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Impact of Caffeine on Ethanol-Induced Stimulation and Sensitization: Changes in ERK and DARPP-32 Phosphorylation in Nucleus Accumbens

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Background: Caffeine is frequently consumed with ethanol to reduce the impairing effects induced by ethanol, including psychomotor slowing or incoordination. Both drugs modulate dopamine (DA)-related markers in accumbens (Acb), and Acb DA is involved in voluntary locomotion and locomotor sensitization. The present study determined whether caffeine can affect locomotion induced by acute and repeated ethanol administration in adult male CD-1 mice.

Methods: Acute administration of caffeine (7.5 to 30.0 mg/kg) was evaluated for its effects on acute ethanol-induced (1.5 to 3.5 g/kg) changes in open-field horizontal locomotion, supported rearing, and rearing not supported by the wall. DA receptor-dependent phosphorylation markers were assessed: extracellular signal-regulated kinase (pERK), and dopamine-and cAMP-regulated phosphoprotein Mr32kDa phosphorylated at threonine 75 site (pDARPP-32-Thr75) in Acb core and shell. Acutely administered caffeine was also evaluated in ethanol-sensitized (1.5 g/kg) mice.

Results: Acute ethanol decreased both types of rearing. Caffeine increased supported rearing but did not block ethanol -induced decreases in rearing. Both substances increased horizontal locomotion in a biphasic manner, and caffeine potentiated ethanol-induced locomotion. Although ethanol administered repeatedly induced sensitization of locomotion and unsupported rearing, acute administration of caffeine to ethanol-sensitized mice in an ethanol-free state resulted in blunted stimulant effects compared with those seen in ethanol-naïve mice. Ethanol increased pERK immunoreactivity in both subregions of the Acb, but coadministration with caffeine blunted this increase. There were no effects on pDARPP-32(Thr75) immunoreactivity.

Conclusions: The present results demonstrated that, after the first administration, caffeine potentiated the stimulating actions of ethanol, but did not counteract its suppressant or ataxic effects. Moreover, our results show that caffeine has less activating effects in ethanol-sensitized animals.

Key Words: Alcohol, Caffeine, Stimulation, Sensitization, Accumbens.

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, increased alertness and energy (Astorino and Roberson, 2010; Duncan et al., 2012, 2013; Smirmaul et al., 2017). Moreover, its use in association with EtOH has, in the last decades, become widespread in order to counteract the sedative effects and the locomotor impairment of high, intoxicating, doses of EtOH (Attwood et al., 2012; Drake et al., 2003; Hasenfratz et al., 1993). While under laboratory conditions, participants sometimes report feeling less motorically impaired when EtOH is consumed with caffeine (Ferreira et al., 2006; Marczinski and Fillmore, 2006), others do not report feeling less sedated or incoordinated (Arria et al., 2011; Peacock et al., 2014). Moreover, objective measures of motor function have been demonstrated to be largely unaffected by coadministration of caffeine with EtOH (Ulbrich et al., 2013; Weldy, 2010), although some mild stimulating effects have also been observed, such as reductions in reaction time after their combined consumption (Howland et al., 2010).

In animal studies, it has been shown that when acutely administered, both EtOH and caffeine are able to stimulate or suppress locomotor activity in a dose-dependent manner, typically with bell-shaped (or inverted-U) dose–response

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functions (Arizzi-LaFrance et al., 2006; Correa et al., 2001a; Hilbert et al., 2013; López-Cruz et al., 2013). Low doses of caffeine seem to potentiate psychomotor stimulation induced by EtOH (López-Cruz et al., 2014; Waldeck, 1974). However, the impact of higher doses, such as the ones found in energy drinks, is much less known. Moreover, the repeated administration of a stimulant substance may produce sensitization, measured as a potentiation of locomotion (Kawa and Robinson, 2019; Steketee and Kalivas, 2011). Cross-sensitization also has been described, which is said to occur when a new drug shows potentiated stimulating effects in subjects that show sensitization to another drug, for example, among EtOH and cocaine in rats (O'Neil et al., 2015; Xu and 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 EtOH and caffeine induced significantly greater locomotor sensitization than the drugs alone (May et al., 2015), and repeated consumption of both substances produces a robust potentiation of locomotor stimulation in adolescent mice (Fritz et al., 2016).

However, cross-sensitization between both drugs remains largely unexplored. It has been observed, in adolescent mice, that repeated exposure to caffeine-mixed alcohol causes locomotor sensitization not observed in mice exposed to caffeine or alcohol alone (Robins et al., 2016). Sensitization in locomotor exploration is related to the motivational properties of drugs of abuse (Robinson and 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 (DA) system (Robinson and Berridge, 2000; Salamone et al., 2016). EtOH and caffeine act on the DA systems via different mechanisms: EtOH preferentially increases the firing of dopaminergic neurons in the ventral tegmental area leading to an increase in DA transmission in nucleus accumbens (Acb) (Carboni et al., 2000; Di Chiara and Imperato, 1988; Gessa et al., 1985; Vena et al., 2016) while caffeine causes an increase in extracellular DA in prefrontal cortex, but not in Acb (Acquas et al., 2002). 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 EtOH 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 (Acquas et al., 2010; Valjent et al., 2004), and this effect is mediated by D_1 receptors (Acquas et al., 2010).

These 2 drugs also act on the adenosinergic system; EtOH 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; Ferré, 2008; Fuxe et al., 2003), such as Dopamine- and cAMP-regulated phosphoprotein Mr 32 kDa phosphorylated at the threonine 75 (Thr-75) site (pDARPP-32-Thr75), which is associated with activation of DA D_2 receptors (Nunes et al., 2013; Svenningsson et al., 1999).

The present experiments were developed to determine the effects of acute administration of caffeine (7.5, 15, or 30 mg/kg), EtOH (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) produces cross-sensitization in animals sensitized to a repeated dose of EtOH (1.5 g/kg). Furthermore, we assessed the effects of an acute challenge with caffeine on EtOH-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 to 40 g, Janvier, France S.A.) were 8 to 10 weeks old at the beginning of the study. Mice were housed in groups of 3 or 4 per cage, with standard laboratory rodent chow and tap water available *ad libitum*. The colony was kept at a temperature of 22±2°C with lights on from 08:00 to 20:00 hours. 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

EtOH (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, Madrid, Spain) was dissolved in 0.9% w/v saline and administered IP 30 minutes 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., 2014, 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 EtOH on spontaneous locomotion and locomotor sensitization (Correa et al., 2004). The floor of the cylinder was divided into 4 equal quadrants by 2 intersecting lines drawn on the floor. The behavioral test room was illuminated with a soft light, and external noise was attenuated. Animals were placed in the center of the cylinder and videotyped for 10 minutes. Horizontal and vertical locomotion in the OF was simultaneously recorded. In a posterior session, researchers, blind to the experimental condition, evaluated manually these variables. Interreliability between researcher was set at 95%. For horizontal locomotion, an activity count was registered each time the animal crossed from 1 quadrant to another with all 4 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), 2 groups of animals received either vehicle or EtOH and were introduced in the OF (session 1). This treatment was repeated in alternating days until a total of 5 sessions were performed. In the cross-sensitization test (experiment 6), these same animals were further divided into 3 groups, and 2 days after session 5, they received a single dose of vehicle, caffeine 15 mg/kg, or caffeine 30 mg/kg.

Blood-EtOH Determinations. Additional mice were used to determine whether caffeine influenced blood-EtOH 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 EtOH (1.5 or 2.5 g/kg). Trunk blood samples (20 µl) were collected 10 and 20 minutes after EtOH 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 were added. After centrifugation at 4°C (5 minutes, 12,000 rpm), the supernatant was removed and blood-EtOH concentrations were determined by headspace gas chromatography with a flame-ionized detector (CE Instruments GC 8000, HS 850).

pERK Immunohistochemistry. Mice (n = 5 for SAL-SAL, n = 7 for SAL-EtOH, n = 7 for CAF15-EtOH and n = 7 for CAF30-EtOH) were anesthetized with carbon dioxide for 30 s and perfused 15 minutes after the last treatment. The time interval between EtOH administration and anesthesia was selected on the basis of the time of the peak effect on DA transmission (Ibba et al., 2009; Melis et al., 2007). Brains were collected and stored in paraformaldehyde solution during 24 hours and refrigerated in sucrose (30%), sodium azide (2%) and phosphate buffer PB (0.1 M) solution prior to slicing. Free floating coronal sections (40 µm) were serially cut using a microtome cryostat (Weymouth, MA) according to plates 21 to 23 (approximately from AP 1.18 to AP 0.98 mm from bregma for the AcbC and AcbSh) of the mouse brain atlas (Paxinos and Franklin, 2001). After rinsing in 0.01 M phosphate-buffered saline (PBS) (pH 7.4) (3 times for 10 minutes) and incubating for 30 minutes with 1% hydrogen peroxide and after 3 rinses of 30 minutes each 1, the slices were incubated for 1 hour 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, catalog number #9101; Cell Signaling Technology, Beverly, MA) 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 Copenhagen, Denmark) for 1.5 hour on a rotating shaker at room temperature. Finally, sections were washed and rinsed for 1 to 3 minutes in 3,3-diaminobenzidine chromogen (DAKO Copenhagen, Denmark).

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 (n = 5 for SAL-SAL, n = 8 for SAL-EtOH, n = 8 for CAF15-EtOH and n = 7 for CAF30-EtOH). Brain slices were rinsed in 0.01 M PBS (pH 7.4) and incubated in 1% hydrogen peroxide for 30 minutes to block endogenous staining. Sections were then rinsed in 0.01 M PBS (pH 7.4) (3 times for 5 minutes). 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; catalog number #2301; Cell Signaling Technology). The antibody was dissolved in solutions that also contained 3% BSA and 0.1% T.X in PBS for 24 hour of incubation at 4°C. After the primary antibody treatment, the sections were rinsed in PBS (3 times for 5 minutes) and incubated in the secondary antibody, anti-rabbit HRP conjugate envision plus (DAKO) for 1.5 hour on a rotating shaker at room temperature. Finally, sections were washed and rinsed for 1 to 3 minutes in 3,3-diaminobenzidine chromogen (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-Aldrich, Merck KGaA) as a mounting medium. The sections were examined and photographed using a Nikon Eclipse E600 (Melville, NY) upright microscope equipped with an Insight Spot digital camera (Diagnostic Instruments, Sterling Heights, MI, USA Inc). Images of the regions of interest were magnified at 20× 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 3 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, nonorthogonal 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 1. A probability level of 0.05 or smaller was used to indicate statistical significance. Statistics were done using STATISTICA 8 (StatSoft Inc., Tulsa, OK) 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 minutes 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 1*A*). These same doses of caffeine also produced significant increases in the number of supported rears ($p < 0.05$) (Fig 1*B*). No significant effect of caffeine treatment on unsupported rearing was observed, $F(3, 30) = 0.45$, n.s. (Fig 1*C*).

Experiment 2. Effects of Acute Administration of EtOH on Several Measures of Locomotion

Figure 2 shows the effects of EtOH (0.0, 1.5, 2.5, or 3.5 g/kg) administered 10 minutes before the OF test (N = 40).

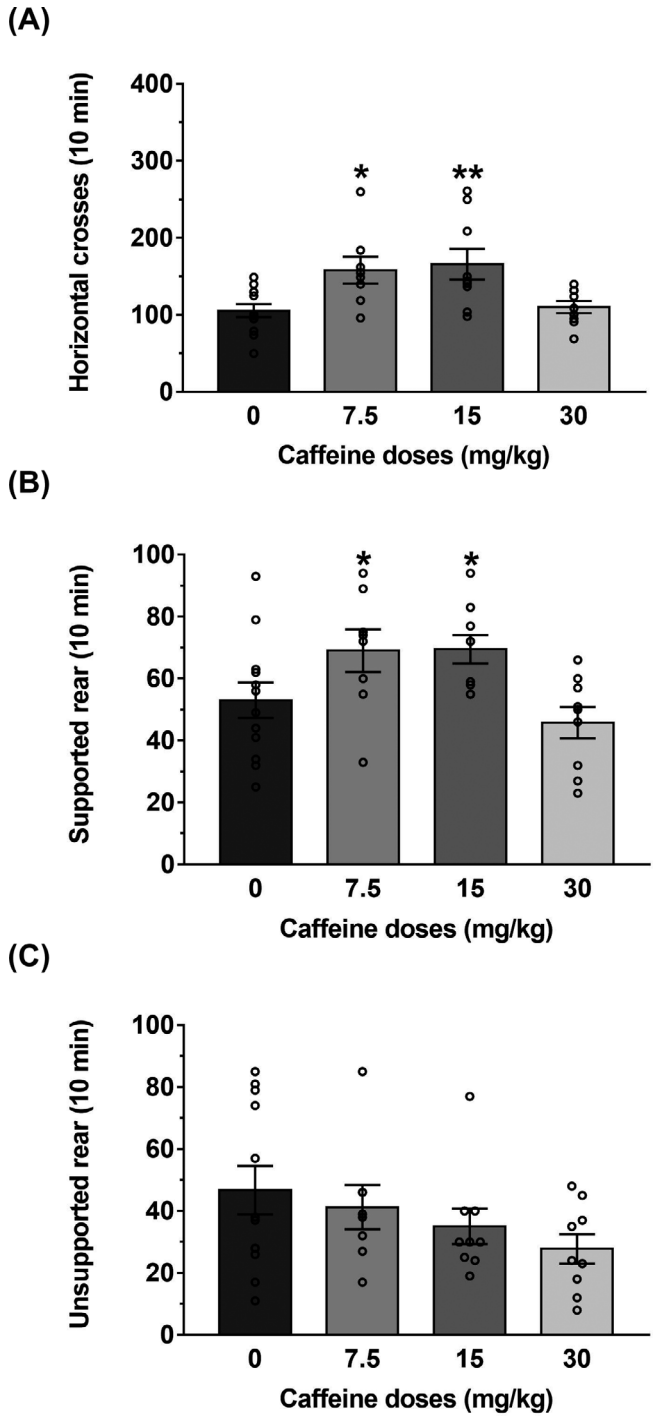

Fig. 1. Effects of acute administration of caffeine (0, 7.5, 15, or 30 mg/kg) on horizontal locomotion (**A**), supported rearing (**B**), and unsupported rearing (**C**) in the OF. Data are expressed as mean (±SEM) number of counts during 10 minutes. $^{**}p < 0.01$, $^{*}p < 0.05$ significantly different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

One-way ANOVA revealed an overall effect of EtOH 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 EtOH significantly increased horizontal crosses at the dose of 2.5 g/kg ($p < 0.05$) (Fig 2*A*). Supported rearing was decreased by the highest doses of EtOH (2.5 and 3.5 g/kg, $p < 0.01$) (Fig 2*B*). All EtOH doses significantly decreased unsupported rearing ($p < 0.01$) (Fig 2*C*).

Experiment 3. Effects of Acute Administration of Caffeine and EtOH on Several Measures of Locomotion

Figure 3 shows the effects of caffeine (0, 15, or 30 mg/kg) and EtOH (0.0, 1.5, 2.5, or 3.5 g/kg) combination in mice ($N = 115$) evaluated in the OF. Factorial ANOVA (Caffeine x EtOH) showed an overall effect of caffeine, $F(2, 112) = 11.18$, $p < 0.01$, EtOH, $F(3, 112) = 59.35$, $p < 0.01$, and caffeine– EtOH 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, EtOH 1.5 g/kg produced a significant increase in locomotion compared with caffeine (0 mg/kg) + EtOH (1.5 g/kg)-treated mice ($p < 0.05$). In addition, caffeine (15 and 30 mg/kg) + EtOH (2.5 g/kg) groups were significantly different from caffeine (0 mg/kg) + EtOH (2.5 g/kg)-treated mice ($p < 0.01$). Moreover, caffeine (0 mg/kg) + EtOH (2.5 g/kg) was significantly different compared with vehicle + vehicle group ($p < 0.01$) (Fig 3*A*). The factorial ANOVA (Caffeine × EtOH) for the variable supported rearing, as a measure of vertical locomotion, also showed an overall effect of caffeine, $F(2, 112) = 3.81$, $p < 0.05$, EtOH, $F(3, 112) = 62.26$, $p < 0.01$, 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 with control ($p < 0.01$). Among EtOH 1.5 g/kg treated mice, both caffeine (15 and 30 mg/kg)-treated groups increased supported rearing compared with caffeine (0 mg/kg) + EtOH (1.5 g/kg) group ($p < 0.01$). In addition, caffeine (0 mg/kg) + EtOH (2.5 and 3.5 g/kg) groups were significantly different from vehicle control group ($p < 0.01$) (Fig 3*B*). Finally, the factorial ANOVA (Caffeine x EtOH) for unsupported rearing (Fig 3*C*) showed a significant effect of EtOH treatment, $F(3, 112) = 66.89$, $p < 0.01$. However, there was no significant effect of caffeine, $F(2, 112) = 0.94$, n.s., and no significant interaction, $F(6, 112) = 0.83$, n.s.

Experiment 4. Effects of Caffeine Administration on Blood-EtOH Levels

Additional mice ($N = 24$) were used to determine whether caffeine influenced blood-EtOH levels after motor stimulating doses. Animals received caffeine (0 or 30 mg/kg) and 20 minutes later EtOH (1.5 or 2.5 g/kg) was administered. Two-way factorial ANOVA (EtOH x Caffeine) showed a significant effect of EtOH, $F(1, 25) = 326.82$, $p < 0.01$, 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 EtOH coadministered with caffeine are not due to changes in blood-EtOH concentration.

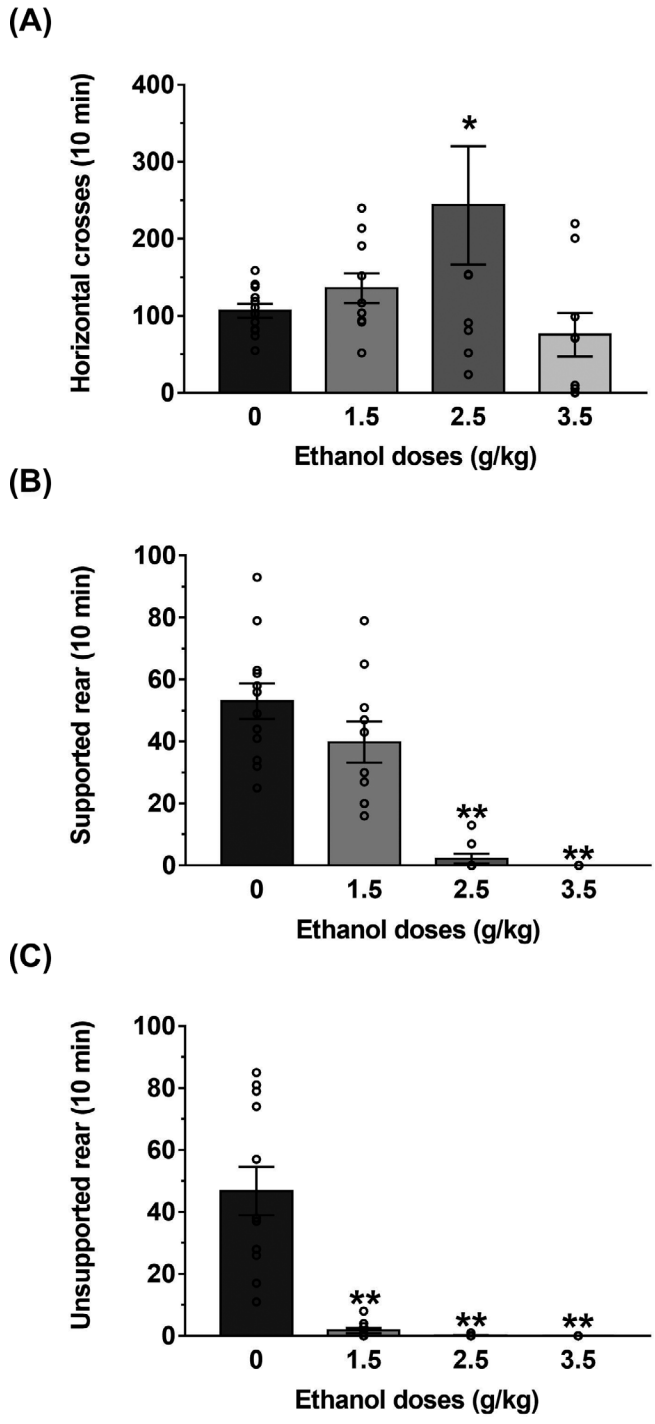

Fig. 2. Effects of acute administration of ethanol (EtOH) (0, 1.5, 2.5, or 3.5 g/kg) on horizontal locomotion (**A**), supported rearing (**B**), and unsupported rearing (**C**) in the OF. Data are expressed as mean (±SEM) number of counts during 10 minutes. $^{**}p < 0.01$, $^{*}p < 0.05$ significantly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

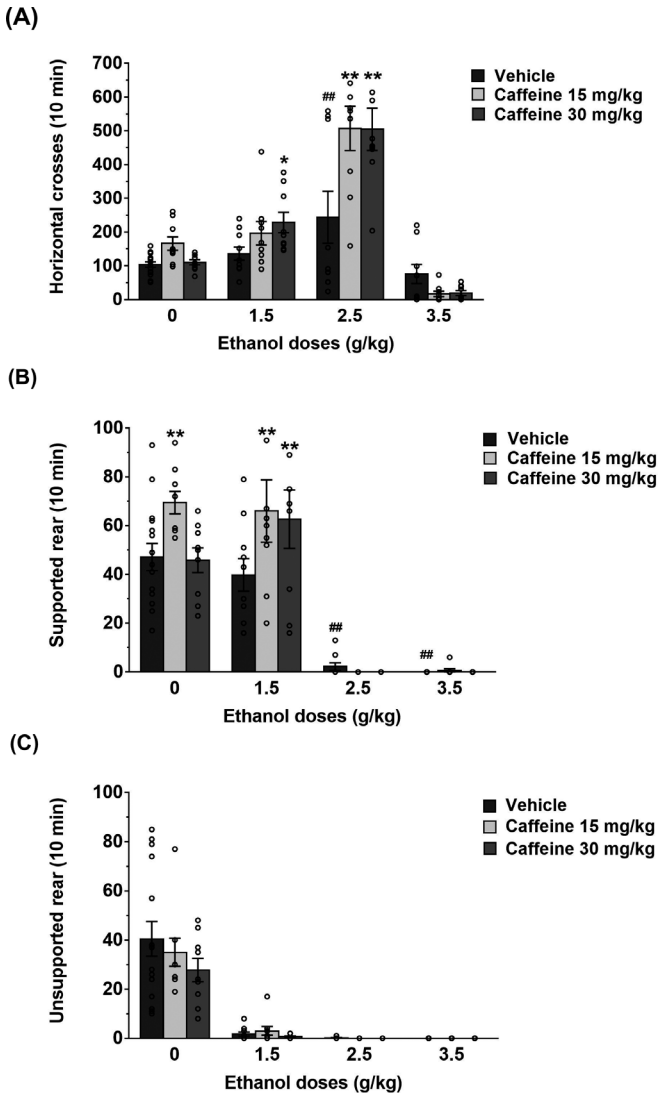

Fig. 3. Effects of acute coadministration of caffeine (0, 15, or 30 mg/kg) and ethanol (EtOH) (0, 1.5, 2.5, or 3.5 g/kg) on horizontal locomotion (**A**), supported rearing (**B**), and unsupported rearing (**C**) in the OF. Mean (±SEM) number of counts during 10 minutes. $^{**}p < 0.01$, $^{*}p < 0.05$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

Experiment 5. Effects of Repeated Administration of EtOH on Several Measures of Locomotion

Figure 4 shows the effects of repeated EtOH (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 (EtOH dose: 0 or 1.5 g/kg EtOH) 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 EtOH (1.5 g/kg) compared with vehicle-treated mice in the first session ($p < 0.01$), and the EtOH-treated group in session 5 was also significantly different compared with the vehicle group in the same session ($p < 0.01$). Moreover, EtOH in session 5 further increased locomotion compared with session 1 ($p < 0.01$). This

Table 1. Effect of Caffeine on Blood-Ethanol (EtOH) Levels

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

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

increase in locomotion over sessions was not observed in the vehicle-treated groups, suggesting a sensitization of locomotion induced by EtOH (Fig 4*A*). The factorial ANOVA for the variable supported rearing showed a significant effect of EtOH 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 EtOH dose x session interaction, $F(1, 64) = 4.61$, $p < 0.05$ (Fig 4*B*). Planned comparisons showed that although EtOH 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 EtOH 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 4*C*).

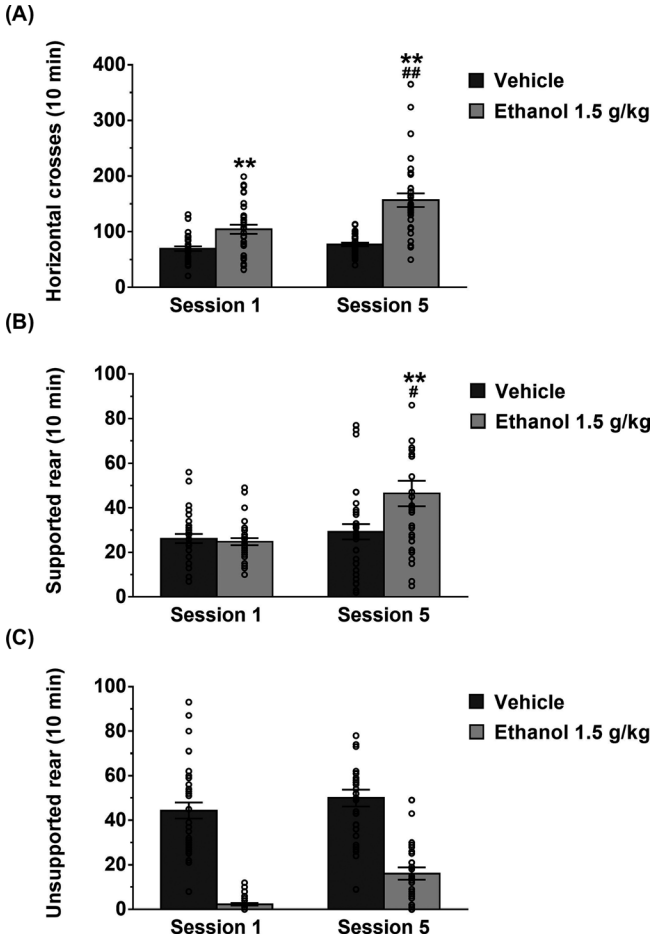

Fig. 4. Effects of repeated administration of ethanol (EtOH) (0.0 or 1.5 g/kg) on horizontal locomotion (**A**), supported rearing (**B**), and unsupported rearing (**C**) in the OF. Data are expressed as mean (±SEM) number of counts during 10 minutes. $^{**}p < 0.01$, $^{*}p < 0.05$ significantly different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

Experiment 6. Effects of Acute Administration of Caffeine on EtOH-Induced Locomotor Sensitization in Several Behavioral Measures

Figure 5 shows the effects of an acute administration of caffeine on EtOH-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 whether there was a cross-sensitization effect. The factorial ANOVA, previous EtOH treatment (0 or 1.5 g/kg) × caffeine dose (0, 15, or 30 mg/kg), showed an overall effect of previous EtOH 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 5*A*). 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 EtOH (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 EtOH (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 5*A*).

The same pattern of results was observed on the other 2 variables, supported (Fig 5*B*) and unsupported rearing (Fig 5C). Thus, for supported rearing the results were as follows: EtOH 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 EtOH-pretreated group ($p < 0.01$) compared with its vehicle group, and this dose produced a blunted induction of supported rearing that showed significant differences between the 2 caffeine 15 mg/kg groups ($p < 0.05$). In addition, caffeine 30 mg/kg decreased supported rearing in the EtOH (1.5 g/kg)-pretreated group compared with the same dose of caffeine in the EtOH (0 g/kg)-pretreated group ($p < 0.05$) (Fig 5*B*).

Finally, the same pattern of results was observed on unsupported rearing (Fig 5*C*), $F(1, 65) = 4.87$, $p < 0.05$; $F(2, 65) = 10.90$, $p < 0.01$; $F(1, 65) = 5.36$, $p < 0.01$, respectively. While caffeine at both doses increased unsupported rearing

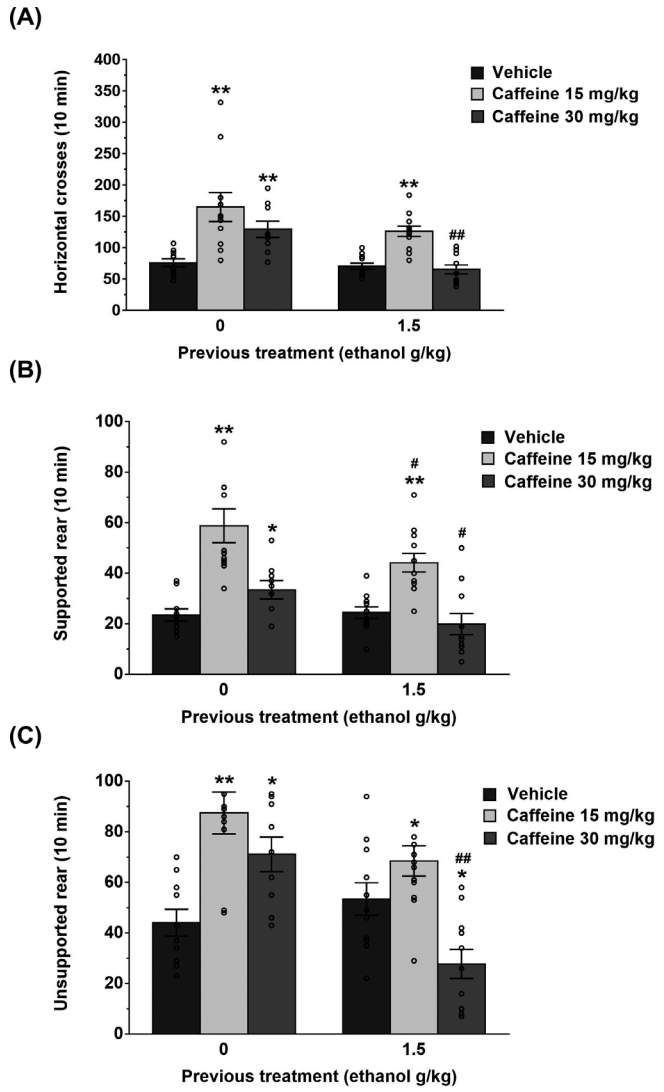

Fig. 5. Effects of caffeine (0, 15, or 30 mg/kg) in mice treated with ethanol (0 or 1.5 g/kg) in previous days on horizontal locomotion (**A**), supported rearing (**B**), and unsupported rearing (**C**) in the OF. Data are expressed as mean (±SEM) number of counts during 10 minutes. $^{**}p < 0.01$, $^{*}p < 0.05$ 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.

in the vehicle-pretreated group ($p < 0.01$ and $p < 0.05$, respectively), only caffeine 15 mg/kg increased unsupported rearing in the EtOH (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 with its vehicle. Moreover, the effect of caffeine 30 mg/kg in the EtOH (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 EtOH-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 minutes before the administration of EtOH (0 or 1.5 g/kg) on the number of pERK- and pDARPP-32(Thr75)-positive neurons in the AcbC and AcbSh ($N = 28$). 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 in pERK expression after caffeine (0 mg/kg) + EtOH (1.5 g/kg) treatment relative to caffeine (0 mg/kg) + EtOH (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) + EtOH (1.5 g/kg) in the AcbC and AcbSh ($p < 0.01$, for both doses in both structures).

Conversely, 1-way ANOVA did not reveal 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 adult male CD-1 mice, we investigated the acute interaction of caffeine and EtOH, 2 of the most widely used psychoactive drugs by humans, on different measures of locomotor exploration in an OF: horizontal and vertical locomotion. Moreover, vertical locomotion was separated in 2 parameters, one more dependent on postural coordination (nonsupported rearing) and another less so (wall-supported rearing).

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

Accordingly, in the present studies, acute intraperitoneal administration of caffeine or EtOH showed a dose-dependent effect on locomotion, with low and moderate doses (caffeine 7.5 and 15 mg/kg, and EtOH 2.5 g/kg) stimulating, and high doses (caffeine 30 mg/kg and EtOH 3.5 g/kg) reducing horizontal locomotion compared with 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, EtOH dose dependently decreased both types of rearing.

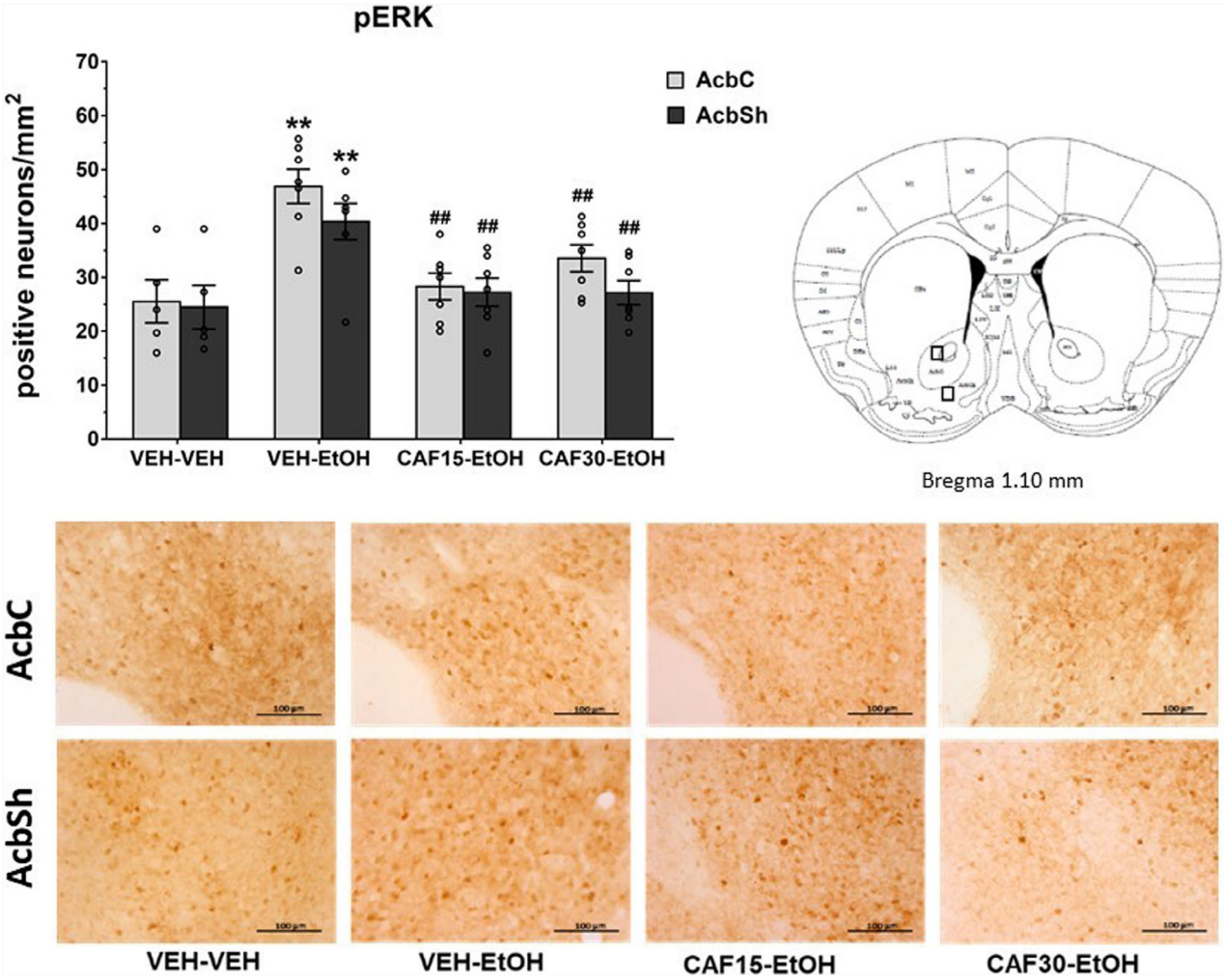

Fig. 6. Effects of acute administration of caffeine on the expression of ethanol (EtOH)-elicited pERK-positive neurons in Acb. Data are expressed as mean (±SEM) of the number of positive neurons/mm^2. Right upper parts: effect of caffeine acute treatment (0, 15, and 30 mg/kg) on the expression of pERK-positive neurons in mice treated with EtOH (0, 1.5 g/kg). Left upper part: diagram of a coronal section with bregma coordinates from Paxinos and Franklin (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 VEH/VEH (caffeine 0 mg/kg + EtOH 0 g/kg) groups in the AcbC and AcbSh. $^{\#\#}p < 0.01$ significantly different from VEH/ETOH (caffeine 0 mg/kg + EtOH 1.5 g/kg) groups in the AcbC and AcbSh.

Interestingly, acute administration of both drugs revealed that stimulant (15 mg/kg) and nonstimulant (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 EtOH. Moreover, caffeine (30 mg/kg) increased horizontal locomotion and supported rearing in combination with a dose of EtOH (1.5 g/kg) that administered alone was not stimulatory. However, at the highest dose of EtOH (3.5 g/kg) caffeine was not able to reverse EtOH's effects in any of the 3 locomotion parameters.

Our results are similar to previous results, in terms of the effects of drug combinations. Caffeine in combination with low doses of EtOH (1.75 g/kg) potentiated stimulation and at higher EtOH doses (2.5 and 3.25 g/kg) caffeine potentiated reductions in locomotion (Hilbert et al., 2013). Thus, the synergistic activity of the 2 substances is revealed at low doses, while if 1 of the 2 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 (Hilbert et al., 2013; Waldeck, 1974). For example, 100 mg/kg of caffeine totally suppresses the locomotor activity induced by a low dose of EtOH (1 g/kg) (Waldeck, 1974).

In terms of coordination, the oral administration of a low dose of caffeine (10 mg/kg) has demonstrated to reduce EtOH-induced ataxia (Kuribara et al., 1992), and, when both substances are coconsumed, caffeine was also found to mitigate alcohol-induced sedation and ataxia (Fritz et al., 2014). It has been suggested that EtOH-induced ataxia may be regulated by cerebellar adenosinergic A1 and GABAergic A receptors (Dar, 2014). There is a functional similarity between GABA(A) and adenosine A(1) receptors acting as comodulators of EtOH ataxia, even though both receptor

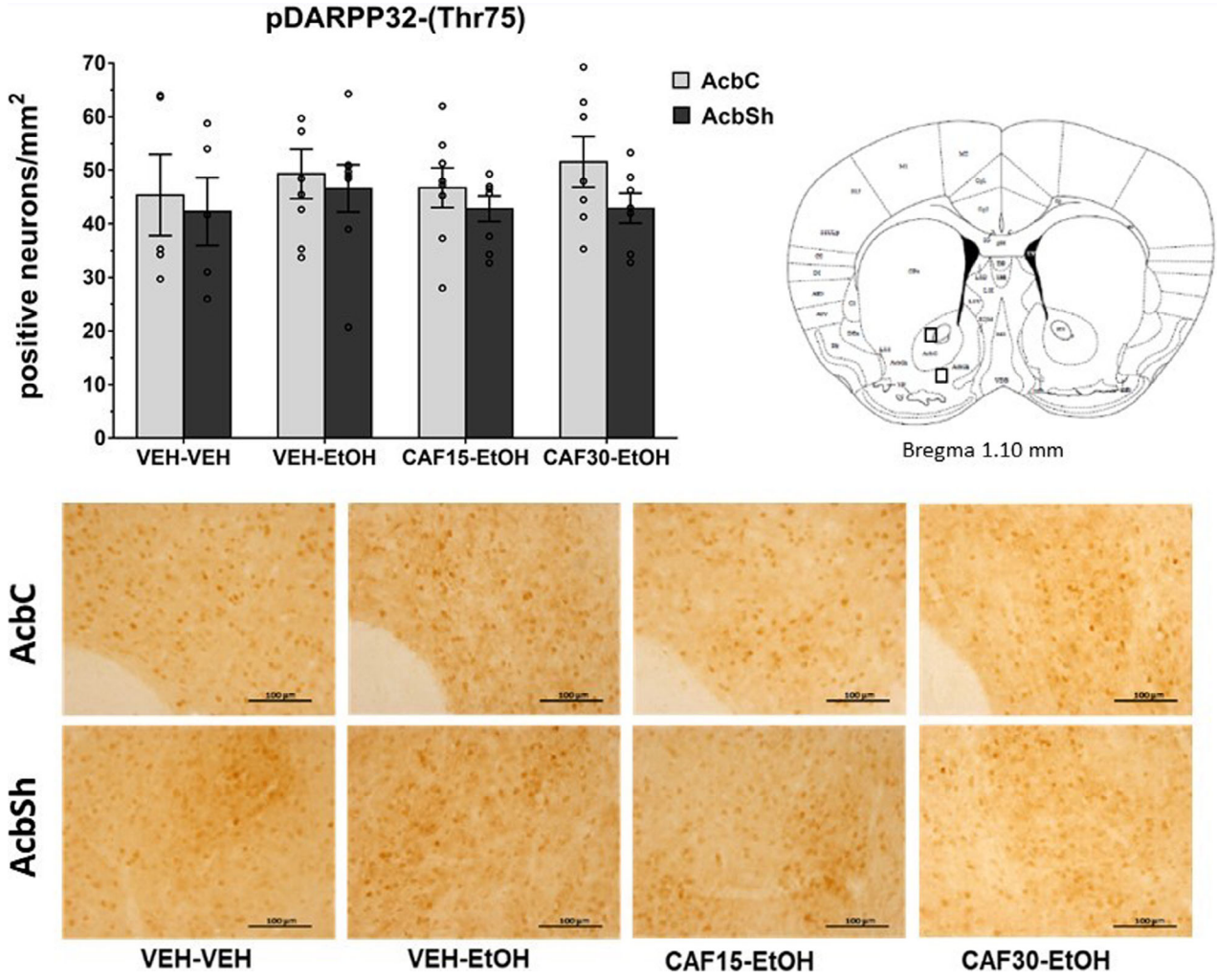

Fig. 7. Effects of acute administration of caffeine on the expression of ethanol (EtOH)-elicited pDARPP-32(Thr75)-positive neurons in Acb. Data are expressed as mean (±SEM) of the number of positive neurons/mm^2. Right upper parts: effect of caffeine acute treatment (0, 15, and 30 mg/kg) on the expression of pDARPP-32(Thr75)-positive neurons in mice treated with EtOH (0, 1.5 g/kg). Left upper part: diagram of a coronal section with bregma coordinates from Paxinos and Franklin (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).

types are known to couple to different signaling systems (Dar, 2006). Thus, caffeine acting as an antagonist of A1 receptors (Ferré et al., 2008) could be reversing part of the ataxic effects of EtOH mediated by the cerebellum. However, in our studies caffeine was not only not effective at blocking EtOH-induced ataxia, but it further suppressed rearing, indicating that it must be a different mechanism or an additional brain area involved for the synergistic actions of both substances at some doses. Thus, in our study, a high dose of EtOH (3.5 g/kg) coadministered with moderate doses of caffeine (15 or 30 mg/kg) produced an even deeper suppression. Similarly, intracerebroventricular administration of caffeine at low doses (less than 25 micrograms) dose dependently reduced EtOH-elicited incoordination in mice, while a high dose (75 μg) it potentiated it (Dar, 1988).

In addition, we also evaluated the impact of acute administration of caffeine on EtOH-induced sensitization, which involves a progressive increase in the motor response resulting from repeated, intermittent EtOH exposure (Camarini and Pautassi, 2016). There is very little evidence of cross-sensitization between EtOH 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 EtOH (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 EtOH exposure in this particular context. On the other hand, there was no cross-sensitization after acute administration of caffeine to EtOH-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 EtOH-preexposed group these different stimulatory effects were blunted significantly for the low dose of caffeine and they completely 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.

EtOH acutely and repeatedly has been shown to increase DA release in Acb in mice (Pavón et al., 2019) and in rats (Bassareo et al., 2019; Vena et al., 2016). 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; De Luca *et al*. 2007). We have previously observed in rats that EtOH (at an intragastric dose of 1.0 g/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 2 Acb subregions (Acquas et al., 2010; Valjent et al., 2004). Interestingly, in the present study using mice, EtOH (1.5 g/kg) significantly induced pERK, and this effect was counteracted by caffeine at both doses in both AcbC and AcbSh. These results are consistent with a recent paper from our laboratory in which caffeine (3 and 15 mg/kg) blocked EtOH (2.0 g/kg) induction of pERK in both subregions of Acb as well as 2 nuclei in the amygdala in this same strain of mice (Porru et al., 2020).

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 EtOH would lead to an increase in pDARPP-32-Thr75 in D_2 containing neurons. Consistent with models of striatal function and DA-related signal transduction, EtOH (1.5 g/kg) in rats has shown to increase phosphorylation of DARPP-32 at Thr34 in striatum (Nuutinen et al., 2011), and EtOH -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). However, in our study, pDARPP-32(Thr75) was not affected by EtOH or by the EtOH plus caffeine combination.

In conclusion, despite the popular assumptions about the ability of caffeine in energy drinks to counteract the motor impairments induced by EtOH, the present results using animal models of motor stimulation and of coordination demonstrated that, after the first administration, caffeine can potentiate the stimulating actions of EtOH but did not counteract its suppressant and ataxic effects. Moreover, our results show that the activating actions of caffeine are blunted in EtOH-sensitized animals. The relevance of choosing a range of doses and behavioral tests that in animal models mimic the effects of both drugs on human behavior is required in order to extrapolate conclusions. However, although there are obvious translational limitations due to species differences, the 2 highest doses (15 and 30 mg/kg) used in the present studies are considered moderate and high according to previous mice studies showing a range of normal behavioral effects that go from mild motor stimulation with little incoordination and mild anxiety (López-Cruz et al., 2014). However, it is important to recognize that animal doses should not simply be extrapolated to a human equivalent dose (HED) by a simple conversion based on body weight (Reagan-Shaw et al., 2008). The US Food and Drug Administration has proposed that to use the body surface area (BSA), normalization method gives a better estimate when converting a dose for translation from animals to humans (Reagan-Shaw et al., 2008). BSA correlates across several mammalian species with several parameters such as oxygen utilization, caloric expenditure, basal metabolism, blood volume, circulating plasma proteins, and renal function (Reagan-Shaw et al., 2008). Thus, according to this conversion, a dose of 15 mg/kg in a mouse would be a HED of 1.2 mg/kg. This means that in a person that weights 100 kg, a dose of 1.2 mg/kg would be a total amount of 120 mg, which is a normal amount of caffeine consumption in a day (less than 2 cups of coffee), and which is below the maximum recommended for the European Food Safety Authority and Health Canada (400 mg per day for adults), and is very close to 144.2 mg which corresponds to the average dose seen in representative European surveys who examined young adults (Mackus et al., 2016).

Our studies also have identified at least 1 potential brain area in which caffeine can block the stimulating actions of EtOH, that is, the nucleus Acb, a critical area for the regulation of the activational component of motivation in humans and other animals (Salamone and Correa, 2012). This brain region also appears to be very important for the regulation of voluntary locomotion, novelty-induced exploration, and reinforcement-seeking behaviors for natural reinforcers and drugs such as EtOH, which are phenomena that are associated with EtOH consumption, abuse, and addiction.

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CONFLICT OF INTEREST

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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