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Effects of Caffeine on Ethanol-elicited place preference, place aversion and ERK phosphorylation in CD-1 mice

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Running title: Caffeine prevents ethanol-elicited motivation

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56

57 ## Abstract

58 Epidemiological studies indicate a rise in the combined consumption of caffeinated and alcoholic
59 beverages which can lead to increased risk of alcoholic beverages overconsumption. However, the
60 effects of the combination of caffeine and ethanol in animal models related to aspects of drug
61 addiction are currently still underexplored.

62 To characterize the pharmacological interaction between caffeine and ethanol and establish if
63 caffeine can affect the ability of ethanol (2 g/kg) to elicit conditioned place preference (CPP) and
64 conditioned place aversion (CPA), we administered caffeine (3 or 15 mg/kg) to male CD-1 mice
65 before saline or ethanol. Moreover, we determined if these doses of caffeine could affect ethanol
66 (2 g/kg)-elicited Extracellular-signal Regulated Kinase (ERK) phosphorylation (pERK) in brain areas,
67 nucleus accumbens, bed nucleus of stria terminalis, central nucleus of the amygdala and basolateral
68 amygdala, previously associated to this type of associative learning.

69 In the place conditioning paradigm, caffeine did not have an effect on its own, whereas ethanol
70 elicited significant CPP and CPA. Caffeine (15 mg/kg) significantly prevented the acquisition of
71 ethanol-elicited CPP and, at both doses, also prevented the acquisition of ethanol-elicited CPA.
72 Moreover, both doses of caffeine also prevented ethanol-elicited pERK expression in all brain areas
73 examined.

74 The present data indicate a functional antagonistic action of caffeine and ethanol on associative
75 learning and ERK phosphorylation after an acute interaction. These results could provide exciting
76 grounds for further studies**, also in translational perspective,** of their pharmacological interaction
77 modulating other processes involved in drug consumption and addiction.

78

79 **Keywords:** Caffeine; Conditioned Place Preference (CPP); Conditioned Place Aversion (CPA);
80 Ethanol; Extended amygdala; Extracellular-signal Regulated Kinase (ERK).

81

82 ## Introduction
83
84 Ethanol and Caffeine are two of the most widely consumed **recreational and psychotropic**
85 substances in the world. Ethanol, depending on the dose, can have stimulant and also sedative
86 effects that may lead to alcohol use disorders (AUD), including ethanol dependence, and compulsive
87 ethanol intake (Koob and Volkow, 2009). Caffeine is a minor stimulant that is often consumed in the
88 form of drinks like coffee, tea, herbal teas or sodas and in recent years also as an ingredient of
89 "energy drinks" (Peacock et al., 2015; Reissig et al., 2009; Scholey and Kennedy, 2004). Energy drinks
90 contain relatively high caffeine concentrations (e.g. 50–500 mg per serving), and are frequently
91 consumed by teenagers and young adults in order to reduce fatigue and to improve cognitive
92 performance by increasing memory and concentration (Lalanne et al., 2017). Thus, combined intake
93 of alcoholic beverages and energy drinks is grounded on the popular belief that caffeine antagonizes
94 the intoxicating and sedative effects of high ethanol concentrations (Marczinski, 2011; Reissig et al.,
95 2009; Weitzman et al., 2003).

96 However, very little is known on the subtle consequences of caffeine-ethanol pharmacological
97 interactions at low doses on motivated behaviors and learning processes that can lead to the
98 development of maladaptive patterns of behavior, thus increasing the risk for alcoholic beverages
99 overconsumption and addiction. The results reported in the preclinical literature are often complex
100 and contradictory, and the nature of the interaction between caffeine and ethanol varies across
101 doses and behavioral tasks used. For instance, in mice, low doses of caffeine (5-10 mg/kg) increase
102 ethanol consumption, but higher doses (20 mg/kg) reduce intake (SanMiguel et al., 2019). On the
103 other hand, although low doses of caffeine are able to improve memory acquisition and retention
104 in different learning models (Angelucci et al., 2002; Dash et al., 2004; Spinetta et al., 2008), caffeine
105 in a wide range of doses (5-40 mg/kg) does not reverse the learning deficits caused by low doses of
106 ethanol (1.0-1.4 g/kg) in a plus-maze discriminative avoidance task (Gulick and Gould, 2009) or in
107 social recognition task (López-cruz et al., 2016).

108 Conditioned place preference (CPP) and conditioned place aversion (CPA) are associative
109 processes developed as a consequence of drug pairings with neutral stimuli that, then, can acquire
110 motivational properties which are similar to those of the drug (Tzschentke, 2007). These
111 associations are key factors in the development of stimuli and contextual salience that can trigger
112 drug seeking and drug intake (Blanco-Gandía et al., 2018). Ethanol, **as discussed originally**
113 **(Cunningham et al., 1997) following appropriate inter stimulus intervals (ISI),** has consistently

114 demonstrated to induce both CPP and CPA (Cunningham et al., 2002, 2003; Cunningham and
115 Henderson, 2000; Font et al., 2006; Peana et al., 2008; Rosas et al., 2017; Spina et al., 2015). **This
116 peculiarity was interpreted arguing that ethanol, immediately after its intraperitoneal
117 administration, may exert an initial aversiveness (due to peritoneal irritation or sudden transition
118 from the sober to the intoxicated state) succeeded shortly after by pleasant feelings. Thus,
119 depending on the contiguity (i.e. length of ISI) and direction (backward or forward) of
120 unconditioned and conditioned stimuli association, this could result in conditioned preference or
121 aversion, respectively (Cunningham et al., 1997)**. In contrast, studies on the effects of caffeine on
122 place conditioning have reported inconclusive results, mostly due to substantially different doses,
123 different animal species and schedules of administration across studies (Brent Bedingfield et al.,
124 1998; Brockwell et al., 1991; Hsu et al., 2009; Patkina and Zvartau, 1998). Moreover, the studies that
125 investigated the effects of caffeine and ethanol co-administration on place conditioning reported
126 that caffeine, administered either alone (3 mg/kg) (Brockwell et al., 1991) or, in the same injection,
127 with ethanol (3 mg/kg of caffeine and 1.75 g/kg of ethanol), did induce a significant CPP, although
128 this effect was more modest than that of ethanol itself (Hilbert et al., 2013). In addition, a single
129 administration of caffeine was reported as being able to reduce the expression of cocaine-elicited
130 CPP (Poleszak and Malec, 2002), both acquisition and expression of ethanol-elicited CPP as well as
131 reinstatement of ethanol-elicited CPP (Okhuarobo et al., 2019).

132 The Extracellular signal-Regulated Kinase (ERK) is part of the Mitogen-Activated Protein Kinase
133 (MAPK)-signaling cascade that plays a critical role in signal transduction, neuroplasticity, gene
134 expression, and behavioral changes underlying the reinforcing processes induced by substances of
135 abuse (Valjent et al., 2005). In particular, the active form of this protein, phosphorylated ERK (pERK),
136 plays a key role in the acute effects of ethanol. Increases in ERK expression following acute ethanol
137 administration has been demonstrated in several brain areas, including both the core (AcbC) and
138 shell (AcbSh) subregions of the nucleus accumbens (Ibba et al., 2009), basolateral amygdala (Spanos
139 et al., 2012) and in other nuclei that are part of the extended amygdala, such as the bed nucleus of
140 stria terminalis (BNST), and the central nucleus of the amygdala (CeA) (Ibba et al., 2009). These brain
141 areas are involved in positive and negative effects of ethanol on motivational processes and in the
142 development of dependence (Koob et al., 1998). Moreover, pERK expression is related to associative
143 properties of drugs as demonstrated by CPP experiments (Gerdjikov et al., 2004; Lu et al., 2006;
144 Rosas et al., 2017; Salzmann et al., 2003; Spina et al., 2010; Valjent et al., 2001, 2000) or by self-
145 administration studies (Peana et al., 2013) whereby phosphorylated ERK appears involved in the

146 acquisition of motivational valence by neutral stimuli paired with the primary effects of drugs of
147 abuse (Gerdjikov et al., 2004; Rosas et al., 2017; Valjent et al., 2001).

148 Hence, in order to shed light on the psychopharmacological consequences of the interaction
149 between caffeine and ethanol, the aims of this study were: **1)** determining if caffeine pre-treatment,
150 at the doses of 3 and 15 mg/kg, may affect the acquisition of ethanol (2 g/kg)-elicited CPP and CPA
151 (Cunningham et al., 1997; Rosas et al., 2017; Spina et al., 2015) and **2)** investigating if caffeine, at
152 these doses, may have the ability to affect ethanol-elicited increases of pERK expression in the brain
153 areas (Ibba et al., 2009) mentioned above. We choose a dose of ethanol (2 g/kg) that in CD-1 mice
154 has demonstrated to reliably elicit robust CPP and CPA (Cunningham et al., 1997; Rosas et al., 2017;
155 Spina et al., 2015). Moreover, the study was also undertaken to verify if the stimuli associated with
156 ethanol during CPP or CPA may induce a differential expression of pERK in the brain areas examined,
157 and if the effects of caffeine on the acquisition of place conditioning may also be reflected in the
158 differential expression of phosphorylated ERK in these brain areas important for associative and
159 motivational processes involved in drug addiction.

160

161 Materials and Methods

162

163 Animals

164 Adult male CD-1 mice (22-24 g, Charles River, Calco, Italy) (N=188) were housed in groups of four
165 per cage for at least 6 days before the experiments began, under a 12:00/12:00 h light/dark cycle
166 (lights on at 08:00 a.m.) with food (Mucedola Srl, Settimo Milanese (Milan) Italy) and water available
167 *ad libitum*. All the experiments were carried out during the light phase, between 09:00 and 16:00 h.
168 The total numbers of mice were n=89 and n=80 in the CPP and CPA experiments, respectively. From
169 those animals, a group of n=19 and n=20 were used in the immunohistochemistry experiments after
170 CPP and CPA expression, respectively. **Different subjects were used for the CPP and CPA**
171 **experiments.** In addition, a new group of mice (n=19) was used in the immunohistochemical
172 experiments upon acute drug administrations. All the experimental procedures were performed in
173 accordance with the Principles of laboratory animal care, with the guidelines and protocols
174 approved by the European Union (2010/63/UE L 276 20/10/2010) and with the approval
175 (1177/2016) of the local Committee. Every possible effort was made to minimize animal pain and
176 discomfort and to reduce the number of experimental subjects.

Drugs

Ethanol (Sigma-Aldrich, Milan, Italy) 20% (v/v) was dissolved in isotonic saline, and was administered at the dose of 2 g/kg (12 ml/kg volume injection). Caffeine (Sigma-Aldrich, Milan, Italy) was dissolved in isotonic saline (10 ml/kg volume injection), and was administered at the doses of 3 and 15 mg/kg. All drugs and vehicle (saline) solutions were administered intraperitoneally (IP). Doses and times were selected based on previous experiments (Acquas et al., 2010; Hilbert et al., 2013; Ibba et al., 2009; López-cruz et al., 2016; Rosas et al., 2017; SanMiguel et al., 2019; Spina et al., 2015).

Apparatus

The apparatus consisted of two rectangular Plexiglas boxes (48L x 20W x 30H cm) separated by a **double-faced** guillotine door. The apparatus was placed in a sound-proof room with a constant light of 37.5 Lux (ELD 9010 Luxmeter, Eldes Instruments, Italy) provided by a 40W lamp placed above each compartment. Different visual and tactile cues distinguished the two compartments: vertically striped black and white walls and white smooth floor for one compartment (A), and horizontally striped black and gray walls and fine grid floor for the other compartment (B). The spontaneous preference was randomly distributed between compartments (45% for compartment A and 55% for compartment B in CPP experiments; 49% for compartment A and 51% for compartment B in CPA experiments). **Hence, for CPP experiments the assignment of mice to compartment A or B was based on their individual spontaneous preference being, irrespective of compartment A or B, <450 seconds whereas for CPA experiments the assignment of mice to compartment A or B was based on their individual spontaneous preference being, irrespective of compartment A or B, >450 seconds. Hence both in CPP and in CPA experiments there were mice belonging to the same experimental group for which the conditioned stimulus was compartment A and some others for which the conditioned stimulus was compartment B. Thus, in CPP experiments the drug-paired compartment was always the less-preferred (A or B) compartment; similarly, in CPA experiments the drug-paired compartment was always the preferred (A or B) compartment.** Moreover, mice that had spontaneous preference times at the pre-conditioning test ~~were~~ between 441 and 459 sec/900 were randomly assigned half to compartment A and half to compartment B.

207 ## Conditioned Place Preference and Conditioned Place Aversion: procedure and
208 experimental design

209 Each experiment consisted of three phases. During the first phase (pre-conditioning test, day 1), the
210 guillotine door was kept raised and each mouse was placed randomly in one or the other
211 compartment and given access to both compartments of the apparatus for 15 minutes (900 sec.).
212 The time spent in one compartment was recorded and taken as indication of spontaneous
213 preference. Behavioral schedules for backward (CPP) and forward (CPA) conditioning (figure 1A and
214 1B, respectively) were designed based on Rosas et al. (2017) and Spina et al. (2015), with some
215 modifications related to the timing of pre-treatment with caffeine. In particular, during the second
216 phase (conditioning, days 2-5) of CPP experiments, mice from the different experimental groups
217 were administered caffeine (3 or 15 mg/kg) or saline 20 minutes before ethanol (2 g/kg) or saline,
218 and were returned to their home cage. 10 minutes after ethanol administration mice were exposed
219 for 5 minutes to a given compartment and returned to their home cage. 6 hours later mice were
220 administered saline or caffeine (3 or 15 mg/kg), then returned to their home cage 20 minutes before
221 the second administration (ethanol, 0 or 2 g/kg). 10 minutes after this second administration mice
222 were placed for 5 minutes in the compartment opposite to that of the morning exposure. **Caffeine**
223 **was administered only during conditioning and hence its effects on ethanol-elicited side**
224 **preference shift was restricted to the conditioning phase hence no caffeine's effects were tested**
225 **on mice performing in the ethanol-free post-conditioning test either for CPP and for CPA. An**
226 **interval of 6 hours between conditioning sessions was applied in order to be sure that all possible**
227 **carry over effects had wear off. According to this schedule of administration each mouse in group**
228 **saline + ethanol, and in group caffeine (3 or 15 mg/kg) + ethanol was administered ethanol only**
229 **once a day, and placed in the compartment opposite to that in which was placed after being**
230 **administered saline.**

231 During the second phase (conditioning, days 2-5) of CPA experiments, mice from different
232 experimental groups were administered caffeine (3 or 15 mg/kg) or saline and returned to their
233 home cage for 30 minutes. At the end of this period mice were exposed for 5 minutes to the given
234 compartment. Upon removal from the compartment (i.e. immediately before being returned to
235 their home cage) mice were administered the second injection (ethanol, 0 or 2 g/kg). 6 hours later,
236 mice were administered caffeine (0, 3 or 15 mg/kg) or saline 30 minutes before the 5 minutes
237 exposure to the opposite compartment. Upon removal from the apparatus (i.e. before being
238 returned to their home cage) mice were administered the second injection (ethanol 0 or 2 g/kg).

According to this schedule of administration each mouse in group saline + ethanol, and in group caffeine (3 or 15 mg/kg) + ethanol was administered ethanol only once a day after being placed in a compartment different from that in which was placed before being administered saline.

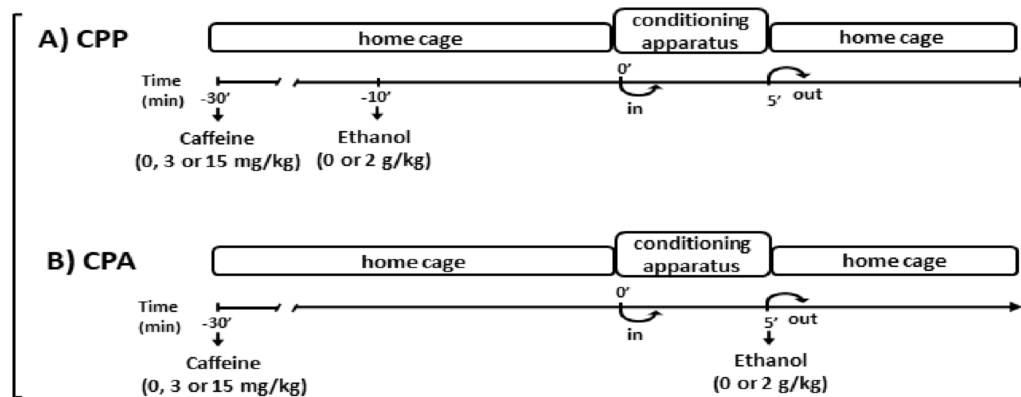
During conditioning days for both CPP and CPA the order of saline and ethanol administration was counterbalanced [i.e. the order of the combined pre-treatment~~/treatment~~ [caffeine (0, 3 and 15 mg/kg)~~/~~ **and treatment [**ethanol (0 or 2 g/kg)] administrations were opposite on even days with respect to that one on odd days] **(for instance, for the experimental group caffeine 3 mg/kg + ethanol 2 g/kg, if on days 1 and 3 of conditioning caffeine + ethanol 0 g/kg administrations in the "morning" were associated to compartment A and caffeine + ethanol 2 g/kg administrations in the "afternoon" were associated to compartment B, on days 2 and 4 of conditioning caffeine + ethanol 2 g/kg administrations in the "morning" were associated to compartment B and caffeine + ethanol 0 g/kg administrations in the "afternoon" were associated to compartment A)**; similarly, the number of mice receiving saline and ethanol was counterbalanced over the 4 days of conditioning. The same counterbalanced design was also applied to the assignment of mice to compartments A and B. As a result of these conditioning schedules, saline (ethanol, 0 g/kg) ~~and ethanol (2 g/kg) were~~ **was** paired four times **(once a day)** with the given compartment **and, similarly, ethanol (2 g/kg) was paired four times (once a day) with the opposite compartment.**

During the third phase of both CPP and CPA experiments (post-conditioning test, day 6), 24 h after the last conditioning treatment, the guillotine door was kept raised and the time spent by each mouse in the drug-paired (backward conditioning, CPP) and in the drug-assigned (forward conditioning, CPA) compartment during 15 minutes was recorded. **However, no caffeine effects were tested on mice performing the ethanol-free post conditioning test either for CPP and for CPA.** The conditions of the post-conditioning test were identical to those of the pre-conditioning test. Pre- and post-conditioning recordings were done with a stopwatch by observers, blind to pharmacological treatments**, present in the experimental room**. Hence, a statistically significant difference between the time spent during pre- and post-conditioning tests (side preference shift) of the drug group with respect to that of the saline group was taken as indication of the development of place conditioning.

Figure 1

Phase 1: PRE-CONDITIONING TEST (day 1; drug-free)

Phase 2: CONDITIONING (days 2-5; drug-environment association)



Phase 3: POST-CONDITIONING TEST (day 6; drug-free)

CPP and CPA conditioning procedures

Schematic representation of the place conditioning procedures used in the CPP (**A**) and CPA (**B**) experiments. (**A**) Mice were administered caffeine (0, 3 or 15 mg/kg) 20 min before the administration of ethanol (0 or 2 g/kg) and were returned to their home cage. 10 min after ethanol (0 or 2 g/kg) administration, mice were exposed for 5 min to the given compartment of the conditioning apparatus and returned to their home cage. (**B**) Mice were administered caffeine (0, 3 or 15 mg/kg) and returned to their home cage for 30 min. At the end of this period, each mouse was exposed for 5 min to the given compartment of the conditioning apparatus. Upon removal from the apparatus, i.e. before being returned to their home cage, mice were administered ethanol (0 or 2 g/kg).

Immunohistochemistry

These experiments were performed on mice of **two** distinct experimental groups:~~(i)~~ **the first group (acute experiments) was made of** drug-naïve mice that were acutely administered caffeine (0, 3 or 15 mg/kg) 20 minutes before ethanol (0 or 2 g/kg) (data shown in figure 4)**; the second group was made of mice that underwent the conditioning procedures as described above (one subgroup of mice for CPP and another subgroup for CPA) and that were**~~; and (ii) ethanol-conditioned,~~ drug-free ~~mice~~ **while** expressing either CPP or CPA (data shown in figures 6 and 7, respectively). Mice **of the "acute experiment"** were deeply anesthetized (fentanyl 0.1 mg/kg) 15 minutes after the last administration (~~acute drugs effects,~~ data shown in figure 4) (Ibba et al., 2009) ~~or~~ **whereas mice that performed the post-conditioning test for CPP or CPA were deeply anesthetized (fentanyl 0.1 mg/kg)** immediately ~~at~~ **after** the completion of the post-conditioning test (15 minutes) (~~caffeine-and ethanol~~**drug**-free animals; effects of ethanol-conditioned stimuli, data shown in figures 6 and

296 7) (Rosas et al., 2017). **The behavioral data of these latter subgroups were pooled with those of**
297 **animals that performed only the CPP or CPA experiments.** Under deep anesthesia, animals were
298 subjected to trans-cardial perfusion with ice-cold Phosphate Buffered Saline (PBS: 137 mM NaCl, 2.7
299 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4, pH 7.4) and 4% paraformaldehyde (PFA) solutions. After
300 perfusion, brains were removed and post-fixed overnight in 4.0% PFA. Brain slices (40 μm) of the
301 regions of interest were cut, on ice-cold PBS with a vibratome (Leica VT1000, Leica, Germany)
302 according to plates 21-23 (approximately from AP 1.18 to AP 0.98 mm from bregma for the nucleus
303 accumbens core and shell), to plates 30-32 (approximately from AP 0.14 to AP -0.10 mm from
304 bregma for the bed nucleus of stria terminalis) and to plates 40-41 (approximately from AP -1.06 to
305 AP -1.34 mm from bregma for the basolateral and central nucleus of the amygdala) of the Paxinos
306 and Franklin (2001) mouse brain atlas. Slices were kept in ice-cold PBS and processed for
307 immunohistochemistry according to a protocol for free-floating slices. After a 30 minutes incubation
308 period in 1% H_2O_2, slices were incubated for 1 hour with 3% BSA. The incubation with the primary
309 anti pERK antibody (phosphorylated ERK, Cell Signalling Technology, Beverly, MA, USA (1:350)) was
310 conducted overnight. On the following day, after rinsing, slices were incubated for 1 hour with the
311 biotinylated secondary antibody (1:800). After three rinses the slices were incubated in avidin biotin
312 peroxidase complex prepared according to the manufacturer's suggestions (Vectastain ABC kit,
313 Vector Laboratories, Burlingame, CA, USA) and a 3-3'-diaminobenzidine solution (10 mg/ml) was
314 added until development of brown staining. Slices were rinsed and mounted onto gelatin-coated
315 slides and processed through alcohol-xylene for light microscopy examination. pERK-positive
316 neurons were identified in the regions of interest of both hemispheres at the lowest magnification
317 (10X) and quantitative analysis was performed using a Zeiss Axioskop 40 light microscope, equipped
318 with PL Fluotar 10X (na=0.3), 40X (na=1.00–0.5) and 100X oil (na=1.3) objectives, coupled with a
319 Nikon D5000 digital camera (Melville, NY, USA). Images (average of three) of the regions of interest
320 were obtained at the lowest magnification (10X) from 40 μm thick slices and used to automatically
321 count the number of pERK-positive neurons/area (pERK expression) by application of the software
322 ImageJ (v. 1.42, National Institutes of Health sponsored image analysis program). Each individual
323 data point shown in figures 4, 6 and 7 is the average number of pERK-positive neurons/brain region
324 measured in both hemispheres in at least two slices taking **two or more non-sequential slices**
325 **(separated by at least one slice in between, if allowed by the size of the brain region of interest)**
326 ~~one slice every other/~~ **per** brain region **of interest**.
327

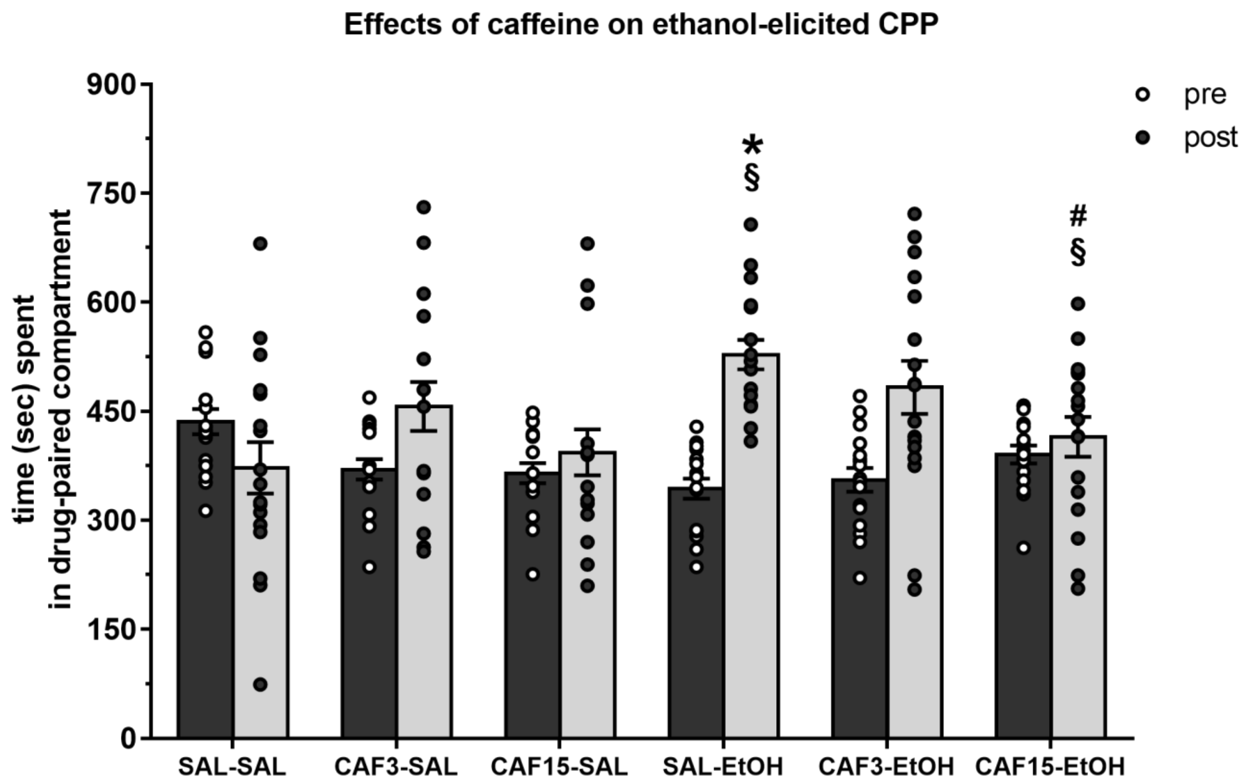
Statistical analysis

To determine statistically significant differences between pre-conditioning values of the experimental groups depicted in figures 2 and 3, one-way Analysis of Variance (ANOVA) was applied (StatSoft, v. 8.0, StatSoft Inc., Tulsa (OK), USA). To determine the effect of pre-treatment (3 levels: caffeine 0, 3 or 15 mg/kg) on conditioning and the effect of treatment (2 levels: EtOH 0 or 2 g/kg) on acquisition of ethanol-elicited CPP or CPA, data were analyzed by three-way ANOVAs with pre-treatment and treatment as independent factors (between subjects), and with pre-conditioning and post-conditioning values as a within-subjects factor (repeated measures). All statistical analyses were carried out using data from the experimental groups depicted in each figure. Post hoc analyses **also between pre- and post-conditioning times within each conditioning group** (with multiple comparisons), carried out using Newman-Keuls post-hoc **tests** ~~analyses~~, were undertaken if significant effects were found ($p<0.05$).

pERK-positive neurons/area following each treatment were expressed as the average number of pERK-positive neurons/area of each experimental group and indicated as pERK-positive neurons/area (pERK expression). These values were used as dependent variables for statistical analyses by one-way ANOVAs with pERK-positive neurons/area as dependent variables and with pre-treatment (3 levels: caffeine 0, 3 or 15 mg/kg)/treatment (2 levels: EtOH 0 or 2 g/kg) used as independent variables. **Although more liberal than the rather conservative Bonferroni's test (Lee and Lee, 2018), for multiple comparisons allowed by ANOVAs significant main effects we applied here the** Fishers Least Significant Difference (LSD) post-hoc analyses~~, which were allowed by ANOVAs significant main effects, were applied for multiple comparisons,~~ with the statistical significance set at $p<0.05$. **Moreover, although the Acb, BNST and CeA are brain areas containing several nuclei, pERK expression was sampled in these nuclei as a whole without considering their several anatomical subdivisions.**

The experiments were planned to require the least possible number of subjects (obtained by calculation with the statistical software G Power 3.1) on the basis of statistical tests applied (ANOVAs).

356 # Results



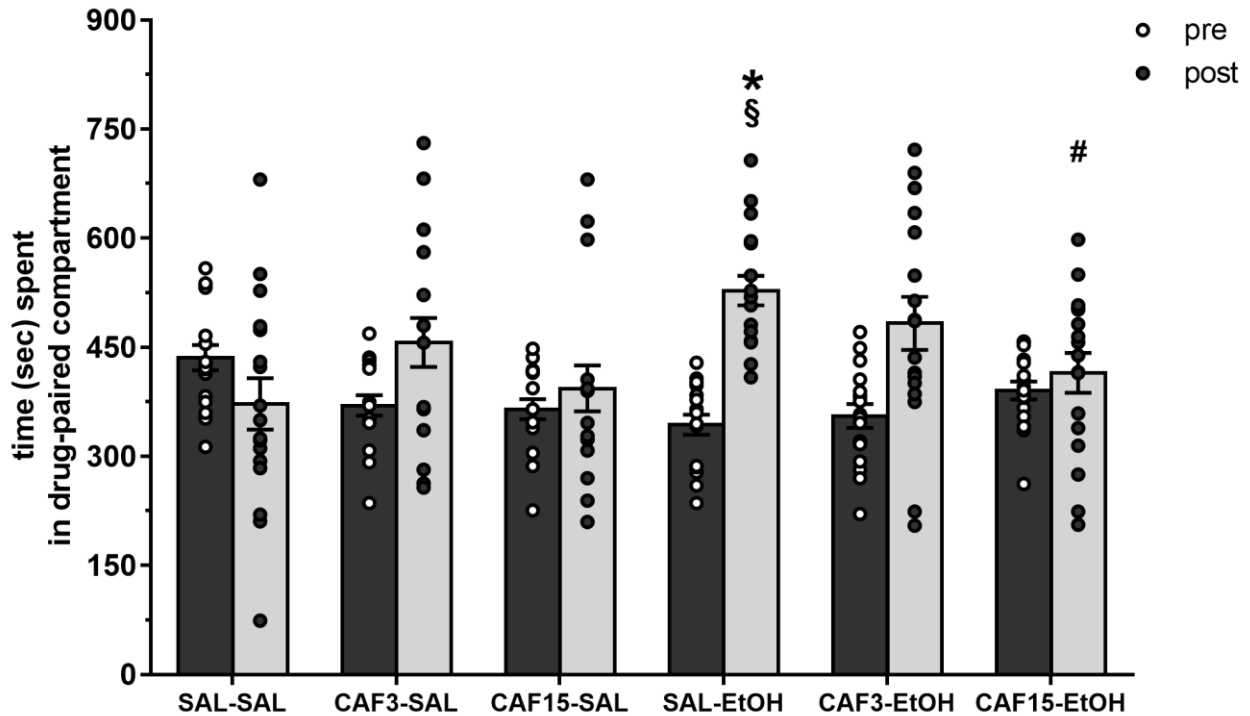
357
358

359 ## Effects of caffeine on acquisition of ethanol-elicited CPP

360 Figure 2 shows the effects of pre-treatment with caffeine (0, 3 or 15 mg/kg) 20 minutes before
361 the administration of ethanol (EtOH, 0 or 2 g/kg) and exposure to the associated compartment for
362 5 minutes (see figure 1A). Pre-conditioning preference times did not significantly differ between
363 experimental groups [$F_{5,81}$=0.06, NS]. Repeated measures three-way ANOVA with preference times
364 (pre- and post-conditioning) as dependent factors, and with pre-treatment (caffeine 0, 3 or 15
365 mg/kg) and treatment (EtOH 0 or 2 g/kg) doses as independent factors, revealed a significant effect
366 of time [$F_{1,83}$=19.21, p<0.001], and significant treatment by time [$F_{1,83}$=10.37, p<0.05] and pre-
367 treatment by treatment by time [$F_{2,83}$=7.24, p<0.05] interactions. Post-hoc analysis using the
368 Newman-Keuls test revealed 1) that EtOH stimulates a significant preference shift from 343±15 to
369 528±23 sec/900 (p<0.05), 2) that 3 and 15 mg/kg caffeine are devoid of conditioning properties and
370 3) that caffeine 15 but not 3 mg/kg significantly prevents the acquisition of CPP induced by EtOH
371 (p<0.05).

372

373 Figure 2



374

375 Effects of caffeine pre-treatment on acquisition of ethanol-elicited CPP
376 Data are shown as average time spent (sec.) ± SEM in the drug-paired compartment. Pre-conditioning
377 preference times were 435±17 for caffeine 0 mg/kg + EtOH 0 g/kg (SAL-SAL) (n=17), 370±20 for caffeine 3
378 mg/kg + EtOH 0 g/kg (CAF3-SAL) (n=12), 365±19 for caffeine 15 mg/kg + EtOH 0 g/kg (CAF15-SAL) (n=12),
379 343±15 for caffeine 0 mg/kg + EtOH 2 g/kg (SAL-EtOH) (n=15), 356±16 for caffeine 3 mg/kg + EtOH 2 g/kg
380 (CAF3-EtOH) (n=17), 390±13 for caffeine 15 mg/kg + EtOH 2 g/kg (CAF15-EtOH) (n=16). *Indicates a
381 significant difference (p<0.05) of time spent during post-conditioning test as compared to SAL-SAL group;
382 #indicates a significant difference (p<0.05) in time spent during the post-conditioning test as compared to
383 SAL-EtOH group**; §indicates a significant difference (p<0.05) between pre- and post-conditioning.**
384 Individual data points are indicated in the corresponding histogram for each experimental
385 group/conditioning phase.

386

387 Effects of caffeine on acquisition of ethanol-elicited CPA

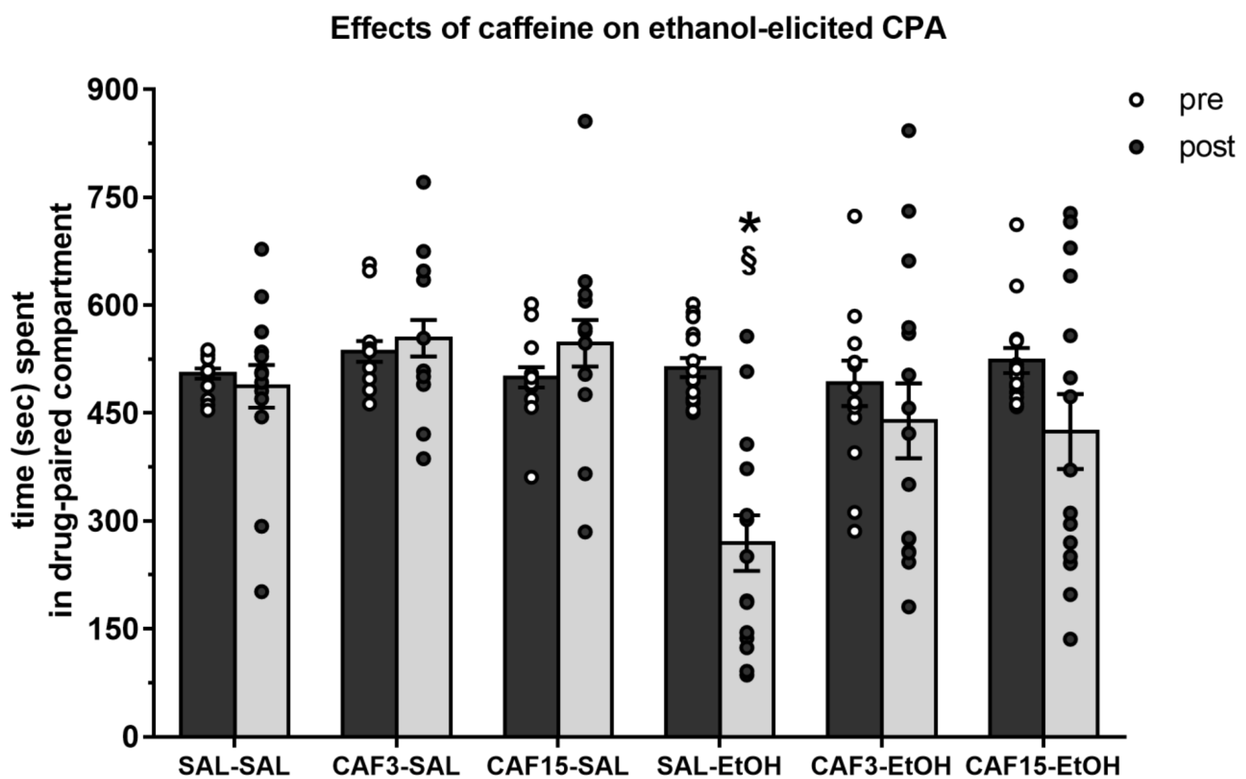
388 Figure 3 shows the effects of pre-treatment with caffeine (0, 3 or 15 mg/kg) 30 minutes before
389 the 5-minute exposure to the assigned compartment, and of ethanol (EtOH 0 or 2 g/kg)
390 administration upon removal from the place conditioning apparatus (see figure 1B). Pre-
391 conditioning preference times did not differ significantly between experimental groups [$F_{5,74}$=0.03,
392 NS]. Repeated measures three-way ANOVA with preference times (pre- and post-conditioning) as
393 dependent factors, and with pre-treatment (caffeine 0, 3 or 15 mg/kg) and treatment (EtOH 0 and
394 2 g/kg) doses as independent factors, revealed significant effects of pre-treatment [$F_{2,74}$=3.51,
395 p<0.05], treatment [$F_{1,74}$=13.10, p<0.001] and time [$F_{1,74}$ =8.87, p<0.05], and significant pre-

396 treatment by time [$F_{2,74}$=3.83, p<0.05] and treatment by time [$F_{1,74}$=14.54, p<0.001] interactions.
397 Post-hoc analysis using Newman-Keuls test revealed 1) that EtOH stimulates a significant CPA from
398 513±13 to 269±38 sec/900 (p<0.001) 2) that caffeine (3 and 15 mg/kg) is devoid of conditioning
399 properties and 3) that caffeine (3 and 15 mg/kg) significantly prevents the acquisition of CPA
400 induced by EtOH (p<0.05).

401

402 Figure 3

403



404

405 Effects of caffeine pre-treatment on acquisition of ethanol-elicited CPA
406 Data are shown as average time spent (sec.) ± SEM in the drug-paired compartment. Pre-conditioning
407 preference times were 505±7 for caffeine (0 mg/kg) + EtOH (0 g/kg) (SAL-SAL) (n=15), 536±22 for caffeine (3
408 mg/kg) + EtOH (0 g/kg) (CAF3-SAL) (n=10), 500±21 for caffeine (15 mg/kg) + EtOH (0 g/kg) (CAF15-SAL) (n=10),
409 513±13 for caffeine (0 mg/kg) + EtOH (2 g/kg) (SAL-EtOH) (n=15), 491±31 for caffeine (3 mg/kg) + EtOH (2
410 g/kg) (CAF3-EtOH) (n=15) and 523±17 for caffeine (15 mg/kg) + EtOH (2 g/kg) (CAF15-EtOH) (n=15). *Indicates
411 a significant difference (p<0.05) of time spent during the post-conditioning test between SAL-EtOH group and
412 all other groups; **§indicates a significant difference (p<0.05) between pre- and post-conditioning**. Individual
413 data points are indicated in the corresponding histogram for each experimental group/conditioning phase.

414

415 Effects of acute administration of caffeine on the expression of ethanol-elicited pERK-
416 positive neurons in the Acb, BNST, CeA and BLA

417 Figure 4 shows the effects of pre-treatment with caffeine (0, 3 or 15 mg/kg) 20 minutes before
418 the administration of ethanol (EtOH 0 or 2 g/kg) on the number of pERK-positive neurons (pERK
419 expression) in the AcbC and AcbSh, BNST, CeA and BLA of drug-naive CD-1 mice.

420 The administration of EtOH (2 g/kg) increased the number of pERK-positive cells in the AcbC and
421 AcbSh ($p<0.05$) (from 41±2 and 50±10 to 149±13 and 168±34, respectively) and caffeine pre-
422 treatment reduced the ability of EtOH to stimulate ERK phosphorylation in both Acb subregions (to
423 78±10 and 101±21 at the dose of 3 mg/kg and to 51±3 and 82±15 at the dose of 15 mg/kg; figure
424 4A). One-way ANOVA, with pre-treatment/treatment factor as independent variable and with the
425 number of pERK-positive cells/area as dependent variable, revealed significant pre-
426 treatment/treatment effects in AcbC [$F_{3,15}=6.91$, $p<0.05$] and AcbSh [$F_{3,15}=5.12$, $p<0.05$]. Post hoc
427 analysis using Fishers Least Significant Difference (LSD) test revealed 1) that EtOH stimulates a
428 significant increase of the number of pERK-positive neurons both in the AcbC and in the AcbSh
429 ($p<0.05$) and 2) that pre-treatment with caffeine at both doses significantly reduces this effect in
430 both areas.

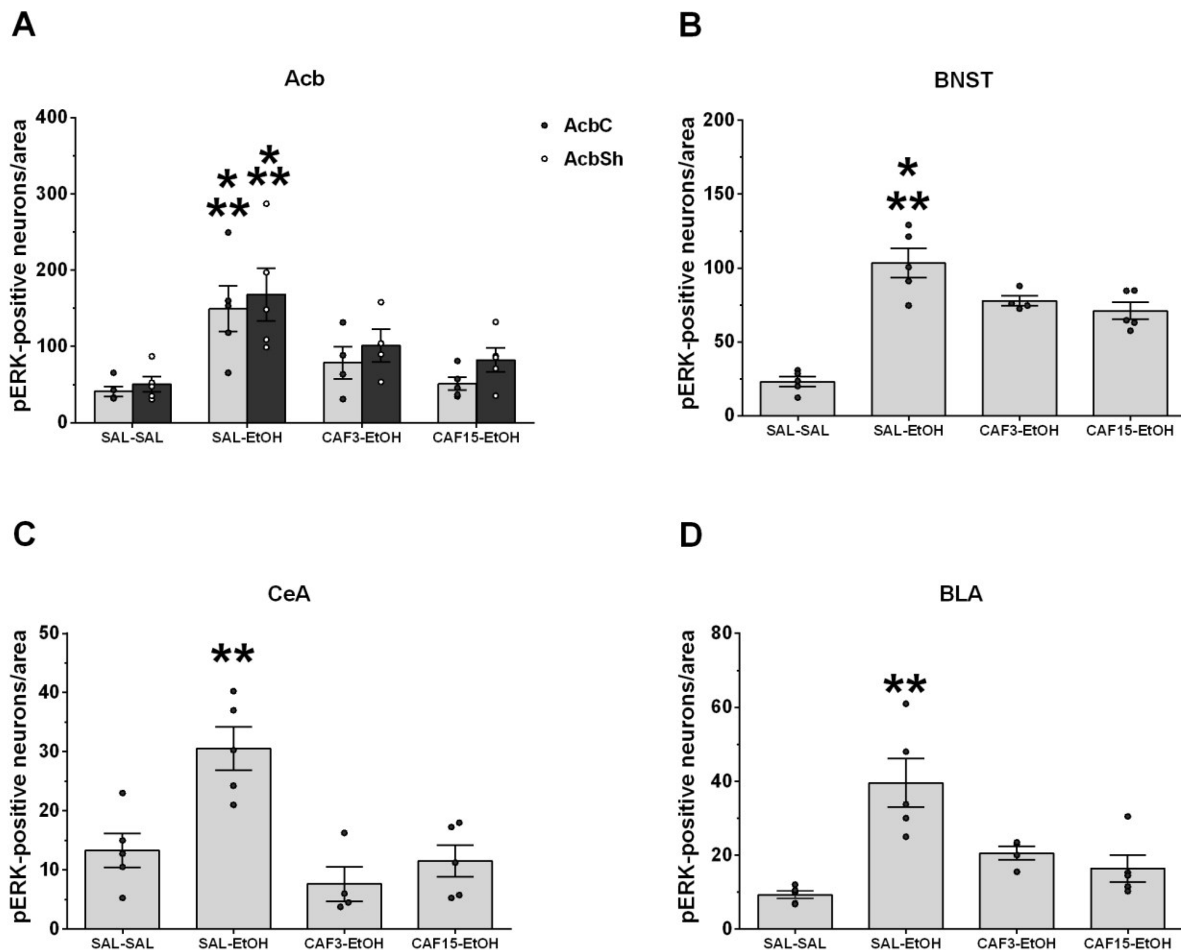
431 Moreover, the administration of EtOH (2 g/kg) increased pERK-positive cells in the BNST ($p<0.05$)
432 (from 23±3 to 103±9), and caffeine pre-treatment reduced the ability of EtOH to stimulate ERK
433 phosphorylation in this area (to 77±3 at the dose of 3 mg/kg and to 72±6 at the dose of 15 mg/kg;
434 figure 4B). One-way ANOVA, with pre-treatment/treatment as independent variable and with the
435 number of pERK-positive cells/area as dependent variable, revealed significant effects of pre-
436 treatment/treatment [$F_{3,15}=28.04$, $p<0.001$]. Post hoc analysis using Fishers Least Significant
437 Difference (LSD) test revealed 1) that EtOH stimulates a significant increase of the number of pERK-
438 positive neurons in the BNST ($p<0.001$) and 2) that pre-treatment with caffeine at both doses
439 significantly reduces ($p<0.05$) this effect.

440 Finally, the administration of EtOH (2 g/kg) also increased the number of pERK-positive cells in
441 the CeA and BLA ($p<0.05$), respectively, from 13±2 to 30±3 in the CeA and from 9±1 to 39±6 in the
442 BLA; pre-treatment with caffeine, at the doses of 3 and 15 mg/kg, reduced to 7±2 and 11±3
443 respectively, in the CeA and to 20±1 and 16±4, respectively, in the BLA, the number of pERK-positive
444 neurons elicited by ethanol (figures 4C and 4D). One-way ANOVA, with pre-treatment/treatment as
445 independent variables and with the number of pERK-positive cells/area as dependent variable,
446 revealed significant effects of pre-treatment/treatment [$F_{3,15}=10.76$, $p<0.05$]. Post hoc analysis
447 using Fishers Least Significant Difference (LSD) test revealed 1) that EtOH stimulates a significant

448 increase of the number of pERK-positive neurons in the CeA and BLA ($p<0.05$) and 2) that pre-
449 treatment with caffeine at both doses significantly reduces ($p<0.001$) this effect in both areas.
450 Representative images of these results are shown in Figure 5.

451

452 Figure 4

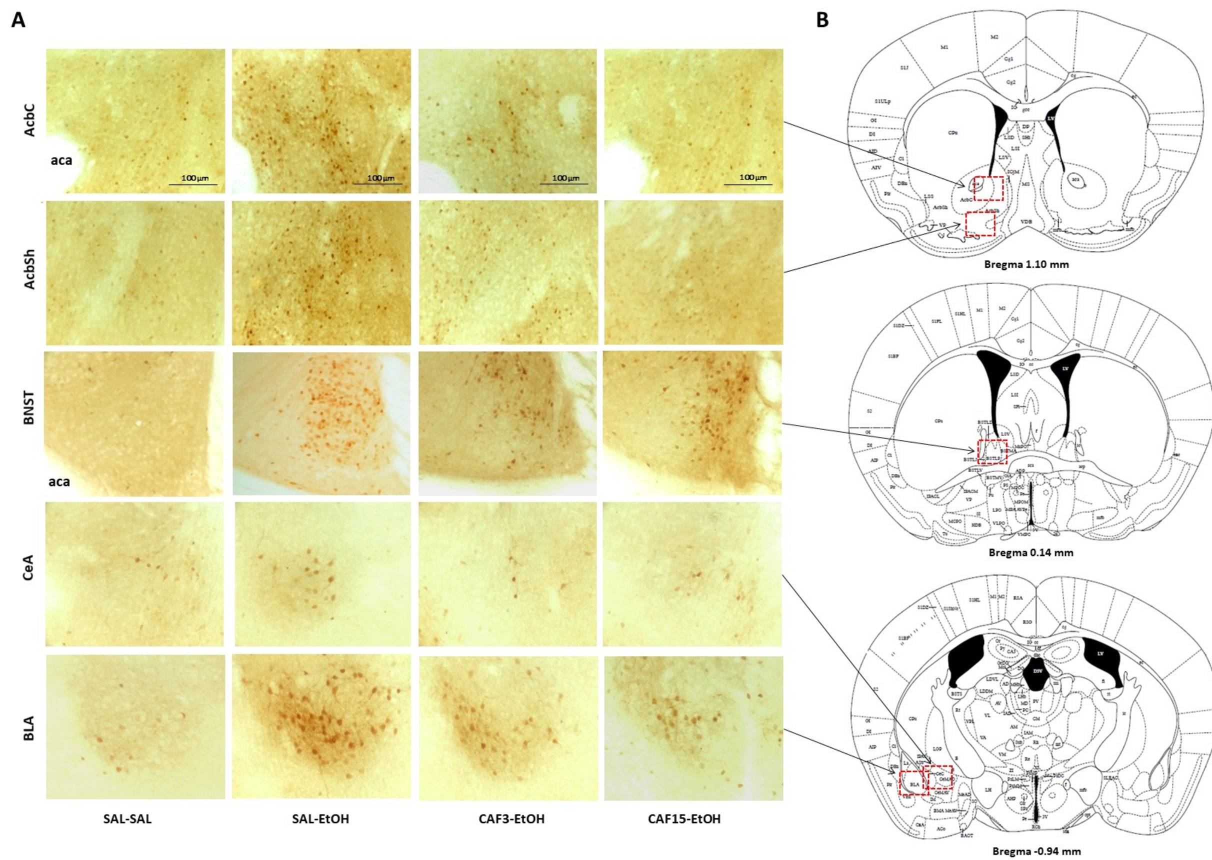


453

454 Effects of acute administration of caffeine on the expression of ethanol-elicited pERK-positive neurons in the
455 Acb, BNST, CeA and BLA
456 Data are shown as mean ± SEM of pERK-positive neurons/area. The number of animals of each experimental
457 group were: n=5 for SAL-SAL (i.e. caffeine 0 mg/kg + ethanol 0 g/kg) group; n=5 for SAL-EtOH (i.e. caffeine 0
458 mg/kg + ethanol 2 g/kg) group; n=4 for CAF3-EtOH (i.e. caffeine 3 mg/kg + ethanol 2 g/kg) group and n=5 for
459 CAF15-EtOH (i.e. caffeine 15 mg/kg + ethanol 2 g/kg) group. Figure 4A: *indicates a significant difference
460 ($p<0.05$) between SAL-**EtOH**~~SAL~~ and CAF3-EtOH and **between SAL-EtOH and** CAF15-EtOH groups in AcbC and
461 AcbSh; **indicates a significant difference ($p<0.001$) between SAL-EtOH and SAL-SAL groups in AcbC and
462 AcbSh; figure 4B: *indicates a significant difference ($p<0.05$) between SAL-EtOH and CAF3-EtOH and **between**
463 **SAL- EtOH and** CAF15-EtOH groups in BNST; **indicates a significant difference ($p<0.001$) between SAL-EtOH
464 and SAL-SAL groups in BNST; figure 4C: **indicates a significant difference ($p<0.001$) between SAL-EtOH and
465 all other groups in CeA; figure 4D**:** ~~*indicates a significant difference (p<0.05) between SAL-EtOH and CAF3-~~
466 ~~EtOH and CAF15-EtOH groups in BLA;~~ **indicates a significant difference ($p<0.001$) ~~in the number of pERK-~~
467 ~~positive neurons/area~~ between SAL-EtOH and all other groups in BLA. Individual data points are indicated in
468 the corresponding histogram for each experimental group.

469

470 Figure 5



471

472 **Representative images of** **the effects of acute administration of caffeine on the expression of ethanol-**
473 **elicited** **pERK-immunostaining** **in the Acb, BNST, CeA and BLA**. (**A**) Low (20X) magnification images of pERK-
474 positive neurons from mice representative of ~~each~~ treatment **groups (saline + saline, SAL-SAL; saline +**
475 **ethanol (2 g/kg), SAL-EtOH; caffeine (3 mg/kg) + ethanol (2 g/kg), CAF3-EtOH; and caffeine (15 mg/kg) +**
476 **ethanol (2 g/kg), CAF15-EtOH)**. (**B**) Coronal sections with bregma coordinates taken from the mouse brain
477 atlas of Paxinos and Franklin (2001) showing**, framed by the red squares,** the location of the areas (AcbC,
478 AcbSh, BNST, CeA and BLA) for pERK immunoreactivity counting.

479

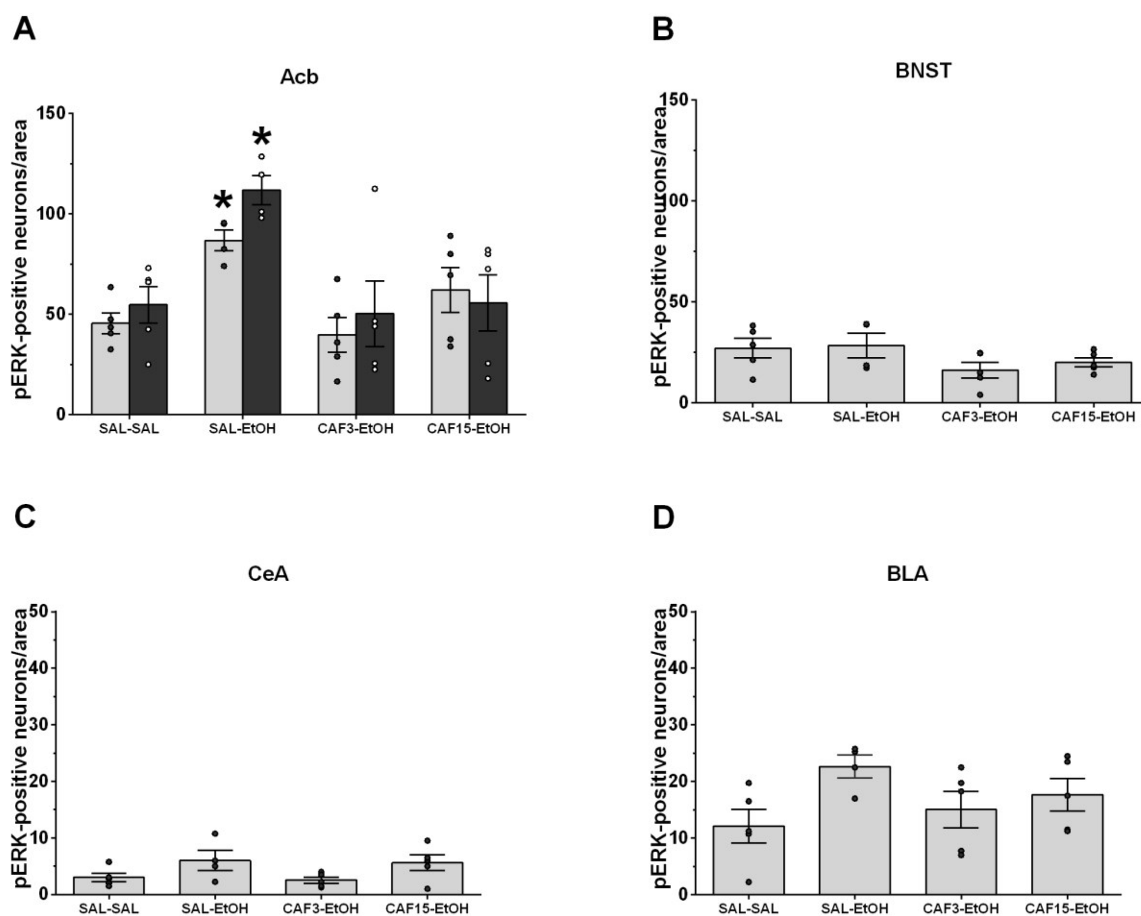
480 Effects of the expression of ethanol-elicited CPP on ERK phosphorylation in the Acb, 481 BNST, CeA and BLA

482 Figure 6 shows the effects of the expression of ethanol-elicited CPP on the number of pERK-
483 positive neurons/area (pERK expression) in AcbC and AcbSh, BNST, CeA and BLA of CD-1 mice.

484 As shown in figure 2, administration of ethanol (2 g/kg) during conditioning (see figure 1A)
485 resulted in a significant CPP. The expression, during the post-conditioning test, of this acquired
486 preference was associated with an increase in the number of pERK-positive cells in the AcbC and
487 AcbSh ($p<0.05$) (respectively from 45±2 to 86±2 and from 54±9 to 111±7). Pre-treatment with
488 caffeine, during conditioning, reduced the ability of the EtOH-conditioned stimulus to increase ERK

489 phosphorylation in both subregions of nucleus accumbens (figure 6A; to 39±6 in AcbC and to 50±16
490 in AcbSh at the dose of 3 mg/kg and to 62±5 in AcbC and 56±14 in AcbSh at the dose of 15 mg/kg).
491 A one-way ANOVA, with pre-treatment/treatment as independent variables and the number of
492 pERK-positive neurons/area as dependent variable, revealed significant pre-treatment/treatment
493 effects in the AcbC [$F_{3,15}$=5.98, p<0.05] and AcbSh [$F_{3,15}$=4.62, p<0.05] but not significant effects in
494 BNST [$F_{3,15}$=2.04, NS], CeA [$F_{3,15}$=2.41, NS] and BLA [$F_{3,15}$=2.23, NS]. Post-hoc analysis using Fishers
495 Least Significant Difference (LSD) test for the Acb revealed 1) that the expression of EtOH-elicited
496 CPP is associated with a significant increase of the number of pERK-positive neurons/area both in
497 the AcbC and in the AcbSh (p<0.05) and 2) that caffeine at both doses, during conditioning,
498 significantly reduced this effect.

499 Figure 6



500
501 Effects of the expression of ethanol-elicited CPP on ERK phosphorylation in the Acb, BNST, CeA and BLA
502 Animals of each group, in a drug-free state, performed the post-conditioning test (15 minutes) and
503 immediately afterwards were anesthetized and perfused for immunohistochemical analysis. Data are shown
504 as mean ± SEM of pERK-positive neurons/area. The number of animals of each experimental group were: n=5

505 for SAL-SAL (caffeine 0 mg/kg + ethanol 0 g/kg) group; n=4 for SAL-EtOH (caffeine 0 mg/kg + ethanol 2 g/kg)
506 group; n=5 for CAF3-EtOH (caffeine 3 mg/kg + ethanol 2 g/kg) group and n=5 for CAF15-EtOH (caffeine 15
507 mg/kg + ethanol 2 g/kg) group. Figure 6A: *indicates a significant difference between SAL-EtOH and SAL-SAL
508 groups and between SAL-EtOH and CAF3-EtOH and CAF15-EtOH groups in AcbC and AcbSh. Individual data
509 points are indicated in the corresponding histogram for each experimental group.
510

511 Effects of the expression of ethanol-elicited CPA on ERK phosphorylation in the Acb, 512 BNST, CeA and BLA

513 Figure 7 shows the effects of the expression of ethanol-elicited CPA on the number of pERK-
514 positive neurons/area (pERK expression) in AcbC and AcbSh, BNST, CeA and BLA of CD-1 mice.

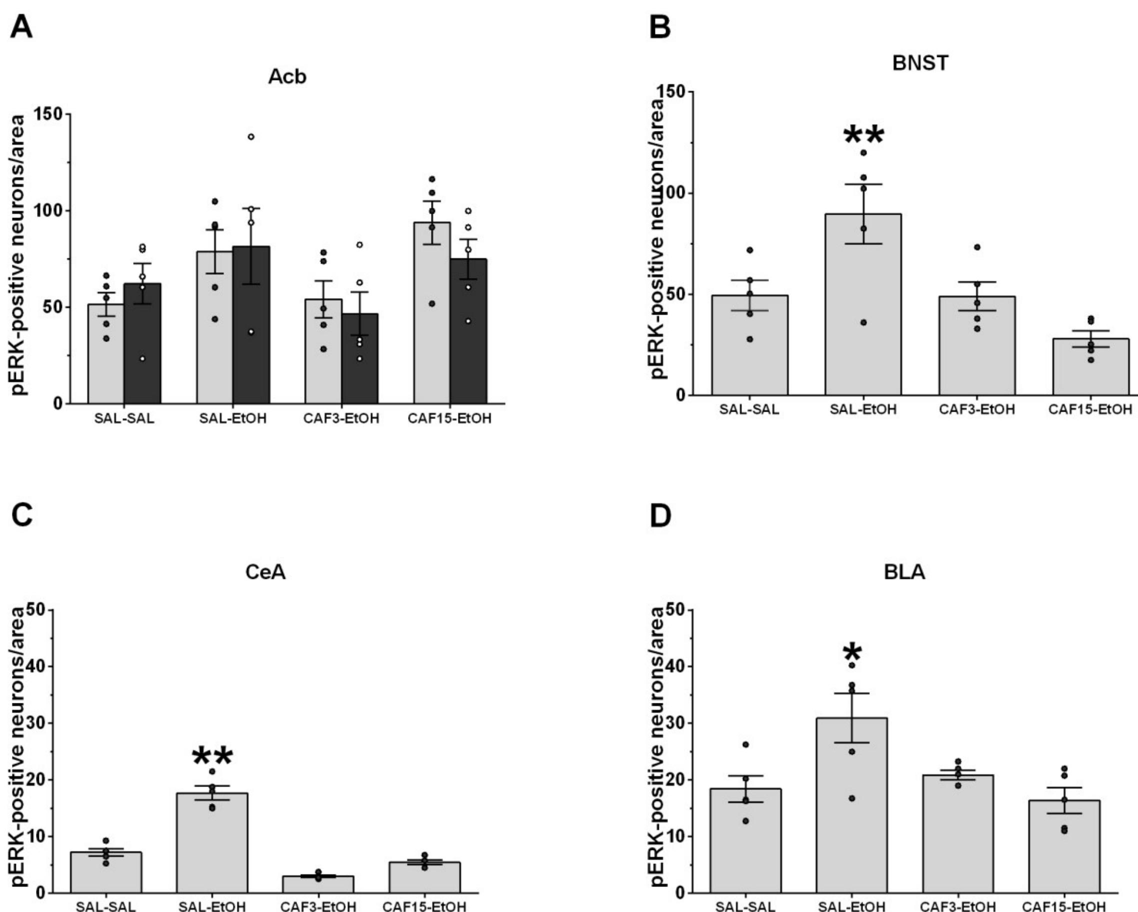
515 As shown in figure 3, administration of ethanol (2 g/kg) during conditioning (see figure 1B)
516 resulted in a significant CPA. The expression, during the post-conditioning test, of this acquired
517 aversion failed to affect the number of pERK-positive cells in the AcbC and AcbSh ($p>0.05$) (figure
518 7A). One-way ANOVA with pre-treatment/treatment as independent variables and the number of
519 pERK-positive neurons/area as a dependent variable, reveled significant pre-treatment and
520 treatment effects in the AcbC [$F_{1,16}=4.25$, $p<0.05$] but not in the AcbSh [$F_{3,16}=1.30$, NS] (figure 7A).
521 Post-hoc tests using Fishers Least Significant Difference (LSD) revealed that the expression of
522 ethanol-induced CPA is not associated with a significant increase of the number of pERK-positive
523 neurons/area in AcbSh and AcbC ($p>0.05$) with respect to caffeine (0 mg/kg) + EtOH (0 g/kg)
524 conditioned group.

525 However, the expression, during the post-conditioning test, of the acquired aversion was
526 associated with increases in the number of pERK-positive cells in the BNST ($p<0.001$) (from 37±4 to
527 85±14). Caffeine pre-treatment, during conditioning, at both doses reduced the ability of EtOH-
528 elicited CPA expression to stimulate ERK phosphorylation in this area [to 49±7 (caffeine 3 mg/kg)
529 and 28±4 (caffeine 15 mg/kg) pERK-positive cells/area; figure 7B]. One-way ANOVA with pre-
530 treatment/treatment as independent variable and with the number of pERK-positive cells/area as
531 dependent variable, revealed significant pre-treatment and treatment effects [$F_{3,16}=9.06$, $p<0.001$].
532 Post hoc analysis using Fishers Least Significant Difference (LSD) test revealed 1) that expression of
533 EtOH-elicited CPA stimulated a significant increase of the number of pERK-positive neurons in the
534 BNST ($p<0.001$) and 2) that pre-treatment with caffeine, given during conditioning, at both doses
535 significantly reduced ($p<0.001$) this effect.

536 Finally, the expression, during the post-conditioning test, of the acquired aversion was associated
537 with increases in pERK-positive cells in the CeA ($p<0.001$) and BLA ($p<0.05$) of the amygdala complex

538 (respectively from 7±1 to 17±1 for CeA and from 18±2 to 31±4 for BLA). Both doses of caffeine pre-
539 treatment, given during conditioning, significantly reduced pERK expression to 3±1 and 5±1 pERK-
540 positive cells for the doses of caffeine of 3 and 15 mg/kg, respectively, in the CeA, and to 21±1 and
541 16±2 for the doses of caffeine of 3 and 15 mg/kg respectively in the BLA (figures 7C and 7D). One-
542 way ANOVA with pre-treatment/treatment as independent variable and with the number of pERK-
543 positive cells/area as dependent variable, revealed significant pre-treatment and treatment effects
544 in CeA [$F_{3,16}$=79.57, p<0.001] and BLA [$F_{3,16}$=5.52, p<0.05]. Post hoc analysis using Fishers Least
545 Significant Difference (LSD) test revealed that 1) expression of EtOH-elicited CPA stimulates a
546 significant increase of the number of pERK-positive neurons in both regions of amygdala complex
547 (p<0.05) and that 2) pre-treatment with caffeine, given during conditioning, at both doses
548 significantly reduced (p<0.05) this effect.

549 Figure 7



550

551 Effects of ethanol-elicited CPA expression on ERK phosphorylation in the Acb, BNST, CeA and BLA

552 Animals of each group, in a drug-free state, performed the post-conditioning test (15 minutes) and
553 immediately afterwards were anesthetized and perfused for immunohistochemical analysis. Data are shown
554 as mean ± SEM of pERK-positive neurons/area. The number of animals of each experimental group were: n=5
555 for SAL-SAL (caffeine 0 mg/kg + ethanol 0 g/kg) group; n=5 for SAL-EtOH (caffeine 0 mg/kg + ethanol 2 g/kg)
556 group; n=5 for CAF3-EtOH (caffeine 3 mg/kg + ethanol 2 g/kg) group and n=5 for CAF15-EtOH (caffeine 15
557 mg/kg + ethanol 2 g/kg) group. Figures 7B and 7C: **indicates a significant difference ($p<0.001$) between
558 SAL-EtOH and all other groups in BNST and CeA; figure 7D: *indicates a significant difference ($p<0.05$)
559 between SAL-EtOH and all other groups in BLA. Individual data points are indicated in the corresponding
560 histogram for each experimental group.

561

562 ## Discussion

563 Drug interactions may have substantial consequences both in the therapeutic practice and in
564 relation to substance abuse and addiction. The interaction between caffeine and ethanol makes no
565 exception in this regard, and is especially important because involves a very large number of
566 individuals all over the world. The present work was designed and carried out in order to start laying
567 the foundation for systematically studying the consequences of ethanol-caffeine interaction, with
568 the explicit aim of investigating the possible alterations that the conditioning properties of ethanol
569 may undergo as a consequence of such interaction.
570 In particular, the present study was aimed at establishing whether the administration of caffeine at
571 low to moderate doses (3 and 15 mg/kg), thought to be borderline for eliciting arousal (Acquas et
572 al., 2002; De Luca et al., 2007; Hasenfratz et al., 1993) and locomotor activity (Dar, 1988; López-Cruz
573 et al., 2013), could affect the conditioning properties of ethanol at a dose (2 g/kg) utilized in place
574 conditioning studies in mice (Cunningham et al., 2002, 2003; Cunningham and Henderson, 2000;
575 Font et al., 2006; Pati et al., 2019; Rosas et al., 2017; Spina et., 2015; Zuniga and Cunningham, 2019).
576 To this end, and also in order to address a possible mechanism through which this interaction could
577 take place, we adopted a behavioral and biochemical approach. For the behavioral measures, in the
578 place conditioning experiments we assessed the shift from spontaneous preference for a given
579 environment (Acquas et al., 1989; Rosas et al., 2017, 2018; Spina et al., 2015) after conditioning with
580 the administration of caffeine or ethanol or of their combination. The biochemical measures were
581 aimed at detecting, in specific brain regions involved in the affective and motivational responses to
582 drug stimuli, including ethanol (Ibba et al., 2009), the expression of phosphorylated ERK, a cellular
583 marker related to neural plasticity and short- and long-term adaptive responses to substances of
584 abuse (Acquas et al., 2007, 2010; Gerdjikov et al., 2004; Ibba et al., 2009; Rosas et al., 2017; Sun et
585 al., 2016; Sweatt, 2001, 2004; Valjent et al., 2004).
586 Our behavioral model, which involved slight modifications of the procedures introduced by
587 Cunningham and Colleagues (Cunningham et al., 2003, 2006), had the value added of emphasizing
588 that the same dose of the same unconditioned stimulus, ethanol, can have both preferring and
589 aversive conditioning properties. In fact, in agreement with others (Pati et al., 2019) we regard this
590 approach also useful for characterizing the potentially differential impact that another substance,
591 caffeine, may have on such opposite effects.
592 In agreement with previous extensive data (Cunningham et al., 2002, 2003; Cunningham and
593 Henderson, 2000; Pati et al., 2019; Rosas et al., 2017; Spina et al., 2015), the results of the behavioral

experiments confirmed that ethanol elicits strong and significant CPP (figure 2) and CPA (figure 3). The study also reveals that caffeine, at either doses, failed to alter the spontaneous preference of the animals as shown by the results of the post-conditioning tests of the CAF3-SAL and CAF15-SAL groups. Notably, in agreement with Kaplan et al., (1990), the timing observed in our CPP and CPA schedules for the combined administrations of caffeine and ethanol suggest that in both circumstances plasma concentrations of caffeine could have reached a plateau at the time saline or ethanol were administered. Thus, the observation that caffeine affects ethanol-elicited conditioning (figures 2 and 3) suggests that failure of caffeine to exert conditioning properties on its own, cannot be attributed to pharmacokinetic reasons, at least under the present experimental conditions. **Notably, i**~~I~~n other studies, ~~the~~ **a** significant CPP elicited by caffeine was observed only after test one **(performed, after eight conditioning days), but not after test two, (performed after eight additional conditioning days),** (Hilbert et al., 2013) or only after test three **(after the eighth conditioning trials). However, there was no CPP after either the first or the second test was performed, respectively, after four and six conditioning trials)** (Zuniga and Cunningham, 2019)~~, while that for ethanol remained constant across tests (Hilbert et al., 2013; Zuniga and Cunningham, 2019)~~ suggesting that~~, at least in those experimental conditions,~~ caffeine exerts, **if any,** weak **and inconsistent** reinforcing properties (Liu et al., 2008).

Moreover, the combination of caffeine and ethanol administrations significantly altered the outcome of ethanol-elicited conditioning: in particular, 15 mg/kg caffeine prevented the acquisition of ethanol-elicited CPP (figure 2), and both doses significantly prevented the acquisition of ethanol-elicited CPA (figure 3). These results appear at variance with those of the study by Hilbert and Colleagues (2013), who found that caffeine (3 mg/kg) exerted conditioning effects in C57BL/6J mice, although they were weaker compared to those of ethanol (1.75 g/kg), and that their combination resulted in conditioning properties indistinguishable from those of ethanol alone. **Data from our group indicate that, at doses and timing very similar to the ones used in the present experiments, caffeine increases ethanol-elicited locomotion in an open field (Porru et al., in preparation). In the present experiments we did not measure mice locomotor activity either during the conditioning or during the post-conditioning tests. However, although an increased locomotor activity might bring about an increased side-preference shift (Tzschentke, 2007), we could rule out the possibility of such non specific effect of locomotion on conditioning since the interaction between caffeine and ethanol on place conditioning resulted indeed in the ability of caffeine to prevent ethanol-elicited conditioning.** Moreover, our results are also partly at variance with those of the

626 study by Zuniga and Cunningham (2019), who reported that caffeine (3 or 30 mg/kg), administered
627 in combination with ethanol (2 g/kg), fails to affect ethanol-elicited CPP. However, a number of
628 critical differences may be taken into account to interpret these discrepant results. For example,
629 the experimental design of the studies by Hilbert and Colleagues (2013) and Zuniga and Cunningham
630 (2019) were quite different from that of the present one, including different route and modality of
631 administration (a single injection vs two separate injections at different times), different strains of
632 animals (C57BL/6J or DBA/2J vs CD-1), different acquisition times (16 vs 8 conditioning sessions),
633 different numbers of post-conditioning tests and different time intervals between the
634 administration of the unconditioned stimulus and the exposure to the apparatus. These substantial
635 experimental differences could reasonably have led to different results.
636 Overall, the behavioral results of our study indicate that the combination of appropriate low doses
637 of caffeine with a dose of ethanol capable of exerting conditioning properties (Cunningham et al.,
638 2002, 2003; Cunningham and Henderson, 2000; Font et al., 2006; Rosas et al., 2017; Spina et al.,
639 2015), prevented the establishment of learning the CS-US association and, consequently, in a drug-
640 free condition in the post-conditioning test, prevented its expression. This indicates that the ability
641 of ethanol to transfer its properties to the environment (acquisition of place conditioning), is
642 prevented by caffeine, suggesting that its acute administration can interfere with the conditioning
643 effects of ethanol. This evidence is in agreement with previous observations that caffeine prevents
644 **acquisition and expression of** cocaine- (Poleszak and Malec, 2002) and **expression of** ethanol-
645 elicited CPP (Okhuarobo et al., 2019), **suggesting further that the effects of caffeine might be
646 attributed to an interference with the cognitive processes involved in the acquisition and
647 expression of conditioned responses to the behavioral paradigm.**
648 Consistent with previous studies (Ibba et al., 2009), the results of the immunohistochemical
649 experiments confirmed that ethanol significantly increased the number of pERK-positive neurons in
650 the AcbC and AcbSh, in the bed nucleus of stria terminalis, in the central nucleus of the amygdala
651 and in the basolateral amygdala. **However, in these experiments we did not run the saline-caffeine
652 groups based on the application of the 3R principle and on the observation that** ~~P~~previous studies
653 (Valjent et al., 2004) have reported that caffeine, at a dose (10 mg/kg) similar to the one used in the
654 present study, fails to increase pERK in the AcbC and AcbSh as well as in nuclei of the extended
655 amygdala, while doing so in the medial prefrontal cortex and other cortical areas (Acquas et al.,
656 2010; Valjent et al., 2004). ~~In the present experiments, t~~Their combined administration revealed
657 that pre-treatment with caffeine at both 3 and 15 mg/kg significantly prevented the effects of

658 ethanol on ERK phosphorylation (figures 4 and 5). However, while such mechanistic interpretation
659 of the behavioral results shown in figures 2 and 3 may hold true for the effect of caffeine on ethanol-
660 elicited CPP, this may not be the case for interpreting the effect of caffeine on ethanol-elicited CPA.
661 In fact, while ERK phosphorylation in the Acb has been reported to be critical for the acquisition of
662 place preference conditioning (Beninger and Gerdjikov, 2004; Gerdjikov et al., 2004), in our previous
663 studies we reported that blockade of **mitogen-activating extracellular kinase (**MEK**)** by the MEK
664 inhibitor SL327, although able to prevent the acquisition of ethanol-elicited CPP (Rosas et al., 2017)
665 and lithium-elicited CPA (Longoni et al., 2011), failed to affect ethanol-elicited CPA (Rosas et al.,
666 2017). Hence, although we cannot exclude that prevention of ethanol-elicited ERK phosphorylation
667 by caffeine may play a role in the mechanism by which caffeine affects ethanol-elicited CPA (figure
668 3 of the present study), the data from our previous studies (Longoni et al., 2011; Rosas et al., 2017)
669 suggest that other~~, presently unknown,~~ mechanisms **(for instance lithium-elicited CPA in Longoni
670 et al., (2011) was obtained as a consequence of backward conditioning)** should be taken into
671 consideration to interpret these behavioral results.
672 Interestingly, as we have demonstrated using Roman rats psychogenetically selected for poor vs
673 rapid acquisitions of the two-way active avoidance response in a shuttle box (Martin et al., 1982),
674 there are instances in which an addictive drug, morphine in that case, may act differentially **(i)** by
675 eliciting CPP but decreasing ERK phosphorylation in the Acb of Roman Low Avoidance (RLA) rats and
676 **(ii)** by eliciting CPP and not affecting ERK phosphorylation in the Acb of Roman High Avoidance (RHA)
677 rats (Rosas et al., 2018). Strikingly, Rosas and Colleagues (2018) also found that when administered
678 during conditioning to RLA rats, morphine fails to decrease ERK phosphorylation suggesting that the
679 procedure of conditioning in itself is able to affect the way morphine impacts on ERK
680 phosphorylation.
681 The present study also aimed at evaluating the expression of pERK after exposure to the conditioned
682 stimulus (ethanol-paired compartment) in animals that were sacrificed immediately after the
683 completion of the post-conditioning test. The results of these experiments clearly demonstrate a
684 differentiation between the behavioral expression (positive or negative side preference shift
685 compared to pre-test) and the brain region-dependent ERK activation. In particular, in animals
686 receiving ethanol during conditioning under the schedule expected to elicit CPP, we found that the
687 performance of the post-conditioning test resulted in a significant increase of pERK-positive neurons
688 in the AcbC and AcbSh but not in the bed nucleus of stria terminalis, in the central nucleus of the
689 amygdala and in the basolateral amygdala. On the contrary, in animals receiving ethanol during

690 conditioning under the schedule expected to elicit CPA, the performance of the post-conditioning
691 test resulted in a significant increase of pERK-positive neurons in the bed nucleus of stria terminalis,
692 the central nucleus of the amygdala and the basolateral amygdala, suggesting a critical involvement
693 of these areas (McDonald et al., 2010; Pati et al., 2019; Wscieklica et al., 2019), but not in the AcbC
694 and AcbSh. These results, shown in figures 6 and 7, indicate that the conditioned stimulus, i.e. the
695 environment associated with ethanol (forward conditioning, CPP) and that assigned to ethanol
696 (backward conditioning, CPA), has a different impact on the phosphorylation of ERK in these brain
697 areas. Interestingly, the results of the present conditioned aversive stimulus experiments appear at
698 variance with respect to a previous study in which we found that expression of CPA elicited by
699 lithium (Longoni et al., 2011) was associated with increased ERK phosphorylation in the dorsal
700 striatum but not in the bed nucleus of stria terminalis, the central amygdala and the basolateral
701 amygdala. This discrepancy might be attributed to profound differences between these two
702 unconditioned stimuli (ethanol and lithium). In contrast, in the present study, pERK expression was
703 activated in nuclei of the extended amygdala and in the basolateral amygdala, suggesting an overall
704 complex and differential involvement of this kinase in brain areas involved in associative learning,
705 reinforcement and emotion (McDonald et al., 2010; Pati et al., 2019; Wscieklica et al., 2019).
706 In conclusion, these results cast a new light on a critical topic that has considerable translational
707 significance. In fact, given the role attributed to conditioned stimuli in determining drug-taking
708 reinstatement, although difficult to interpret under a unitary hypothesis, these results offer an
709 original view for the potential usefulness of appropriate combined administrations of caffeine and
710 ethanol. In this regard, we recognize of particular interest the observation made by (Okhuarobo et
711 al., 2019) that caffeine administration before the post-conditioning test in ethanol-conditioned CD-
712 1 mice prevents CPP expression. Future experiments are needed to identify and further characterize
713 the mechanism(s) at the basis of these complex interactions between caffeine and ethanol. **All this
714 notwithstanding, the present results might contribute to increase the awareness on the critical
715 role that the knowledge of the exact conditions at which the interaction between caffeine and
716 ethanol take place. They are also important in order to prevent the consequences of the
717 oversimplification that their combined consumption may have, leading to increased impulsivity,
718 risk-taking behaviors as well as the potential risk of developing addictive behaviors (Snipes et al.,
719 2015).**

720 ## Declaration of conflicting interests

721 The author(s) declared no potential conflicts of interest with respect to the research, authorship,
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