types of locomotor responses in the vehicle exposed mice (effects that were different from the lack of stimulation induced by 30 mg/kg of caffeine administered acutely in a novel context, experiment 1), in the ethanol preexposed group these different stimulatory effects were blunted significantly for the low dose of caffeine and they completedly disappeared with the highest dose of caffeine, even further than in experiment 1 when animals were exposed for the first time to this dose of caffeine and to the OF.

Ethanol acutely and repeatedly has been shown to increase DA release in Acb in mice (Pavón et al. 2019) and in rats (Rosas et al. 2014; Vena et al. 2016; Bassareo et al. 2019). However, the effects of caffeine on DA release in Acb are not very conclusive. Using microdialysis it has been shown that caffeine can induce DA release in AcbSh (Solinas *et al.* 2002), and in the medial prefrontal cortex (Acquas *et al.* 2002), although not in AcbC (Acquas *et al.* 2002).

We have previously observed in rats that ethanol (at an intragastric dose of 1.0 mg/kg) significantly increased postsynaptic intracellular markers related to DA D_1 signaling, pERK in AcbC and AcbSh (Ibba *et al.* 2009). However, caffeine (10 mg /kg) alone did not have an effect on this parameter in the two Acb subregions (Acquas et al. 2010). Interestingly, in the present study using mice, ethanol (1.5 mg/kg) significantly induced pERK, and this effect was counteracted by caffeine at both doses in both AcbC and AcbSh.

Caffeine (15 mg/kg) in mice has previously been demonstrated to suppress the increase in pDARPP-32(Thr34) produced by a DA depleting agent (López-Cruz et al. 2018) potentially in D_2 receptor containing neurons. Reduced DA transmission potentiates pDARPP-32-Thr75 in D_1 containing neurons and pDARPP-32-Thr34 in D_2 containing neuron of AcbSh and AcbC in rats (Nunes *et al*. 2013). Thus, it would have been expected that an increase in DA levels induced by ethanol would lead to an increase in pDARPP-32-Thr75 in D_2 contining neurons. Consistent with models of striatal function and DA-related signal transduction, ethanol (1.5 g/kg) in rats has shown to increase phosphorylation of DARPP-32 at Thr34 in striatum (Nuutinen *et al.* 2011), and ethanol sensitized mice have shown functional hyperresponsiveness of D_1 receptors in Acb, which induced higher

pDARPP-32(Thr34) in sensitized mice (Abrahao et al. 2014). In our study, pDARPP-32(Thr75) was not affected by ethanol or by the ethanol plus caffeine conbination, suggesting a lack of DA D_2 receptor transmission in Acb under these conditions. In this regard, it has been suggested that caffeine acts predominantly on adenosine A_1 receptors (Ferré 2008). These receptors are widely distributed in the brain, but in Acb they are colocalized with DA D_1 receptors (Svenningsson *et al.* 1999; Nunes *et al.* 2013).

In conclusion, despite the popular assumptions about the ability of caffeine in energy drinks to counteract the motor impairments induced by ethanol, the present results using animal models demonstrated that, after the first administration, caffeine can potentiate the stimulating actions of ethanol, but can not counteract its suppressant and ataxic effects. Moreover, our results show that caffeine has less activating actions in ethanol sensitized animals. Our studies also have identified at least one potential brain area in which caffeine can block the stimulating actions of ethanol i.e., the nucleus accumbens. This brain region also appears to be very important for the regulation of behavioral activation, induction of locomotion and ethanol seeking behavior, which can lead to phenomena such as ethanol consumption, abuse and addiction.

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EtOH (g/kg)	Caffeine (mg/kg)	
	0	**30**
1.5	0.89 ± 0.04	1.00 ± 0.07
2.5	2.12 ± 0.07	2.03 ± 0.05

Table 1. Effect of caffeine on blood ethanol levels. Mean ± SEM of blood ethanol levels (in milligrams per deciliter) after acute IP administration of ethanol (1.5 or 2.5 g/kg) and caffeine (0 or 30 mg/kg).

Figure captions.

Figure 1 Effects of acute administration of caffeine (0, 7.5, 15 or 30 mg/kg) on horizontal locomotion (A), supported rearing (B), and unsupported rearing (C) in the OF. Data are expressed as mean (±SEM) number of counts during 10 minutes. **p<0.01, *p<0.05 significantly different from vehicle (caffeine 0 mg/kg) control group.

Figure 2. Effects of acute administration of ethanol (0, 1.5, 2.5 or 3.5 g/kg) on horizontal locomotion (A), supported rearing (B), and unsupported rearing (C) in the OF. Data are expressed as mean (±SEM) number of counts during 10 minutes. **p<0.01, *p<0.05 significantly different from vehicle (ethanol 0 g/kg) control group.

Figure 3. Effects of acute co-administration of caffeine (0, 15 or 30 mg/kg) and ethanol (0, 1.5, 2.5 or 3.5 g/kg) on horizontal locomotion (A), supported rearing (B), and unsupported rearing (C) in the OF. Mean (±SEM) number of counts during 10 minutes. **p<0.01, *p<0.05 significantly different from caffeine (0 mg/kg) in the same ethanol dose group; ##p<0.01 significantly different from vehicle (caffeine 0 mg/kg + ethanol 0 g/kg) control group.

Figure 4. Effects of repeated administration of ethanol (0.0 or 1.5 g/kg) on horizontal locomotion (A), supported rearing (B), and unsupported rearing (C) in the OF. Data are expressed as mean (±SEM) number of counts during 10 minutes. **p<0.01, *p<0.05 significantly different between vehicle (ethanol 0 g/kg) and ethanol (1.5 g/kg) in the same session. ##p<0.01 significantly different between sessions in the same ethanol (1.5 g/kg) group.

Figure 5. Effects of caffeine (0, 15 or 30 mg/kg) in mice treated with ethanol (0 or 1.5 g/kg) in previous days on horizontal locomotion (A), supported rearing (B) and unsupported rearing (C) in the OF. Data are expressed as mean (±SEM) number of counts during 10 minutes. **p<0.01, *p<0.05 significantly different from vehicle in the same pretreatment group. ##p<0.01, #p<0.05 significantly different from the same dose of caffeine in animals that received vehicle in previous sessions.

Figure 6. Effects of acute administration of caffeine on the expression of ethanol-elicited pERK-positive neurons in Acb. Data are expressed as mean (±SEM) of the number of positive neurons/mm^2. Right upper parts: effect of caffeine acute treatment (0, 15 and 30 mg/kg) on the expression of pERK-positive neurons in mice treated with ethanol (0, 1.5 g/kg). Left upper part: diagram of a coronal section with bregma coordinates from Franklin and Paxinos (2001) showing location of the brain areas for pERK immunoreactivity counting. Lower part: photomicrographs of

pERK staining in AcbC and AcbSh from representative mice in each treatment group. Low power images (20X). **p<0.01 significantly different from vehicle (caffeine 0 mg/kg + ethanol 0 g/kg) groups in the AcbC and AcbSh. ##p<0.01 significantly different from caffeine (15 and 30 mg/kg) + ethanol (1.5 g/kg) groups in the AcbC and AcbSh.

Figure 7. Effects of acute administration of caffeine on the expression of ethanol-elicited pDARPP-32(Thr75)-positive neurons in Acb. Data are expressed as mean (±SEM) of the number of positive neurons/mm^2. Right upper parts: effect of caffeine acute treatment (0, 15 and 30 mg/kg) on the expression of pDARPP-32(Thr75) positive neurons in mice treated with ethanol (0, 1.5 g/kg). Left upper part: diagram of a coronal section with bregma coordinates from Franklin and Paxinos (2001) showing location of the brain areas for pDARPP32(Thr75) immunoreactivity counting. Lower part: photomicrographs of pDARPP-32(Thr75) staining in AcbC and AcbSh from representative mice in each treatment group. Low power images (20X).