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Behavioral and neurochemical assessment of the role of ERK pathway in the psychopharmacological effects of ethanol, caffeine and of their interaction

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Summary

SUMMARY

The intracellular signaling cascades constitute the means by which addictive substances induce the remodeling of the circuits involved in motivated behaviors which underlie the learning processes and the development of memories at the basis of the progression of addiction. A protagonist in such signaling cascades is the Extracellular signal-Regulated Kinase (ERK), a member of the mitogen-activated protein kinase, that constitutes an important biochemical factor common to many cellular functions. The abundant expression of ERK in brain areas of addiction circuits emphasizes the relevance of this kinase in modulating behavioral functions mediated by these circuits. These findings led to the development of new research lines aimed at understanding the involvement of ERK in the processes related to the onset of addiction and at characterizing their role in various aspects of this pathology, including substances' consumption, sensitization as well as substances' involvement in its cognitive and motivational aspects. Another biochemical factor involved in this process is the Dopamine and cAMP-regulated phosphoprotein Mr 32,000 kDa (DARPP-32), which acts upstream of the MEK/ERK cascade, and regulates the activity and the phosphorylation state of this pathway. Thus, the general aim of the present doctoral thesis was to study the role of ERK in terms of protein kinase expression induced by ethanol, caffeine and their association, and its relationship with some behavioral responses in validated animal models of drug addiction such as the response to stimuli associated to their intrinsic properties and conditioned to the environment (place conditioning), or the psychomotor activation mediated by these substances.

The first and second chapters of this dissertation examined the involvement of protein kinases ERK in different aspects of ethanol-induced place conditioning. Specifically, in the first chapter we used SL327, a compound that inhibits MEK, to study how the blockade of this cascade could affect the acquisition and expression of Conditioned Place Preference (CPP) and Conditioned Place Aversion (CPA) induced by ethanol. Furthermore, we also studied the expression of ERK phosphorylation (pERK) in different areas of the motivation circuit in response to acute SL327 and ethanol administration. In the second chapter we explored the results of the pharmacological

relationship between caffeine, ethanol and their association in the experimental paradigm of ethanol-elicited CPP and CPA previously utilized for experiments described in chapter 1. We also investigated the expression of pERK in various brain areas as a result of either the acute administration of both substances and of the presentation of the positively (CPP) or negatively (CPA) stimuli conditioned to ethanol. The last chapter examines the effects of caffeine, ethanol and their interaction through the analysis of horizontal and vertical locomotion. Moreover, using the open field, we studied the effects of these substances and their interaction in novelty-induced exploration in order to observe whether a cross-sensitization effect could develop. Finally, we studied the phosphorylation in the nucleus accumbens of ERK and DARPP-32(Thr75), related to D_1 and D_2 receptor activation, respectively. The findings of these studies revealed that:

- The MEK/ERK pathway is differentially involved in distinct phases of associative learning behavior expressed in the CPP and CPA elicited by ethanol; specifically, the acquisition phase, as well as the appetitive motivation (CPP), seems to be more sensitive to the blockade of the intracellular signaling cascade. However, the ability of the kinase inhibitor, SL327, to prevent ethanol-induced ERK phosphorylation is not reflected in the differential results of behavioral experiments.

- Caffeine was devoid of conditioning effects but significantly impaired ethanol-elicited place conditioning (both CPP and CPA), which could be referred to a functional antagonistic action exerted by these two drugs on the adenosinergic system. Furthermore, the results of acute administration experiments support this hypothesis of a functional antagonistic interaction since caffeine is able to prevent ethanol-induced pERK expression in several brain areas. Nevertheless, the evaluation of pERK expression following the presentation of the conditioned stimuli reflects a differential activation patterns depending on the brain area examined.

- Caffeine and ethanol, administered acutely, affected horizontal and vertical locomotion in the open field in a biphasic manner and caffeine potentiated acute ethanol-induced locomotion. However, the acute administration of

caffeine to mice that were previously sensitized to the effects of ethanol had reduced stimulating effects compared to ethanol naïve mice. Finally, caffeine prevented the ethanol-elicited pERK expression in the nucleus accumbens whereas there were no effects on pDARPP-32(Thr75), suggesting an effect mediated by D_1-A_1 receptors on this area.

Taken together the results of the present thesis offer new insights into the complexity of the involvement of ERK cascade in the acquisition of associative learning and in the expression of acquired responses related to models of drug addiction. These data provide new information about the antagonistic interaction between caffeine and ethanol in the motivational properties expressed in the place conditioning paradigm and in the ability of ethanol sensitization to blunt the stimulatory effects of caffeine.

RESUMEN

Las cascadas de señalización intracelular constituyen el medio por el cual las sustancias adictivas inducen la remodelación de los circuitos involucrados en comportamientos motivados que subyacen en los procesos de aprendizaje y desarrollo de memorias en base a la progresión de la adicción. Una molécula protagonista en tales cascadas de señalización es "Extracellular signal-Regulated Kinase" (ERK), un miembro de la proteína quinasa activada por mitógenos, que constituye un importante factor bioquímico común a muchas funciones celulares. La abundante expresión de ERK en áreas cerebrales implicadas en el circuito de la adicción, enfatiza la relevancia de estas quinasas en la modulación de las funciones del comportamiento mediadas por estos circuitos. Estos hallazgos condujeron al desarrollo de nuevas líneas de investigación destinadas a caracterizar el papel de la ERK en varios aspectos de la adicción a las drogas, incluido el consumo, la sensibilización y la participación de las sustancias en aspectos cognitivos y motivacionales de esta patología, para comprender la participación de la ERK en los procesos relacionados con su inicio. Otro factor bioquímico involucrado en este proceso es "Dopamine and cAMP-regulated phosphoprotein Mr 32,000 kDa (DARPP-32)", que actúa como parte de la cascada MEK/ERK, regulando la actividad y el estado de fosforilación de la ERK. De este modo, el objetivo general de la presente tesis doctoral fue estudiar el papel de ERK, en términos de expresión de proteína quinasa inducida por drogas (etanol, cafeína y su asociación), así como su relación con respuestas conductuales relacionadas con el desarrollo de la adicción tales como la respuesta a estímulos asociados a las propiedades intrínsecas y condicionadas al entorno (condicionamiento de lugar). Finalmente, también investigamos la actividad psicomotora mediada por estas sustancias.

Los capítulos primero y segundo de esta tesis doctoral examinan la participación de la proteína quinasa ERK en diferentes aspectos del condicionamiento de lugar inducido por etanol. Específicamente, en el primer capítulo usamos SL327, un compuesto que inhibe la MEK, para estudiar cómo el bloqueo de esta cascada podría afectar la adquisición y la expresión de la preferencia de lugar condicionada (CPP) y la aversión de lugar condicionada (CPA) inducidas por etanol. Además, también estudiamos la

expresión de la fosforilación de ERK (pERK) en diferentes áreas de circuitos motivacionales tras la administración aguda de SL327 y etanol. El segundo capítulo explora los resultados de la relación farmacológica entre cafeína, etanol y su asociación en el mismo paradigma experimental de CPP y CPA inducidos por etanol. También investigamos la expresión de pERK en diversas áreas del cerebro como resultado de la administración aguda de ambas sustancias y su impacto en la expresión del condicionamiento positivo (CPP) o negativo (CPA) al etanol. El último capítulo examina los efectos de la cafeína, el etanol y su interacción a través del análisis de la locomoción horizontal y vertical. Además, utilizando el campo abierto, estudiamos los efectos de estas sustancias y su interacción en la exploración inducida por novedad para observar si se desarrolla un efecto de sensibilización cruzada. Finalmente, estudiamos la fosforilación en el núcleo accumbens de pERK y pDARPP-32(Thr75), relacionado con la activación de los receptores D_1 y D_2, respectivamente. Los resultados de estos estudios revelaron que:

- La vía MEK/ERK participa de manera diferencial en distintas fases del comportamiento de aprendizaje asociativo expresado en el CPP y el CPA inducido por el etanol; específicamente, la fase de adquisición, así como la motivación apetitiva (CPP), parece ser más sensible al bloqueo de la cascada de señalización intracelular. Sin embargo, la capacidad del inhibidor de la quinasa SL327, para prevenir la fosforilación de ERK inducida por etanol, no se refleja en los diferentes resultados de los experimentos conductuales.

- La cafeína no produce efectos por sí misma en condicionamiento, pero afecta significativamente el condicionamiento de lugar inducido por el etanol (tanto CPP como CPA), lo que podría reflejar una acción antagonista funcional ejercida por estos dos fármacos sobre el sistema adenosinérgico. Además, los resultados de los experimentos de administración aguda respaldan esta hipótesis de una interacción antagónica ya que la cafeína puede prevenir la expresión de pERK inducida por etanol en varias áreas del cerebro. Sin embargo, la evaluación de la expresión de pERK después de la expresión de los estímulos condicionados refleja diferentes patrones de activación dependiendo del área del cerebro examinada.

- La cafeína y el etanol, administrados de manera aguda, afectan la locomoción horizontal y vertical en campo abierto de manera bifásica y la locomoción inducida por etanol potenciada por cafeína. Sin embargo, la administración aguda de cafeína a ratones que previamente habían estado sensibilizados a los efectos del etanol reduce los efectos estimulantes en comparación con los ratones sin tratamiento previo con etanol. Finalmente, la cafeína evita la expresión de pERK provocada por etanol en el núcleo accumbens, mientras que no hay efectos sobre pDARPP-32(Thr75), lo que sugiere un efecto mediado por los receptores D_1-A_1 en esta área cerebral.

Tomados en conjunto, los resultados de la presente tesis ofrecen nuevos conocimientos sobre la complejidad de la participación de la cascada ERK en la adquisición del aprendizaje asociativo y en la expresión de las respuestas adquiridas. Estos datos proporcionan nueva información sobre la interacción antagónica entre la cafeína y el etanol en las propiedades motivacionales expresadas en el paradigma del condicionamiento de lugar y en la capacidad de sensibilización del etanol para mitigar los efectos estimulantes de la cafeína.

General Introduction

GENERAL INTRODUCTION

MOTIVATED BEHAVIOR

Since the dawn of psychology, the study of the mechanisms underlying the concept of motivation has been considered essential for understanding human behavior. In general, the term motivation refers to the set of conditions that convey our behavior, determining the orientation towards a specific goal. Motivation is a necessary ingredient of interaction for all the species. In fact, all animals are guided by motivation to satisfy natural needs essential for survival and these behaviors are the result of biological and psychological processes that have been subjected to evolution at various levels (Simpson & Balsam 2016). Motivated behavior can be described by activational and directional aspects. The activational aspects concern the fact that the beginning and the maintenance of motivated behaviors can be represented by high levels of activity and vigor and persistence in the working results (Salamone *et al.* 2018). Moreover, the directional aspects are related to the fact that the behavior can be directed towards or away from a specific stimulus, in order to search for some (i.e., water, food, social and sex interaction) and avoid others (i.e., pain, discomfort) (Salamone 2010; Salamone & Correa 2012). This difference in the emotional response reflects the motivational valence of the stimulus and can be positive or negative, depending on the purely instinctive perception of reacting to stimuli. Natural stimuli are characterized by a positive motivational value and can be considered appetitive stimuli and, due to their intrinsic nature, the descending behavior will be indeed "motivated" to their research. On the contrary, in case of unpleasant stimuli, the reaction will be avoidance (aversive behavior). Operationally, a motivated behavior can be defined as the result of two consecutive phases: the initial phase consisting mainly of approaching or seeking the reinforcing goal stimulus (e.g., seeking behavior), while in the subsequent phase the organism gains access to the stimulus and directly interacts with it (consummatory behavior) (Salamone & Correa 2012). However, motivated behaviors are widely adaptable, even though they can become habitual. Both the first motivation to emit the behavior and the subsequent behaviors are influenced by past and present experience with the reinforcing stimulus (Volkow *et al.*

2017). Therefore, the perception of a stimulus can be manipulated through learning processes, such as in the associative learning.

A MODEL OF ASSOCIATIVE LEARNING: PAVLOVIAN CONDITIONING

The associative learning can be described as a process in which the subject learns the relationships between two stimuli, or between a stimulus, and a behavior. In the classification of the experimental procedures, the associative learning can be distinguished in classic conditioning, that involves learning the relationships between two stimuli, and operant conditioning, that includes learning the relationship existing between one or more stimuli and the reaction derived from interacting with it. The modern neuroscientific study of classical conditioning, also called "Pavlovian conditioning", was originally described by Russian physiologist Ivan Pavlov (1927). In Pavlov's seminal experiment two stimuli were presented paired to a dog: a bell tone simultaneously with some food. The repeated association (conditioning procedure) between these two stimuli changed the dog's behavior in response to the presentation of the first stimulus. In fact, the tone never would have caused a reaction (salivation) until it would have been paired with the food. In this study, the tone is called conditioned stimulus (CS), whereas the food is the unconditioned stimulus (US) and the learned response, as a consequence of a repeated exposure to the appetitive stimulus, is named the conditioned response (CR) to differentiate it from the innate unconditioned response (UR), because dog salivated less or more depending on its hunger status. In fact, the CS upon the learning experience acquires the property of eliciting a new response, the CR, strictly related to and dependent from the conditioning process (Fanselow & Wassum 2016).

CONDITIONED PLACE PREFERENCE AND CONDITIONED PLACE AVERSION

A model of Pavlovian conditioning is the experimental procedure of place conditioning in its two variants, Conditioned Place Preference (CPP) and Conditioned Place Aversion (CPA). The CPP paradigm is characterized by the pairing of two distinct sets of environmental cues (e.g., apparatus with different wall color or pattern, size or shape) with the US (e.g., drug, food). The conditioning itself is represented by the repeated exposure to the US in one compartment of the CS (conditioned stimulus paired with

drug; CS+), while the exposure to the other compartment occurs without the US (conditioned stimulus paired without the drug; CS-). At the end of the conditioning, the animal, in US absence, is exposed to both contexts (CS+ and CS-) and a choice test is performed. This condition is one of the greatest advantages of place conditioning as, being the animal tested in a drug-free state, there cannot be interference with the emitted choice as a consequence of the pharmacological effects of the drug. A significant increase in time spent in the paired compartment relative to a control value is an indication that the US was provided of positive motivational properties (Bardo & Bevins 2000; Tzschentke 2007). A significant reduction in time spent in the paired compartment relative to a control value is an indication that the US was provided of negative motivational properties (Bardo & Bevins 2000; Tzschentke 2007). The CPP, under appropriate conditions, is a method for measuring drug reinforcement. Drugs of abuse have been widely used for CPP experiments, such as cocaine (Sellings 2006), ecstasy (Robledo *et al.* 2004), morphine (Ruiu *et al.* 2013; Zhu *et al.* 2015), nicotine (Ahsan *et al.* 2014), THC (Vann *et al.* 2008) and ethanol (Cunningham *et al.* 2003, 2006; Spina *et al.* 2015), although this latter, similarly to others such as nicotine, is endowed with the property, under appropriate conditions, to elicit both CPP and CPA (Cunningham *et al.* 2006; Spina *et al.* 2015). Moreover, if the learning process resulting in CPP is grounded on the ability of animals to transfer the positive biological significance (wellness and euphoria), that related to CPA is founded on the opposite principle, i.e. to the transposition of negative (sickness and dysphoria) feelings to motivationally neutral components of the environment (Bardo and Bevins 2000; Tzschentke 2007). In addition, upon an appropriate manipulation of the experimental conditions it is possible to shift the ability of a given US from that of resulting in a preference state, in which the animals choose to spend more time in the context associated with the drug, to that of resulting in an aversion state, such as in case of high doses of the same drug, e.g. nicotine that has dose-dependent rewarding and aversive effects (Risinger & Oakes 1995). With due caution, also the condition of withdrawal (from a drug) states have been shown to elicit CPA (Stinus *et al.* 2005).

FROM MOTIVATION TO ADDICTION

The mesolimbic system: neurophysiology

The mesolimbic system plays a pivotal role in the coordination of behaviors, emotions and memories, and interconnects cortical and subcortical structures devoted to linking primordial states and emotion to learning and behavior. The stimulation of the circuit is involved in the acquisition of motivational properties to neutral stimuli (Catani *et al.* 2013). This system has been described for the first time in 1954 by Olds and Milner. In their experiment these Authors discovered that rats responded to reinforcing stimuli, brain electrical stimulation, only when applied in specifically brain areas (Olds & Milner 1954). Subsequently, it was established that the area of mesolimbic system most involved in reinforcement processes and related behavior is the nucleus accumbens (Acb). Natural stimuli, such as food, water and sex, but also drugs of abuse, have the common property to enhance the release of dopamine in the Acb (Di Chiara & Imperato 1988). However, the continuous stimulation of dopaminergic neurons and the consequent increases of extracellular dopamine in the Acb elicited by addictive drugs, unlike the natural stimuli, does not develop the adaptive mechanisms of habituation but, on the contrary, strongly encourages the acquisition of motivational properties by drug conditioned stimuli (Di Chiara & Bassareo 2007). Furthermore, drugs of abuse promote an adaptive function, the incentive learning, defined as the acquisition by previously neutral stimuli of the capacity to elicit effects and other responses and occurs in association with the presentation of reinforcing stimuli (Berridge & Robinson 1998). Thus, abnormal changes in the motivation are a key element to develop disorders of motivated behaviors that can be divided into two groups. The first group is characterized by pathological deficits in motivation frequently in patients with schizophrenia and affective disorders. The second group consists in problematic excesses in behavior as well as addiction disorder elicited by addictive drugs (Simpson & Balsam 2016). Indeed, in susceptible subjects, repeated use of drugs of abuse carries the risk of developing dependence and addiction, defined as a chronic disease characterized by compulsive drug use despite adverse consequences, loss of control over drug-seeking behavior and high risk of relapse (Koob & Volkow 2009). Since ancient times humans began to use psychoactive

substances, initially in a ritual and/or religious context and then inspired by personal ''recreational'' desires. These substances have the common characteristic to induce a set of emotional and physical feelings that, by mimicking the reinforcing properties of natural stimuli, are recognized as pleasurable (pleasure). The perception of pleasure, due to the stimulation of the mesolimbic system, leads a motivated behavior aimed to seek and re-experience the same gratifying feeling perceived during the consumption of the drug. Consequently, the substance acquires a positive value (primary reinforcement) in the individual who carries out a series of behaviors with the sole purpose of obtaining the drug of abuse (Spanagel & Weiss 1999; Koob & Volkow 2009). Addictive drugs can reinforce previous intake behavior by inducing pleasant effects (positive reinforcement) or by ending an unpleasant feeling (negative reinforcement). In this regard, the development of physical addiction/dependence could be explained by the possibility of another form of reinforcement: whenever the use of a drug relieves the unpleasant effect of the withdrawal syndrome, the previous behavior is further supported. Even when the development of tolerance attenuates the initial reinforcing effects, drugs of abuse can elicit a recurrent and regular sense of dysphoria or suffering, which is immediately eliminated, and sustained, by another administration (Di Chiara 2002; Koob & Volkow 2009).

The nucleus accumbens: neuroanatomy

The Acb is the central area of the limbic system and it is supposed to play a critical role in the motivated behavior, and, specifically, in the translation of limbic information into goal-directed behavior. It receives glutamatergic projections from the hippocampus, basolateral amygdala, prefrontal cortex and dopaminergic inputs from the ventral tegmental area (VTA) (Charara & Grace 2003). The Acb and VTA receive glutamatergic output from the prefrontal cortex and, moreover, a reciprocal glutamatergic connection has been described between the amygdala and prefrontal cortex. All these regions, that make up a circuit whose key role is played by dopamine, appear to perform an important role in learning processes, motivation, memory and adaptive behavior (Kelley 2004). The Acb is characterized by two functionally distinct subregions termed core and shell. The core is the dorsolateral part, associated with the anterior and orbitofrontal cortex, and regulates the motor expression of motivated

behavior. The medioventral compartment, the shell, is interconnected with the hypothalamus and VTA and is mostly involved in the perception of gratification. Additionally, the shell is implicated in the relationship between motivational events and simultaneous environmental perceptions that contribute to learning establishment (Kalivas & Volkow 2005). In the Acb the main cell types are GABAergic and the majority of these are medium spiny neurons (MSNs), on which dendrites converge dopaminergic and glutamatergic inputs (Cahill *et al.* 2014). Based on the peptide that contain, the MSNs can be divided into two groups, one subpopulation of neurons that express substance P and dynorphin, and another group containing enkephalin. Furthermore, MSNs express different types of receptors, predominantly glutamatergic and dopaminergic. Studies of receptor localization have shown that D_1 dopaminergic receptors in the striatum are located predominantly at the postsynaptic level (Altar & Marien 1987), are coupled with a G-protein that stimulates adenylate cyclase activity (G_s) and co-express substance P (Lu *et al.* 1997), while dopaminergic D_2 receptors are located both in the presynaptic and postsynaptic terminals (De Mei *et al.* 2009), are coupled to G-proteins that inhibit adenylate cyclase ($G_{i/olf}$) and co-express mainly enkephalin (Lu *et al.* 1997). When D_2 receptors are in homologous terminations are inhibitory auto-receptors, but also can be localized in glutamatergic, cholinergic and GABAergic neurons. However, there are biochemical and physiological findings that support the hypothesis that a subpopulation of MSNs that express both D_1 and D_2 receptors is also present (Lu *et al.* 1997; Svenningsson *et al.* 1999).

BIOCHEMICAL BASIS OF ADDICTION

ERK signaling

The Extracellular signal-Regulated Kinases (ERK) are part of the Mitogen-Activated Protein Kinase (MAPK)-signaling cascade, which is a family of serine-threonine kinases expressed in all eukaryotic cells, where they play an essential role in several cellular processes, such as cell proliferation, differentiation and apoptosis (Sweatt 2004). There are several ERK isoforms, but the most characterized and more involved in neurophysiological processes as well as in drug addiction are ERK_1 and ERK_2, with molecular weight of 44 and 42 kDa, respectively (Girault *et al.* 2007). Their activation

requires the dimerization and phosphorylation on two regulatory residues, Thr202/Tyr204 for ERK_1 and Thr183/Tyr185 for ERK_2 (Khokhlatchev *et al.* 1998). This double phosphorylation is commonly detected by antibodies, and is thereby considered as a reliable index of ERK activation. In neurons ERK are activated through phosphorylation by various extracellular signals and, consequently, by the stimulation of different cellular receptors, including metabotropic G protein-coupled receptors (GPCRs) and tyrosine kinase receptors. The two pathways converge on the phosphorylation of one common kinase, the mitogen-activated protein kinase/ERK kinase (MEK) which is responsible of ERK phosphorylation. Once activated, ERK can phosphorylate other cytosolic proteins or move into the nucleus where they play a key role in gene regulation, thereby they can phosphorylate a number of transcription factors and control the transcription of immediate early genes (e.g. c-Fos) (Valjent *et al.* 2001; Davis & Laroche 2006; Girault *et al.* 2007; Sun *et al.* 2016).

ERK and drugs of abuse

ERK phosphorylation is elicited also by drugs of abuse in a subset of neurons in brain areas related to addiction. The first study which showed an increase of ERK expression in response to morphine or cocaine reported an increase in the VTA of rats after a chronic treatment (Berhow *et al.* 1996). In particular in the MSNs of the striatum, the most expressed GPCRs are the dopaminergic D_1 and D_2, receptors positively and negatively coupled to cyclic adenosine monophosphate (cAMP)/protein kinase A (PKA) pathway, respectively. Nevertheless, ERK activation requires the activation of both dopamine D_1 and N-methyl-D-aspartate (NMDA) glutamate receptors, that converge onto the dendrites of the MSNs, in fact, the stimulation of D_1 receptors cannot elicit ERK phosphorylation *per se*, but requires an increase of calcium influx through NMDA receptors as a consequence of endogenous glutamate (Pascoli *et al.* 2011). D_1 receptors are represented in the cerebral cortex, limbic system, thalamus and dorsal and ventral regions of the striatum (Girault *et al.* 2007; Cahill *et al.* 2014). It has been demonstrated that pretreatment with a D_1 receptor antagonist (SCH23390) is able to prevent cocaine-elicited ERK phosphorylation (Fontana *et al.* 1993) and raclopride, a D_2 receptor antagonist, fails to prevent this increase (Valjent *et al.* 2000). Moreover, studies using D_1 receptors knock-out mice demonstrated that both the locomotor

activation (Xu *et al.* 2000) and the self-administration (Caine *et al.* 2007) elicited by cocaine have been prevented. Furthermore, also glutamatergic inputs, which attain in the striatum from cortical and subcortical regions, are required for ERK activation. Glutamate receptors, both metabotropic and ionotropic, are co-expressed closely to the dopaminergic receptors. In particular, studies with NMDA receptors antagonist (MK801), as well as with GluN1-knockdown mice, demonstrated a complete prevention of cocaine-induced sensitization (Ramsey *et al.* 2008; Valjent *et al.* 2010). Thus, the convergence of dopaminergic and glutamatergic signals on the ERK pathway in response to binding of DA to D_1 receptor involves the activation of PKA, that quickly phosphorylates the NMDA receptor at Ser897 of the GluN1 subunit. Notably, however, the trigger event for the ERK activation depends on a non-canonical signaling pathway that involves the phosphorylation of GluN2B by the protein-tyrosine kinase Fyn with a consequent increase of Ca^{2+} influx into the neuron (Pascoli *et al.* 2011). This last event causes the activation of the calcium-sensitive Ras-guanine releasing factor (Ras-GRF1) that turns on the MEK/ERK pathway, whereas the maintenance of ERK phosphorylation depends on cAMP/PKA pathway. Specifically, the maintenance of activation is due to the deactivation of MEK/ERK-related phosphatases via dopamine and cAMP-regulated phosphoprotein M_r 32,000 kDa (DARPP-32) and striatal-enriched tyrosine phosphatase (STEP). In fact, the activation of PKA via D_1 receptors can phosphorylate DARPP-32 at Thr-34 site, which converts it in a strong inhibitor of the protein phosphatase PP-1, which dephosphorylates the other substrate, STEP (Valjent *et al.* 2005; Baik 2013; Cahill *et al.* 2014) (see figure 1).

Moreover, ERK pathway is activated in response to acute (Valjent *et al.* 2000, 2004; Salzmann *et al.* 2003; Zhang *et al.* 2004; Brami-Cherrier *et al.* 2005; Acquas *et al.* 2007; Ibba *et al.* 2009) and chronic (Berhow *et al.* 1996; Muller & Unterwald 2004) exposure to addictive drugs in mesolimbic circuit, that play a critical role on neuroplasticity, gene expression and behavioral changes underlying the reinforcing processes induced by these substances. Moreover, the role of these kinases has also been correlated to the motivational properties of drugs evaluated through CPP procedures (Valjent *et al.* 2000, 2001; Salzmann *et al.* 2003; Gerdjikov *et al.* 2004; Lu *et al.* 2006; Spina *et al.* 2010) but also to the ability of contextual stimuli to evoke the expression of acquired

CPP (Miller & Marshall 2005) and of conditioned stimuli on reinstatement of ethanol-seeking behavior in self-administration experiments (Radwanska *et al.* 2008; Schroeder *et al.* 2008; Peana *et al.* 2013).
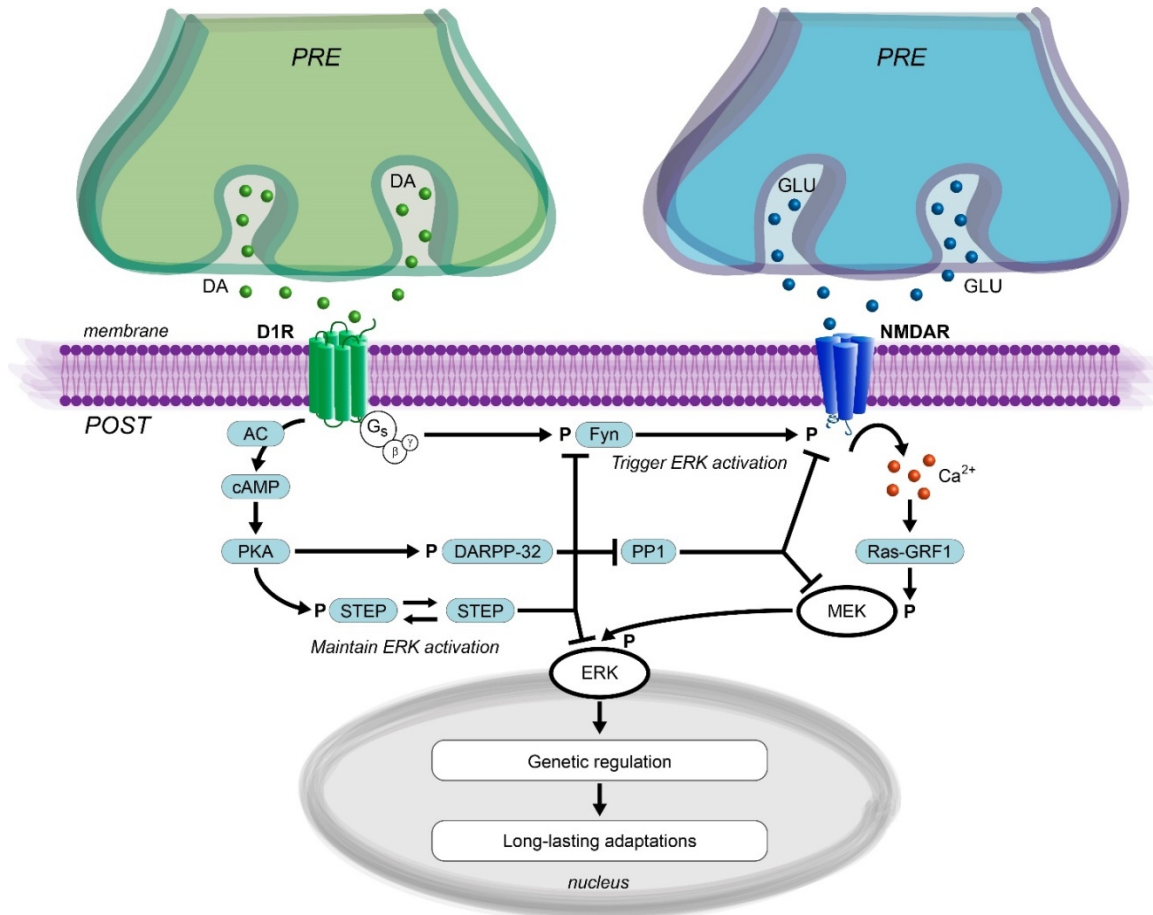

Figure 1. Schematic representation of ERK activation signaling pathway. *The simultaneous stimulation of dopaminergic D$_1$ (D1R) and glutamatergic NMDA receptors (NMDAR) in the striatum mediates ERK phosphorylation.* ***AC****: adenylate cyclase;* ***cAMP****: cyclic adenosine monophosphate;* ***PKA****: protein kinase A;* ***DARPP-32****: dopamine and cAMP-regulated phospho-protein M$_r$ 32,000 kDa;* ***STEP****: striatal-enriched tyrosine phosphatase;* ***PP1****: protein phosphatase 1;* ***Ras-GRF1****: Ras-guanine releasing factor;* ***MEK****: mitogen-activated protein kinase/ERK kinase;* ***ERK****: Extracellular signal-Regulated Kinase.*

DARPP-32 signaling

DARPP-32 is a protein kinase/phosphatase inhibitor highly expressed in dopaminergic brain areas, the major levels of DARPP-32 being found in the striatum (caudate-putamen and Acb), olfactory tubercle and nuclei of amygdala (Ouimet *et al.* 1998). Moreover, DARPP-32 is abundantly expressed in all parts of MSNs including dendrites, axons and terminals. Given its ability to be phosphorylated on different sites, and their different resulting effects, DARPP-32 appears of fundamental importance for the

integration of biochemical and behavioral responses regulated by dopamine and other neurotransmitters (Svenningsson *et al.* 2005). Interestingly, the phosphorylation of DARPP-32 (pDARPP-32) at various sites and their effects on several substrates, as well as on DARPP-32 itself, produces a multifaceted system of positive and negative feedbacks (Yger & Girault 2011). Indeed, it has been hypothesized that DARPP-32 is a sensor of glutamatergic and dopaminergic inputs, allowing to assume a role in the processing of responses in the MSNs (Barbano *et al.* 2007). Dopaminergic signaling is controlled by the phosphorylation of DARPP-32 at various residues in D_1 and D_2 receptors of MSNs (Nishi & Shuto 2017). One of the most widely studied sites is the phosphorylation at Threonine-34 (Thr-34) by PKA that converts DARPP-32 in a potent inhibitor of the serine/threonine protein phosphatase-1 (PP1). This event has important consequences in the amplification of D_1 dopaminergic signaling because allows to increase the phosphorylation state of all the substrates PKA/PP1 downstream regulated and, moreover, is crucial to maintain the ERK activation elicited by addictive drugs through the regulation of STEP, a potent inhibitor of ERK (Yger & Girault 2011). Furthermore, DARPP-32 is a physiological target of the cyclin-dependent kinase 5 (Cdk5) which phosphorylates it at Threonine-75 (Thr-75) residue with the resulting effect of PKA inhibition and consequent reduction of D_1 signaling cascade. Specifically, the phosphorylation at Thr-75 is more potent than that at Thr-34 in terms of PKA inhibition and related substrates. On the contrary, the inactivation of pDARPP-32 at Thr-75 is caused by dopamine through the phosphatase PP2A/B56δ via D_1 receptor/PKA, which results in the disinhibition of PKA, i.e. in the removal of their tonic inhibition (Bibb *et al.* 1999) (see figure 2).

However, also glutamate can regulate the DARPP-32 signal. In fact, via NMDA and AMPA receptors glutamate reduces pDARPP-32(Thr-34) with the resulting effects to activate PP1. Nevertheless, when pDARPP-32(Thr-34) is low, the dephosphorylation of pDARPP-32(Thr-75) and the consequent disinhibition of PKA could act to potentiate dopamine D_1 receptor/PKA/pDARPP-32(Thr34) cascade. Furthermore, as mentioned above, pDARPP-32(Thr-34) plays a critical role in the regulation of the ERK pathway, not only with the inhibition of PP1, but also preventing ERK deactivation through the maintaining of the tyrosine phosphatase STEP in an inactive, phosphorylated, state

(Svenningsson *et al.* 2005). Thus, as a consequence of D$_1$ receptors activation, pDARPP-32(Thr-34) moves to the nucleus and enables the phosphorylation of histone H3 on Ser- 10 which promotes gene expression (Stipanovich *et al.* 2009).
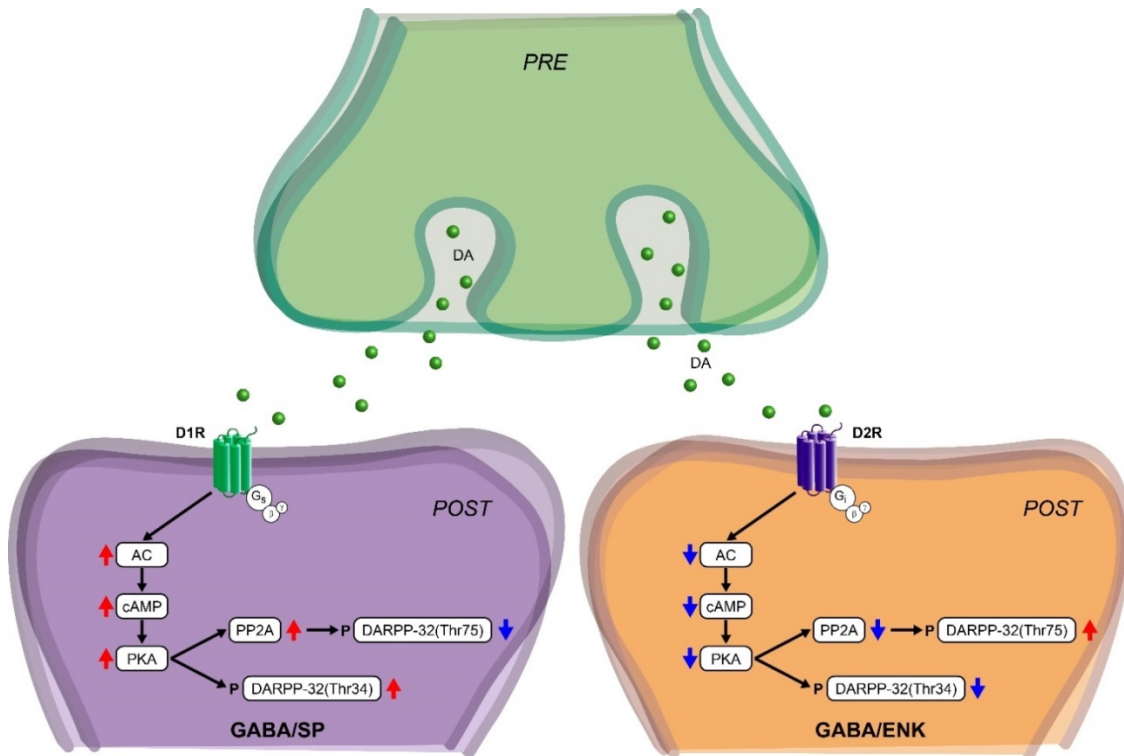

*Figure 2. Schematic representation of the intracellular cascade and effect of dopamine on the phosphorylation of DARPP-32 at -Thr34 and -Thr75 residues. The different stimulation of D$_1$ and D$_2$ dopamine receptor is critical to exert opposing effects on the two different types of MSNs. **D1R**: D$_1$ receptor; **D2R**: D$_2$ receptor; **AC**: adenylate cyclase; **PKA**: protein kinase A; **DARPP-32**: dopamine and cAMP-regulated phospho-protein M$_r$ 32,000 kDa; **PP2A**: protein phosphatase 2A; **GABA/SP**: GABAergic medium spiny neurons co-express substance P; **GABA/ENK**: GABAergic medium spiny neurons co-express enkephaline.*

DARPP-32 and drugs of abuse

DARPP-32 plays an essential role in addictive disorders mediating or modulating short- and long-term effects of drugs of abuse. This implication has been shown in several studies using DARPP-32 knockout and transgenic mice (Svenningsson *et al.* 2005; Borgkvist & Fisone 2007). DARPP-32 is ubiquitously expressed in two types of MSNs but seems to be subjected to different regulatory mechanisms in D$_1$ and D$_2$ receptors. It has been demonstrated that acute cocaine administration increases pDARPP-32(Thr34) and reduces pDARPP-32(Thr75) expression in MSNs containing D$_1$ receptors, while opposite results were found upon the activation of MSNs that express D$_2$

receptors (Bateup *et al.* 2008). Furthermore, in mutant mice selective deletion of D_1 receptors in MSNs elicited a reduction of cocaine-induced locomotor stimulation, while the deletion of D_2 receptors caused an increase of locomotor activity (Bateup *et al.* 2010). Moreover, in a CPP paradigm DARPP-32 knockout and DARPP-32(Thr34) mutant mice show a reduction in cocaine-elicited place preference compared to the DARPP-32(Thr75) mutant mice (Zachariou *et al.* 2006). In addition, also acute morphine treatment shows an increase of pDARPP-32(Thr34) via D_1 receptors in the Acb but not of pDARPP-32(Thr75) (Borgkvist & Fisone 2007). This different activation of DARPP-32 seems to be correlated to the duration of the pharmacological treatment: indeed it has been observed that in acute psychostimulant treatment, the expression of pDARPP-32(Thr34) sustains the reinforcing effects through D_1/PKA signaling, whereas in a schedule of repeated administration the inhibition of PKA, as a consequence of pDARPP-32(Thr75) expression, appears as a homeostatic event against the development of addiction (Nishi & Shuto 2017).

ETHANOL MOTIVATIONAL PROPERTIES

Ethanol is the psychopharmacological active component of alcoholic drinks and it is responsible for several disorders and alcohol-related chronic diseases that strongly interest individuals and society. Ethanol is a small molecule with lipophilic and hydrophilic characteristics, that render it able to distribute rapidly in all the body districts. Curiously, the precise target(s) of ethanol have not yet been established, but it is well known that its effects in neurons and synapses of the brain are due on one hand to an increase of inhibitory transmission via γ-aminobutyric acid-A (GABA_A) receptors, and on the other hand to an inhibition of excitatory transmission via NMDA glutamate receptors (Ron & Wang 2009; Trudell *et al.* 2014). The characteristic acute behavioral effects of ethanol range from disinhibition to sedation, and acute intoxication with hypnosis at high doses. The reinforcing properties of ethanol can be attributed to the activation of mesolimbic dopaminergic system, indeed, ethanol preferentially increases firing of dopaminergic neurons in the VTA and dopamine transmission in the Acb and other brain regions of extended amygdala (Gessa *et al.* 1985; Di Chiara & Imperato 1988; Carboni *et al.* 2000; Gonzales *et al.* 2004) through a mechanism of disinhibition of GABAergic control on dopaminergic neurons of the VTA

(Brodie & Appel 1998). This mechanism is supported with intra-VTA ethanol self-administration experiments (Gatto *et al.* 1994; Rodd *et al.* 2004).

Moreover, through place conditioning studies it has been widely confirmed the ability of ethanol to induce a significant CPP and CPA for the drug-paired compartment. The literature describes several studies of ethanol place conditioning in rats and mice, using different routes of administration and different doses of ethanol. The intraperitoneal administration of the drug at the dose of 2 g/kg produces a significant CPP in mice (Cunningham *et al.* 1997; Itzhak *et al.* 2009; Bhutada *et al.* 2010; Spina *et al.* 2015; Rosas *et al.* 2017) and rats (Cole *et al.* 2003), as well as the intragastric administration (Peana *et al.* 2008). However, the final result of the conditioning process is closely dependent on the timing of administration; in fact, in mice an intraperitoneal injection of ethanol (2 g/kg), immediately before the exposure for 5 min in a given compartment of the conditioning apparatus, is able to elicit CPP, while the same dose administered immediately after a 5 min exposure to the paired compartment elicits CPA (Cunningham & Henderson 2000; Cunningham 2019). Similarly, intragastric administration of ethanol can produce CPP or CPA (Cunningham *et al.* 2002; Fidler *et al.* 2004). The ability to give CPA or CPP, independently from the route, intraperitoneal or intragastric administration, is dependent on the dose of ethanol and the temporal relationship between its administration and the exposure to the compartment. In both modalities of administration, the CPA can be due to an initial, but temporary, adverse effect of ethanol, while the CPP has a delayed gratifying effect. Furthermore, the CPA produced by the intraperitoneal administration of ethanol is attributed to the burning sensation and irritation given by the substance, while the CPA induced by the intragastric administration can be correlated with the rapid passage from the condition of sobriety to that of the state of intoxication (Cunningham *et al.* 2002).

As previously described, the key role of dopamine in the reinforcing effects of ethanol has been widely demonstrated, and, furthermore, the literature contains several studies that relate the role of dopaminergic receptors in the learning of stimuli associated with ethanol. It has been shown that the D_1 receptor antagonist SCH-23390 is able to impair the acquisition of conditioned taste aversion elicited by ethanol in

mice (Risinger *et al.* 1999) and the self-administration in rats (Hodge *et al.* 1997). Moreover, it has also been demonstrated a positive correlation between the capacity of ethanol to induce a significant CPP for the drug-paired compartment and dopaminergic D_1 receptor activation, which has been fully prevented by the pre-treatment with the D_1 antagonist SCH39166 (Spina *et al.* 2010). The differential role of dopaminergic receptor subtypes in the Acb in the effects of ethanol has been widely confirmed with several studies. It has been demonstrated that the down-regulation of D_1 receptors using lentiviral vectors in the Acb of mice significantly reduced ethanol consumption, ethanol-elicited-locomotor sensitization and acquisition, but not expression, of ethanol-elicited CPP (Bahi & Dreyer 2012). Moreover, in other studies with D_2 knockout mice, ethanol-elicited CPP is reduced (Cunningham & Henderson 2000) and in a more recent study it has also been observed that the bilateral intra-Acb infusions of the D_1 receptor antagonist, SCH23390, but not the use of D_2 antagonist raclopride, is able to prevent the acquisition of ethanol-elicited CPP (Young *et al.* 2014).

However, the individual response to ethanol depends on the additive actions exercised on the integration of the glutamate-dopamine signal at MSNs level (Tabakoff & Hoffman 2013). In this sense, the activation of the ERK pathway plays a key role since it depends on both glutamate and dopaminergic receptors activation and is one of the focal points of adaptive behaviors in response to substances of abuse (Girault *et al.* 2007). Thereby, the study of Ibba et al. (2009) demonstrated for the first time that ethanol, in a dose-dependent manner, is able to activate ERK in the core and shell of Acb and in the nuclei of extended amygdala, through a mechanism mediated by D_1 receptors. The pre-treatment with the D_1 antagonist SCH39166, administered 10 min before ethanol, totally prevented the expression of ERK (Ibba *et al.* 2009). Thus, the activation of ERK in these areas and the involvement of D_1 receptors suggests a contribution of the brain structures implicated in positive reinforcement processes.

Moreover, also the DARPP-32 seems to be implicated in both acute and long-term responses to ethanol. Different ethanol-related behaviors have been evaluated using DARPP-32 knockout mice. These mice showed a reduction of the motivational properties of ethanol measured in CPP and self-administration experiments (Risinger

et al. 2018). Nevertheless, in the same study ethanol induced sensitivity to locomotor activation produced by a single dose (Risinger *et al.* 2018), suggesting that the DARPP-32 signaling may be a critical intracellular mechanism regulating ethanol motivated behaviors. A further study with knockout mice showed that DARPP-32 is involved in the reinforcing properties of ethanol through a mechanism mediated by NMDA receptors. Under normal conditions, ethanol reduces NMDA synaptic currents; in DARPP-32 knockout mice the regulation of ethanol sensitivity of NMDA receptors via D_1 receptors is absent and the D_1 activation does not impair the ethanol ability to inhibit NMDA receptors (Maldve *et al.* 2002).

Taken together, all these evidences support the idea that dopaminergic activation of D_1 receptors in the Acb is involved in developing and mediating the rewarding effects of ethanol or in learning of motivated behaviors related to its effects.

CAFFEINE AND ETHANOL INTERACTION: EFFECTS ON ADENOSINERGIC SYSTEM

Caffeine (1,3,7-trimethylxanthine) is a natural alkaloid derived from xanthine and is contained in the seeds, nuts, or leaves of a various plant species. The most well-known source of caffeine is the coffee bean and is, actually, the most widely taken psychoactive stimulant in the world (Evans & Battisti 2019). It is rapidly absorbed in humans and the process is not influenced by the ingestion of food or alcohol. Moreover, rapidly crosses the body membranes and is uniformly distributed in all district and fluids (Ferré & O'Brien 2011).

The impact of caffeine on ethanol consumption and abuse has become a topic of great interest due to the rise in popularity of "energy drinks", whose main, although not the only, active ingredient is caffeine. These drinks are frequently taken in combination with ethanol (i.e. alcoholic drinks), with the belief that caffeine can offset some of the intoxicating effects of ethanol. However, scientific research has not universally supported the idea that caffeine can antagonize the effects of ethanol in humans or in rodents, and the mechanisms mediating caffeine-ethanol interactions are not well understood.

Caffeine and ethanol act on different neurotransmitters and neuromodulators, but the adenosinergic and the dopaminergic systems are the ones more evidently affected by these two substances. Adenosine is a central neuromodulator and performs its effects through four subtypes of GPCRs; A_1, A_{2A}, A_{2B}, and A_3, with the A_1 and A_{2A} receptor subtypes being the most abundant in the central nervous system (Fuxe *et al.* 2003). It has been demonstrated with binding studies that A_1 receptors are largely distributed in the brain, with a quite high concentration in the hippocampus, whereas A_{2A} subtypes are highly expressed, almost exclusively, in the striatum and olfactory bulbs and tubercle (Svenningsson *et al.* 1999). Caffeine is a non-selective A_1/A_{2A} receptor antagonist and, as such, mediates its stimulant (Ferré 2008), anxiogenic (Correa & Font 2008) and motivational effects (López-Cruz *et al.* 2018). If an increase of adenosine concentration induces sleep and fatigue, caffeine has the opposite effect, in fact it is popularly consumed to increase alertness (Johnson *et al.* 1990).

Adenosine A_1 and A_{2A} receptors are co-localized with DA D_1 and D_2 receptors in striatal areas, including the Acb, but they are differentially segregated in distinct populations of neurons (Nunes *et al.* 2013). In particular, D_2 and adenosine A_{2A} receptors are co-localized on enkephalin-containing MSNs, whereas D_1 and A_1 receptors are co-localized on substance P-containing MSNs (Fuxe *et al.* 2003). These pairs of receptors can form functional heteromers and can converge on common mechanisms, such as intracellular signaling cascades with opposite effects (Fuxe *et al.* 2003; Ferré 2008). Indeed, the relationship between these pairs of receptors is antagonistic: it has been shown that the adenosine stimulation of presynaptic A_1 receptors shunts DA release, whereas that of postsynaptic A_1 antagonistically interacts with D_1 receptors and the result is a minor binding and coupling ability of G_s proteins. Furthermore, the activation of A_{2A} reduces the binding availability of D_2 receptors (Ferré 2008).

Moreover, caffeine and adenosine A_1 or A_{2A} antagonists elicit opposite effects to DA antagonists on markers such as DARPP-32 (Nunes *et al.* 2013). In particular, through the blockade of A_{2A} receptors, caffeine can modulate the neural substrates through which dopamine produces excitation (Lazarus *et al.* 2011). Caffeine can also increase dopaminergic neurotransmission through additional block-related mechanisms of presynaptic A_1 striatal receptors that modulate the release of dopamine and glutamate

(Ferré 2016). Furthermore, from the analysis of the literature on this specific aspect it emerges that in very few studies A_1 and A_{2A} receptors antagonists and agonists were used in order to investigate their role in the effects derived from the co-administration of caffeine and ethanol. Specifically, it has been shown that caffeine acts preferentially through A_1 receptors as regards the reduction of motor incoordination due to ethanol, although this effect of caffeine is evident only when it is administered at low doses (3 mg/kg), because at moderate concentrations (30 mg/kg) it supports the negative effects of ethanol (López-Cruz *et al.* 2013). Moreover, regarding the effects of ethanol on the adenosinergic system, although ethanol does not act directly on adenosine receptors, it can enhance adenosine extracellular concentrations by increasing the release and the synthesis, or decreasing Equilibrative Nucleoside Transporters type-1 (ENT-1)-dependent adenosine uptake (Nagy *et al.* 1990; Nagy 1992; Fredholm & Wallman-Johansson 1996) (see figure 3).
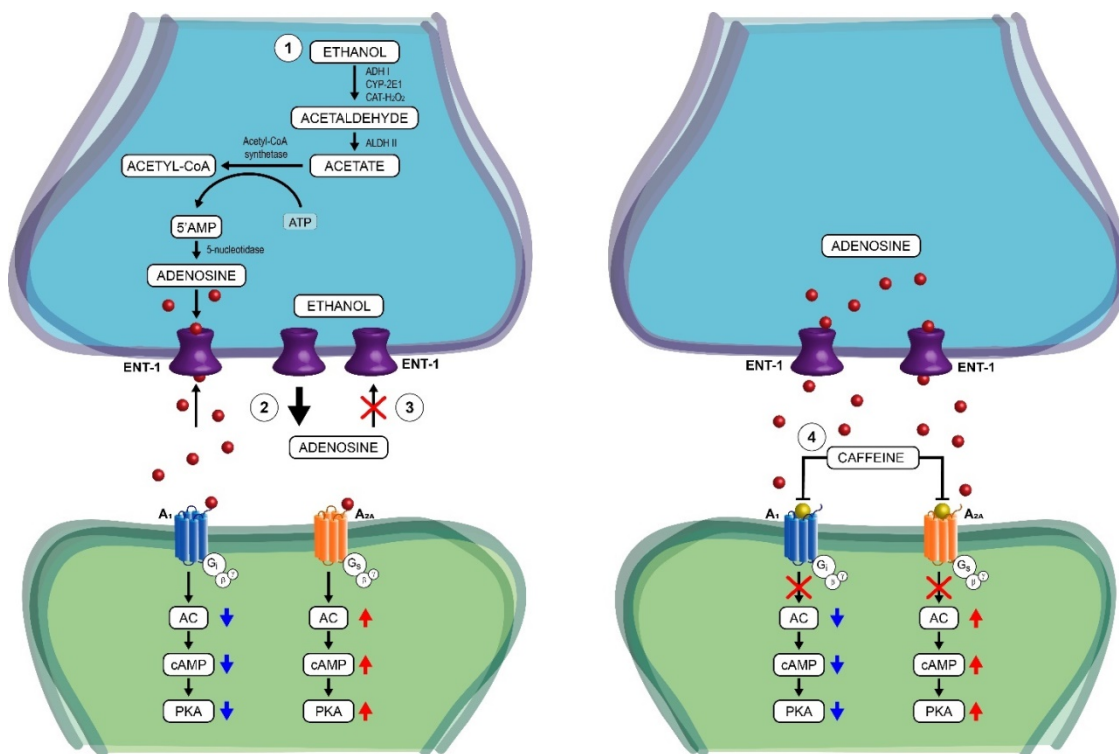

*Figure 3. Schematic representation of opposite effects of ethanol and caffeine on brain adenosinergic system. Ethanol regulates adenosine's level by increasing the its production (1), release (3) and preventing the uptake (3), whereas caffeine acts as a non-selective adenosine A_1 and A_{2A} receptors antagonist (4). **ADH**: alcohol dehydrogenase; **CYP-2E1**: cytochrome P-4502E1; **CAT-H₂O₂**: catalase; **ALDH II**: aldehyde dehydrogenase class 2; **ATP**: adenosine triphosphate; **AMP**: adenosine monophosphate; **ENT-1**: equilibrative nucleoside transporters type 1; **A_1 and A_{2A}**: adenosine A_1 and A_{2A} receptors; **AC**: adenylate cyclase; **cAMP**: cyclic adenosine monophosphate; **PKA**: protein kinase A.*

Thus, from the available pre-clinical studies that have investigated the interaction between caffeine and ethanol in rodents, it emerges that it has effects on locomotor activation, learning and memory in different experimental paradigms, as well as in ethanol self-administration paradigm (López-Cruz *et al.* 2013). The literature reveals also a significant lack of data, especially as regards the combined treatment of the two substances in the associative model of ethanol-elicited CPP. Specifically, a work investigated the effect of co-administration of caffeine (3 mg/kg) and ethanol (1.75 g/kg) in C57BL/6J male mice subjected to the CPP paradigm. The results have showed that both ethanol and caffeine administered individually increase the time spent in the compartment paired with the drug, although the effect of caffeine was significantly more modest than that of ethanol. This study also revealed that the combination of the two drugs results in a significant place preference, even if more modest than that determined by ethanol alone (Hilbert *et al.* 2013). Both the motivational properties of ethanol highlighted through the experimental model of place conditioning and the acute effects, not associated with conditioned stimuli, have also been correlated to the expression of pERK but, also in this case, the combined acute effects of the two drugs have not been extensively investigated.

IS CAFFEINE AN ADDICTIVE DRUG?

The dopaminergic theory of addiction predicts that all drugs of abuse, although with different molecular mechanisms, are able to directly activate the dopamine transmission in the mesolimbic system (Pontieri *et al.* 1995; Berridge & Robinson 1998). It has been demonstrate that caffeine causes an increase of extracellular dopamine (Acquas *et al.* 2002) and enhances the number of positive pERK neurons in the superficial and deep layers of the prefrontal cortex, but not in the Acb of rats (Valjent et al., 2004; Acquas et al. 2010) as well as in the other regions of extended amygdala of mice (Valjent *et al.* 2004). Furthermore, the phosphorylation of ERK in prefrontal cortex seems to depend on D_1 receptors, in fact their blockade prevents the activation of caffeine-induced pERK expression. In this way D_1 receptors appear to modulate caffeine-mediated activation of pERK and it has been hypothesized that these mechanisms are at the basis of the behavioral consequences of caffeine in the cognitive and memory processes (Acquas *et al.* 2010). These effects could be linked to

the psychostimulant properties of the substance, but it is not yet clear if this is the cause or effect of these properties, raising a still controversial topic about the behavioral and pharmacological properties of caffeine and whether it can be considered a substance of abuse, despite its psychostimulant effects (Acquas *et al.* 2002, 2010). Indeed, caffeine has both positive effects that drive humans to its continuous intake, but also negative ones. However, the withdrawal symptoms it determines are limited and not particularly severe, such as headache and lethargy (Satel 2006).

Furthermore, while the reinforcing properties of ethanol have been widely highlighted in studies of CPP and CPA (Cunningham & Henderson 2000; Cunningham *et al.* 2002, 2003; Font *et al.* 2006) and self-administration (Peana *et al.* 2014; Faccidomo *et al.* 2015; Lorrai *et al.* 2019) in rodents, likewise this does not occur with regard to caffeine. In fact, the studies reporting on caffeine-elicited CPP are inconclusive for different doses and experimental schedules (Steigerwald *et al.* 1988; Brockwell *et al.* 1991; Brent Bedingfield *et al.* 1998; Patkina & Zvartau 1998). Furthermore, it has been observed that caffeine, in rats, is able to elicit CPP at the dose of 3 mg/kg (Patkina & Zvartau 1998) and CPA at the dose of 30 mg/kg (Brockwell *et al.* 1991). These results suggest that low doses have probably a weak and inconsistent gratifying effect, while high doses produce aversion to associated environmental stimuli (Patkina & Zvartau 1998). These data support the biphasic effects expressed by caffeine in both humans and animals. At low doses it determines a stimulation of the central nervous system with positive and desirable effects: increase in concentration, alertness and a state of well-being, while at high doses it determines negative consequences, such as tension, nervousness, anxiety, restlessness, palpitations, sweating and depressive effects (Daly & Fredholm 1998). Notably, studies investigating the effects of co-administration of the two substances are even less, describing that caffeine, administered either alone (3.0 mg/kg) (Brockwell *et al.* 1991) or in the same injection with ethanol (1.75 g/kg of ethanol and 3.0 mg/kg of caffeine) induced a significant CPP, but the effect of caffeine was more modest than that of ethanol itself (Hilbert *et al.* 2013).

However, as a consequence of the interaction on the adenosinergic and dopaminergic system, caffeine could amplify and act synergistically enhancing the effects of other

more powerful drugs, such as psychostimulants as cocaine and amphetamine (Gasior *et al.* 2000). It has been investigated that administration of caffeine enhanced intravenous cocaine self-administration in rats (Horger *et al.* 1991; Schenk *et al.* 1994) and reinstatement of cocaine self-administration (Schenk & Partridge 1999), whereas the self-administration of caffeine alone did not show consistent results (Briscoe et al. 1998). It has been recently demonstrated that caffeine increased the motivation to obtain non-drug rewards, such as saccharin, in a dose-dependent manner. In fact, intravenous caffeine (at moderate doses of 0.5–1 mg/kg/infusion) when combined with oral saccharin significantly increased the operant responding to it at, whereas oral caffeine increased responding at all doses tested (2.5, 3.5, 5, 7.5 mg/ml) when it was combined with saccharin. In contrast, oral caffeine administration without saccharin did not increase responding at any dose (Bradley & Palmatier 2019).

Taken together it appears that the results made available so far from animal studies are not conclusive to define caffeine as a drug of abuse although it produces behavioral and physiological effects similar to other addictive substances. Furthermore, neither the DSM-4 nor the DSM-5, the Diagnostic and Statistical Manual of Mental Disorders, officially recognizes caffeine as a drug that elicits typical addiction symptoms and an addictive-like disorder similar to that elicited by other substances of abuse, although the DSM-5 recognizes the "Caffeine Use Disorder" as a condition that deserves further investigation (Meredith *et al.* 2013).

Aims

AIMS

The general aim of the present doctoral thesis was to investigate the involvement and significance of the ERK pathway in the psychopharmacological effects of ethanol, caffeine and of their interaction in terms of behavioral and biochemical responses. The motivational properties of ethanol have long been studied in validated experimental models, among which the place conditioning paradigm can be considered a valid investigation tool to model the conditions of acquiring and recalling of drug-associated affective memories. Thus, the same drug is able to elicit and transfer both positive and negative motivational properties to the environment during the conditioning process, which can be highlighted in the behavioral model of CPP and CPA, respectively. Furthermore, while the growing trend of mixing caffeine, often contained in caffeinated beverages, with ethanol is increasingly common, the knowledge of the pharmacological bases of this interaction and the related mechanisms are still unknown. The experimental approach taken to challenge this research question is translated in the structure of the present thesis that sees the experimental part divided into the following three chapters:

Chapter 1: describes the involvement of MEK/ERK protein kinases cascade in the acquisition and expression of CPP and CPA induced by a single dose of ethanol and the effects of the blockade of the kinases downstream ERKs, MEK, by the use of the MEK inhibitor SL327 in both experimental phases of place conditioning. Moreover, following the acute treatment with SL327, chapter 1 describes the results and discusses the experiments aimed at investigating the biochemical results of ethanol-elicited phosphorylated ERK expression in different regions constituting the mesolimbic system.

Chapter 2: explores the interaction of ethanol and caffeine in the acquisition of ethanol-induced place conditioning and, in particular, the effects of caffeine pre-treatment on the validated motivational properties of ethanol expressed in CPP and CPA paradigms. Furthermore, it investigates whether caffeine may prevent the ethanol-elicited pERK in different brain regions. Chapter 2 also discusses the results of the experiments aimed at verifying if the stimuli (positive and negative) conditioned to

ethanol and the drugs combination are related to a differential pERK expression in all brain areas examined.

Chapter 3: evaluates the effects of different doses of ethanol and caffeine administration and their interaction on psychomotor performance measured as horizontal and vertical locomotion in the open field apparatus. Chapter 3 also describes the results of caffeine on mice previously sensitized with ethanol, to investigate if there may be cross sensitization effect. Finally, it discusses the results of the expression of two biochemical markers of neural activation, pERK and pDARPP-32(Thr75), in the nucleus accumbens of mice acutely treated with both substances.

Experimental Studies

Chapter 1

CHAPTER 1: Differential effects of the MEK inhibitor SL327 on the acquisition and expression of ethanol-elicited conditioned place preference and aversion in mice

ABSTRACT

The involvement of mitogen-activating extracellular kinase (MEK) in place conditioning may vary depending on the motivational sign (positive or negative) and nature (pharmacological or nociceptive) of the unconditioned stimulus (US) and on the phase (acquisition or expression) of the learning process. This study investigated the role of MEK on the acquisition and expression of ethanol-elicited (given 2 g/kg) backward (preference, CPP) and forward (aversion, CPA) place conditioning. The MEK inhibitor SL327 (50 mg/kg for CPP, and 50 and 100 mg/kg for CPA) was administered to CD-1 mice 60 min before an ethanol dose (acquisition) or 60 min before the post-conditioning tests (expression). Ethanol significantly elicited CPP and CPA; SL327 (50 mg/kg) significantly blocked the acquisition of ethanol-elicited CPP, but not that of CPA. Moreover, SL327 (50 and 100 mg/kg) significantly reduced the expression of ethanol-elicited CPP, but not that of CPA. Finally, SL327 also prevented ethanol-elicited (given 2 g/kg) increases of phosphorylated extracellular signal regulated kinase (pERK)-positive neurons in the nucleus accumbens and other nuclei of the extended amygdala. Overall, these results confirmed the differential involvement of MEK in the acquisition and expression of drug-elicited place conditioning and suggested its differential involvement in distinct behavioral outcomes, depending on the motivational sign of the (same) US and on the significance of the experimental phase of the learning process.

INTRODUCTION

The place conditioning is an experimental model widely used to characterize the motivational properties of drugs (both positive and negative) and the role of neural substrates underlying this process. The result of a conditioning paradigm of associative learning leads to the establishment of memories, and more specifically, the CPP aroused by the drug is based on the establishment of positive memories and as such represents a valid experimental model for studying the drug-seeking behavior (Tzschentke 2007). Conversely, the elicited-CPA determines the onset of negative memories and is a model to study negative reinforcement, since the resulting behavior is aimed to prevent adverse experiences previously conditioned to drug-associated stimuli (Tzschentke 2007). In addition, acquiring and recalling of drug-associated memories can be modeled by different phases of place conditioning, the acquisition and the expression, respectively. Interestingly, the ERK pathway represents one of the most investigated molecular mechanisms that have been related to place conditioning. Several previous studies have demonstrated that these kinases cascade is involved in different learning paradigm, such as conditioned taste aversion (Marotta *et al.* 2014), conditioning fear (Atkins *et al.* 1998; Villarreal & Barea-Rodriguez 2006), spatial learning (Blum *et al.* 1999; Selcher *et al.* 1999), recognition memory (Kelly *et al.* 2003), appetitive-reward seeking behavior (Kirschmann *et al.* 2014) and drug-elicited place conditioning (Valjent *et al.* 2000; Gerdjikov *et al.* 2004; Girault *et al.* 2007; Spina *et al.* 2010; Longoni *et al.* 2011; Rosas *et al.* 2018). Moreover, phosphorylated ERK (pERK) are widely expressed in the mesolimbic circuit areas, such as Acb and nuclei of extended amygdala (bed nucleus of stria terminalis and central and basolateral nuclei of the amygdala) (Valjent *et al.* 2004; Ibba *et al.* 2009; Vinci *et al.* 2010). Furthermore, the involvement of MEK, the kinases that are upstream of ERK, in the acquisition of drug-elicited CPP has been demonstrated with several drugs of abuse, such as d-amphetamine (Gerdjikov *et al.* 2004), ecstasy (Salzmann *et al.* 2003), morphine (Mazzucchelli *et al.* 2002; Spina *et al.* 2010) and cocaine (Valjent *et al.* 2000) but in the case of ethanol, their involvement is not satisfactorily clear (Groblewski *et al.* 2011). Additionally, in contrast to the evidence supporting the role of the MEK on the

acquisition of CPP, their contribution in the expression of drug-elicited CPP and CPA awaits further clarifications.

There are evidences regarding the involvement of MEK in the acquisition of cocaine-elicited CPP (Valjent *et al.* 2000; Miller & Marshall 2005), but not to its expression (Miller & Marshall 2005). Similarly, in the CPA paradigm, it has been confirmed that the reversible MEK inhibitor, SL327 (Selcher *et al.* 1999), is able to prevent the acquisition of lithium-elicited CPA (Longoni *et al.* 2011) but not the expression of acquired (conditioned) response. However, the literature described that blockade of MEK in the anterior cingulate cortex prevents both phases of CPA procedure induced by intra-plantar formalin but not by foot shock nor by the κ opioid agonist, U69,593 (Cao *et al.* 2009). Taken together these results suggest that the involvement of MEK in aversive memories could depend on the force and nature (nociceptive vs pharmacological) of the unconditioned stimulus (US) as well as on the brain regions involved. Furthermore, these different results are also probably due to different neurobiological mechanisms at the basis of the behaviors modeled by these different phases of place conditioning and may have a common but also distinct and unique mechanisms with respect to MEK involvement (Sanchis-segura & Spanagel 2006; Shiflett & Balleine 2011).

To avoid these possible discrepancies, in the present study we decided to characterize the role of MEK/ERK cascade in the place conditioning paradigm using an identical US, the effect of a pharmacologically active, non intoxicating, dose of ethanol, that under appropriate experimental conditions is able to elicit either CPP and CPA (Cunningham *et al.* 1997). Indeed, in the experiments of Cunningham and Co-workers (1997) it has been established that ethanol, after its intraperitoneal administration, may initially exert an aversive reaction succeeded shortly after by pleasant feelings. As a consequence, depending on the experimental schedule followed during conditioning, the association of the US to the paired compartment (conditioned stimulus, CS) results in CPP if the CS follows the US (backward conditioning) or results in CPA if the CS anticipates the US (forward conditioning) (Cunningham *et al.* 1997). Therefore, to characterize the role of this kinase cascade in the acquisition and expression of the CS, elicited by the identical US, ethanol, we will establish first whether in our experimental conditions the administration of the MEK inhibitor SL327 can affect the acquisition and

expression of ethanol-elicited (given 2 g/kg) backward (preference, CPP) and forward (aversion, CPA) place conditioning. The MEK inhibitor SL327 (50 mg/kg for CPP, and 50 and 100 mg/kg for CPA) will be administered to CD-1 mice 60 min before an ethanol dose (acquisition) or 60 min before the post-conditioning tests (expression). Finally, we will assess if SL327 pre-treatment also prevented ethanol-elicited pERK-positive neurons in the nucleus accumbens and other nuclei of the extended amygdala.

MATERIALS AND METHODS

Animals

Male CD-1 mice (22-24 g, Envigo, Udine, Italy) were housed in groups of four per cage for at least 6 days before use and maintained on a 12:00/12:00 h light/dark cycle (lights on at 08:00 h) with food and water freely available. All the experiments were carried out during the light phase, between 09:00 and 16:00 h. The total numbers of mice were n=42 and n=50 in the CPP and CPA acquisition experiments, respectively, and n=46 and n=40 in the CPP and CPA expression experiments, respectively. The total numbers of mice used in the immunohistochemical experiments were: n=9 for the group vehicle/saline, n=7 for the group SL327/saline, n=8 for the group vehicle/ethanol and n=8 for the group SL327/ethanol. All the experimental procedures were performed in accordance with the Principles of laboratory animal care and with the guidelines and protocols approved by the European Union (2010/63/UE L 276 20/10/2010). Every possible effort was made to minimize animal pain and discomfort and to reduce the number of experimental subjects.

Drugs

Ethanol (Sigma-Aldrich, Milan, Italy) 20% (v/v) in isotonic saline, was administered at the dose of 2 g/kg (12 mL/kg volume injection). The reversible MEK inhibitor, SL327 (Ascent Scientific Ltd, Bristol, UK), was dissolved at 50 and 100 mg/kg in a vehicle made as follows: dimethyl sulfoxide (DMSO) (Sigma-Aldrich, Milan, Italy), cremophor (Sigma Aldrich, Milan, Italy) and isotonic saline 30/30/40% (v/v) (injection volume: 20 mL/kg). The dose of 50 mg/kg of SL327 was selected in agreement with previous studies (Selcher *et al.* 1999; Valjent *et al.* 2000, 2006; Salzmann *et al.* 2003; Longoni *et al.* 2011) 2006), whereas the dose of 100 mg/kg was selected after Longoni et al. (2011). Sodium pentobarbital (Carlo Sessa Spa, Sesto San Giovanni, Milan, Italy) (50 mg/kg) was dissolved in isotonic saline. All drugs and vehicle were administered intraperitoneally (IP).

Apparatus

The apparatus consisted of two rectangular Plexiglas boxes (48Lx20Wx30H cm) separated by a 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 (49.2 % A and 50.8% B) and average spontaneous preference times (sec./900±SEM) were not significantly different between compartments (Paired t-test: t=1.68, p=0.093, df=354, number of pairs=178; with average total session times being 443±5.89 sec./900 and 457±5.89 sec./900 respectively). However, in spite of such unbiased apparatus, the experimental groups for CPP experiments were made by grouping mice whose spontaneous preferences were, no matter the compartment, A or B, closer to 400 sec. Similarly (but opposite), the experimental groups for CPA experiments were made by grouping mice whose spontaneous preferences were, no matter the compartment, closer to 500 sec.

Acquisition of Conditioned Place Preference and Conditioned Place Aversion

Each experiment consisted of three phases. During the first phase (day 1), the guillotine door was kept raised and each mouse was placed randomly in one or the other compartment and given access to both compartments of the apparatus for 15 min (900 sec.). The time spent in one compartment was recorded and taken as an indication of spontaneous preference. Behavioral schedules for backward and forward conditioning (figure 1) were designed after Cunningham et al. (1997). In particular, during the second phase (days 2-5) of CPP experiments, mice of distinct experimental groups, were administered SL327 (50 mg/kg) or vehicle, 60 min before ethanol (2 g/kg) or saline, and were returned to their home cage. Ten minutes after ethanol administration mice were exposed for 5 min to a given compartment and returned to their home cage. Four hours later, mice were administered vehicle (SL327, 0 mg/kg) or SL327 (50 mg/kg), then returned to their home cage 60 min before the second administration (ethanol, 0 or 2 g/kg). Ten min after this second administration mice were placed in the compartment opposite to that of the morning exposure. During the second phase (conditioning days 2-5) of CPA experiments, mice of distinct experimental groups, were administered SL327 (50 and 100 mg/kg) or vehicle, and

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returned to their home cage for 60 min. At the end of this period mice were exposed for 5 min to the given compartment. Upon removal from the compartment (i.e. before being returned to their home cage) mice were administered the second injection (ethanol, 2 g/kg) or vehicle. Four hours later, mice were administered vehicle (SL327, 0 mg/kg) or SL327 (50 mg/kg), 60 min before the 5 min exposure to the opposite compartment. Upon removal from the apparatus, i.e. before being returned to their home cage, mice were administered the second injection (ethanol 0 or 2 g/kg). During conditioning days for both CPP and CPA the order of vehicle and ethanol administration was counterbalanced [i.e. the order of the combined pre-treatment/treatment [SL327 (50-100 or 0 mg/kg)/ethanol (0 or 2 g/kg)] administrations was opposite on even days with respect to that one on odd days]; similarly, the number of mice receiving saline and ethanol was counterbalanced over the four 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 paired four times with the given compartment. The four hours interval between conditioning sessions was chosen in order to avoid carryover effects of both SL327, whose ability to prevent MAPK activation is most effective between 30 min and 2 hours (Selcher *et al.* 1999), and ethanol. During the third phase of both CPP and CPA experiments (post-conditioning test), 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 min was recorded. The conditions of the post-conditioning test were identical to those of the pre-conditioning test. Pre- and post-conditioning recordings were done with stop watch by observers blind to pharmacological treatments. Hence, a statistically significant difference between the time spent during pre- and post-conditioning tests of the drug group with respect to that of the saline group was taken as indication of the development of place conditioning.

A

Conditioned Place Preference (CPP)

B
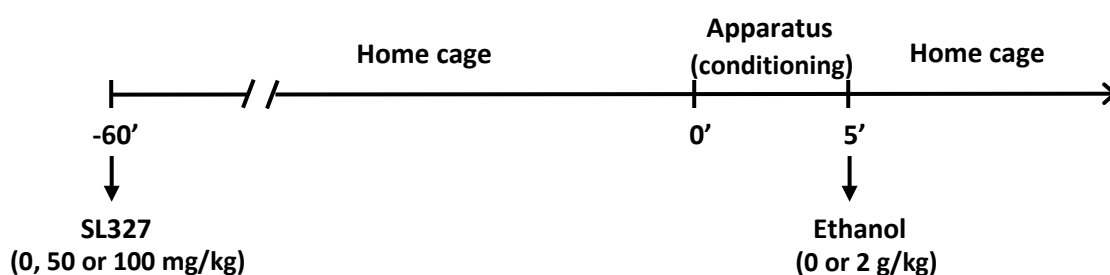
Conditioned Place Aversion (CPA)

Figure 1. CPP and CPA conditioning procedures. *Diagrammatic representation of the place conditioning procedures used in the CPP (A) and CPA (B) experiments. (A) Mice were administered vehicle (SL327 0 mg/kg) or SL327 (50 and 100 mg/kg) 60 min before administration of saline or EtOH (2 g/kg) and were returned to their home cage. After 10 min of saline or EtOH 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 vehicle (SL327 0 mg/kg) or SL327 (50 and 100 mg/kg) and returned to their home cage for 60 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 saline (0 g/kg EtOH) or EtOH (2 g/kg EtOH).*

Expression of Conditioned Place Preference and Conditioned Place Aversion

In the expression experiments, in agreement with Cunningham et al. (1997), 60 min before the beginning of the post-conditioning test, distinct groups of ethanol-conditioned mice were administered SL327 (0, 50 and 100 mg/kg). Testing for CPP or CPA expression was done on the first day following conditioning in the time frame corresponding to the interval between "morning" and "afternoon" conditioning sessions of days 2-5. The difference between the time spent in the drug-paired (CPP) and drug-assigned (CPA) compartments during the post-conditioning test and that

spent during the pre-conditioning test (side preference shift) was taken as a measure of the degree of place conditioning induced by the drug (Carr *et al.* 1989). Hence, a statistically significant difference between the time spent during pre- and post-conditioning tests of the drug group with respect to that of the saline group indicates the development of place conditioning.

Immunohistochemistry

For these experiments mice of distinct experimental groups were anesthetized with sodium pentobarbital (Valjent *et al.* 2001) 15 min after the administration of saline or ethanol (Ibba *et al.* 2009). Doses and timing of pre-treatment and treatment administrations exactly reproduced those followed on day 2 (first conditioning day) of the behavioral experiments. Following pretreatment administrations, mice were placed back in their home cages with their cage-mates. Under deep anesthesia, animals were subjected to trans-cardiac perfusion with ice-cold Phosphate Buffered Saline (PBS): 137 mM NaCl, 2.7 mM KCl, 10 mM Na2HPO4, 2 mM KH2PO4, and 4% paraformaldehyde (PFA). After perfusion, brains were removed and post-fixed overnight in 4% PFA. Brain slices (40 µm) of the regions of interest were cut, on ice-cold PBS with a vibratome (Leica VT1000, Leica, Germany) according to the mouse brain atlas (Paxinos & Franklin 2001). Slices were kept in ice-cold PBS and processed for immunohistochemistry according to a protocol for free-floating slices. Immunoreactions for pERK-positive cells detection were applied to at least two every other slices obtained from each brain. After incubation for 30 min in 1% H_2O_2, slices were incubated for 1 hour with 3% BSA. The incubation with the primary anti pERK antibody (phosphorylated ERK, Cell Signalling Technology, Beverly, MA, USA (1:350)) was conducted overnight. On the following day, after rinsing, slices were incubated for 1 hour with the biotinylated secondary antibody (1:800). After three rinses the slices were incubated in avidin biotin peroxidase complex prepared according to the manufacturer's suggestions (Vectastain ABC kit, Vector Laboratories, Burlingame, CA, USA) and a 3-3'-diaminobenzidine solution (10 mg/mL) was added until development of brown staining. Slices were rinsed and mounted onto gelatine-coated slides and processed through alcohol-xylene for light microscopy examination. pERK-positive neurons were identified in the regions of interest of both hemispheres at the lowest

magnification (10X) and quantitative analysis was performed using a Zeiss Axioskop 40 light microscope, equipped with PL Fluotar 10X (na=0.3), 40X (na=1.00–0.5) and 100X oil (na=1.3) objectives, coupled with a digital camera (Nikon D5000, Melville, NY, USA). Images of the regions of interest were obtained at the lowest magnification (10X) from at least two every other 40 µm thick slices, according to the atlas Paxinos and Franklin (2001), and used to automatically count the number of pERK-positive neurons/area (pERK expression) by application of the software ImageJ (v. 1.42, National Institutes of Health sponsored image analysis program) in conjunction with automated background to avoid experimenter bias, and entropy threshold plug-in. pERK-positive neurons/area were counted in the regions of interest whose borders in the slices were depicted/recognized according to plates 15-17 (approximately from AP 1.94 to AP 1.70 mm from bregma for the prefrontal cortex), to plates 21-23 (approximately from AP 1.18 to AP 0.98 mm from bregma for the nucleus accumbens), to plates 31-32 (approximately from AP 0.02 to AP -0.1 mm from bregma for the bed nucleus of stria terminalis) and to plates 36-38 (approximately from AP -0.58 to AP -0.82 mm from bregma for the central nucleus of the amygdala) of the Paxinos and Franklin (2001) brain atlas. These brain regions were selected for their involvement in learning and motivation and their relationship with pERK in these processes (Valjent *et al.* 2004; Ibba *et al.* 2009; Marotta *et al.* 2014). The reported numbers for each brain region (table 1 and figure 6) are the average ± SEM of average counts/slice from each animal.

Statistical analysis

To determine statistically significant differences between pre-conditioning values of the experimental groups depicted in figures 2-5, one-way Analysis of Variance (ANOVA) was applied (Statistica, v. 8.0, StatSoft Inc., Tulsa (OK), USA). To determine the effect of pre-treatment (2 levels: SL327 0 or 50 (or 100) mg/kg) on conditioning and those 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). Two-way ANOVA, with pre-treatment as an independent factor (between subjects) and pre-conditioning and post-conditioning values as within subjects factors (repeated

measures), was also applied to assess the statistical significance of data from the expression experiments. All statistical analyses were carried out using data from the experimental groups depicted in each figure. Post hoc analyses (with multiple comparisons), carried out using Newman-Keuls tests, 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-, two- and three-way ANOVAs with pERK-positive neurons/area as dependent variables and with the factors brain area (2 levels: shell and core), pre-treatment (2 levels: vehicle or SL327) and treatment (2 levels: EtOH 0 or 2 g/kg) used as independent variables. Newman-Keuls post-hoc analyses, whereby allowed by ANOVAs significant main effects, were applied for multiple comparisons, with the statistical significance set at $p < 0.05$.

RESULTS

Effects of SL327 on acquisition of ethanol-elicited CPP and ethanol-elicited CPA

Figure **2** shows the effects of pre-treatment with SL327 (0 or 50 mg/kg), 60 min before the administration of ethanol (0 and 2 g/kg) and 10 min before exposure to the assigned compartment for 5 min (see figure 1A). Pre-conditioning preference times did not significantly differ between experimental groups [$F_{3,38} = 1.52$, NS]. Repeated measures, three-way ANOVA revealed significant effects of pre-treatment [$F_{1,38} = 9.52$, $p < 0.05$], time [$F_{1,38} = 22.28$, $p < 0.001$] and treatment [$F_{1,38} = 8.23$, $p < 0.05$], and significant pre-treatment by time [$F_{1,38} = 11.46$, $p < 0.001$], treatment by time [$F_{1,38} = 7.849$, $p < 0.05$] and pre-treatment by treatment by time [$F_{1,38} = 8.76$, $p < 0.05$] interactions. Newman-Keuls' post hoc analysis revealed a significant difference in the time spent in drug-paired compartment in post-conditioning between the group SL327 (0 mg/kg)/ethanol (0 g/kg) and the group SL327 (0 mg/kg)/ethanol (2 g/kg) ($p < 0.05$).
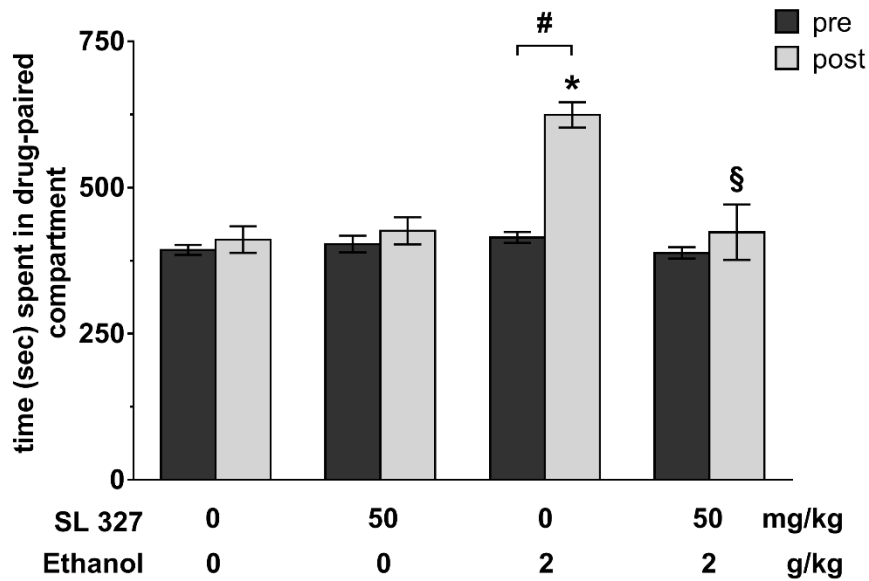
*Figure 2. Effects of SL327 pre-treatment on acquisition of ethanol-elicited CPP. Data are shown as average time spent (sec.) ± SEM in the drug-paired compartment. Pre-conditioning preference times were 393±8 for SL327 (0 mg/kg) + ethanol (0 g/kg) (n=12), 403±14 for SL327 (50 mg/kg) + ethanol (0 g/kg) (n=7), 414±9 for SL327 (0 mg/kg) + ethanol (2 g/kg) (n=13) and 388±9 for SL327 (50 mg/kg) + ethanol (2 g/kg) (n=10) groups. *Indicates a significant difference (p<0.05) of time spent during post-conditioning test as compared to SL327 (0 mg/kg) + ethanol (0 g/kg) group; §indicates a significant difference (p<0.05) of time spent during post-conditioning test as compared to SL327 (0 mg/kg) + ethanol (2 g/kg) group; #indicates a significant difference (p<0.05) between pre- and post-conditioning.*

Figure **3** shows the effects of pre-treatment with SL327 (0, 50 and 100 mg/kg), 60 min before the exposure to the assigned compartment for 5 min, and of ethanol (0 and 2 g/kg) administration upon removal from the place conditioning apparatus (see figure 1B). Pre-conditioning preference times did not significantly differ between experimental groups [$F_{5,44}=0.99$, NS]. Repeated measures, three-way ANOVA revealed significant effects of pre-treatment [$F_{1,44}=46.44$, p<0.001] and time [$F_{1,44}=56.66$, p<0.001], and significant pre-treatment by time [$F_{1,44}=63.53$, p<0.001] and treatment by time [$F_{2,44}=3.99$, p<0.05] interactions. Newman-Keuls' post hoc analysis revealed a significant difference in the time spent in drug-paired compartment in post-conditioning between the group SL327 (0 mg/kg)/ethanol (0 g/kg) and the group SL327 (0 mg)/kg/ethanol (2 g/kg), that SL327 (50 and 100 mg/kg) was devoid of motivational properties and that SL327 (50 and 100 mg/kg) failed to prevent, but significantly (p<0.05) reduced, the acquisition of ethanol-elicited CPA.
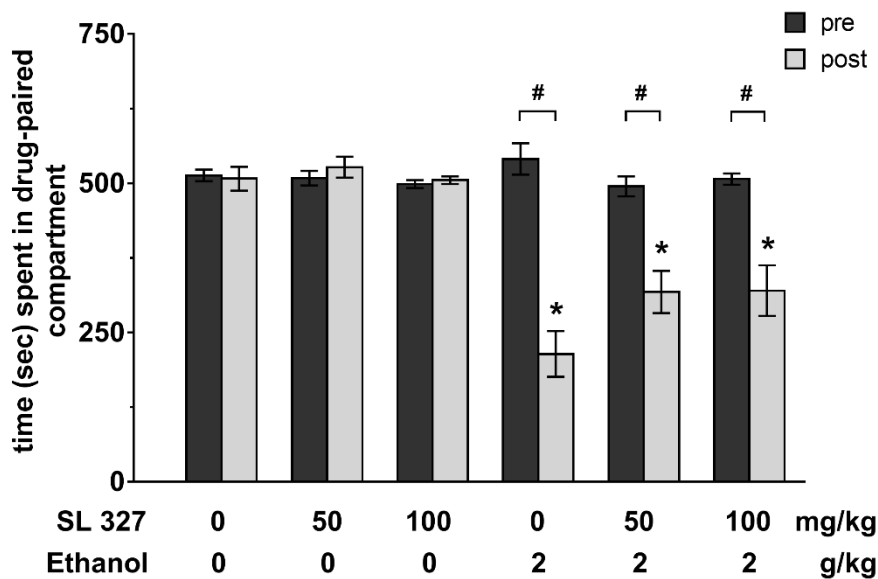
Figure 3. Effects of SL327 pre-treatment on acquisition of ethanol-elicited CPA. *Data are shown as average time spent (sec.) ± SEM in the drug-paired compartment. Pre-conditioning preference times were 513±9 for SL327 (0 mg/kg) + ethanol (0 g/kg) (n=10), 508±1 for SL327 (50 mg/kg) + ethanol (0 g/kg) (n=7), 498±6 for SL327 (100 mg/kg) + ethanol (0 g/kg) (n=6), 540±2 for SL327 (0 mg/kg) + ethanol (2 g/kg) (n=11), 494±1 for SL327 (50 mg/kg) + ethanol (2 g/kg) (n=10) and 507±9 for SL327 (100 mg/kg) + ethanol (2 g/kg) (n=6) groups. *Indicates a significant difference (p<0.05) of time spent during post-conditioning test as compared to SL 327 (0 mg/kg) + ethanol (0 g/kg) group; #indicates a significant difference (p<0.05) between pre- and post-conditioning.*

Effects of SL327 on expression of ethanol-elicited CPP and ethanol-elicited CPA

Figure **4** shows the effects of SL327 (0, 50 and 100 mg/kg) administered 60 min before the post-conditioning test of mice of three independent SL327 (0 mg/kg) + ethanol (2 g/kg) conditioned groups. Repeated measures, two-way ANOVA revealed significant effects of time [$F_{1,43}$=56.54, p<0.001] and pre-treatment [$F_{2,43}$=5.48, p<0.05] and a significant time by pre-treatment [$F_{2,43}$=5.13, p<0.05] interaction. Newman-Keuls' post hoc analysis revealed that ethanol elicited a significant CPP, that SL327 pre-treatment (50 and 100 mg/kg) failed to prevent, but significantly (p<0.05) reduced, ethanol-elicited CPP expression.

Figure **5** shows the effects of pre-treatment with SL327 (0, 50 and 100 mg/kg) administered 60 min before the post-conditioning test of mice of three independent SL327 (0 mg/kg) + ethanol (2 g/kg) conditioned groups. Repeated measures, two-way ANOVA of the effects of SL327 revealed a significant effect of time [$F_{1,37}$=110.39, p<0.001] and a significant time by pre-treatment [$F_{2,37}$=3.45, p<0.05] interaction.

Newman-Keuls' post hoc analysis revealed that ethanol elicited a significant CPA and that SL327 pre-treatment at the doses of 50 and 100 mg/kg failed to affect the expression of ethanol-elicited CPA.
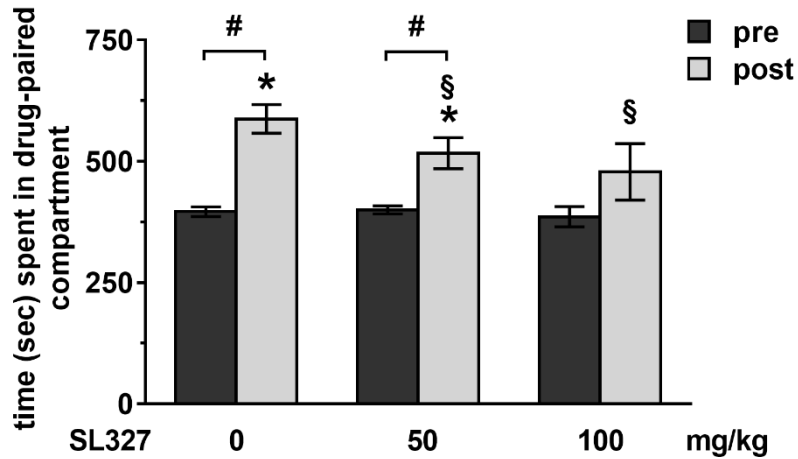

Figure 4. Effects of SL327 pre-treatment on expression of ethanol-elicited CPP. *Data are shown as average time spent (sec.) ± SEM in the drug-paired compartment. Pre-conditioning preference times were 400±8 (n=18), 396±10 (n=17) and 385±21 (n=11) for SL327 (0, 50 and 100 mg/kg, respectively, on SL327 (0 mg/kg) + ethanol (2 g/kg) -conditioned groups). *Indicates a significant difference (p<0.05) of time spent during post-conditioning test as compared to pre-conditioning test of SL327 (0 and 50 mg/kg) + ethanol (2 g/kg) conditioned groups; §indicates a significant difference (p<0.05) of time spent during post-conditioning test between SL327 (0 mg/kg) and SL327 (50 and 100 mg/kg) groups; #indicates a significant difference (p<0.05) between pre- and post-conditioning.*
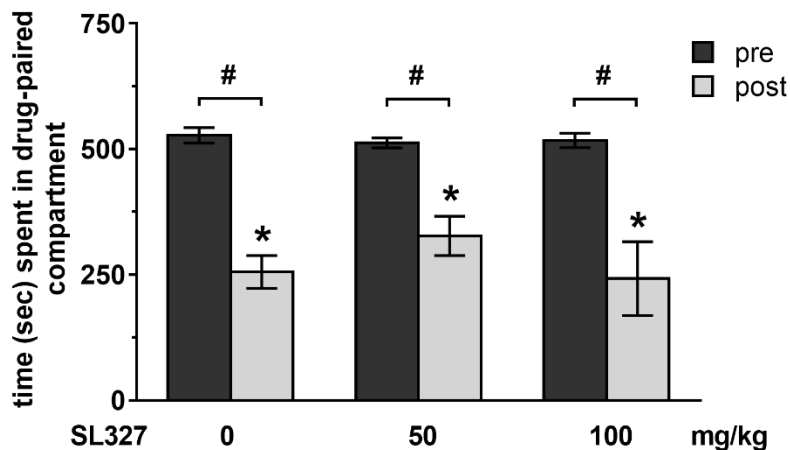

Figure 5. Effect of SL327 pre-treatment on the expression of ethanol-elicited CPA. *Data are shown as average time spent (sec.) ± SEM in the assigned compartment. Pre-conditioning preference times were 527±15 (n=21) 512±10 (n=11) and 517±14 (n=8) for SL327 (0, 50 and 100 mg/kg, respectively, on SL327 (0 mg/kg) + ethanol (2 g/kg) conditioned groups. *Indicates (p<0.05) of time spent during post-conditioning test as compared to pre-conditioning test of SL327 (0, 50 and 100 mg/kg) + ethanol (2 g/kg) conditioned groups; #indicates a significant difference (p<0.05) between pre- and post-conditioning.*

Effects of SL327 on the expression of ethanol-elicited pERK-positive neurons

Figure **6** shows the effects of 60 min pretreatment with SL327 (0 and 50 mg/kg) on the expression of ethanol (2 g/kg)-elicited pERK-positive neurons in the shell and core of the nucleus accumbens. Three-way ANOVA of the results of ethanol administration in the shell and core of the nucleus accumbens, revealed a main effect of pre-treatment [$F_{1,49}$=46.29, p<0.001], treatment [$F_{1,49}$=9.02, p<0.05] and brain region [$F_{1,49}$=8.56, p<0.05] and a significant pre-treatment by treatment interaction [$F_{1,49}$=41.00, p<0.001]. Newman-Keuls' post-hoc test revealed that ethanol increased pERK-positive neurons/area with respect to saline in the shell and core of the nucleus accumbens. Moreover, one-way ANOVA of the results of SL327 administration followed by saline (ethanol vehicle group) administration in the shell and core of the nucleus accumbens yielded not significant effects (F_{shell}1,13=0.04, NS; F_{core}1,12=0.33, NS).
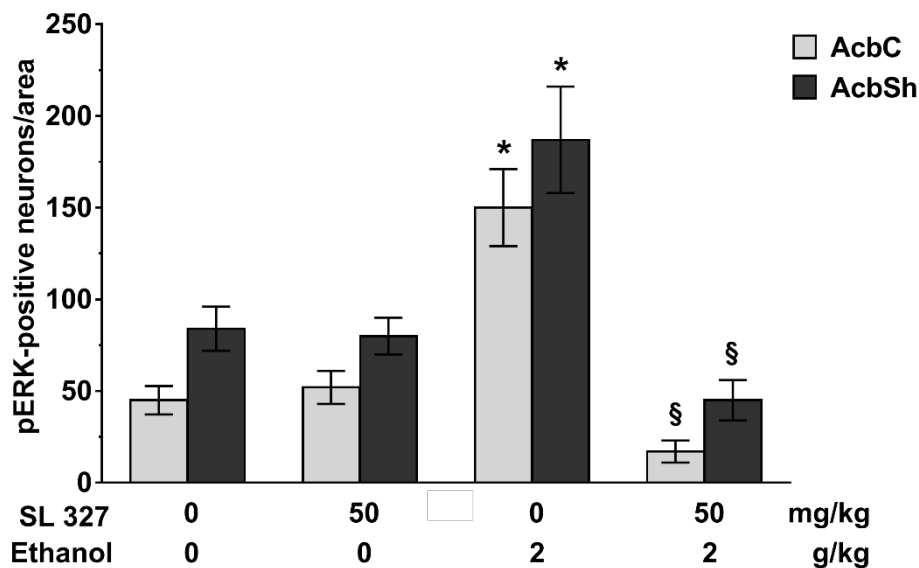

*Figure 6. Effect of SL327 on the expression of ethanol-elicited pERK-positive neurons in the nucleus accumbens. Data are shown as mean ± SEM of pERK-positive neurons/area. Effects of pre-treatment with SL327 (0 and 50 mg/kg) on ethanol (EtOH 0 (sal) and 2 g/kg) -elicited pERK-positive neurons/area in the shell (AcbSh) and core (AcbC) of the nucleus accumbens [n=9, for the group veh/sal; n=6, for the group SL327/sal; n=5, for the group veh/EtOH; n=7, for the group SL327/EtOH]. *indicates a significant difference (p<0.05) with respect to veh/sal (same brain region); §indicates a significant difference (p<0.05) with respect to veh/EtOH (same brain region).*

In addition, table 1 shows the effects of 60 min pretreatment with SL327 (0 and 50 mg/kg) on the expression of ethanol (2 g/kg)-elicited pERK-positive neurons in the

prefrontal cortex (prelimbic and infralimbic), bed nucleus of stria terminalis and central nucleus of the amygdala. One-way ANOVA of the results of SL327 administration followed by saline (ethanol vehicle group) administration in the prefrontal cortex (prelimbic and infralimbic), bed nucleus of stria terminalis and central nucleus of the amygdala yielded not significant effects ($F_{PrL}1,12=0.055$, NS; $F_{IL}1,12=0.11$, NS; $F_{BSTL}1,12=0.25$, NS and $F_{CeA}1,12=0.09$, NS). Furthermore, two-way ANOVAs, followed by Newman-Keuls' post-hoc tests revealed that SL327 significantly prevented the expression of ethanol-elicited pERK-positive neurons/area in all brain regions.

	pERK-positive neurons/area				
	veh/sal	**SL327/sal**	**veh/EtOH**	**SL327/EtOH**	**Two-way ANOVAs**
PrL	42±10	38.2±10.9	156±23.2	54±13**	$F_{pre-treament\,(1.26)}=11.05$ p<0.05 $F_{treatment\,(1.26)}=16.5$, p<0.001 $F_{interaction\,(1.26)}=9.62$, p<0.05
IL	33±8	29.5±7.9	93.5±1*	39±10**	$F_{pre-treament\,(1.26)}=7.27$, p<0.05 $F_{treatment\,(1.26)}=10.6$, p<0.05 $F_{interaction\,(1.26)}=5.48$, p<0.05
BNST	25±8	18.9±8.2	102±14*	29±10**	$F_{pre-treament\,(1.26)}=14.5$, p<0.001 $F_{treatment(1.26)}=17.77$, p<0.001 $F_{interaction\,(1.26)}=10.62$, p<0.05
CeA	3±1	9.3±5.7	55±10*	13±6**	$F_{pre-treament(1.26)}=6.6$, p<0.05 $F_{treatment(1.26)}=15.95$, p<0.001 $F_{interaction(1.26)}=11.94$, p<0.05

Table 1. Effects of SL327 (0 and 50 mg/kg) on ethanol (2 g/kg)-elicited pERK-positive neurons in the pre-limbic (PrL); and infralimbic (IL) prefrontal cortex, in the bed nucleus of stria terminalis (BNST) and in the central nucleus of the amygdala (CeA). Data are the average ± SEM raw numbers of pERK-positive neurons/area from at least three every other slices. For all the brain regions the number of animals was: n=7, for the group veh/sal; n=7, for the group SL327/sal; n=8, for the group veh/EtOH; n=8, for the group SL327/EtOH. *indicates p<0.05 at Newman-Keuls post hoc test with respect to veh/sal; **indicates p<0.05 at Newman-Keuls post hoc test with respect to veh/EtOH.*

DISCUSSION

In agreement with Cunningham and Co-workers (1997), the results of this study confirm the ability of ethanol to elicit CPP and CPA (figure 1). Furthermore, the results demonstrate the differential involvement of MEK/ERK cascade in the acquisition and expression of place conditioning as the consequence of the of the blood brain barrier-penetrant compound, SL327 (Selcher *et al.* 1999), ability to block some of these effects (figures 2-5). The immunohistochemistry experiments also establish that SL327 significantly reduces the MEK mediated expression of pERK-positive neurons elicited by ethanol in the nucleus accumbens shell and core and in other nuclei of extended amygdala, at the same doses and timing of place conditioning experiments (table 1 and figure 6).

Effects on CPP and CPA Acquisition

The administration of SL327 (50 mg/kg) significantly prevents the acquisition of ethanol-elicited CPP, whereas its administration (50 and 100 mg/kg) is not able to prevent, but significantly reduces, the acquisition of ethanol-elicited CPA. The result that pre-treatment with SL327 affects the ability of ethanol to elicit CPP (figure 2) is in agreement with several studies (Valjent *et al.* 2000, 2001; Salzmann *et al.* 2003; Gerdjikov *et al.* 2004; Spina *et al.* 2010). In fact, it has been demonstrated that the local administration of MEK inhibitors, PD98059 in the nucleus accumbens (Gerdjikov *et al.* 2004) or U0126 in the VTA (Lin *et al.* 2010), during conditioning phase, precludes d-amphetamine- and morphine-elicited CPP, respectively. On the same vein, in another study the intracerebroventricular administration of PD98059 impairs the acquisition of morphine-elicited CPP (Spina *et al.* 2010). Moreover, the acute administration of ethanol (Ibba *et al.* 2009; Rosas *et al.* 2014; Agoglia *et al.* 2015) is able to increase the phosphorylation of ERK in the nucleus accumbens, brain region which has been shown to be involved in the acquisition of CPP (Di Chiara 2002; Gerdjikov *et al.* 2004). The involvement of MEK/ERK pathway is also in agreement with self-administration experiments in which the administration of SL327 at a low dose (30 mg/kg) increases the operant behavior, whereas at a higher dose (100 mg/kg) prevents it (Faccidomo *et al.* 2009). Furthermore, it has also been shown that the voluntary consumption of ethanol enhances pERK expression in medial prefrontal cortex, nucleus

accumbens and amygdala (Faccidomo *et al.* 2015). Although different circuits are involved in the self-administration experiments, it is demonstrated that the neurobiological mechanisms and the brain regions that underlie the learning process of operant behavior are in common with Pavlovian learning (Sanchis-segura & Spanagel 2006) and are triggered by the pharmacological effects of ethanol. In this regard, our result that MEK blockade prevents the acquisition of CPP is in agreement with the hint of a pivotal role of the MEK/ERK cascade in positive reinforcement mediated by ethanol (Faccidomo *et al.* 2009, 2015). However, this suggestion appears in contrast with the study of Groblewski, Franken and Cunnigham (2011) reporting that administration of SL327 (50 mg/kg) before two conditioning sessions failed to affect acquisition of ethanol (2 g/kg i.p.)-elicited CPP. These differences could be due to different experimental schedules [unbiased (Groblewski *et al.* 2011) vs biased design (present data)], different strain of animals (DBA/2J), different vehicle for the dissolution of SL327 and time intervals (90 min vs 60 min in the present study) before the exposure to the conditioning apparatus.

Regarding the results of the CPA acquisition, SL327 is unable to fully prevent, but significantly reduces, the ability of ethanol to elicit CPA (figure 3) appears in contrast with the ability of SL327 to prevent the acquisition of ethanol-elicited CPP. The effects of SL327 on the acquisition of ethanol-elicited CPA appear also at variance with the results of Cao and Co-workers (2009) in which the local administration of MEK inhibitor, U0126, in the anterior cingulate cortex, affects the acquisition of CPA induced by intra-plantar formalin (Cao *et al.* 2009). Also, in this case the differences between this study and present data could be due to different routes of administration (local vs systemic) of the MEK inhibitors and nature and strength (pharmacological vs nociceptive) of the US, that play a critical role in determining the ability of MEK blockade to affect CPA acquisition. Similar, but opposite, results on the role of MEK blockade on CPA acquisition were reported by Cao et al. (2009) by showing that other MEK inhibitors prevent intra-plantar formalin- but not foot-shock or U69,593-elicited CPA. Moreover, the immunohistochemical data (table 1 and figure 6), in agreement with Valjent et al. (2000), clearly demonstrate that, at the same dose and timing observed in the place conditioning experiments, SL327 totally prevents the activation

of ERK in brain regions involved in the acquisition of associative learning (Marotta *et al.* 2014) and positive (Gerdjikov *et al.* 2004) and negative conditioning (Deyama *et al.* 2007). However, due to a significantly different experimental design, the immunohistochemical data cannot be offered to provide a consistent explanation about the effect of SL327 on ERK phosphorylation in animals exposed to the CS, but only the effects of SL327 on pERK expression induced by an acute administration of ethanol. Hence, we can only assume that SL327 may prevent ethanol-elicited ERK phosphorylation in the following conditioning sessions as in the first one.

Thus, in light of the ability of SL327 to prevent the acquisition of ethanol-elicited CPP, these observations overall suggest that learning the aversive US-CS association encompasses differentially the MEK/ERK cascade so that its involvement may or may not result critical for the specific behavioral outcome.

In summary, while we cannot offer any conclusive interpretation that may comprehensively explain such experimental differences, including the finding of a significant reduction by SL327 of the acquisition of ethanol-elicited CPA, these observations suggest that MEK blockade may represent the necessary but not sufficient condition to prevent aversive associative learning.

Effects on CPP and CPA Expression

The expression phase of the place conditioning experiments allows establishing whether drug-associated affective memories are able, in a drug-free state and in a state of free choice between CS+ and CS-, to direct animals' behavior toward preferring or avoiding the CS+. This indicates that measurements of place conditioning expression may be the result of both attraction to the CS+ or aversion/avoidance of the CS-. The finding that SL327 significantly reduces, although not fully preventing (figure 4), the expression of ethanol-elicited CPP is in agreement with previous studies showing that MEK inhibitors could prevent retrieval of cocaine (Miller & Marshall 2005) and morphine (Lin *et al.* 2010)-elicited CPP and with the observation that presentation of conditioned stimuli, either in CPP (Nygard *et al.* 2015) and in self-administration experiments (Radwanska *et al.* 2008; Peana *et al.* 2013; Faccidomo *et al.* 2015) is associated with an increased expression of phosphorylated ERK. However,

these studies, at least in terms of brain structures involved, fail to provide a view that may sustain a unifying hypothesis on the role of MEK and the significance of activated ERK in the expression of responses to conditioned stimuli. In fact, the critical involvement of this kinase pathway has been reported in different brain structures such as the VTA (Lin *et al.* 2010), the medial prefrontal cortex (Faccidomo *et al.* 2015), the amygdala (Radwanska *et al.* 2008) and the core of the nucleus accumbens (Miller & Marshall 2005) whereby its blockade results in prevention to express the acquired association.

In contrast with the observation that SL327 significantly reduces CPP expression (figure 4), we found, in agreement with Longoni et al. (2011), that its administration before the post-conditioning test to rats undergone the schedule of aversive conditioning, fails to affect the ability of the CS+ to elicit place aversion (figure 5). Interestingly, although ERK phosphorylation has been reported under a number of experimental conditions related to the establishment of aversive memories such as conditioned taste aversion (Marotta *et al.* 2014) and opiate withdrawal (Wang *et al.* 2015) to our knowledge only few studies, Cao et al. (2009) and Longoni et al. (2011), have addressed, with opposite results, the issue of the role of MEK in the ability of a CS+ to evoke aversive memories. A number of methodological differences might be responsible for discrepancies between these studies and the present data and we can only state at the present that recalling aversive memories by a CS+ may or may not require the involvement of MEK/ERK cascade as a function of the US.

In summary, since the acquisition is more sensitive to MEK/ERK disruption, these results suggest that the signaling kinase under study may be more involved in the initial learning than in the expression of a previously learned association as well as that it may be more involved in appetitive (present data; Salzmann *et al.* (2003); Valjent *et al.* 2000 and 2001)) than in aversive motivation. Furthermore, the observation that SL327 (50 and 100 mg/kg) significantly reduces the expression of ethanol-elicited CPP whereas fully fails to affect the expression of ethanol-elicited CPA allows also to speculate that in CPP expression experiments the attraction toward the CS+ succeeds over the repulsion from the CS-.

In conclusion, given the significance of the place conditioning model, i.e. to highlight the appetitive or aversive motivational properties of USs on one side, and to characterize the strength of CSs to evoke responses of attraction to, or repulsion from, the USs, on the other, the present study provides new insight on the complexity of the involvement of MEK in the establishment of associative learning and in the expression of the acquired responses.

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Experimental Studies

Chapter 2

CHAPTER 2: Caffeine prevents ethanol-elicited place preference and aversion and ERK phosphorylation in the nucleus accumbens and other areas of the extended amygdala

ABSTRACT

Although several epidemiological studies focus on the consequences of the association between caffeinated and alcoholic beverages, the effects of the combination of caffeine and ethanol in animal models of drug addiction are currently still underexplored.

To characterize the pharmacological interaction between caffeine and ethanol and establish if caffeine can affect the ability of ethanol (2 g/kg) of eliciting conditioned place preference (CPP) and conditioned place aversion (CPA), we administered caffeine (3 or 15 mg/kg) to male CD-1 mice before saline or ethanol. Moreover, we assessed whether caffeine, at doses at which fails to affect the expression of Extracellular-signal Regulated Kinase (ERK) phosphorylation (pERK) in the nucleus accumbens, bed nucleus of stria terminalis, central and basolateral amygdala, could prevent ethanol (2 g/kg)-elicited pERK expression in these brain regions.

In the place conditioning paradigm, caffeine was devoid of reinforcing properties whereas ethanol elicited significant CPP and CPA. Moreover, caffeine (15 mg/kg) significantly prevented ethanol-elicited CPP and at both doses also ethanol-elicited CPA. Caffeine (3 and 15 mg/kg) also prevented ethanol-elicited pERK expression in all brain areas examined.

These results, that could be interpreted as due to the functional antagonistic action of caffeine and ethanol on the adenosine-mediated regulation of associative learning and motivated behaviors, disclose exciting insights on the consequences of the acute interaction between two of the most used psychotropic substances and provide exciting grounds to further study their pharmacological interaction.

INTRODUCTION

Ethanol and caffeine are two of the most widely consumed substances in the world. Ethanol is a sedative/hypnotic drug that is recognized as being responsible for initiating and sustaining a wide array of conditions that are fundamental to alcohol use disorders, including alcohol dependence, a progressive escalation from low or moderate to high doses, and compulsive ethanol intake (Koob & Volkow 2009). Caffeine is a minor stimulant that is often consumed in the form of drinks like tea or coffee, but in recent years also as an ingredient in "energy drinks" that contain relatively high caffeine concentrations (e.g., 50–500 mg caffeine per serving) (Scholey & Kennedy 2004; Reissig *et al.* 2009; Peacock *et al.* 2015). Energy drinks are frequently consumed by teenagers and young adults in order to increase athletic performance by reducing fatigue, and/or to improve cognitive performance by increasing memory and concentration (Lalanne *et al.* 2017). Furthermore, the combined intake of alcohol and energy drinks is an emerging phenomenon. The tendency to combine caffeine with ethanol during binge drinking may be due to the popular belief that caffeine antagonizes intoxicating effects of alcohol, and improves social interactions (Weitzman *et al.* 2003; Reissig *et al.* 2009; Marczinski 2011).

Caffeine and ethanol both act on adenosine function, but in opposite ways. Specifically, caffeine acts as a non-selective antagonist of A_1 and A_{2A} adenosine receptors (Cauli & Morelli 2005; Ferré *et al.* 2008), while ethanol acts indirectly via the production of its second metabolite, acetate, to increase adenosine levels (Nagy *et al.* 1990; Nagy 1992; Fredholm & Wallman-Johansson 1996). Interestingly, although epidemiological studies have shown that there can be a positive correlation between the consumption of caffeine and that of ethanol (Marczinski & Fillmore 2014; Kristjansson *et al.* 2015), the results reported in the preclinical literature are often complex and contradictory, and the nature of the interaction varies across the doses and behavioral tasks used. Administration of caffeine at the dose of 5.0 mg/kg to rats subsequently given access to ethanol (10% v/v) facilitated its ingestion, while the administration of doses of 2.5 and 10.0 mg/kg failed to increase ethanol intake (Kunin *et al.* 2000). In mice, high doses of caffeine (20 mg/kg) reduced ethanol intake under restricted access conditions, but increased it when ethanol was available 24 hours.

Lower doses of caffeine (5-10 mg/kg) did also increase ethanol consumption under unrestricted access conditions (SanMiguel *et al.* 2019). Moreover, high doses of ethanol are known to cause amnesia and learning impairments, which are frequently associated with episodes of binge drinking (Wetherill & Fromme 2016), whereas caffeine at moderate doses (5.0-10.0 mg/kg) was able to improve memory acquisition and retention in different learning models (Angelucci *et al.* 2002; Dash *et al.* 2004; Spinetta *et al.* 2008). However, caffeine (5.0-40.0 mg/kg) did not reverse the learning deficits caused by ethanol (1.0-1.4 g/kg) in a plus-maze discriminative avoidance task (Gulick & Gould 2009) or in a social interaction three-chamber test (López-cruz *et al.* 2016). Low doses of ethanol that did not impair social interaction reduced social recognition 24 hours later, and co-administration of caffeine was unable to block these amnesic effects (López-cruz *et al.* 2016).

Ethanol has motivational properties that are consistently highlighted in studies of place conditioning, such as conditioned place preference (CPP) and conditioned place aversion (CPA) (Cunningham et al., 2002, 2003; Cunningham and Henderson, 2000; Font et al., 2006; Rosas et al., 2017; Spina et., 2015), as well as voluntary intake and ethanol-induced reinforcement of operant behavior (Bassareo et al., 2017; Faccidomo et al., 2015; Lorrai et al., 2019; Peana et al., 2014). In contrast, the motivational effects of caffeine are not well characterized. In fact, the few studies focusing on caffeine-induced CPP have reported inconclusive results, mostly due to substantially different doses and schedules of administration across studies (Brockwell *et al.* 1991; Brent Bedingfield *et al.* 1998; Patkina & Zvartau 1998; Hsu *et al.* 2009). Moreover, the studies that investigated the effects of caffeine and ethanol co-administration on place conditioning reported that caffeine, administered either alone (3.0 mg/kg) (Brockwell *et al.* 1991) or in the same injection as ethanol (3.0 mg/kg of caffeine and 1.75 g/kg of ethanol), did induce a significant CPP, although this effect was more modest than that of ethanol itself (Hilbert *et al.* 2013). In addition, a single administration of caffeine (3.0 mg/kg) was reported as being able to reduce the expression of ethanol-elicited CPP and its reinstatement (Okhuarobo *et al.* 2019).

The Extracellular signal-Regulated Kinase (ERK) is part of the Mitogen-Activated Protein Kinase (MAPK)-signaling cascade that plays a critical role in neuroplasticity, gene

expression, and behavioral changes underlying the reinforcing processes induced by substances of abuse (Valjent *et al.* 2005). The active form of this protein, phosphorylated ERK (pERK), plays a key role in the acute effects of ethanol. Increases in ERK expression following acute ethanol administration has been demonstrated in several brain areas, including both the core (AcbC) and shell (AcbSh) subregions of the nucleus accumbens, nuclei that are part of the extended amygdala (bed nucleus of stria terminalis (BNST) and central nucleus of the amygdala (CeA)) (Ibba *et al.* 2009), and basolateral amygdala (Spanos *et al.* 2012). These brain areas are involved in positive and negative effects of ethanol reinforcement and in the development of dependence (Koob *et al.* 1998). Moreover, ERK expression is related to the motivational properties of drugs as demonstrated by CPP experiments (Valjent *et al.* 2000, 2001; Salzmann *et al.* 2003; Gerdjikov *et al.* 2004; Lu *et al.* 2006; Spina *et al.* 2010). ERK appears to be involved in the acquisition of motivational valence by neutral stimuli paired with the primary effects of addictive drugs (Valjent *et al.* 2001; Gerdjikov *et al.* 2004).

In order to shed light on the interaction between low but pharmacologically significant doses of caffeine and the motivational properties of ethanol, the aims of this study were: **1)** determining if pre-treatment with different doses of caffeine (3.0 and 15.0 mg/kg) may affect the acquisition of ethanol (2 g/kg)-elicited CPP and CPA (Cunningham *et al.* 1997; Spina *et al.* 2015; Rosas *et al.* 2017), and **2)** investigating if caffeine has the ability to affect the increases of pERK expression elicited by ethanol in the nucleus accumbens, extended amydgala (bed nucleus of stria terminalis and central nucleus of the amygdala), and basolateral amygdala. Moreover, the study was also undertaken to verify if the stimuli (positive or negative) associated with ethanol may induce a differential expression of pERK in the brain areas examined, and if the effects of caffeine on the acquisition of place conditioning may also be reflected in the differential expression of ERK in brain areas involved in ethanol-conditioned reinforcement.

MATERIALS AND METHODS

Animals

Adult male CD-1 mice (22-24 g, Charles River, Calco, Italy) were housed in groups of four per cage for at least 6 days before use and maintained on a 12:00/12:00 h light/dark cycle (lights on at 08:00 a.m.) with food and water *ad libitum*. All the experiments were carried out during the light phase, between 09:00 and 16:00 h. The total numbers of mice were n=89 and n=80 in the CPP and CPA experiments, respectively, and n=19 and n=20 in the immunohistochemistry experiments after CPP and CPA expression, respectively. Finally, the total number of mice used in the immunohistochemical experiments were n=19.

All the experimental procedures were performed in accordance with the Principles of laboratory animal care and with the guidelines and protocols approved by the European Union (2010/63/UE L 276 20/10/2010). Every possible effort was made to minimize animal pain and discomfort and to reduce the number of experimental subjects.

Drugs

Ethanol (Sigma-Aldrich, Milan, Italy) 20% (v/v) in isotonic saline, 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) were administered intraperitoneally (IP).

Apparatus

The apparatus consisted of two rectangular Plexiglas boxes (48L x 20W x 30H cm) separated by a 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 (45,4% for compartment A and 54,6%

for compartment B in CPP experiments; 49% for compartment A and 51% for compartment B in CPA experiments). Moreover, mice whose spontaneous preference times, at the pre-conditioning test, were between 441 and 459 sec were randomly assigned half to compartment A and half to compartment B.

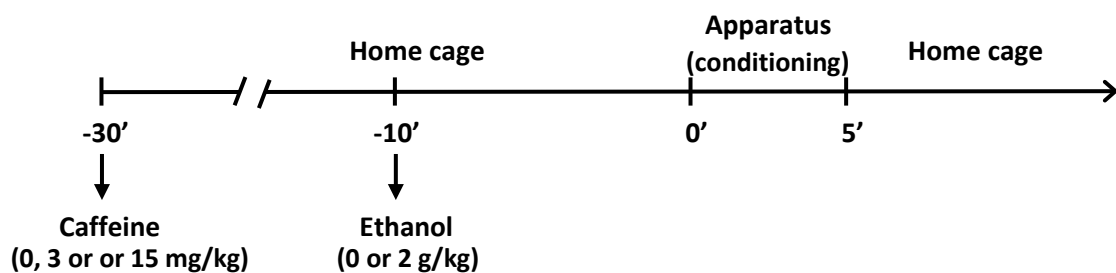## Conditioned Place Preference and Conditioned Place Aversion: procedure and experimental design

Each experiment consisted of three phases. During the first phase (pre-test, day 1), the guillotine door was kept raised and each mouse was placed randomly in one or the other compartment and given access to both compartments of the apparatus for 15 minutes (900 sec). The time spent in one compartment was recorded and taken as indication of spontaneous preference. Behavioral schedules for backward (CPP) and forward (CPA) conditioning (figure 1A and 1B, respectively) were designed after Rosas et al. (2017) and Spina et al. (2015) with some modifications related to the timing of pre-treatment with caffeine. In particular, during the second phase (conditioning, days 2-5) of CPP experiments, mice of distinct experimental groups, were administered caffeine (3 or 15 mg/kg) or saline 20 min before ethanol (2 g/kg) or saline, and were returned to their home cage. 10 min after ethanol administration mice were exposed for 5 min to a given compartment and returned to their home cage. 6 hours later mice were administered saline or caffeine (3 or 15 mg/kg), then returned to their home cage 20 min before the second administration (ethanol, 0 or 2 g/kg). 10 min after this second administration mice were placed (for 5 min) in the compartment opposite to that of the morning exposure.

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

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)/ethanol (0 or 2 g/kg)] administrations was opposite on even days with respect to that one on odd days]; 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 paired four times with the given compartment.

A

Conditioned Place Preference (CPP)

B
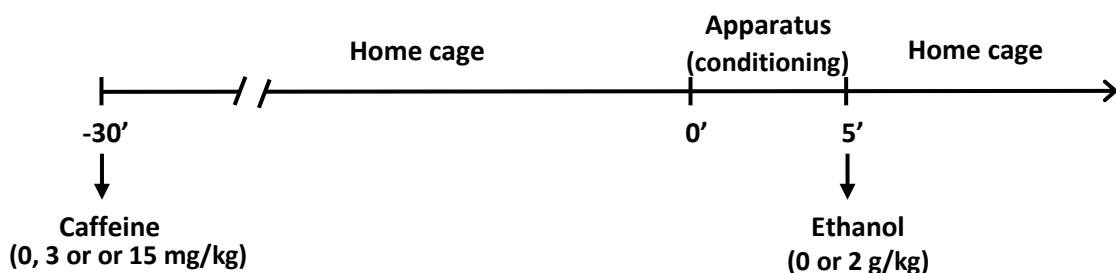
Conditioned Place Aversion (CPA)

Figure 1. 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 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).*

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 min was recorded. The conditions of the post-conditioning test were identical to those of the pre-conditioning test. Pre- and post-conditioning recordings were done with stop watch by observers blind to pharmacological treatments. Hence, a statistically significant difference between the time spent during pre- and post-conditioning tests of the drug group with respect to that of the saline group was taken as indication of the development of place conditioning (Carr *et al.* 1989).

Immunohistochemistry

For these experiments mice of distinct experimental groups were administrated caffeine (0, 3 or 15 mg/kg, IP) 20 min before ethanol (0 or 2 g/kg, IP) and deeply anesthetized 15 min after the last administration (Ibba *et al.* 2009). Under deep anesthesia, animals were subjected to trans-cardiac perfusion with ice-cold PBS (Phosphate Buffered Saline: 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4, pH 7.4) and 4% paraformaldehyde (PFA) solutions. After perfusion, brains were removed and post-fixed overnight in 4% PFA. Brain slices (40 μm) of the regions of interest were cut, on ice-cold PBS with a vibratome (Leica VT1000, Leica, Germany) according to plates 21-23 (approximately from AP 1.18 to AP 0.98 mm from bregma for the nucleus accumbens core and shell), to plates 30-32 (approximately from AP 0.14 to AP -0.10 mm from bregma for the bed nucleus of stria terminalis) and to plates 40-41 (approximately from AP -1.06 to AP -1.34 mm from bregma for the basolateral and central nucleus of the amygdala) of the mouse brain atlas (Paxinos & Franklin 2001). Slices were kept in ice-cold PBS and processed for immunohistochemistry according to a protocol for free-floating slices. Immunoreactions for pERK-positive cells detection were applied to at least two every other slice obtained from each brain. After an incubation for 30 min in 1% H_2O_2, slices were incubated for 1 hour with 3% BSA. The incubation with the primary anti pERK antibody (phosphorylated ERK, Cell Signalling Technology, Beverly, MA, USA (1:350)) was conducted overnight. On the following day, after rinsing, slices were incubated for 1 hour with the biotinylated secondary antibody

(1:800). After three rinses the slices were incubated in avidin biotin peroxidase complex prepared according to the manufacturer's suggestions (Vectastain ABC kit, Vector Laboratories, Burlingame, CA, USA) and a 3-3'-diaminobenzidine solution (10 mg/mL) was added until development of brown staining. Slices were rinsed and mounted onto gelatine-coated slides and processed through alcohol-xylene for light microscopy examination. pERK-positive neurons were identified in the regions of interest of both hemispheres at the lowest magnification (10X) and quantitative analysis was performed using a Zeiss Axioskop 40 light microscope, equipped with PL Fluotar 10X (na=0.3), 40X (na=1.00–0.5) and 100X oil (na=1.3) objectives, coupled with a Nikon D5000 digital camera (Melville, NY, USA). Images of the regions of interest were obtained at the lowest magnification (10X) from at least three every other 40 μm thick slices and used to automatically count the number of pERK-positive neurons/area (pERK expression) by application of the software ImageJ (v. 1.42, National Institutes of Health sponsored image analysis program).

Statistical analysis

To determine statistically significant differences between pre-conditioning values of the experimental groups depicted in figures 2-6, one-way Analysis of Variance (ANOVA) was applied (Statistica 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 those 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 (with multiple comparisons), carried out using Newman-Keuls post-hoc 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 variable. Fishers Least Significant Difference (LSD) post-hoc analyses, whereby allowed by ANOVAs significant main effects, were applied for multiple comparisons, with the statistical significance set at $p<0.05$.

RESULTS

Effects of caffeine on acquisition of ethanol-elicited CPP

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

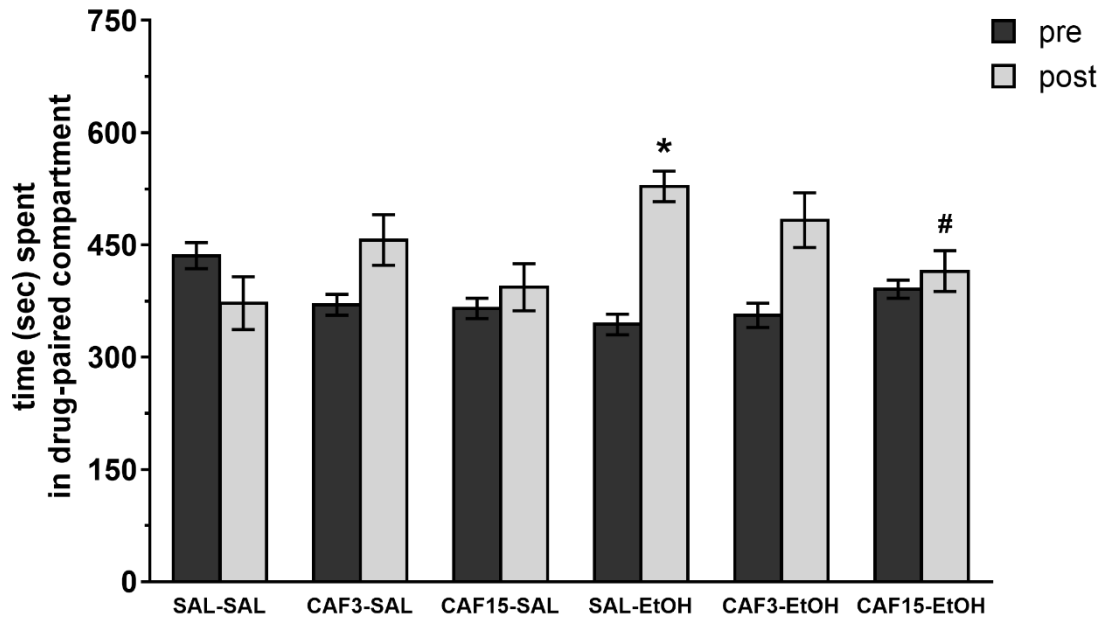**Figure 2. Effects of caffeine pre-treatment on acquisition of ethanol-elicited CPP.** *Data are shown as average time spent (sec.) ± SEM in the drug-paired compartment. *Indicates a significant difference (p<0.05) of time spent during post-conditioning test as compared to caffeine (0 mg/kg) + EtOH (0 g/kg) group; #Indicates a significant difference (p<0.05) in time spent during the post-conditioning test as compared to caffeine (0 mg/kg) + EtOH (2 g/kg) group.*

Effects of caffeine on acquisition of ethanol-elicited CPA

Figure **3** shows the effects of pre-treatment with caffeine (0, 3 or 15 mg/kg) 30 min before the exposure to the assigned compartment for 5 min, and of ethanol (EtOH 0 or 2 g/kg) administration upon removal from the place conditioning apparatus (see figure 1B). Pre-conditioning preference times did not differ significantly between experimental groups [$F_{5,74}$=0.03, NS]. Repeated measures three-way ANOVA with preference times (pre- and post-conditioning) as dependent factors and with pre-treatment (caffeine 0, 3 or 15 mg/kg) and treatment (EtOH 0 and 2 g/kg) doses as independent factors, revealed significant effects of pre-treatment [$F_{2,74}$=3.51, 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-treatment by time [$F_{2,74}$=3.83, p<0.05] and treatment by time [$F_{1,74}$=14.54, p<0.001] interactions. Post-hoc analysis according to Newman-Keuls test revealed that 1) EtOH stimulated a significant CPA from 513±13 to 269±38 sec/900 (p<0.001) 2) caffeine (3 and 15 mg/kg) is devoid of motivational properties and 3) caffeine (3 and 15 mg/kg) significantly prevents the acquisition of CPA induced by EtOH (p<0.05).
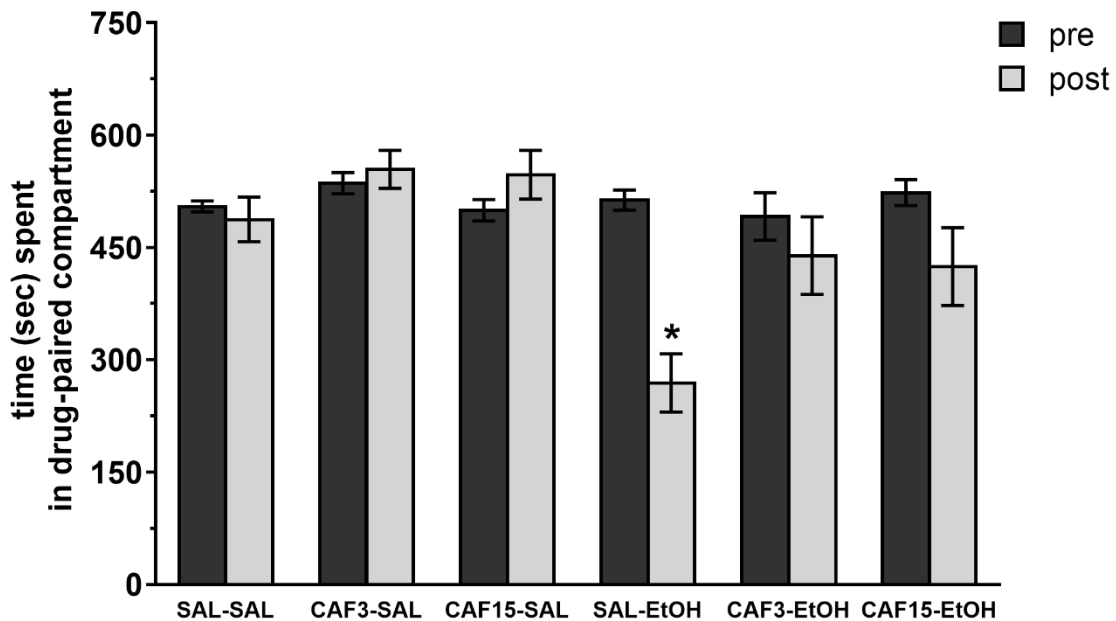
Figure 3. Effects of caffeine pre-treatment on acquisition of ethanol-elicited CPA. *Data are shown as average time spent (sec.) ± SEM in the drug-paired compartment. *Indicates a significant difference (p<0.05) of time spent during the post conditioning test between caffeine (0 mg/kg) + EtOH (2 g/kg) group and all other groups.*

Effects of acute administration of caffeine on the expression of ethanol-elicited pERK-positive neurons

Figure **4** shows the effects of pre-treatment with caffeine (0, 3 or 15 mg/kg) 20 min before the administration of ethanol (EtOH 0 or 2 g/kg) on the number of pERK-positive neurons in AcbC and AcbSh, BNST, CeA and BLA.

The administration of EtOH (2 g/kg), according to previous studies (Ibba *et al.* 2009) increases the number of pERK-positive cells in the AcbC and AcbSh (p<0.05) (from 41±2 and 50±10 to 149±13 and 168±34, respectively) and caffeine pretreatment reduces to 78±10 and 101±21 at the doses of 3 mg/kg and to 51±3 and 82±15 at the dose of 15 mg/kg, the ability of EtOH to stimulate ERK phosphorylation in both Acb subregions (figure **4A**). One-way ANOVA, with pre-treatment/treatment factor as independent variable and with the number of pERK-positive cells/area as dependent variable, revealed significant pre-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 analysis according to Fishers Least Significant Difference (LSD) test revealed that 1) EtOH stimulates a significant increase of the

number of pERK-positive neurons both in the AcbC and in the AcbSh (p<0.05) and 2) pre-treatment with caffeine at both doses significantly reduces this effect in both areas.

Moreover, the administration of EtOH (2 g/kg) increases pERK-positive cells in the BNST (p<0.05) (respectively from 23±3 to 103±9 and caffeine pretreatment reduces, to 77±3 at the dose of 3 mg/kg and to 72±6 at the dose of 15 mg/kg, the ability of EtOH to stimulate ERK phosphorylation in this area (figure **4B**). One-way ANOVA, with pre-treatment/treatment as independent variable and with the number of pERK-positive cells/area as dependent variable, revealed significant effects of pre-treatment/treatment [$F_{3,15}$=28.04, p<0.001]. Post hoc analysis according to Fishers Least Significant Difference (LSD) test revealed 1) that EtOH stimulates a significant increase of the number of pERK-positive neurons in the BNST (p<0.001) and 2) that pre-treatment with caffeine at both doses significantly reduces (p<0.05) this effect.
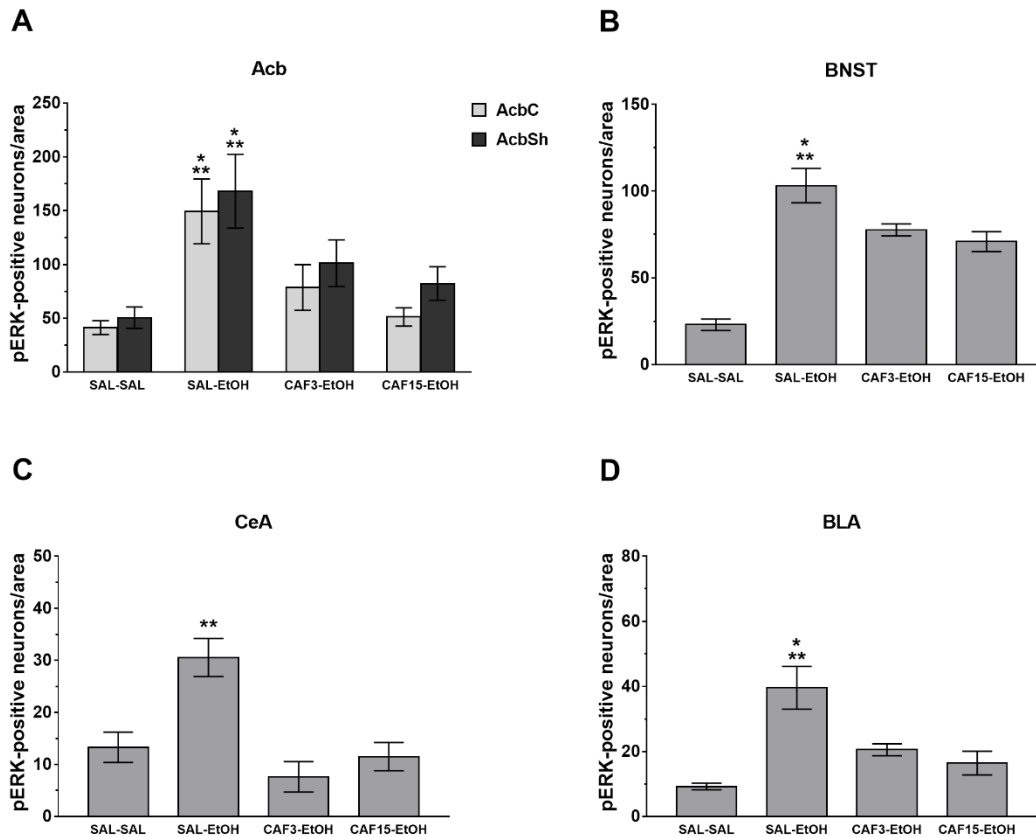
Figure 4. Effects of acute administration of caffeine on the expression of ethanol-elicited pERK-positive neurons in the Acb, BNST, CeA and BLA. *Data are shown as mean ± SEM of pERK-positive neurons/area. Figure 4A: *indicates a significant difference (p<0.05) between caffeine (0 mg/kg) + EtOH (2 g/kg) and caffeine (3 and 15 mg/kg) + EtOH (2 g/kg) groups in AcbC and AcbSh; **indicates a significant difference (p<0.001) between caffeine (0 mg/kg) + EtOH (2 g/kg) and caffeine (0 mg/kg) + EtOH (0 g/kg) groups in AcbC and AcbSh. Figure 4B: *indicates a significant difference (p<0.05) between caffeine (0 mg/kg) + EtOH (2 g/kg) and caffeine (3 and 15 mg/kg) + EtOH (2 g/kg) groups in BNST; **indicates a significant difference (p<0.001) between caffeine (0 mg/kg) + EtOH (2 g/kg) and caffeine (0 mg/kg) + EtOH (0 g/kg) groups in BNST. Figure 4C: **indicates a significant difference (p<0.001) between caffeine (0 mg/kg) + EtOH (2 g/kg) and all other groups in CeA. Figure 4D: *indicates a significant difference (p<0.05) between caffeine (0 mg/kg) + EtOH (2 g/kg) and caffeine (3 and 15 mg/kg) + EtOH (2 g/kg) groups in BLA; **indicates a significant difference (p<0.001) between caffeine (0 mg/kg) + EtOH (2 g/kg) and caffeine (0 mg/kg) + EtOH (0 g/kg) groups in BLA.*

Finally, the administration of EtOH (2 g/kg) also increases the number of pERK-positive cells in 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 BLA; pretreatment with caffeine, at the doses of 3 and 15 mg/kg, reduces to 7±2 and 11±3 respectively, in the CeA and to 20±1 and 16±4, respectively, in the BLA, the number of pERK-positive neurons elicited by ethanol (figures **4C** and **4D**). One-way ANOVA, with pre-treatment/treatment as independent variables and with the number of pERK-positive cells/area as dependent variable, revealed

significant effects of pre-treatment/treatment [$F_{3,15}=10.76$, $p<0.05$]. Post hoc analysis according to Fishers Least Significant Difference (LSD) test revealed that 1) EtOH stimulates a significant increase of the number of pERK-positive neurons in the CeA and BLA ($p<0.05$) and 2) pre-treatment with caffeine at both doses significantly reduces ($p<0.001$) this effect in both areas. Representative images of these results are shown in Figure **5**.
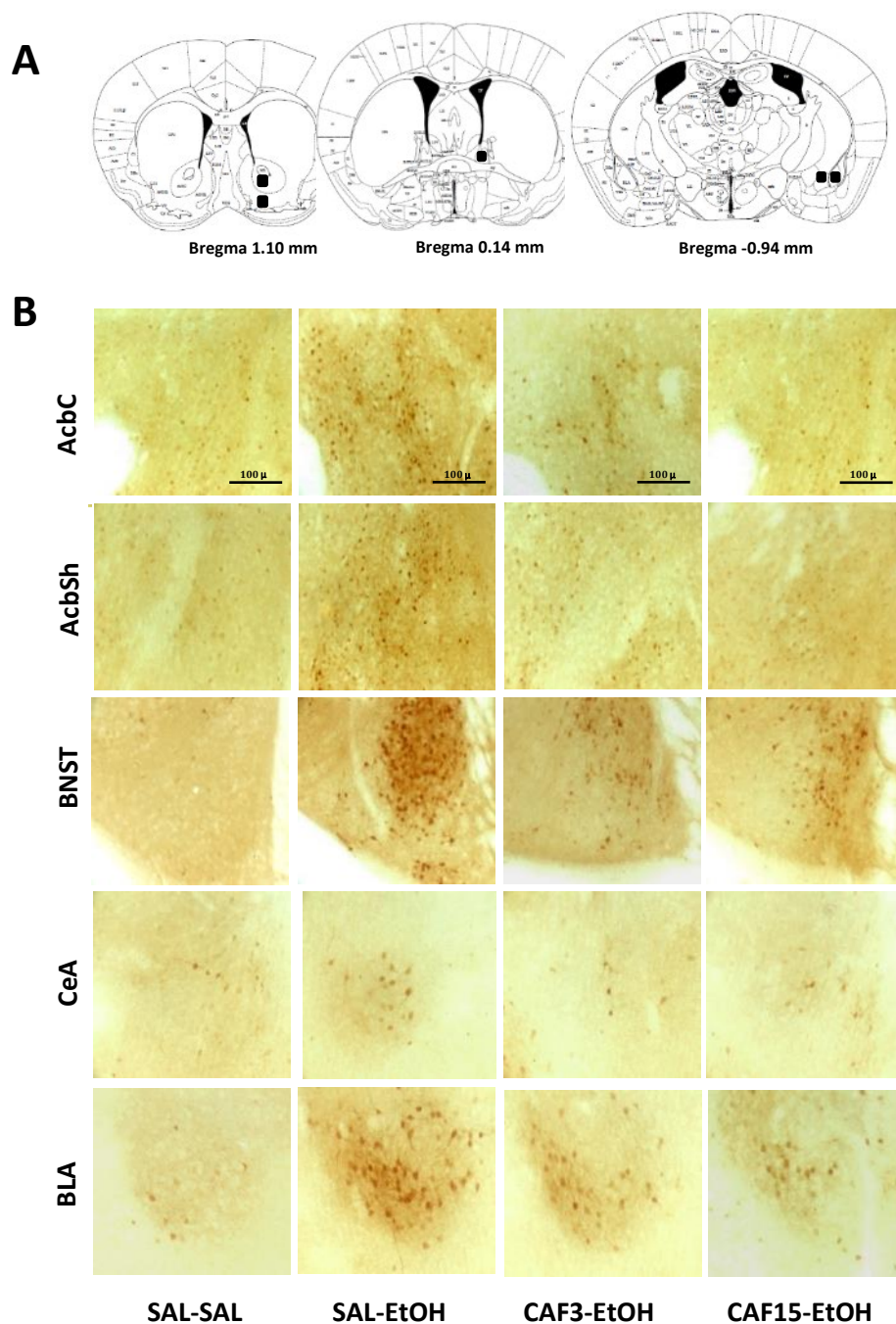

Figure 5. Coronal sections with bregma coordinates taken from the mouse brain atlas of Paxinos and Franklin (2001) showing the location of the areas (AcbC and AcbSh, BNST, CeA and BLA) for pERK immunoreactivity counting (figure 5A). Low (20X) magnification images of pERK-positive neurons from mice representative of each treatment group (figure 5B).

Effects of the expression of ethanol-elicited CPP on ERK phosphorylation

Figure **6** shows the effects of ethanol-elicited CPP expression behavior on the number of pERK-positive neurons/area in AcbC and AcbSh, BNST, CeA and BLA.
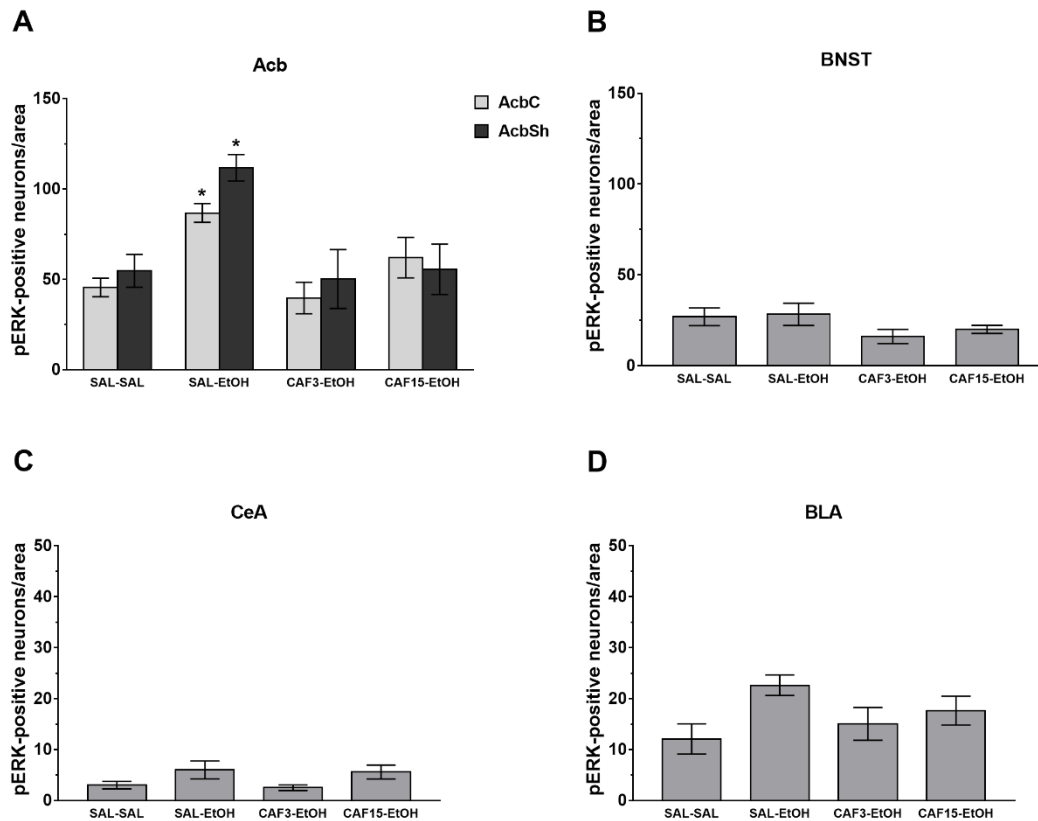

Figure 6. Effects of the expression of ethanol-elicited CPP on ERK phosphorylation in the Acb, BNST, CeA and BLA. *Animals of each group, in a drug-free state, performed the post-conditioning test (15 min) and immediately afterwards were anesthetized and perfused for immunohistochemical analysis. Data are shown as mean ± SEM of pERK-positive neurons/area. Figure **6A**: *indicates a significant difference (p<0.05) between caffeine (0 mg/kg) + EtOH (2 g/kg) and caffeine (0 mg/kg) + EtOH (0 g/kg) groups and between caffeine (0 mg/kg) + EtOH (2 g/kg) and caffeine (3 and 15 mg/kg) + EtOH (0 g/kg) groups in AcbC and AcbSh.*

The administration of ethanol (2 g/kg) during conditioning, according to the schedule depicted in figure 1A, resulted, as also shown in figure 2, in a significant CPP; the expression of this acquired preference during the post conditioning test, in turn, increases the number of pERK-positive cells in the AcbC and AcbSh (p<0.05), respectively from 45±2 to 86±2 and from 54±9 to 111±7; pretreatment with caffeine during conditioning reduces to 39±6 in AcbC and to 50±16 in AcbSh at the dose of caffeine 3 mg/kg and to 62±5 in AcbC and 56±14 in AcbSh at the dose of caffeine 15

mg/kg, the ability of the EtOH-conditioned stimulus to increase ERK phosphorylation in both subregions of nucleus accumbens (figure **6A**). One-way ANOVA, considering pretreatment/treatment as independent variables and the number of pERK-positive neurons/area as dependent variable, revealed significant pre-treatment/treatment 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 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 according to Fishers Least Significant Difference (LSD) test for the Acb revealed that 1) the expression of EtOH-elicited CPP is associated with a significant increase of the number of pERK-positive neurons/area both in the AcbC and in the AcbSh (p<0.05) and that 2) caffeine at both doses, during conditioning, significantly reduced this effect.

Effects of ethanol-elicited CPA expression on ERK phosphorylation

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

The administration of ethanol (2 g/kg) during conditioning, according to the schedule depicted in figure 1B, resulted, as also shown in figure 3, in a significant CPA; the expression of this acquired aversion during the post conditioning test, fails to increase the number of pERK-positive cells in the AcbC and AcbSh (p>0.05) (figure **7A**). One-way ANOVA, considering the number of pERK-positive neurons/area as a dependent variable, and pretreatment/treatment as independent variables, reveled significant pre-treatment and 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**). Post-hoc test according to Fishers Least Significant Difference (LSD) revealed that 1) the expression of ethanol-elicited CPA is not associated with a significant increase of the number of pERK-positive neurons/area in AcbSh and AcbC (p>0.05) with respect to caffeine (0 mg/kg) + EtOH (0 g/kg) conditioned group.

However, the expression of the acquired aversion during the post conditioning test, increases the number of pERK-positive cells in the BNST (p<0.001) (respectively from 37±4 to 85±14) and caffeine pretreatment at both doses reduces, to 49±7 (caffeine 3

mg/kg) and 28±4 (caffeine 15 mg/kg) pERK-positive cells/area, the ability of EtOH-elicited CPA expression to stimulate ERK phosphorylation in this area (figure **7B**). One-way ANOVA, with pre-treatment/treatment as independent variable and with the number of pERK-positive cells/area as dependent variable, revealed significant pre-treatment and treatment effects [$F_{3,16}$=9.06, p<0.001]. Post hoc analysis according to Fishers Least Significant Difference (LSD) test revealed that 1) expression of EtOH-elicited CPA stimulated a significant increase of the number of pERK-positive neurons in the BNST (p<0.001) and that 2) pre-treatment with caffeine at both doses significantly reduced (p<0.001) this effect.

Finally, the expression of the acquired aversion during the post conditioning test, increases of pERK-positive cells in the CeA (p<0.001) and BLA (p<0.05) of the amygdala complex (respectively from 7±1 to 17±1 for the CeA and from 18±2 to 31±4 for BLA) and caffeine pretreatment at both doses, during conditioning, significantly reduces it to 3±1 and 5±1 for the doses of caffeine of 3 and 15 mg/kg, respectively in the CeA and to 21±1 and 16±2 for the doses of caffeine of 3 and 15 mg/kg, respectively, in the BLA (figure **7C** and **7D**). One-way ANOVA, with pre-treatment/treatment as independent variable and with the number of pERK-positive cells/area as dependent variable, revealed a significant pre-treatment and treatment effects in CeA [$F_{3,16}$=79.57, p<0.001] and BLA [$F_{3,16}$=5.52, p<0.05]. Post hoc analysis according to Fishers Least Significant Difference (LSD) test revealed that 1) expression of EtOH-elicited CPA stimulates a significant increase of the number of pERK-positive neurons in both regions of amygdala complex (p<0.05) and that 2) pre-treatment with caffeine at both doses significantly reduced (p<0.05) this effect.
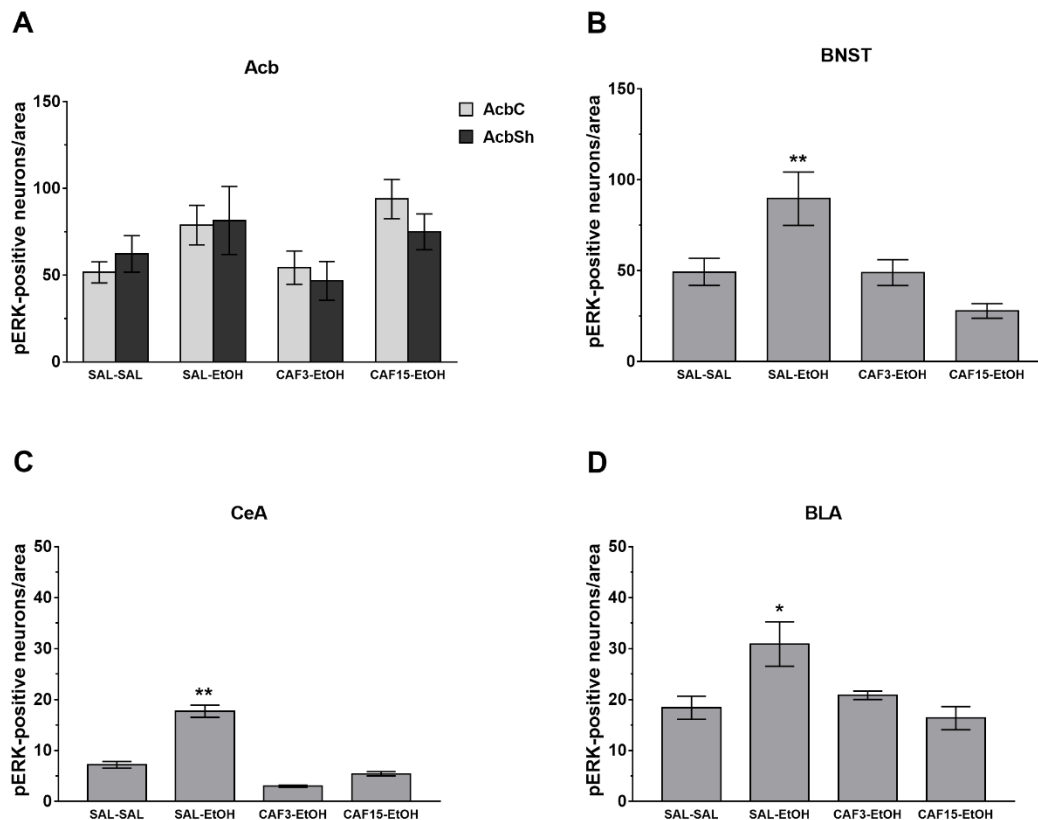
Figure 7. Effects of ethanol-elicited CPA expression on ERK phosphorylation in the Acb, BNST, CeA and BLA. *Animals of each group, in a drug-free state, performed the post-conditioning test (15 min) and immediately afterwards were anesthetized and perfused for immunohistochemical analysis. Data are shown as mean ± SEM of pERK-positive neurons/area. Figures **7B** and **7C**: **indicates a significant difference (p<0.001) between caffeine (0 mg/kg) + EtOH (2 g/kg) and all other groups in BNST and CeA. Figure **7D**: *indicates a significant difference (p<0.05) between caffeine (0 mg/kg) + EtOH (2 g/kg) and all other groups in BLA.*

DISCUSSION

The consequences of drug interactions have an enormous importance both for substance abuse and therapeutic practice. Depending on the substances these interactions can lead to numerous and, *a priori* unsuspected, problems that can occur from pharmacokinetic, pharmacodynamic, metabolic and toxicological factors. The interaction between caffeine and ethanol is no exception in this regard. Moreover, not only does this interaction involve a very large number of individuals all over the world, but it also involves the functional antagonistic effects of two substances that share a pharmacological target represented by the adenosinergic system (López-Cruz *et al.* 2013). The present work was designed and carried out in order to start laying a

foundation for systematically studying the consequences of the interaction between these two broadly-used psychoactive substances, especially with specific reference to the motivational effects descending from such interaction.

In particular, the study was aimed at establishing whether the administration of caffeine at low to moderate doses that are thought to be on the borderline for altering behavior in terms of eliciting arousal (Acquas et al., 2002; De Luca et al., 2007; Hasenfratz et al., 1993) and locomotor activity (Dar 1988; López-Cruz *et al.* 2013) could affect the reinforcing properties of a moderate dose of ethanol (2.0 g/kg) as measured by the place conditioning method in adult male CD-1 mice (Cunningham *et al.* 2006; Spina *et al.* 2015; Rosas *et al.* 2017). To this end we adopted a dual approach, currently applied in our laboratory, which involves both behavioral and biochemical determinations. For the behavioral measure we assessed spontaneous preference for a given environment in terms of place conditioning (Acquas *et al.* 1989; Spina *et al.* 2015; Rosas *et al.* 2017). The neurochemical measure involved the detection of pERK expression in specific brain regions involved in the affective and motivational responses to drug stimuli. Phosphorylated ERK is important to assess because it is a cellular marker related to neural plasticity and short- and long-term adaptive responses to substances of abuse (Sweatt 2001, 2004; Gerdjikov *et al.* 2004; Acquas *et al.* 2007, 2010; Ibba *et al.* 2009; Sun *et al.* 2016; Rosas *et al.* 2017).

Our behavioral model, which involved slight modifications of the methods introduced by Cunningham and Colleagues (Cunningham *et al.* 2003, 2006), had the added benefit of highlighting the fact that the same dose of the unconditioned stimulus (ethanol), can have both positive and negative motivational properties. This is useful for characterizing the opposite motivational aspects elicited by the same substance. In agreement with previous data (Cunningham & Henderson 2000; Cunningham *et al.* 2002, 2003; Spina *et al.* 2015; Rosas *et al.* 2017), the results of the behavioral experiments confirmed that ethanol at the dose of 2.0 g/kg elicits strong and significant CPP (figure 2) and CPA (figure 3). The study also reports that caffeine at either dose (3.0 and 15.0 mg/kg) failed to alter the spontaneous preference of the animals as shown by the results of the post-conditioning tests of the caffeine (3.0 mg/kg) + EtOH (0 g/kg) and caffeine (15.0 mg/kg) + EtOH (0 g/kg) groups. Nevertheless, the combination of caffeine and ethanol administration significantly altered the

outcome of ethanol elicited conditioning. In particular, 15.0 mg/kg caffeine significantly 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 of Hilbert and Colleagues (2013), who found that caffeine (3.0 mg/kg) exerted reinforcing effects in C57BL/6J mice that were generally weaker compared to ethanol (1.75 g/kg) and that their combination resulted in motivational properties indistinguishable from those of ethanol alone. Moreover, our results are also partly at variance with those of the study by Zuniga and Cunningham (2019), who reported that caffeine (3.0 or 30.0 mg/kg) administered in combination with ethanol (2.0 g/kg) fails to affect ethanol-elicited CPP. However, a number of significant differences may be taken into account to interpret these discrepant results. For example, the experimental design of the studies by Hilbert and Colleagues (2013) and Zuniga and Cunningham (2019) were quite different than that of the present one, including a different route of administration (single injection vs two separate injections at different times), different strains of animals (C57BL/6J or DBA/2J vs CD-1), different acquisition times (16 vs 8 conditioning sessions), different time intervals between administration of the US and exposure to the conditioning apparatus, and different numbers of post-conditioning tests. These substantial experimental differences could reasonably have led to different results. In addition, the significant CPP elicited by caffeine was observed only after test one in the study by Hilbert et al., (2013) and only after test three in the study by Zuniga and Cunningham (2019), while that for ethanol remained constant across tests (Hilbert *et al.* 2013; Zuniga & Cunningham 2019), and in this regard these results overall confirmed that caffeine has weak reinforcing properties (Liu *et al.* 2008).

The results of our study on the whole indicate that the combination of appropriate doses of caffeine with the 2.0 g/kg of ethanol, a dose of ethanol which was capable of exerting motivational properties, prevented the establishment of learning the CS-US association and consequently its expression. This suggests that the acute effect of ethanol, i.e. its ability to transfer motivational properties to the environment (acquisition of place conditioning), is prevented by the blockade of A_1 and/or A_{2A} adenosinergic receptors by caffeine. In other words, these data show for the first time

that the acute administration of caffeine can interfere with the motivationally relevant conditioning effects of ethanol.

Moreover, these present results also appear at variance with those of the recent study by SanMiguel et al., (2019) reporting that low to moderate doses of caffeine actually increased ethanol intake, in particular, in moderate ethanol-consumers (on average between 10.0 to 15.0 g/kg/24 hours) in C57BL6J mice. However, in the same study, a high dose of caffeine was indeed able to reduce high levels of ethanol intake in the drinking in the dark paradigm (SanMiguel *et al.* 2019).

The results of the immunohistochemical experiments revealed that, consistent with previous studies (Ibba *et al.* 2009), ethanol stimulated a significant increase of the number of pERK-positive neurons both in the AcbC and in the AcbSh, and that pre-treatment with caffeine at both doses significantly reduced these effects. These results also extend to the other brain areas examined (figures 4A-D), thus providing a possible interpretative mechanism for the behavioral results illustrated in figures 2 and 3 of this work. These findings also are in agreement with the observations that the expression of this phosphorylated kinase is, in fact, increased in the Acb and in other nuclei of the extended amygdala (central nucleus of the amygdala, bed nucleus of the stria terminalis) following the administration of numerous substances with addictive properties (Valjent *et al.* 2004) but not following that of other psychopharmacologically active, but not addictive, substances such as caffeine itself (Valjent *et al.* 2004; Acquas *et al.* 2010).

In the present study, we also aimed to evaluate the expression of pERK in the core and shell of the Acb, and in the amygdala, after exposure to the conditioned stimulus. The results clearly demonstrate the dissociation between behavioral expression (preference shift compared to pre-test) and the brain regions showing increased expression of ERK phosphorylation in relation to the affective value acquired by the conditioned stimulus. These data also strongly support the specificity of the effects of caffeine on ethanol's ability to activate the behavior (figures 2 and 3) and to elicit expression of phosphorylated ERK in the AcbC, AcbSh, CeA and BLA (figure 4) after acute administration (Ibba *et al.* 2009).

As previously mentioned, caffeine and ethanol act in opposite directions on the adenosinergic system. Caffeine is a non-selective antagonist of the A_1 and A_{2A}

adenosine receptors (Cauli & Morelli 2005; Ferré *et al.* 2008). In contrast, ethanol increases the levels of the endogenous agonist, adenosine, through different mechanisms that include the stimulation of its release, the reduction of its reuptake through inhibition of nucleoside transporter, and the promotion of its synthesis through the increased availability of acetate, an intermediate of adenosine biosynthesis, that accumulates in the cells upon ethanol's oxidative metabolism (Nagy *et al.* 1990; Nagy 1992; Fredholm & Wallman-Johansson 1996). These biochemical actions could provide the basis of the results on pERK expression in animals that received combined administration of caffeine and ethanol, and in fact it can be hypothesized that the increase in adenosine concentrations induced by ethanol is the main factor responsible of the increased stimulation of adenosine A_1 receptor activity. Adenosine A_1 and A_{2A} receptors are co-localized with dopaminergic D_1 and D_2 receptors, respectively, in several areas of the brain including the mesolimbic dopaminergic system (Fuxe *et al.* 2003; Nunes *et al.* 2013). Thus, it is possible that ethanol activates pERK expression in both subregions of Acb, and in the amygdala, through a mechanism mediated by D_1 receptors (Ibba *et al.* 2009). Otherwise ethanol increases alongside dopaminergic transmission in the AcbC and in the AcbSh, either after contingent (Bassareo *et al.* 2017) and non-contingent administration (Bassareo *et al.* 2003, 2019). Thus, a functional antagonism between caffeine and ethanol that is related to actions on adenosine receptors might be at least partially responsible for the behavioral interactions between these two substances seen in the present studies.

Furthermore, the data shown in Figures 6 and 7 indicate that the conditioned stimulus (environment associated with ethanol, CPP; environment assigned to ethanol, CPA) has a different impact on the phosphorylation of ERK in these brain areas. In other words, the same conditioned stimulus, but with opposite motivational properties, stimulated the phosphorylation of ERK in a differential manner in the AcbC and AcbSh. Previous evidence demonstrated that a conditioned aversive stimulus (expression of lithium-elicited CPA) (Longoni *et al.* 2011) was not associated with increased ERK phosphorylation in the Acb. However, pERK expression is activated in nuclei in the amygdala complex, suggesting an involvement of these areas in associative learning and reinforcement (McDonald *et al.* 2010; Pati *et al.* 2019; Wscieklica *et al.* 2019). Thus, the results from the experiments on the effects of conditioned stimuli on pERK

expression appear in agreement with the role attributed to the activation of this kinase in the acquisition and expression of motivated behaviors (Gerdjikov *et al.* 2004; Zhai *et al.* 2008; Sun *et al.* 2016).

Overall, these results cast fresh light on a critical topic that has considerable translational significance. Future experiments need to identify and further characterize the significance of the interaction between caffeine and ethanol.

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Experimental Studies

Chapter 3

CHAPTER 3: Impact of caffeine on ethanol induced sensitization: relation to dopamine metabotropic changes in ERK and DARPP-32

ABSTRACT

In recreational contexts, caffeine is frequently consumed with ethanol to reduce psychomotor slowing, sedation and coordination induced by this drug of abuse. Both drugs modulate dopamine and adenosine receptors, which are co-localized in nucleus accumbens (Acb), an area that regulates behavioral activation.

To determine if caffeine can reverse these ethanol-induced impairments we evaluated in adult male CD-1 mice novelty-induced exploration in an open field. Acute effects of caffeine (7.5, 15 and 30 mg/kg) were evaluated on horizontal and vertical exploration (locomotion and supported rearing) as well as rearing non-supported by the wall (a measure of incoordination) modulated by acute or repeated administration of ethanol (1.5 g/kg). Phosphorylation of markers dependent on dopamine receptor activation were evaluated (increases in pERK and pDARPP-32(Thr75) related to D_1 and D_2 receptor activation, respectively) in Acb.

Acutely, ethanol decreased both types of rearing. Caffeine increased only supported rearing and also reversed ethanol-reduction in supported rearing. Both substances increased locomotion in a biphasic manner, and caffeine potentiated ethanol-induced locomotion. Ethanol administered repeatedly produced locomotion and supported rearing sensitization. However, caffeine administered acutely to those mice on an ethanol-free state, showed blunted stimulating effects compared to ethanol naïve mice. Finally, acute administration of ethanol increased pERK immunoreactivity in Acb, but co-administration with caffeine suppressed that increase. There were no effects on pDARPP-32(Thr75).

Caffeine potentiates stimulation and reduces acute ethanol effects on suppression of vertical exploration, but cannot reverse its incoordinating effects. In ethanol-experienced mice caffeine has a blunted or even impairing effect on horizontal and vertical exploration. Caffeine blocks dopaminergic D_1 receptor-dependent markers induced by ethanol in Acb.

INTRODUCTION

Caffeine is a substance commonly used for its known psychostimulant properties (Temple *et al.* 2017). Preferentially in form of beverages, contained in tea, coffee or more recently in popular drinks rich in caffeine called "energy drinks", it is taken daily with the aim of improving cognitive and physical performance. A large number of studies in humans have highlighted its beneficial effects in terms of fatigue reduction, increase alertness and energy (Astorino & Roberson 2010; Duncan *et al.* 2012, 2013; Smirmaul *et al.* 2017). Moreover, its use in association with ethanol has, in the last decades, become widespread in order to counteract the sedative effects and the locomotor impairment of high, intoxicating, doses of ethanol (Hasenfratz *et al.* 1993; Drake *et al.* 2003; Attwood *et al.* 2012). In animal studies, it has been shown that the administration of caffeine and ethanol affects in different manners behavioral stimulation evaluated as locomotion. In rodents, both ethanol and caffeine are able to stimulate or inhibit the motor activity in a dose dependent manner, typically with bell-shaped (or inverted-U) dose-response functions (Correa *et al.* 2001a; Hilbert *et al.* 2013; López-Cruz *et al.* 2013). However, the long-term risks of repeated exposure to ethanol in interaction with caffeine are not yet well investigated. In animal models, the repeated administration of a stimulant substance may produce sensitization, measured as a progressive increase in locomotion (Steketee & Kalivas 2011). It has been also described cross-sensitization when a new drug shows potentiated stimulating effects in subjects that show sensitization to another drug, for example among ethanol and cocaine in rats (Xu & Kang 2017). Moreover, caffeine induces locomotor sensitization as well as cross-sensitization with other substances such as amphetamine and nicotine (Celik *et al.* 2006). Recently it has been reported the repeated intragastric coadministration of ethanol and caffeine induced significantly greater locomotor sensitization than the drugs alone (May *et al.* 2015). However, cross-sensitization between both drugs has not been assessed.

Sensitization in locomotor exploration is related to the motivational properties of drugs of abuse (Robinson & Berridge 2000), such as the regulation of locomotion, behavioral activation, and processes such as incentive salience and vigor involved in goal directed responses, all of then regulated by the mesolimbic system; dopaminergic

neurons that project from ventral tegmental area to the nucleus accumbens (Acb) (Robinson & Berridge 2000; Salamone *et al.* 2016). Sensitization process may be considered as a drug-dependent behavioral plasticity that can lead to greater vulnerability to the development of addiction (Ferreira *et al.* 2013). Ethanol and caffeine act on the dopaminergic system via different mechanisms: ethanol preferentially increases the firing of dopaminergic neurons in the ventral tegmental area leading to an increase in dopamine (DA) transmission in Acb (Gessa *et al.* 1985; Di Chiara & Imperato 1988; Carboni *et al.* 2000) while caffeine causes an increase of extracellular DA in prefrontal cortex, but not in Acb (Acquas *et al.* 2002, 2010). The two drugs act on the adenosinergic system; ethanol increases endogenous adenosine (López-Cruz *et al.* 2014), while caffeine is an antagonist of adenosine receptors (Ferré 2008). Adenosine A_1 and A_{2A} receptor subtypes, are highly expressed in brain areas rich in DA such as Acb (Ferré 2008; Nunes *et al.* 2013). In particular A_{2A} receptors and dopaminergic D_2 receptors are co-localized on encephalin-positive neurons, whereas adenosine A_1 are co-localized with DA D_1 receptors on substance P-positive neurons (Ferré *et al.* 2008). Adenosinergic and dopaminergic receptors converge on common mechanisms, showing opposite effects on metabotropic intracellular cascades (Agnati *et al.* 2003; Fuxe *et al.* 2003; Ferré 2008), such as Dopamine- and cAMP-regulated phosphoprotein Mr 32 kDa phosphorylated at the Threonine75 (Thr-75) site (pDARPP-32-Thr75), which is associated with activation of DA D_2 receptors (Svenningsson *et al.* 1999; Nunes *et al.* 2013). Their interaction can be studied also on the expression of biochemical parameters related to DA receptor activation such as Extracellular signal-regulated kinase (ERK), protein-kinase important for long-term synaptic plasticity (Valjent *et al.* 2005).

Given these premises, the present experiments were developed with the purpose to determine the effects of acute administration of caffeine (7.5, 15 or 30 mg/kg), ethanol (1.5, 2.5 or 3.5 g/kg) and their interaction on different indicators of novelty induced behavioral activation and exploration measured in an open field apparatus. In addition, we also studied if an acute administration of caffeine at different doses (15 and 30 mg/kg) can reverse the motor sensitization elicited by repeated administration of ethanol (1.5 g/kg). Furthermore, we assessed the effects of an acute challenge with

caffeine on ethanol-elicited pERK and pDARPP-32(Thr75) immunoreactivity in Acb core (AcbC) and shell (AcbSh), as a measure of neuronal markers of DA and adenosine receptor activation.

MATERIALS AND METHODS

Animals

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

Drugs

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

Apparatus and testing procedures

Open Field (OF)

The OF apparatus consists of a clear glass cylinder 25 cm in diameter and 30 cm high previously used to observe the effects of ethanol on spontaneous locomotion and locomotor sensitization (Correa *et al.* 2004). The floor of the cylinder was divided into four equal quadrants by two intersecting lines drawn on the floor. Animals were placed in the center of the cylinder and immediately observed for 10 min. The behavioral test room was illuminated with a soft light, and external noise was attenuated. Horizontal and

vertical locomotion in the OF were simultaneously recorded and registered manually. For horizontal locomotion, an activity count was registered each time the animal crossed from one quadrant to another with all four legs. A count of vertical locomotion was registered each time the animal raised its forepaws in the air higher than its back (unsupported rear) or rested them on the wall (supported rear).

Blood ethanol determinations

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

pERK immunohistochemistry

Mice were anesthetized with carbon dioxide for 30 s and perfused 15 min after the last treatment. The time interval between ethanol administration and anesthesia was selected on the basis of the time of the peak effect on DA transmission (Melis *et al.* 2007; Ibba *et al.* 2009). Brains were collected and stored in paraformaldehyde solution 3.7% during 24 h and refrigerated in sucrose (30%), sodium azide (2%) and phosphate buffer PB (0.1M) solution prior to slicing. Free floating coronal sections (40 µm) were serially cut using a microtome cryostat (Weymouth, MA, USA) according to plates 21-23 (approximately from AP 1.18 to AP 0.98 mm from bregma for the nucleus accumbens core and shell) of the mouse brain atlas (Paxinos & Franklin 2001). After rinsing in 0.01 M 0.01 M phosphate buffer saline (PBS) (pH 7.4) (3 times for 10 min) and incubating for 30 min with 1% hydrogen peroxide and after three rinses of 30 min each one, the slices were incubated for 1 hr with 0.1% Triton X-100 (T.X) in TBS and 3% Bovine Albumin serum (BSA). The incubation with the primary anti-pERK antibody (phosphorylated ERK, Cell

Signaling Technology, Beverly, MA, USA) at 1:350 was conducted overnight at 4°C. On the following day, after rinsing, the slices were incubated with the second antibody, the anti-rabbit HRP conjugate envision plus (DAKO) for 1.5 h on a rotating shaker at room temperature. Finally, sections were washed and rinsed for 1-3 min in 3,3-diaminobenzidine chromagen (DAKO).

pDARPP-32(Thr75) immunohistochemistry

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

Image analysis

Processed brain sections were mounted to microscope slides (Menzel-Gläser, Superfrost ® Plus, Thermo scientific), air dried, processed through alcohol-xylene and cover-slipped using Eukitt® (Sigma AldrichMerck KGaA) as a mounting medium. The sections were examined and photographed using a Nikon Eclipse E600 (Melville, NY, USA) upright microscope equipped with an Insight Spot digital camera (Diagnostic Instruments, Inc). Images of the regions of interest were magnified at 20X and captured digitally using Stereo Investigator software. Cells were quantified with ImageJ software (v. 1.42,

National Institutes of Health sponsored image analysis program) in three sections per animal, and the average value per mm^2 was used for statistical analysis.

Statistical analysis

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

RESULTS

Effects of acute administration of caffeine on locomotion

Figure **1** shows the effects of caffeine (0.0, 7.5, 15.0 or 30.0 mg/kg) administered 30 min before 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 rear [$F_{3,30}$=3.48, $p<0.05$]. Planned comparisons revealed that caffeine at low and moderate doses (7.5 and 15 mg/kg) significantly increased horizontal locomotion ($p<0.05$ and $p<0.01$, respectively) (**Fig 1A**). These doses of caffeine also produced significant increases in the number of supported rears ($p<0.05$) (**Fig 1B**). No significant effect of caffeine treatment on unsupported rear was observed [$F_{3,30}$=0.45, NS] (**Fig 1C**).

Effects of acute administration of ethanol on locomotion

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

p<0.01) (**Fig 2B**). All ethanol doses significantly decreased unsupported rear (p<0.01) (**Fig 2C**).
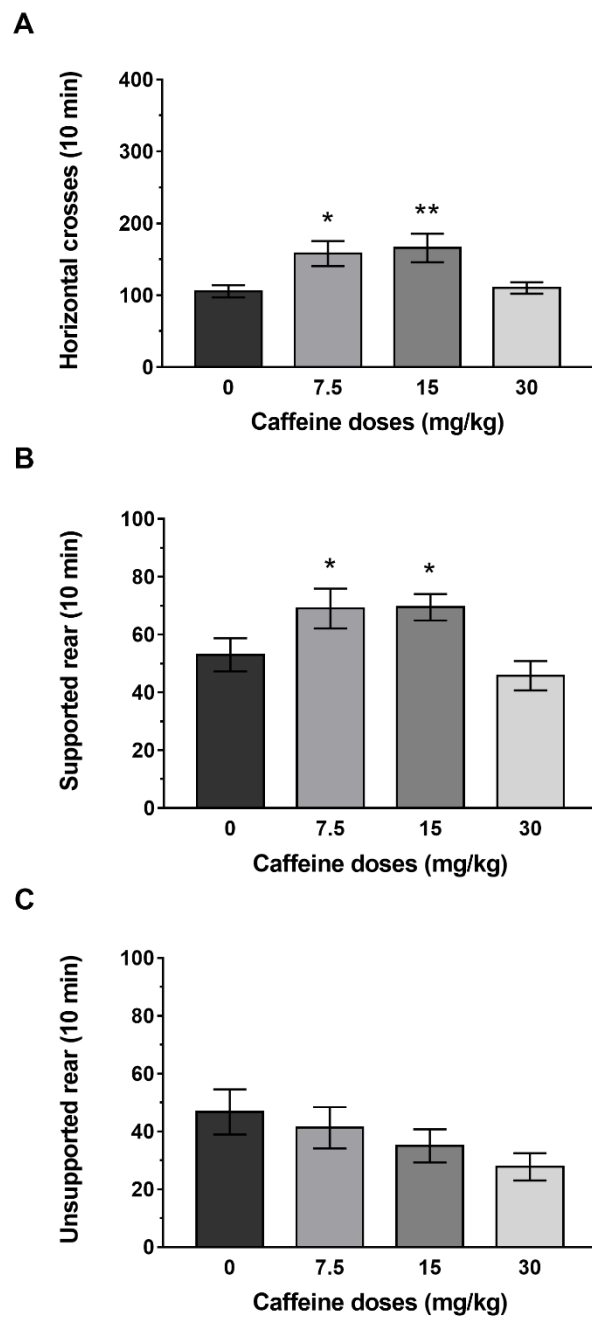

*Figure 1. Effects of acute administration of caffeine (0, 7.5, 15 or 30 mg/kg) on horizontal locomotion (A), supported rear (B), and unsupported rear (C) in the OF. Data are expressed as mean (±SEM) number of counts during 10 minutes. **p<0.01, *p<0.05 significantly different from vehicle (caffeine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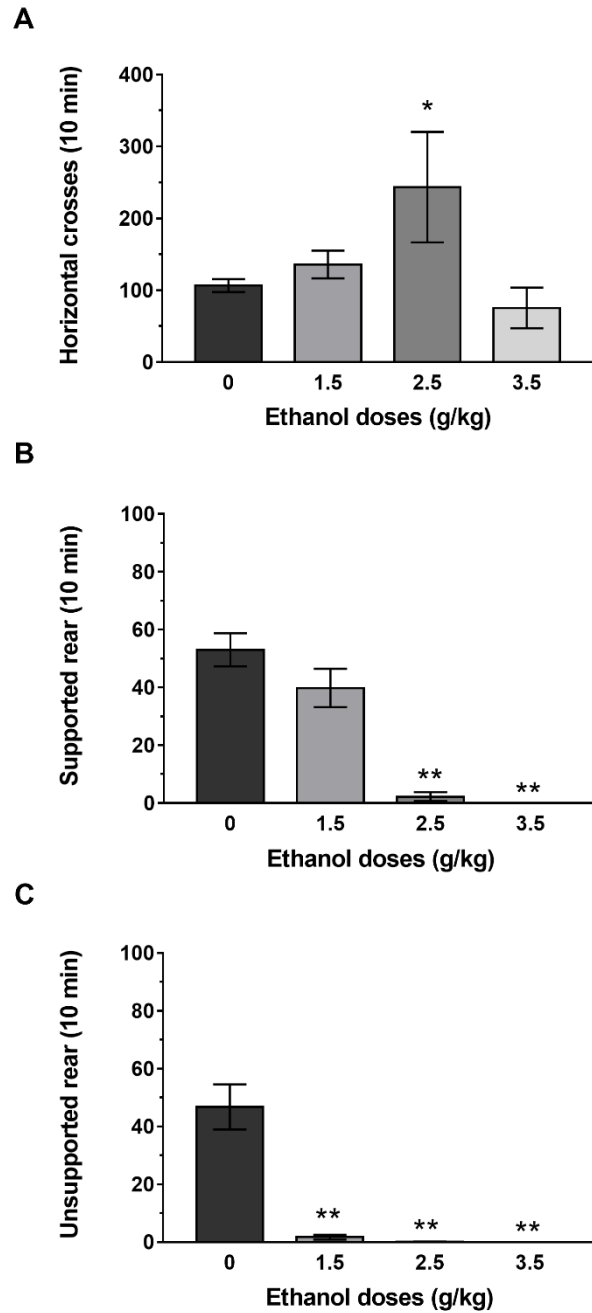
A

B

C

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

Effects of acute administration of caffeine and ethanol on locomotion

Figure **3** shows the effects of caffeine (0, 15 or 30 mg/kg) and ethanol (0.0, 1.5, 2.5 or 3.5 g/kg) combination in mice (n=115) evaluated in the OF. Factorial ANOVA (Caffeine x Ethanol) showed an overall effect of caffeine [$F_{2,112}$=11.18, p<0.01], ethanol [$F_{3,112}$=59.35, p<0.01] and caffeine-ethanol interaction [$F_{6,112}$=6.64, p<0.01] on horizontal crosses in the OF. Planned comparisons revealed that in caffeine 30 mg/kg treated mice, ethanol 1.5 g/kg produced a significant increase in locomotion compared to caffeine (0 mg/kg) + ethanol (1.5 g/kg) treated mice (p<0.05). In addition, caffeine (15 and 30 mg/kg) + ethanol (2.5 g/kg) groups were significantly different from caffeine (0 mg/kg) + ethanol (2.5 g/kg) treated mice (p<0.01). Moreover, caffeine (0 mg/kg) + ethanol (2.5 g/kg) was significantly different compared to vehicle control group (p<0.01) (**Fig 3A**). The factorial ANOVA (Caffeine x Ethanol) for the variable supported rear, as a measure of vertical locomotion, also showed an overall effect of caffeine [$F_{2,112}$=3.81, p<0.05], ethanol [$F_{3,112}$=62.26, p<0.01] and their interaction [$F_{6,112}$=2.29, p<0.05]. Planned comparisons showed that in vehicle treated group, caffeine 15 mg/kg increased supported rear compared to control (p<0.01). Among ethanol 1.5 g/kg treated mice, both caffeine (15 and 30 mg/kg) treated groups increased supported rearing compared to caffeine (0 mg/kg) + ethanol (1.5 g/kg) group (p<0.01) In addition, caffeine (0 mg/kg) + ethanol (2.5 and 3.5 g/kg) groups were significantly different from vehicle control group (p<0.01) (**Fig 3B**). Finally, the factorial ANOVA (Caffeine x Ethanol) for unsupported rear (**Fig 3C**) showed a significant effect of ethanol treatment [$F_{3,112}$=66.89, p<0.01]. However, there was no significant effect of caffeine [$F_{2,112}$=0.94, NS], and no significant interaction [$F_{6,112}$=0.83, NS].

Effects of caffeine administration on blood ethanol levels

Additional mice (n=24) were used to determine whether caffeine influenced blood-ethanol levels after motor stimulating doses. Animals received caffeine (0 or 30 mg/kg) and 20 min later ethanol (1.5 or 2.5 g/kg) was administered. Two-way factorial ANOVA (Ethanol x Caffeine) showed a significant effect of ethanol [$F_{1,25}$= 326.82, p<0.01], but no significant effect of caffeine [$F_{1,25}$=0.31, NS], nor of the interaction [$F_{1,25}$=3.39, NS]. These data suggest that the observed behavioral effects of ethanol co-administered with caffeine are not due to changes in blood ethanol concentration.
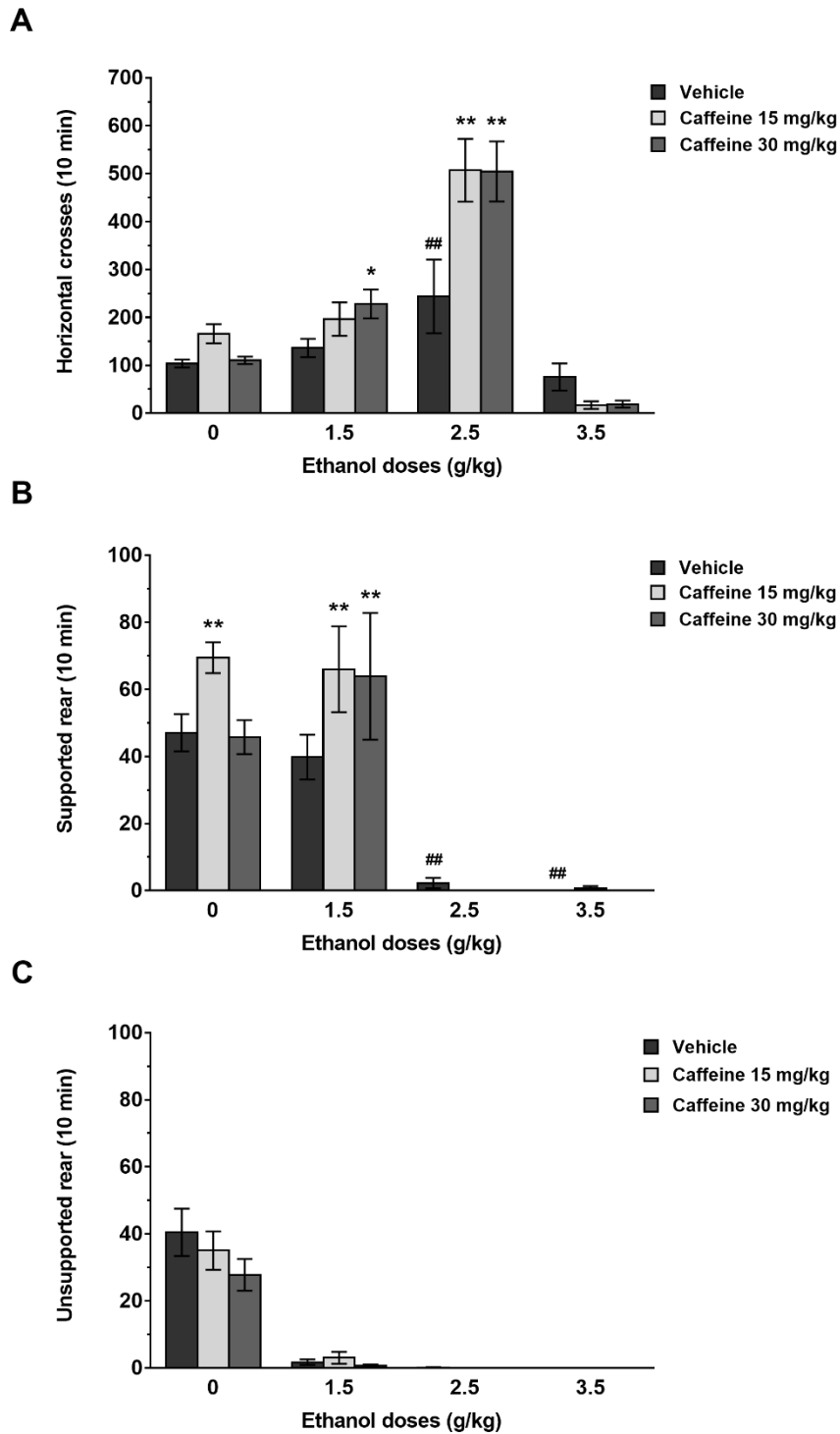
Figure 3. Effects of acute co-administration of caffeine (0, 15 or 30 mg/kg) and ethanol (0, 1.5, 2.5 or 3.5 g/kg) on horizontal locomotion (A), supported rear (B), and unsupported rear (C) in the OF. *Mean (±SEM) number of counts during 10 minutes. **p<0.01, *p<0.05 significantly different from caffeine (0 mg/kg) in the same ethanol dose group; ##p<0.01 significantly different from vehicle (caffeine 0 mg/kg + ethanol 0 g/kg) control group.*

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

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

Effects of repeated administration of ethanol on locomotion

Figure **4** shows the effects of repeated ethanol (0 or 1.5 g/kg) administration in mice (n=32) during 5 sessions in alternating days. Factorial ANOVA with a between factor (session: 1 and 5) and a within factor (ethanol dose: 0 or 1.5 g/kg ethanol) showed a significant effect of treatment [$F_{1,64}$=30.87, p<0.01], a significant effect of session [$F_{1,64}$=18.15, p<0.01] and treatment x session interaction [$F_{1,64}$=8.24, p<0.01]. Planned comparisons showed a locomotor stimulant effect of ethanol (1.5 g/kg) compared with vehicle treated mice in the first session (p<0.01). Ethanol treated group in session 5 was significantly different compared to vehicle group in the same session (p<0.01). Moreover, ethanol in session 5 increased locomotion compared to session 1 (p<0.01). This increase in locomotion over sessions was not observed in the vehicle treated groups, suggesting a sensitization of locomotion induced by ethanol (**Fig 4A**). The factorial ANOVA showed a significant effect of ethanol dose on supported rear [$F_{1,64}$=6.42, p<0.01], no significant effect of session [$F_{1,64}$=3.32, NS], but a significant effect of ethanol dose x session interaction [$F_{1,64}$=4.61, p<0.05] **(Fig 4B).** Planned comparisons showed that although ethanol did not change supported rearing in session 1 when compared to the vehicle group, it increased the number of supported rears when administered in session 5 compared to the vehicle group in the same session (p<0.01), and also compared to its administration in session 1 (p<0.05). The ANOVA for the dependent variable unsupported rear showed a significant effect of ethanol dose [$F_{1,64}$=127.4, p<0.01], a significant effect of session [$F_{1,64}$=21.62, p<0.01], but did not show a significant interaction [$F_{1,64}$=2.60, NS] (**Fig 4C**).
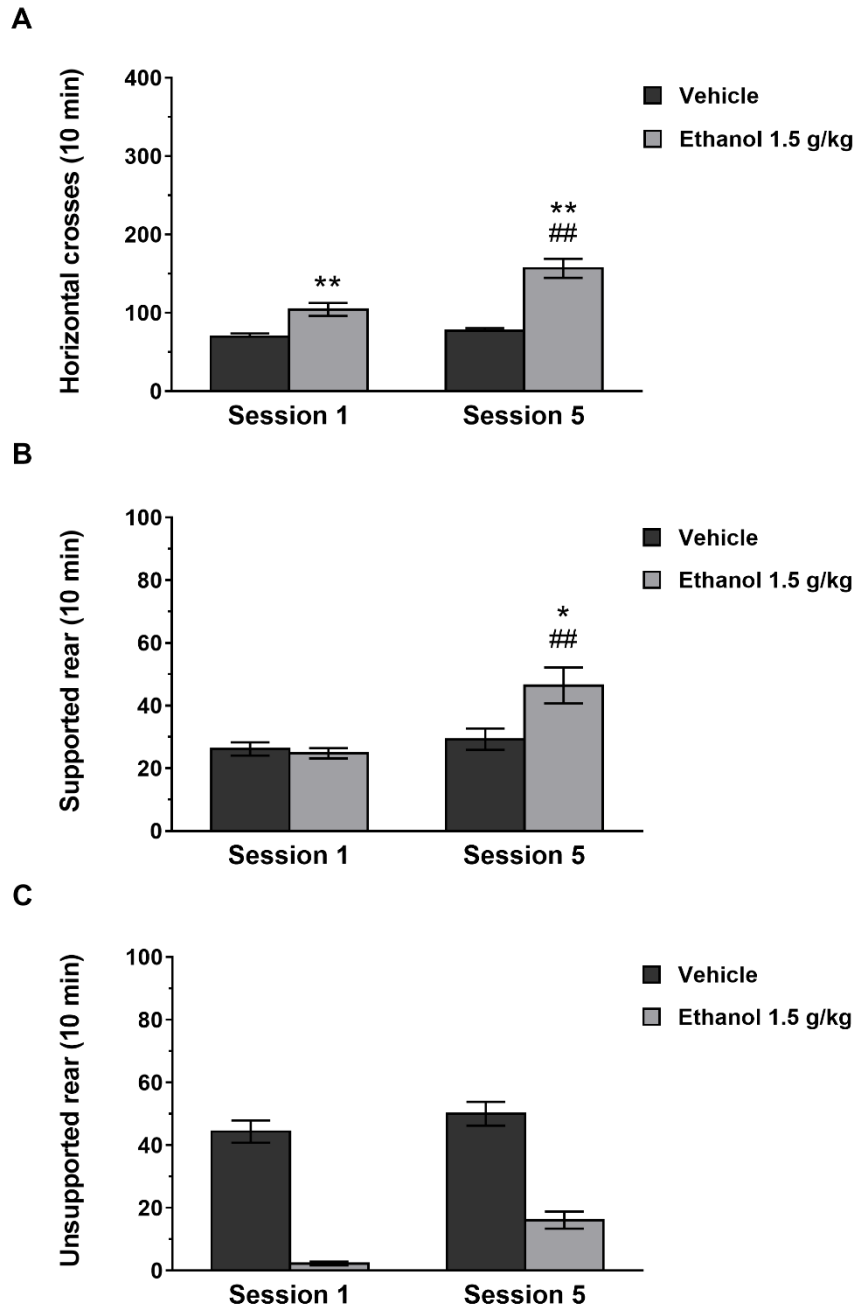
Figure 4. Effects of repeated administration of ethanol (0.0 or 1.5 g/kg) on horizontal locomotion (A), supported rear (B), and unsupported rear (C) in the OF. *Data are expressed as mean (±SEM) number of counts during 10 minutes. ***p<0.01, *p<0.05 significantly different between vehicle (ethanol 0 g/kg) and ethanol (1.5 g/kg) in the same session. ##p<0.01 significantly different between sessions in the same ethanol (1.5 g/kg) group.*

Effects of acute administration of caffeine on ethanol-induced locomotor sensitization

Figure **5** shows the effects of an acute administration of caffeine on ethanol (1.5 g/kg)-sensitized mice. Two days after the last drug administration, animals received an acute administration of caffeine (0, 15 or 30 mg/kg) in order to observe if there was a cross-sensitization effect. The factorial ANOVA; previous ethanol treatment (0 or 1.5 g/kg) x caffeine dose (0, 15 or 30 mg/kg) showed an overall effect of previous ethanol dose [$F_{1,65}$=11.48, p<0.01], caffeine dose [$F_{2,65}$=25.45, p<0.01] and also a significant effect of their interaction [$F_{1,65}$=3.82, p<0.05] on horizontal locomotion **(Fig 5A).** The same pattern of results was observed on supported rear **(Fig 5B)** [$F_{1,65}$=9.66, p<0.01; $F_{2,65}$=29.28, p<0.01; $F_{1,65}$=3.25, p<0.05, respectively] and unsupported rear **(Fig 5C)** [$F_{1,65}$=4.87, p<0.05; $F_{2,65}$=10.90, p<0.01; $F_{1,65}$=5.36, p<0.01, respectively]. Planned comparison showed a stimulant effect of caffeine at both doses (15 and 30 mg/kg) in the ethanol (0 g/kg) pretreated group (p<0.01). Only the dose of 15 mg/kg of caffeine induced locomotion in the ethanol (1.5 g/kg) pretreated group (p<0.01). Interestingly, caffeine at the highest dose (30 mg/kg) significantly decreased locomotion in animals pretreated with ethanol (1.5 g/kg) when compared to the effect of this dose of caffeine in the ethanol (0 g/kg) pretreated group (p<0.01) **(Fig 5A)**. The effect of caffeine on supported rear showed a similar pattern of effects. Caffeine increased supported rear at both doses (15 and 30 mg/kg, p<0.01 and p<0.05, respectively) in the ethanol (0 g/kg) pretreated group. However, only caffeine 15 mg/kg significantly increased this variable in the ethanol pretreated group (p<0.01). Caffeine 30 mg/kg decreased supported rear in the ethanol (1.5 g/kg) pretreated group compared to its effect in the ethanol (0 g/kg) pretreated group (p<0.05) **(Fig 5B)**. Finally, caffeine 15 mg/kg increased unsupported rear in the ethanol (1.5 g/kg) pretreated group, but at the highest dose (30 mg/kg) significantly decreased this variable (p<0.05). Moreover, the effect of caffeine 30 mg/kg in the ethanol (1.5 g/kg) pretreated group was significantly different to the effect observed in the ethanol (0 g/kg) pretreated group (p<0.01).
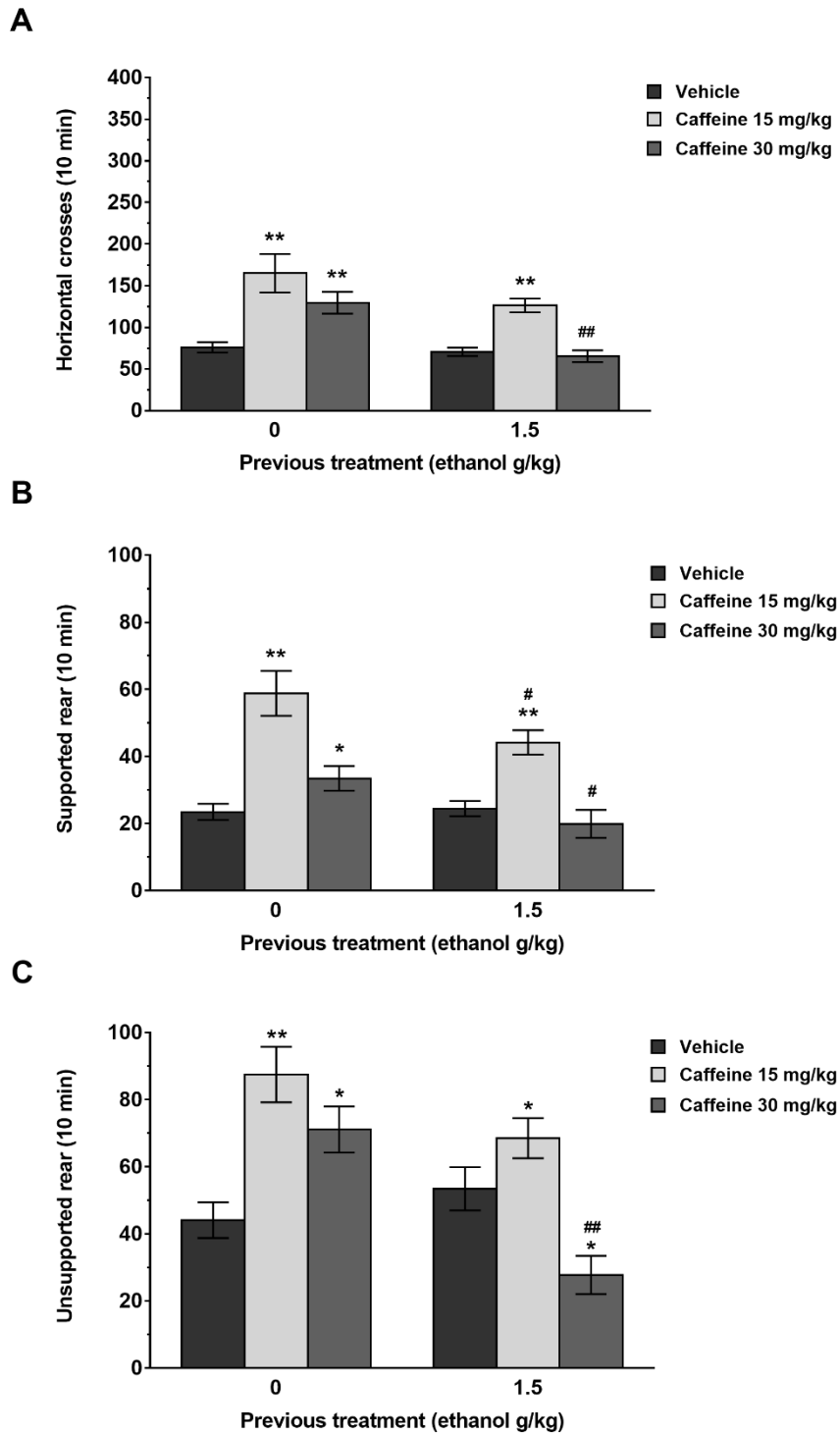
Figure 5. Effects of caffeine (0, 15 or 30 mg/kg) in mice treated with ethanol (0 or 1.5 g/kg) in previous days on horizontal locomotion (A), supported rear (B) and unsupported rear (C) in the OF. *Data are expressed as mean (±SEM) number of counts during 10 minutes. **p<0.01, *p<0.05 significantly different 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.*

Effects of caffeine on the expression of ethanol-elicited pERK and pDARPP32(Thr75) in the nucleus accumbens

Figure **6** shows the effects of caffeine (0, 15 or 30 mg/kg) administered 20 min before of administration ethanol (0 or 1.5 g/kg) on the number of pERK- and pDARPP-32(Thr75)-positive neurons in the AcbC and AcbSh. Immunoreactivity levels were analyzed separately for Acb subregions. One-way ANOVA on the number of pERK–positive cells revealed an overall effect of treatment on AcbC [$F_{3,22}=10.18$; $p<0.01$] and AcbSh [$F_{3,22}=5.69$; $p<0.01$] (**Fig 6A**). Planned comparison's analysis showed a significant increase on pERK expression after caffeine (0 mg/kg) + ethanol (1.5 g/kg) treatment relative to caffeine (0 mg/kg) + ethanol (0 g/kg) groups in the AcbC and AcbSh ($p<0.01$). Furthermore, the administration of caffeine (15 and 30 mg/kg) + ethanol (1.5 g/kg) was significantly different from caffeine (0 mg/kg) + ethanol (1.5 g/kg) in the AcbC and AcbSh ($p<0.01$, both cases). Conversely, one-way ANOVA did not revealed a significant effect of caffeine (0 mg/kg) + ethanol (1.5 g/kg) nor a significant effect of co-administration of caffeine (15 and 30 mg/kg) + ethanol (1.5 g/kg) on the number of pDARPP-32(Thr75)–positive cells in the AcbC [$F_{3,24}=0.29$; NS] and AcbSh [$F_{3,24}=0.27$; NS] (**Fig 6B**).
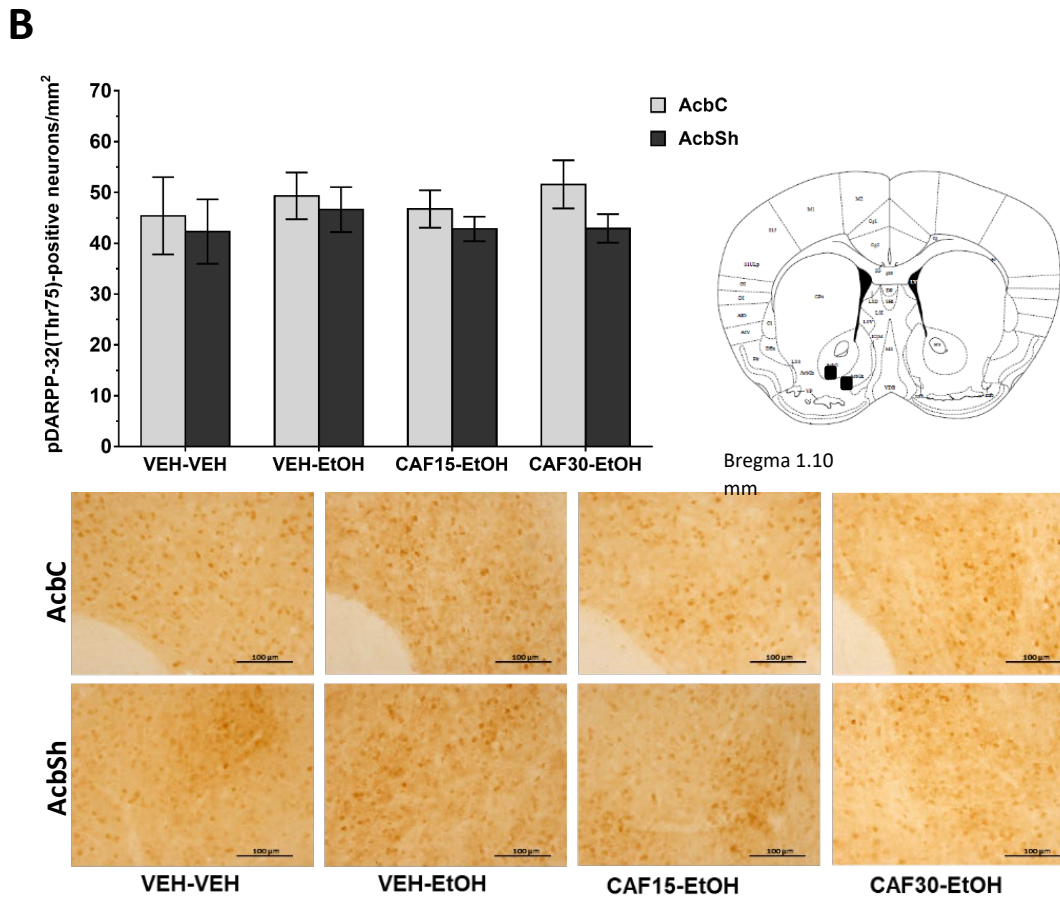

104

B

Bregma 1.10 mm
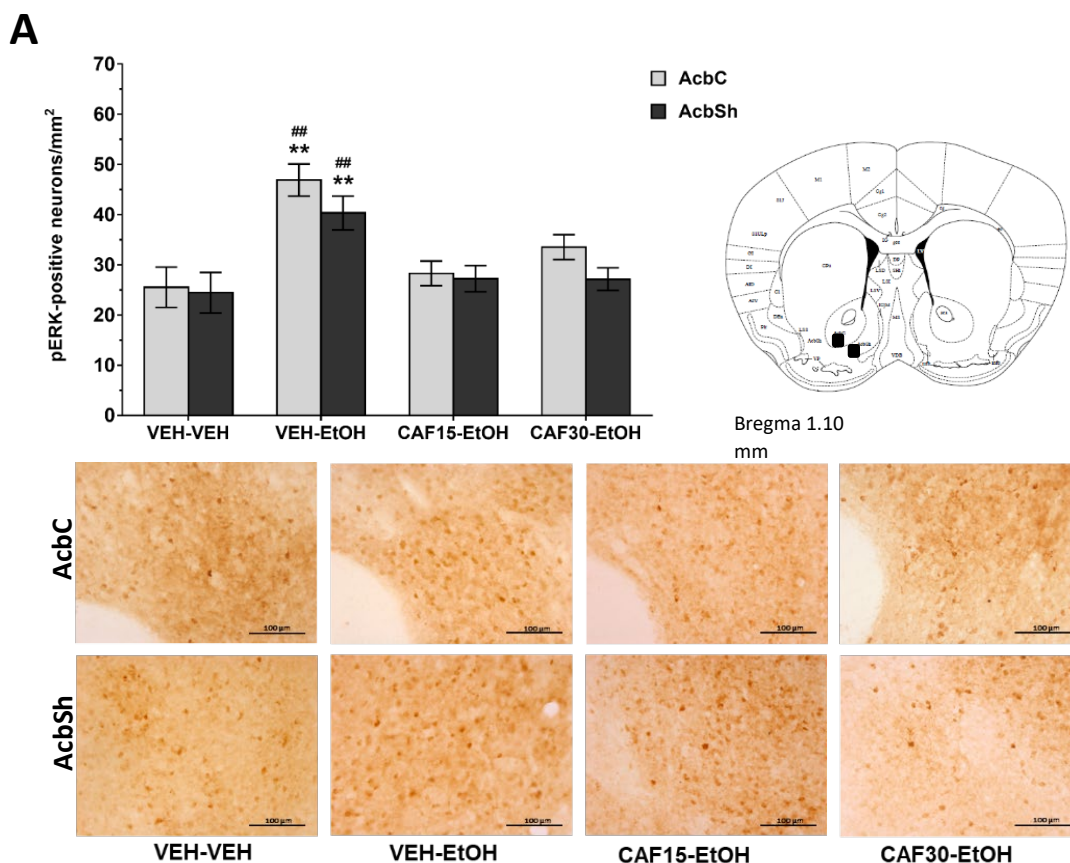
Figure 6. Effects of acute administration of caffeine on the expression of ethanol-elicited pERK and pDARPP-32(Thr75)-positive neurons in the Acb. *Data are expressed as mean (±SEM) of the number of positive neurons/mm^2. Right upper parts: effect of caffeine acute treatment (0, 15 and 30 mg/kg) on the expression of pERK (A) and pDARPP-32(Thr75) (B) positive neurons in mice treated with ethanol (0, 1.5 g/kg). Left upper part: diagram of a coronal section with bregma coordinates from Franklin and Paxinos (2001) showing location of the brain areas for pERK and pDARPP32(Thr75) immunoreactivity counting. Lower part: photomicrographs of pERK and pDARPP-32(Thr75) staining in AcbC and AcbSh from representative mice in each treatment group. Low power images (20X). **p<0.01 significantly different from vehicle (caffeine 0 mg/kg + ethanol 0 g/kg) groups in the AcbC and AcbSh. ##p<0.01 significantly different from caffeine (15 and 30 mg/kg) + ethanol (1.5 g/kg) groups in the AcbC and AcbSh.*

DISCUSSION

In the present studies, using male CD-1 mice, we investigated the acute interaction of caffeine and ethanol, two of the most widely used psychoactive drugs, on different measures of locomotor exploration in an open field; horizontal and vertical locomotion. Moreover, vertical locomotion was separated in two measures; one more dependent on postural coordination (non-supported rearing), and another less so (wall-supported rearing). The biphasic effects of ethanol on locomotion have been widely known, both in mice (Phillips & Shen 1996; Correa *et al.* 2001a; Karlsson & Roman 2016), but also in rats

after central administration (Correa *et al.* 2003a b). Thus, at low doses, ethanol has stimulatory effects, whereas at high doses prevails the suppressant effect on locomotion (Correa *et al.* 2001b; Chuck *et al.* 2006), and then ataxia, incoordination and sedation predominate (Correa *et al.* 2001b; Chuck *et al.* 2006). Furthermore, the biphasic nature of caffeine on general locomotion has previously been described in mice (El Yacoubi *et al.* 2003; Zhang *et al.* 2011; López-Cruz *et al.* 2014) although only very high doses of caffeine (100 mg/kg), much higher than the ones used in the present study, suppress locomotion (Zhang *et al.* 2011). Accordingly, in the present studies, the acute intraperitoneal administration of caffeine or ethanol showed a dose dependent effect on locomotion, with low and moderate doses (caffeine 7.5 and 15 mg/kg, and ethanol 2.5 g/kg) stimulating, and high doses (caffeine 30 mg/kg and ethanol 3.5 g/kg) reducing horizontal locomotion compared to the vehicle group. Furthermore, caffeine showed a bell-shaped dose response curve inducing stimulant effects on supported rear at low and moderate doses (7.5 and 15 mg/kg), but not at the highest (30 mg/kg) dose used, whereas ethanol dose dependently decreased both types of rearing. Interestingly, the acute administration of both drugs revealed that stimulant (15 mg/kg) and non-stimulant (30 mg/kg) doses of caffeine enhanced locomotion in mice treated with low and moderate (1.5 and 2.5 g/kg, respectively) doses of ethanol. Remarkably, caffeine (30 mg/kg) increased horizontal locomotion and supported rearing in interaction with a dose of ethanol (1.5 g/kg) that administered alone was not stimulatory. However, at the highest dose of ethanol (3.5 g/kg) caffeine was not able to reverse ethanol's effects in any of the three locomotion parameters. Our results are similar to previous results, in terms of the drug combination; caffeine in interaction with low doses of ethanol (1.75 g/kg) potentiates stimulation and at higher doses (2.5 and 3.25 g/kg) potentiates reduction in locomotion (Hilbert *et al.* 2013). Thus, the synergistic activity of the two substances is revealed at low doses, while if one of the two is particularly high the antagonistic effect prevails and even at higher doses there is a potentiation in suppression of locomotion (Waldeck 1974; Hilbert *et al.* 2013). For example, 100 mg/kg of caffeine totally suppresses the locomotor activity induced by a low dose of ethanol (1 g/kg) (Waldeck 1974). In our study, a high dose of ethanol (3.5 g/kg) co-administered with moderate doses of caffeine (15 or 30 mg/kg) produced even a deeper suppression of locomotion. In addition, in terms of coordination, the oral administration of low dose of caffeine (10 mg/kg) reduced ethanol induced-ataxia

(Kuribara *et al.* 1992) and local intracerebroventricularly administration of caffeine at low doses (less than 25 micrograms) dose-dependently reduced ethanol-elicited incoordination in mice, while a high dose (75 micrograms) potentiated it (Dar 1988).

In addition, we evaluated the impact of acute administration of caffeine on ethanol-induced sensitization of those exploration parameters after repeated administration. This phenomenon is defined as a progressive increase of motor response measured as a behavioral consequence of a repeated ethanol exposure (Camarini & Pautassi 2016). There are very few evidences of cross-sensitization between ethanol and caffeine after repeated intragastric administration of both drugs which induced significantly greater locomotor sensitization than the substances alone (May *et al.* 2015). However, in our experiment, the repeated administration of a low dose of ethanol (1.5 g/kg) induced locomotor and supported rearing sensitization, and the acute administration of caffeine on ethanol (1.5 g/kg)-induced sensitization increase locomotion only at 15 mg/kg dose, whereas the highest dose (30 mg/kg) significantly decreased locomotion in animals that had been repeatedly exposed to ethanol in previous days, when compared with the effect of this dose of caffeine in the vehicle pretreated group. The impact of caffeine on supported rear showed a similar pattern of effects. Furthermore, although both doses of caffeine increased unsupported rear in naïve animals, in the ethanol pretreated group caffeine at the highest dose (30 mg/kg) significantly decreased this variable compared to vehicle. Thus, a non-stimulant dose of caffeine (30 mg/kg) totally blocked or produced suppression in all behavioral parameters in ethanol sensitized animals.

These behavioral effects induced by the interaction of caffeine and ethanol can be due to neurobiological mechanisms involving neurotransmission systems common to the two substances. Thus, the dopaminergic and adenosinergic systems are the ones more evidently affected by ethanol and caffeine. The stimulatory effect of ethanol can be related to an increase in dopaminergic stimulation in the nucleus accumbens (Di Chiara & Imperato 1988; Ibba *et al.* 2009), whereas caffeine seems to act as a non-selective A_1/A_{2A} receptor antagonist and, as such, mediates its stimulant (Ferré 2008; Pardo et al., 2013), anxiogenic (Correa & Font 2008) and motivational effects (López-Cruz *et al.* 2018). The colocalization of adenosinergic and dopaminergic receptors in brain striatal areas, such as nucleus accumbens (Acb), reflects a possible functional interaction and

can converge on common mechanisms, such as intracellular signaling cascades with opposite effects (Fuxe *et al.* 2003; Ferré 2008).

Finally, as we have previously observed (Ibba *et al.* 2009), ethanol significantly increased postsynaptic intracellular markers related to DA signaling; the phosphorylated ERK and this ethanol-induced increase in markers of DA-related signal transduction were significantly reversed by caffeine at stimulant and non-stimulant doses. In contrast, pDARPP-32(Thr75) was not affected by any of the pharmacological manipulations, indicating that ethanol, at least under the present conditions, does not seem to change DA function in Acb. Caffeine at stimulant dose (15 mg/kg) in mice has previously demonstrated to suppress the increase in pDARPP-32(Thr34) produced by a DA depleting agent (tetrabenazine, TBZ) using western blotting (López-Cruz et a., 2018). Also in rats, using immunohistochemistry, DA suppression potentiate phosphorylation of DARPP-32 (-Thr75 and -Thr34) in Acb shell and core, but in different populations of neurons (Nunes *et al*, 2013).

The effects of caffeine on DA release in Acb are not very conclusive. In microdialysis experiments it has been shown that caffeine can elicit DA release in the shell of Acb (Solinas *et al.* 2002), and in the medial prefrontal cortex (Acquas *et al.* 2002), although not in the core of Acb (Acquas *et al.* 2002). Caffeine, as nonselective adenosine antagonist, can interact with both of adenosine receptors, that are co-localized with DA receptors in striatal medium spiny neurons. Both sets of receptors interact in an antagonistic way; agonism of dopamine D_1 and D_2 receptors leads to actions on the metabotropic cascade that are opposite to those produced by stimulation of adenosine A_1 and A_{2A} receptors respectively (Svenningsson *et al.* 1999). Thus, in a direct or in an indirect way, caffeine can have similar effects on the mesolimbic circuit to drugs that act on the dopaminergic systems. Caffeine seems to act predominantly in A_1 receptors (Ferré 2008), leading to increase phosphorylation of DARPP-32 at Thr34. These receptors are widely distributed in the brain, but in Acb they are colocalized with D_1 receptors typically located in Substance P containing medium spiny neurons (Svenningsson *et al.* 1999; Segovia *et al.* 2012; Nunes *et al.* 2013). Conversely, blockade of adenosine A_{2A} receptors results in increased phosphorylation of DARPP-32 at Thr75 (Lindskog *et al.* 2002), in enkephalin containing neurons (Nunes *et al.* 2013). In the present results, an increase in

pDARPP32(Thr75) expression was not observed after the administration of ethanol, or after co-administration of caffeine plus ethanol. Is also possible that, ethanol at high doses or when enough time has elapsed can increase adenosine levels (Carmichael *et al.* 1991; Diamond & Gordon 1994) and this purine is the endogenous agonist for adenosine receptors. If ethanol at 1.5 g/kg had induced potentiation of adenosine synthesis it would lead to a stimulation of A_1 receptor activity by adenosine, which in turn would have increased pDARPP32(Thr75). In previous studies, ethanol at this dose but in rats which are much more sensitive to ethanol sedative and ataxic effects, was shown to increase phosphorylation of DARPP-32 at Thr34 in striatum (Nuutinen *et al.* 2011) pointing to an increase in adenosine formation and action on A_1 receptors. Those receptors are widely distributed in the brain; thus, it is possible that the stimulating effects of this drug are not mediated by Acb actions.

In summary, despite the popular assumptions about the ability of caffeine in energy drinks to counteract the impact of alcohol, the potential dangers of combining high-caffeine "energy" drinks with ethanol have been demonstrated using the animal models employed in the present experiments. Our results have confirmed how caffeine can exacerbate the already disruptive effects of ethanol, and have identified at least one potential brain area, the nucleus accumbens, in which caffeine and ethanol interact to modulate behavior. This brain region also appears to be very important for the regulation of behavioral processes involved in ethanol seeking behavior and consumption, which can lead to phenomena such as ethanol dependence, abuse and addiction.

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General Discussion

GENERAL DISCUSSION

The present doctoral thesis was aimed at investigating the significance of the role of Extracellular signal-regulated kinase (ERK) expression and in broader perspective of the involvement of the ERK/MAPK signaling cascade in the behavioral and biochemical effects of ethanol. This aim was pursued through different behavioral and biochemical approaches: the behavioral one utilized the place conditioning paradigm (chapters 1 and 2) and the assessment of locomotor activation (chapter 3); the biochemical approach was based on the immunohistochemical quantification of the phosphorylation of ERK-positive neurons in several areas of the addiction circuitry (chapters 1-3).

Addiction can be considered as a result of molecular and cellular adaptations that take place in neurons of specific brain areas following repeated exposure to drugs of abuse (Nestler 2001). The involvement of phosphorylated ERK (pERK) in the action of addictive drugs has been widely demonstrated (Valjent *et al.* 2000, 2004; Cahill *et al.* 2014; Sun *et al.* 2016). Accordingly, although still to be fully understood, ERK/MAPK pathway may be considered as a strategic tool through which these substances exert their addictive potential, generating neuronal changes that may underlie the onset of a series of pathological process which can evolve in addiction (Valjent *et al.* 2000, 2004). This signaling pathway is characterized by a phosphorylation cascade, among which key protagonists are the protein kinases ERK and MEK responsible of ERK's activation. ERK, in turn, by activating transcription factors and early genes such as c-fos (Brami-Cherrier *et al.* 2005), appear to be essential components to exert protein synthesis, changes in gene expression, stabilization of dendritic spines implicated in synaptic plasticity and memory formation (Sweatt 2004). Thus, ERK result important regulators of neuronal function and activity. Accordingly, several studies have correlated these biochemical markers with learning paradigms, such as conditioned taste aversion (Marotta *et al.* 2014), fear conditioning (Atkins *et al.* 1998; Villarreal & Barea-Rodriguez 2006), spatial learning (Blum *et al.* 1999; Selcher *et al.* 1999), recognition memory (Kelly *et al.* 2003), appetitive-reward seeking behavior (Kirschmann *et al.* 2014) and

drug-elicited place conditioning (Valjent *et al.* 2000; Gerdjikov *et al.* 2004; Girault *et al.* 2007; Spina *et al.* 2010; Longoni *et al.* 2011; Rosas *et al.* 2018).

ERK phosphorylation has also been reported to be activated, in a dose-dependent manner, in the core and shell of nucleus accumbens (Acb) and in the nuclei of extended amygdala (bed nucleus of stria terminalis and central nucleus of the amygdala), by ethanol through a mechanism mediated by dopaminergic D_1 receptors (Ibba *et al.* 2009). However still occurs, to date, a number of unanswered questions regarding, in particular, whether and in which brain regions ERK are involved in the effects conditioned to ethanol as well as whether the association of ethanol with other psychotropic substances also extends involving ERK at the basis of their behavioral effects. In this regard, recent evidence, both on the experimental (López-Cruz *et al.* 2013) and on the clinical and epidemiological sides (Marczinski & Fillmore 2014; Kristjansson *et al.* 2015), reveals increasing interest on the combination of ethanol with the most consumed legal psychostimulant in the world, caffeine, often considered in the composition of "energy drinks", as a consequence of the increasingly popular belief that this substance could decrease the adverse effects of ethanol in terms of motor impairment and narcosis (Hasenfratz *et al.* 1993; Drake *et al.* 2003; Attwood *et al.* 2012). Therefore, in the perspective of the general aim of this work as defined above, we considered also critical to investigate the pharmacological consequences of the interaction between caffeine and ethanol in terms of motivational, locomotor and motor coordination effects and, also, in the perspective of the impact of such interaction on ERK expression in different brain regions.

In **chapter 1** we described our investigation on the role of MEK/ERK cascade by studying to what extent these intracellular kinases are involved in the process of acquisition and expression of ethanol-elicited place conditioning. In particular, to investigate a possible differential involvement of this pathway in the acquisition and expression of the positive (conditioned place preference, CPP) and negative (conditioned place aversion, CPA) memories associated with ethanol (the unconditioned stimulus, US), we have chosen to adopt two distinct and appropriate behavioral schedules (Spina *et al.* 2015; Rosas *et al.* 2017) by which ethanol could result able, as a single US, to prompt two opposite effects, CPP or CPA. The results

disclosed, as a consequence of the systemic administration the blood brain barrier-penetrant compound, SL327, unable to affect other kinases such as protein kinase A (PKA) or Ca^{2+}/calmodulin-dependent protein kinase II (CAMKII) (Atkins *et al.* 1998; Selcher *et al.* 1999), a differential involvement of the MEK/ERK pathway in the effects of ethanol. In particular, SL327 (50 mg/kg) significantly prevents the acquisition of ethanol-elicited CPP, whereas its administration (50 and 100 mg/kg) fails to prevent, although significantly reducing, the acquisition of ethanol-elicited CPA. The result of CPP acquisition experiment is in agreement with previous data that showed the ability of different MEK inhibitors to impair the ability of this kinase of transferring ethanol's affective value to conditioned stimuli (Valjent *et al.* 2000, 2001; Salzmann *et al.* 2003; Gerdjikov *et al.* 2004; Spina *et al.* 2010). Furthermore, the results of the expression experiments reveal that SL327 (50 and 100 mg/kg) is able to significantly reduce the expression of ethanol-elicited CPP, but not that of CPA. Moreover, the observation that SL327 (50 and 100 mg/kg) significantly reduces the expression of ethanol-CPP is in agreement with previous studies showing that MEK inhibitors could prevent retrieval of cocaine-(Miller & Marshall 2005) and morphine (Lin *et al.* 2010)-elicited CPP and with the observation that presentation of conditioned stimuli, either in CPP (Nygard *et al.* 2015) and in operant self-administration (Radwanska *et al.* 2008; Peana *et al.* 2013; Faccidomo *et al.* 2015) is related to the enhancement of phosphorylated ERK expression. Conversely, we found, in agreement with previous studies (Longoni *et al.* 2011), that SL327 administration (50 and 100 mg/kg) fails to prevent the expression of ethanol-elicited CPA. The interpretation of the role of ERK in these conditioning effects is supported by the immunohistochemistry experiments demonstrating that pre-treatment with SL327 prevents the increases of pERK-positive neurons elicited by ethanol in the prefrontal cortex, Acb core and shell and in other nuclei of extended amygdala. Interestingly, as mentioned above, the MEK/ERK pathway is involved also in ethanol self-administration experiments (Faccidomo *et al.* 2009) as pERK expression has been demonstrated in the Acb, medial prefrontal cortex and nuclei of amygdala (Faccidomo *et al.* 2015) upon ethanol oral self-administration supporting the hypothesis of the present dissertation that this kinases' cascade is preferentially implicated in the positive reinforcement of ethanol. Overall, the first experimental study of this thesis work reveals that the acquisition phase of a behavior conditioned

to a US, i.e. the initial phase in which learning is established, seems to be more sensitive to the interruption of the MEK/ERK pathway than the expression phase (presentation of the CS after learning the US-CS association). Similarly, appetitive motivation (place preference) discloses a preferential sensitivity of a modulation of this pathway with respect to aversive motivation (place aversion), an observation that further highlights the complexity of the involvement of this pathway in ethanol-sustained associative learning.

In **chapter 2** we studied the effects of pretreatment with caffeine on ethanol-elicited place conditioning with the aim of exploring the consequences of their interaction in terms of motivational properties, a subject of great translational valence, that is still inadequately investigated. In fact, very little is known about the pharmacological outcome of this interaction when using doses of ethanol that result in frank motivational effects devoid of serious intoxicative consequences and of caffeine, at doses with uncertain motivational effect (Liu *et al.* 2008) and considered in the low to moderate range to elicit behavioral activation (Zhang *et al.* 2011). Furthermore, in this chapter we also assessed the results of their combination in terms of pERK expression in different areas of the addiction circuity as well as in terms of the ability of stimuli conditioned to ethanol or to caffeine and ethanol (i.e. upon the expression of ethanol-elicited CPP and CPA) to elicit ERK phosphorylation in those brain regions. Notably, ethanol and caffeine act in opposite manner on the adenosinergic system: the neuromodulator adenosine controls several mechanisms such as neuronal excitability and neurotransmitter release, and the combination of these substances is reflected in the modulation of multiple behavioral effects (López-Cruz *et al.* 2013). However, the literature does not provide sufficient information about the results of pre-treatment with caffeine on ethanol place conditioning and especially with reference to the aversive (negative motivational) effects of ethanol (CPA) there is a total lack of data.

The results of these place conditioning experiments showed that caffeine (3 and 15 mg/kg) was devoid of conditioning effects while, in agreement with previous reports (Cunningham *et al.* 2003; Spina *et al.* 2015; Rosas *et al.* 2017), ethanol (2 g/kg) elicited significant CPP and CPA. Moreover, caffeine (15 mg/kg) significantly prevented ethanol-elicited CPP and, at both doses (3 and 15 mg/kg), significantly impaired

ethanol-elicited CPA. These findings suggest that low and intermediate doses of caffeine are able to interfere with the acute effects of ethanol, expressed in the place conditioning paradigm, by preventing the establishment of the conditioned association and therefore by blocking such ethanol-induced acquisition of CPP and CPA. We postulate that these results can be referred to the antagonistic actions exerted by caffeine on A_1 and A_{2A} receptors. This hypothesis is supported by the results of the acute administrations in the immunohistochemical experiments in which caffeine at both doses also prevented ethanol-elicited increased pERK expression in all brain areas examined. Specifically, also on the basis of the observation made by Ibba *et al.* (2009) that ethanol-elicited ERK expression is mediated by dopaminergic D_1 receptors, it is likely to assume that caffeine exerts the described antagonistic effect on ethanol-elicited acquisition of conditioned responses by acting onto A_1 adenosine receptors are colocalized with dopamine D_1 receptors (Svenningsson *et al.* 1999; Nunes *et al.* 2013). Future experiments devoted to test this hypothesis will allow to confirm or reject this possibility.

Furthermore, the set of results referred to the expression of ethanol-elicited CPP and CPA indicated that ethanol-conditioned stimuli, either positive (CPP) or negative (CPA), have a different impact on the expression of ERK phosphorylation. In particular, the ''identical'' US, but with opposite motivational properties, is able to increase the expression of ERK in the core and shell of Acb as well as in other brain regions of the extended amygdala in opposite manner, in agreement with previous studies of our laboratory in which the expression of lithium-induced CPA is not associated to increased pERK expression in the Acb (Longoni *et al.* 2011). In this study, unfortunately, the expression of pERK was determined only in the Acb and dorsal striatum and therefore we cannot extend the agreement of the interpretation of the present data also to other brain regions. However, pERK increases were detected in others nuclei of extended amygdala suggesting an involvement of these structures in stimulus-stimulus learning and in negative reinforcement (McDonald *et al.* 2010; Pati *et al.* 2019; Wscieklica *et al.* 2019). In summary, the findings of the second chapter demonstrated that caffeine is unable of eliciting pERK expression but interferes with both positive (CPP) and negative (CPA) properties of ethanol. This finding is also

supported by the dissociation between the behavioral expression of CPP and CPA and the differential anatomical localization of pERK expression (Acb in case of CPP expression and bed nucleus of stria terminals, central and basolateral nuclei of the amygdala in case of CPA). We postulate that these effects could be due to the functional antagonistic actions exerted by caffeine and ethanol on adenosinergic system as well as to the antagonistic effect of caffeine onto A_1 adenosine receptors. These interpretations need to be corroborated by future experiments.

Finally, in **chapter 3** we examined the consequences of caffeine and ethanol interaction by exploring the results in terms of locomotor activation and motor coordination. The rationale at the basis of these experiments was the clinical observation of frequently consumed caffeine with ethanol in order to reduce its psychomotor slowing, sedation and motor incoordinating effects. We used an open field to test novelty-induced exploration expressed as horizontal and vertical locomotion. This last parameter was further distinguished into two measures: one more dependent on postural coordination (i.e.: non-supported rearing) and another less so (i.e.: wall-supported rearing). Furthermore, we also evaluated the expression of another biochemical marker of dopamine signaling, upstream of activation of pERK, the Dopamine- and cAMP-regulated phosphoprotein, Mr 32 kDa (DARPP-32) phosphorylated at Threonine75 site (pDARPP-Thr75).

The biphasic effects of caffeine and ethanol have been widely described in literature (Phillips & Shen 1996; Correa *et al.* 2001a b, 2003a b; El Yacoubi *et al.* 2003; Zhang *et al.* 2011; López-Cruz *et al.* 2014; Karlsson & Roman 2016). However, there is a lack of data on further parameters concerning locomotion such as postural stability while the effects of caffeine on ethanol-induced sensitization require further investigation. The results of the present experiments revealed that, acutely, low and moderate doses of caffeine (7.5 and 15 mg/kg) and a moderate dose of ethanol (2.5 g/kg) stimulate the horizontal locomotion whereas high doses of caffeine (30 mg/kg) and ethanol (3.5 g/kg) reduce it. Moreover, ethanol decreased both types of rearing whereas caffeine (7.5 and 15 mg/kg) increased only wall-supported rearing. Interestingly, the acute drugs combination showed that stimulant (15 mg/kg) and non-stimulant (30 mg/kg) doses of caffeine enhanced locomotion in mice treated with the stimulant (2.5 g/kg) dose of ethanol but did not

affect the supported rearing. In addition, a non-stimulant dose of caffeine (30 mg/kg) increased horizontal locomotion and supported rearing with the lowest and non-stimulant dose of ethanol (1.5 g/kg). On the contrary at the highest dose of ethanol (3.5 g/kg) caffeine (30 mg/kg) was not able to reverse the ethanol's ataxic effect in any of the locomotion parameters. These results suggest that caffeine potentiates locomotion at stimulant doses of ethanol but at higher doses or after suppression of locomotion, caffeine at medium to high doses potentiates the incoordinating effects of ethanol. Hence, although this synergistic or antagonist dose-dependent effect of these substances was reported in the literature (Waldeck 1974; Dar 1988; Kuribara *et al.* 1992; Phillips & Shen 1996; Marin *et al.* 2011; Hilbert *et al.* 2013; López-Cruz *et al.* 2013) showing that only very high doses of caffeine (100 mg/kg) suppress locomotion (Zhang *et al.* 2011), this study for the first time evaluates the impact of the combined treatment of stimulant and non-stimulant doses of the two drugs on horizonal and vertical locomotion. Furthermore, we examined also the effects of caffeine pre-treatment (15 and 30 mg/kg) on mice previously sensitized to ethanol (1.5 g/kg), to investigate possible cross-sensitization effects. The results, compared to those from ethanol naïve mice, disclosed that the stimulant dose of caffeine (15 mg/kg) blunted the stimulating effects of ethanol whereas the non-stimulant (30 mg/kg) dose of caffeine produced suppression of locomotion in ethanol sensitized mice. Notably, and in contrast, this high dose of caffeine (30 mg/kg) when administered in combination with ethanol (at the dose of 1.5 g/kg) to non-sensitized animals was able to increase horizontal locomotion and supported rearing. Thus, when caffeine is administered at high doses not only fails to reverse the impairing effects of ethanol, but in ethanol-experienced mice has a blunting or even impairing effect on horizontal and vertical exploration. Finally, the results of immunohistochemical experiments revealed that, in line with previous studies (Ibba *et al.* 2009), ethanol is able to significantly induce pERK-expression in the Acb core and shell and that combination with caffeine at both doses (15 or 30 mg/kg) significantly reversed this increase. Accordingly, caffeine resulted in a reduction of ethanol-induced ERK expression that, similarly to our previous speculation on data of chapter 2, we hypothesize to be mediated by D_1 receptors. This possibility would also be in agreement with the suggestion of the preponderant role of A_1 receptors in these effects of caffeine, which may be a key element in the regulation of the caffeine-mediated modulation of ethanol-induced

behavioral activation. On the contrary, there was no significant effect on pDARPP-32(Thr75).

In conclusion, with the experiments of this doctoral thesis we aimed at investigating the differential involvement of ERK in a number of experimental conditions related to direct and conditioned effects of ethanol, as well as in relation to the effects of ethanol after a previous administration of caffeine. Using ethanol-elicited place conditioning we assessed not only the ability of MEK blockade to interfere with learning the association between US and CS (acquisition) but also the possibility that MEK blockade may also affect the behavioral performance evoked by the presentation of either the CS+ associated to positive, reinforcing effects of ethanol and the CS+ associated to negative, aversive effects of ethanol (that is, associated to the expression of CPP and CPA, respectively). Strikingly, these effects of ERK/MAPK kinase pathway result in a differential involvement of the expression of this phosphorylated kinase in different brain regions (nucleus accumbens core and shell *vs* bed nucleus of stria terminalis, central and basolateral amygdala). Overall, these studies suggest a different and complex involvement of the MEK/ERK pathway in associative learning and in the expression of conditioned responses. Furthermore, we also examined the effects of ethanol and caffeine interaction to explore the consequences on the motivational properties of ethanol assessed in the place conditioning model. The results of these experiments also indicate that the administration of caffeine at appropriate doses prevents the manifestation of the motivational properties of ethanol and suggest that its ability to induce pERK expression could be prevented by caffeine through the blockade of adenosinergic A_1 receptors. Finally, the results of the experiments that evaluated locomotion demonstrate that caffeine does interact in a complex way with the effects of stimulant and non-stimulant doses of ethanol, sometimes increasing the impairing and sometimes blunting the stimulatory effect of ethanol on behavior.

Overall, the impact of the translational value of these findings resides in the significant contribution toward the clarification of the complex interaction between caffeine and ethanol that is mostly highlighted by the dangerousness toward public safety of their prejudice-based use.

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