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Involvement of adenosinergic and dopaminergic systems in the regulation of alcohol or sucrose intake: studies in rodent models of self-administration.

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EXTENDED SUMMARY

Involvement of adenosinergic and dopaminergic systems in the regulation of alcohol or sucrose intake: studies in rodent models of self-administration.

Caffeine is a methylxanthine that acts as a nonselective adenosine antagonist, binding to adenosine A_1 and A_{2A} receptor subtypes. This substance is highly present in different beverages like coffee, tea and in energy drinks. Epidemiology studies have shown a positive correlation between the consumption of energy drinks and that of ethanol. One of the reasons for combining caffeine with ethanol may stem from the popular belief that caffeine antagonizes the intoxicating effects of alcohol. Combination of both substances could affect alcohol consumption patterns and can modulate effects like sedation or motor incoordination.

Both drugs act on the adenosine system but have opposite effects on receptor activation since ethanol can potentiate the amount of extracellular adenosine. Adenosine is a neuromodulator widely distributed throughout the central nervous system and it is involved in several processes. Adenosine acts on A_1 and A_{2A} receptors, which are located in brain areas involved in motivational processes. Adenosine also interacts with other neurotransmitters such as dopamine (DA). Adenosine A_1 and A_{2A} receptors are colocalized with dopamine D_1 and D_2 receptors respectively antagonically interacting in striatal areas.

The first part of the present dissertation (Chapters 1-4) characterize caffeine's actions on ethanol intake under different patterns of access: restricted, unrestricted or after several cycles of ethanol withdrawal. Furthermore, several selective adenosine antagonists have

been used to study the involvement of adenosine receptors on caffeine's actions. Finally, the impact of caffeine on effort-related choice-paradigms has been assessed after the administration of a DA depleting agent. The last two chapters (5-6) focused on the effect of caffeine on sucrose consumption under different patterns of access on palatable food under binge eating inducing, anxiogenic, and effortful conditions. Finally, the present research assessed the impact of caffeine on behavioral procedures that induce individual differences in effort expenditure for food seeking behavior. Effort-related dysfunctions are seen in many psychopathologies, thus the study of individual differences could be useful to optimize treatments and to look for alternative treatments based on the adenosine system.

RESUMEN EXTENSO

Implicación de los sistemas adenosinérgico y dopaminérgico en la regulación de ingesta de alcohol o sucrosa: estudios en modelos animales de auto-administración

La cafeína es una metilxantina que actúa como un antagonista no selectivo de los subtipos de receptores de adenosina A_1 and A_{2A}. Esta sustancia está muy presente en diferentes bebidas como el café, el té y bebidas energéticas. Los estudios epidemiológicos han mostrado una correlación positiva entre el consumo de bebidas energéticas y el de etanol. Una de las razones para combinar la cafeína con etanol puede provenir de la creencia popular de que la cafeína antagoniza los efectos intoxicantes del alcohol. La combinación de ambas sustancias podría afectar los patrones de consumo de alcohol y puede modular efectos como la sedación o la incoordinación motora.

Ambos fármacos actúan sobre el sistema de adenosina pero tienen efectos opuestos sobre la activación del receptor ya que el etanol puede potenciar la cantidad de adenosina extracelular. La adenosina es un neuromodulador ampliamente distribuido a lo largo del sistema nervioso central y está involucrado en varios procesos. La adenosina es el agonista endógeno de los receptores A_1 and A_{2A}, que se localizan en áreas cerebrales involucradas en procesos motivacionales. La adenosina también interactúa con otros neurotransmisores como la dopamina (DA). Los receptores A_1 and A_{2A} de adenosina se colocalizan con receptores D_1 y D_2 de dopamina, respectivamente, que interactúan antagonicamente en áreas estriatales.

La primera parte de la presente tesis doctoral (capítulos 1-4) caracteriza las acciones de la cafeína en el consumo de etanol bajo diferentes patrones de acceso: restringido, sin

restricciones o después de varios ciclos de retirada de etanol. Además, se han utilizado varios antagonistas selectivos de la adenosina para estudiar la implicación de los receptores de adenosina en las acciones de la cafeína. Finalmente, el impacto de la cafeína en los paradigmas de elección relacionados con el esfuerzo se ha evaluado después de la administración de un agente que agota los niveles de DA. Los dos capítulos (5-6) se centran en el efecto de la cafeína en el consumo de sucrosa bajo diferentes patrones de acceso en alimentos sabrosos bajo condiciones de "consumo por atracón", ansiogénesis y esfuerzo. Por último, se evaluó el impacto de la cafeína en los procedimientos conductuales que inducen diferencias individuales en la predisposición a realizar esfuerzo para la búsqueda de alimentos. Las disfunciones relacionadas con el esfuerzo se observan en muchas psicopatologías, por lo que el estudio de las diferencias individuales podría ser útil para optimizar los tratamientos y buscar otros alternativos basados en el sistema de adenosina.

GENERAL INTRODUCTION

1. The CNS neuromodulator Adenosine

Adenosine is an endogenous neuromodulator, which modulates many functions in the CNS and regulates neuronal excitability and neurotransmitter release, and modulates ion channel function through four subtypes of G-protein-coupled receptors; A_1, A_{2A}, A_{2B}, and A_3 (Fredholm et al., 2001). This neuromodulator operates mainly through volume transmission, and concentrations are regulated to a much greater extent by ongoing production and transport (Burnstock, 1972, 2006, 2008).

Adenosine A_1 receptors are present in almost all brain areas and their stimulation can suppress neuronal excitability (Fredholm et al., 1994). A_1 receptor stimulation has the opposite effect to A_{2A} receptors. Furthermore, A_{2A} receptors are almost exclusively concentrated in dopamine (DA) rich areas such as the striatum, where they reach high levels of expression (Fredholm et al., 1994; Vontell et al., 2010). Selective adenosine receptor agonists and antagonists have been used as pharmacological tools as well as the existence of genetic modified animals such as knockout (KO) mice, has permitted the study of the role of A_1 and A_{2A} in the regulation of many behaviors such as psychomotor stimulation or sedation (El Yacoubi et al., 2003; Nagel et al., 2003; Pardo et al., 2015; Farrar et al., 2007; Font et al., 2008; Mingote et al., 2008), memory (Hauber and Bareiss, 2001; Prediger et al., 2004), and in the regulation of affective (Correa and Font, 2008; Prediger et al., 2004; Kaster et al., 2015), and motivational processes (Salamone and Correa, 2002; Pereira et al., 2011; Pardo et al., 2012; Correa et al., 2015).

2. Actions of caffeine and ethanol on adenosine function.

Caffeine and alcohol act on the adenosinergic system. Caffeine is a methylxanthine that acts as a non-selective adenosine antagonist (A_1/A_{2A}) (Fredholm et al., 2001). This mechanism of action mediates its minor stimulant (Ferré, 2008; Urry and Landolt, 2015), anxiogenic (Prediger et al., 2004; Correa and Font, 2008) and motivational effects (Randall et al., 2011; Salamone et al., 2009). On the other hand, ethanol can increase adenosine levels by decreasing adenosine uptake (Diamond and Gordon, 1994) or by increasing adenosine levels, since adenosine is a byproduct of ethanol metabolism (Carmichael et al., 1991; Correa et al., 2012). The sedative and motor incoordinating effects of ethanol may be mediated by this system (Dar, 1990; Meng and Dar, 1995; Correa and Font, 2008; Correa et al., 2012).

Heavy mixing consumption of ethanol and caffeine beverages has grown exponencially in the last years with the appearance of the “energy drinks”. Caffeine and its metabolites such as theophylline, are the main psychoactive components of these drinks. These highly caffeinated beverages are being increasingly consumed, mainly among young populations, in combination with ethanol and under the popular belief that caffeine can compensate the intoxicating effects of alcohol (for a review see Correa et al., 2014). However, data from human studies and animal models show a complex relationship between caffeine and ethanol intake. Moreover, the interaction between high doses of caffeine and ethanol has not been extensively characterized in relation to some behaviors traditionally regulated by ethanol, such as anxiety. Thus, the impact of caffeine and selective adenosine receptor antagonism alone or in combination with ethanol is studied on several behavioral effects and on alcohol consumption. Several reporst have suggested that the use of energy drinks may reduce the intensity of the depressant effects of alcohol, although a growing set of studies have indicated opposite

results. The study of these two drugs in combination can reveal the nature of their interaction and shed light on the role of A_1 and A_{2A} adenosine receptors on these actions.

3. Impact of DA-Adenosine receptor interaction on the activational component of motivation.

Evidence from animal literature indicates that mesolimbic DA, and consequently nucleus accumbens is an important component of the neural circuitry that regulates behavioral activation, energy expenditure, and the ability of organisms to overcome work-related response costs in motivated behaviors (Salamone and Correa, 2002, 2009, 2012; Robbins and Everitt, 2007; Floresco et al., 2008; Mai et al., 2012, Beeler et al., 2012, 2015). The activational aspect of motivation can be evaluated with tasks that offer the choice for distinct reinforcers that can be obtained by instrumental. Such tasks include operant procedures offering choices between responding on ratio schedules for preferred reinforcers versus approaching and consuming a less preferred food (Salamone et al., 1991, 2002; Randall et al., 2012; Sommer et al., 2014), and a T-maze barrier task (Salamone et al., 1994; Mott et al., 2009; Pardo et al., 2012). DA antagonists and accumbens (NAcb) DA depletions have been demonstrated to shift choice behavior, decreasing selection of high effort/high reward options, and increasing selection of low effort/low reward choices (Salamone and Correa, 2002, 2012; Salamone et al., 2015; Mai et al., 2012; Sommer et al., 2014, Yohn et al., 2016), leaving the primary value of the reinforcer intact (Salamone and Correa, 2002, 2012). The study of this activational aspect of motivation has clinical significance. Symptoms such as lethargy, tiredness or anergia are observed in depression and in other pathologies (Salamone et al., 2016).

Considerable evidence indicates that brain adenosine receptor mechanisms interact with DA systems in the regulation of motivational processes (Salamone and Correa, 2009; Farrar et al., 2007). In this regard, several recent studies have focused upon the functional significance of adenosine receptors and their interactions with DA receptors, in relation to aspects of behavioral activation and effort-related processes (Correa et al., 2015; Pardo et al., 2012; Yohn et al., 2015; Farrar et al., 2007; Ferré, 2008).

As mentioned before, adenosine receptors are highly expressed in DA rich brain areas such as neostriatum and nucleus accumbens (Vontell et al., 2010), and adenosine receptors interact in those areas with DA receptors, having antagonistic effects on metabotropic intracellular signaling cascades (Ferré et al., 2004, 2008; Ferré, 2008). In this sense, adenosine A_{2A} agonists have been shown to induce effects that resemble those produced by DA antagonists or DA depletions, inducing anergia-like effects in an effort-based decision making task (Font et al., 2008), whereas selective adenosine antagonists of A_{2A} receptors (and to a much lesser extent of A_1 receptors), have been shown to attenuate anergia-like effects induced by DA antagonists or depletors (Salamone et al., 2009; Pardo et al., 2012; Nunes et al., 2013; Yohn et al., 2015). In this regard, a selective A_{2A} receptor antagonist MSX-3 reversed the anergia-like effects induced by a DA depletor agent (tetrabenazine, TBZ) in concurrent operant /chow feeding choice tasks and in a T-maze barrier task (Nunes et al., 2013; Randall et al., 2014; Yohn et al., 2015). The same pattern of results has been observed in different effort-choice tasks using D_2 antagonists combined with the non-selective (A_1/A_{2A}) antagonists caffeine and theophylline (Salamone et al., 2009; Pardo et al., 2012). These agents restored totally or partially the shift on the choice behavior from the low effort/low reward option to the high effort/high reward option induced by a DA

antagonist or DA depletor in concurrent choice tasks (Salamone et al., 2009; Pardo et al., 2012; Nunes et al., 2013; Randall et al., 2014; Yohn et al., 2014). Consistent with these results, A_{2A} KO mice were protected from the anergia-like effects induced by the DA D_2 antagonist haloperidol (Pardo et al., 2012; Correa et al., 2015). Although several adenosine antagonists have been tested and are effective preventing or blocking the effects of DA interferences on motivation, the effects of caffeine have not been widely explored in rodents (Salamone et al., 2009). Caffeine is proposed as a therapeutic agent to reverse or attenuate the anergia-like effects induced by DA depletions.

4. Intracellular cascade activated by DA and Adenosine receptors.

Adenosine receptors and DA receptors interact at the cellular level (Ferré, 2008; Ferré et al., 2008; Salamone et al., 2010; Santerre et al., 2012; Nunes et al., 2013). Striatal areas such as neostriatum and nucleus accumbens are very rich in adenosine A_{2A} receptors and DA D_2 receptors, and these two receptors are co-localized on encephalin positive medium spiny neurons (Demet et al., 2002; Ferré et al., 2004; 2008). There also is co-localization of DA D_1 receptors and adenosine A_1 receptors in these brain regions, and these receptors also interact (Ferré, 2008; Ferré et al., 2008). This neuronal co-localization and intracellular convergence can explain why A_{2A} receptor antagonists are effective in reversing the effort-related actions of D_2 antagonists such as haloperidol and eticlopride, and why it is more difficult for adenosine A_1 receptor antagonists to reverse the effects of D_2 receptor blockade (Salamone et al., 2009; Pardo et al., 2012; Hauber et al., 2001).

DARPP-32 phosphorylation at threonine 34 and 75 as an index of DA receptor D_1 or D_2 activation. A wide range of behavioral studies have been performed in order to study DA-adenosine interactions, and more specifically characterize the ability of D_1-A_1 and D_2-A_{2A} receptor interactions to modulate the brain circuitry regulating effort-related decision making (Salamone et al., 2010; Pardo et al., 2012, 2015; Yohn et al., 2014). Some of these studies have also focused on the effects of this interaction at the intracellular level (Santerre et al., 2012; Nunes et al., 2013; Svenningson et al., 1999).

It has been observed that a D_2 antagonist, haloperidol, induced an increase on cFos protein synthesis (an index of neuronal activation) and this increase was reversed by selective A_{2A} antagonists (Santerre et al., 2012; Pardo et al., 2012). This interaction on cFos was also observed after the administration of haloperidol to KOA_{2A} mice (Correa et al., 2015; Pardo et al., 2012). Haloperidol induced a shift in effort-based choice in WT animals but not in KOA_{2A} mice, and it also increased cFos synthesis in WT but not in KOA_{2A} mice, showing again a relation between intracellular markers of neural activity and motivated behavior (Correa et al., 2015; Pardo et al., 2012). However, in order to identify a specific pathway of activation, that is, to understand the involvement of D_1 or D_2 DA receptors and A_1 or A_{2A} receptors, more specific intracellular markers should be evaluated.

Dopamine- and cAMP-regulated phosphoprotein, Mr 32 kDa (DARPP-32) is highly present in medium spiny neurons (MSNs) in dorsal and ventral striatum projection neurons (Walaas, 1984; Ouimet et al., 1998; Greengard et al., 1999). There are two subtypes of MNS, which selectively express one of two peptides; enkephalin or dynorphin. Enkephalinergic MSNs predominantly express dopamine D_2 and A_{2A} receptors, while dynorphinergic MSNs, which also express the peptide substance P, predominantly express dopamine D_1 receptors and adenosine receptors of the A_1

subtype (Ferré, 1997; Agnati et al., 2003). DARPP-32 is phosphorylated after activation of D_1-A_1 or D_2-A_{2A} receptors and can be used as an index of DA activation (Svenningsson et al., 1997, 2004; Nunes et al., 2013). DARPP-32 function depends on its relative state of phosphorylation at two main regulatory sites, threonine 34 and 75 (Thr34 and Thr75). When DARPP-32 is phosphorylated at Thr34 by protein kinase A (PKA) it becomes a potent inhibitor of protein phosphatase 1 (PP-1), which in turn regulates the phosphorylation state of several classes of effector proteins including transcription factors, ionotropic receptors, and ion channels (Greengard et al.,1999). When phosphorylated at Thr75 by cdk5, DARPP-32 becomes an inhibitor of PKA signaling, thereby relieving inhibition of PP-1 (Bibb et al., 1999). The phosphorilation of DARPP-32 at Thr34 or Thr75, seems to be directly related with activation or DA D_1 or D_2 receptors and also is modulated by adenosine receptors (Nunes et al., 2013; Svenningsson et al., 1998, 1999, 2004).

Activation of either D_1 or A_{2A} receptors increases the activity of adenylyl cyclase and the resulting increase in cyclic AMP levels activates cyclic AMP-dependent protein kinase (cAMP-PK), which, in turn results in an increase of the phosphorylated form of DARPP-32 (pDARPP-32(Thr34). In this sense, DA D1 receptor agonist SKF 81297, or A_{2A} receptor agonist CGS21680, increased pDARPP-32(Thr34) (Svenningson et al., 1998). This effect was blocked by D_2 receptor agonist quinpirole (Svenningson et al., 1998). However, the D_2 antagonist eticlopride increased pDARPP-32(Thr34), and such effect was not observed in A_{2A}KO mice and in animals pre-treated with a selective adenosine A_{2A} antagonist SHC58261 (Svenningsson et al., 1999). Etriclopride-induced increases in pDARPP-32(Thr34) was also decreased by pretreatment with the D_1 antagonist SHC23390 (Svenningson et al., 1999). Moreover, the D_1 antagonist SHC23390 but not the A_{2A} receptors antagonist SHC58261 was able to abolish the

pDARPP-32(Thr34) increase induced by cocaine (Svenningson et al., 1999). On the other hand, activation of D_2 receptors decreases cAMP levels, thereby increasing pDARPP-32(Thr75), however this pathway has received less attention (Greengard et al., 1999). It seem that opposite modulation of D_1 and D_2 and also A_{2A} receptor agonism or antagonism on DARPP-32 phosphorylation is taking place in different populations of neurons (Nunes et al., 2013; Svenningon et al., 1998; 1999).

5. Implications for behavioral analysis and psychopathology

Research on effort-related choice behavior has implications for understanding the neural basis of psychiatric symptoms such as psychomotor slowing, anergia, fatigue and apathy, which are seen in depression as well as other psychiatric or neurological conditions (Salamone et al., 2006, 2007). These symptoms, which can have devastating behavioral manifestations (Stahl, 2002; Demyttenaere et al., 2005), essentially represent impairments in aspects of instrumental behavior, exertion of effort and effort-related choice. The neural circuitry involved in effort-related functions in animals and the brain systems that have been implicated in psychomotor slowing and anergia in depression (Salamone et al., 2006, 2007, 2009, 2010) show and imbricated connection, making a target in which research on effort-related behavioral processes could produce a different point to act on addiction, depression, and other disorders. Finally, manipulations of the adenosinergic system aiming at modulating ethanol intake could be usefull to understand some of the problems derived from risky alcohol consumption.

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OBJECTIVES

Adenosine is ubiquitously distributed throughout the central nervous system and exerts a broad spectrum of physiological and pathophysiological functions. It is also well known that adenosine receptors are co-localized with DA receptors and their activation leads to functionally opposite effects. Thus, drugs acting on adenosine receptors would be able to attenuate the effects of DA depletion on behavioral activation, and effort based decision-making. Caffeine acts as a non-selective adenosine A_1 and A_{2A} receptor antagonist. This methylxantine is commonly ingested in the normal diet and in beverages with high sucrose content. Furthermore, it is usually combined with alcohol, which also acts on the adenosine system. Thus the present dissertation provides different studies in rodent models to assess the involvement of adenosinergic and dopaminergic systems in the regulation of alcohol (chapters 1-4) or sucrose (chapters 5-6) intake.

Chapter 1 reviews the impact of caffeine, the main component of “energy drinks”, on ethanol consumption and withdrawal.

Chapter 2 studies the interaction of caffeine and ethanol on psychomotor performance evaluated in locomotor activity measured in the running wheel, impaired coordination in the rotarod and sedative effects in the loss of the righting reflex. Neural markers of Adenosine-DA interaction are also assessed.

Chapter 3 analyzes the effect of a broad range of doses of caffeine and selective adenosine antagonists on voluntary ethanol intake under different patterns of access condition restricted (2 hours), unrestricted (24 hours) or after 4 days of deprivation in an unrestricted condition. In addition, intracellular markers of DA receptor activity are also evaluated in animals drinking ethanol during 24 hours.

Chapter 4 evaluates two different access condition differentiated by work output: operant (fixed ratio 5) or free condition. Several pharmacological manipulations related to adenosine and DA systems would modulate rats performance. Contingent and non-contingent variables would be assessed in each condition. Striatal areas are analyzed for DA-Adenosine receptor dependent intracellular effects.

Chapter 5 explores the role of caffeine on consumption of highly palatable food in mice under binge eating, anxiogenic or effortful conditions. The ability of caffeine to reverse DA depletion in a T-maze for effort-based decision-making is also assessed.

Chapter 6 characterized the effort-related effects of caffeine in a concurrent progressive ratio (PR)/free reinforcer choice procedure that requires high levels of work to obtain the preferred reinforcer and generates great variability among different animals.

CHAPTER 1.

CAFFEINE MODULATION OF ALCOHOL INTAKE: IMPACT ON ITS PSYCHOMOTOR EFFECTS AND WITHDRAWAL

Abstract

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”. Energy drinks have many different components, although the main active ingredient is caffeine. These drinks are frequently taken in combination with alcohol under 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 reduce the effects of ethanol in humans or in rodents, and the mechanisms mediating caffeine-ethanol interactions are not well understood. Caffeine and ethanol have a common biological substrate; both act on neurochemical processes related to the neuromodulator adenosine. Caffeine acts as a non-selective adenosine A_1 and A_{2A} receptor antagonist, while ethanol has been demonstrated to increase the basal adenosinergic tone via multiple mechanisms. Since adenosine transmission modulates multiple behavioral processes, the interaction of both drugs can regulate a wide range of behavioral effects, which can have an impact on alcohol consumption and the development of alcohol addiction. In the present review we discuss epidemiological studies and laboratory animal work that have assessed the impact of caffeine on alcohol consumption. In addition, we evaluate how caffeine can also affect the consumption of other drugs of abuse. Finally we present data on human and animal studies analyzing the impact of caffeine on alcohol withdrawal, and psychomotor performance.

Caffeine as a "new" drug of abuse

Caffeine intake, even in excess, is well accepted socially because methylxanthines have activating and attention-preserving properties that can help productivity and enhance performance. However, interest in caffeine abuse has grown ever since the introduction to the market of the so-called "energy drinks". Although energy drinks contain several components with clear psychoactive effects, such as taurine or glucose, recent studies show that caffeine is the active ingredient responsible for the behavioral and cognitive effects associated with these beverages (Giles et al., 2012). In general, energy drinks contain caffeine in quite high concentrations. A cup of coffee contains about 100 mg of caffeine and a can of a traditional cola drink contains arround 35 mg of caffeine. However, although the caffeine content of energy drinks varies considerably, the concentration of caffeine can be much higher than coffee or most sodas; it ranges from as low as 50 mg to ten times more, up to 500 mg of caffeine per unit (Reissig et al., 2009).

The aggressive marketing of energy drinks targets young consumers, with advertising emphasizing that these drinks induce states of arousal and psychological 'highs'. In fact, some slogans of well known energy drinks emphasize the idea that these drinks procure energy, increase endurance, and produce a sense of invincibility. It is quite common to see campaigns that offer free samples on college campuses and venues where this segment of the population concentrates. In the United States 34% of young people aged between 18 and 24 are consumers of energy drinks (O'Brien et al. 2008; Wells et al., 2013), and among college students percentages of consumption rise to 60% (Price et al., 2010). In addition, these drinks are often consumed in combination with other substances that have abuse potential (Morelli and Simola, 2011).

Caffeine: Synergy with effects other drugs of abuse

Caffeine has a facilitating effect on the self-administration of other drugs. Energy drink users are significantly more likely than nonusers to initiate nonmedical use of prescription stimulants and prescription analgesics (Arria et al., 2010). Several reports indicate that cigarette smokers consume more caffeine than nonsmokers (Parsons and Neims, 1978; Swanson et al., 1994), an effect that may be partially due to increased caffeine metabolism among cigarette smokers (Parsons and Neims, 1978). However, in a laboratory context acute high doses of caffeine given to smokers did not increase cigarette smoking, probably because they report increases in anxiety and dysphoric somatic effects (Chait and Griffiths, 1983). Results obtained in animals show that squirrel monkeys that received intramuscular injections of caffeine increased lever-pressing for nicotine (Prada and Goldberg, 1985; Yasar et al., 1997) and in rats, adding caffeine to the drinking water also increased intravenous nicotine self-administration (Shoaib et al., 1999).

Similar experimental results have been observed for cocaine. Caffeine administered in the food to rhesus monkeys produced a modest increase in self-administration of smoked cocaine (Comer and Carroll, 1996). In rats, it has been demonstrated that intraperitoneal (IP) injections of caffeine potentiated intravenous self-administration of cocaine (Horger et al., 1991; Schenk et al., 1994), and reinstated cocaine self-administration after the animal had stoped seeking for the drug (Schenk and Partridge 1999).

The interaction of caffeine with opiates presents a different picture; in humans there seems to be little correlation between heroin abuse and caffeine consumption (Kozlowski et al., 1993). Similarly in animals, it has been demonstrated that both, acute

and chronic caffeine intake, decreased morphine self-administration in rats, possible due again to an increased in anxiety (Sudakov et al., 2002). However, high doses of caffeine induced withdrawal signs in morphine-dependent monkeys (Aceto et al., 1978), and mice (Ahlijanian and Takemori 1985; 1986), as well as rats (Khalili et al., 2001; Capasso and Gallo, 2009), and also increased the naloxone-precipitated withdrawal effect (Capasso and Gallo, 2009).

In contrast, the severity of alcoholism was directly related to various measures of caffeinated beverage use (Kozlowski et al., 1993). Among all drugs of abuse studied, the one that has been demonstrated to be coadministered most frequently with caffeine or energy drinks is alcohol. Thus, this chapter will focus on the interaction between caffeine and alcohol.

Effect of caffeine on alcohol consumption: epidemiological studies

Although the sporadic consumption of energy drinks, caffeinated sodas or coffees typically is not a problem in itself, combined with alcohol consumption can have many added risks. The combined intake of alcohol and energy drinks is a relatively new phenomenon that is increasing in frequency. Within the last 3 years there has been a proliferation of epidemiological studies assessing the incidence of combined consumption of energy drinks and alcohol, especially among teenagers and young adults from many different countries. Typically, combined consumption of these two drugs occurs in young social drinkers on a night-out who are motivated to drink alcohol heavily and to become intoxicated. For instance, around 50-65% of college students report consuming energy drinks to stay awake and study longer hours, but they also report that the main reason to use energy drinks is to be able to last longer when consuming alcohol at parties (Malinauskas et al., 2007; Oteri et al., 2007). Moreover,

the combined use of energy drinks and alcohol in young adults that are not college students in nightclubs (17.1%) is also lower than that generally found in college student samples (Wells et al., 2013).

The reasons for combining caffeine with ethanol may stem from the popular belief that caffeine can antagonize the intoxicating effects of alcohol (Hasenfratz et al., 1993). It has been described that these energy drinks reduce sleepiness, increase energy and also the perceived sense of wellbeing, when combined with alcohol (Malinauskas et al., 2007; O'Brien et al., 2008). This very popular idea may be a factor contributing to the positive correlation between consumption of caffeine and that of ethanol (Kalodner et al., 1989; Spencer et al., 1999). People who use energy drinks consume alcohol more frequently than people who do not. Around half of the college students who consume alcohol regularly report that they do mixed it with energy drinks (O'Brien et al., 2008; Malinauskas et al., 2007; Oteri et al., 2007; Attila and Çakir 2011), and, among those students who drink alcohol, the amount consumed is greater if they do it with energy drinks (Price et al., 2010; Patrick and Maggs 2013). High frequency users of energy drinks consume alcohol more frequently and in higher quantities, increasing the risk of alcohol overdose (Patrick and Maggs 2013). The consumption of alcohol mixed with energy drinks in students is strongly associated with high-risk drinking behavior, including increased binge drinking, more frequent episodes of weekly drunkenness, and elevated blood alcohol content (O’Brien et al., 2008; Patrick and Maggs 2013). High frequency users of energy drinks and ethanol were also twice as likely to meet Diagnostic and Statistical Manual of Mental Disorders 4th edition (DSM-IV) criteria for alcohol dependence, compared with low frequency users (Arria et al., 2011; American Psychiatric Association, 2000).

Furthermore, it appears that energy drinks change the palatability of alcohol when used as a mixer; the high glucose content of these drinks makes beverages with high alcohol content easier to drink, especially for naïve and early consumers with little experience in the consumption of alcohol. Compared to low-frequency energy drink users, high-frequency users were reported to be significantly more likely to have gotten intoxicated at an early age (Arria et al., 2011). Thus, one of the clear risks of the combined consumption is that young people seem to end up consuming more alcohol and doing so earlier in life.

Effect of caffeine on alcohol consumption: laboratory studies

A limited number of studies employing experimental animal models have been performed to elucidate the impact of caffeine on alcohol consumption. Studies in rodents have shown a complex relationship between caffeine administration and ethanol intake. Studies of chronic administration show that caffeine administered in the diet facilitates voluntary ethanol drinking in rats in a free access two-bottle paradigm (Gilbet 1976, 1979), and removal of caffeine from the diet restored alcohol consumption to baseline levels. However, slow-release caffeine pellets failed to alter ethanol intake in a similar paradigm (Potthoff et al., 1983). The presence of caffeine in alcoholic solutions did not increase ethanol consumption in rats exposed to a free-choice procedure (Carvalho et al., 2012). Interestingly, it did prevent the alcohol deprivation effect, blocking the typical increase of ethanol intake after an abstinent period (Carvalho et al., 2012). Caffeine administered acutely did not produce a consistent pattern of effects either; a low dose of caffeine (5.0 mg/kg, IP) promoted ethanol drinking in rats using a limited-access two-bottle choice paradigm (Kunin et al., 2000). However, a high acute

dose of caffeine (50.0 mg/kg, IP) decreased ethanol as well as food intake in rats (Dietze and Kulkosky, 1991).

Caffeine has been shown to indirectly modulate the activity of many neurotransmitters and neuromodulators, among which the most direct action is on adenosine receptors (Fredholm et al., 1999). Caffeine acts as a nonselective antagonist for A_1 and A_{2A} receptor subtypes in the central nervous system (CNS). (Fredholm et al., 1999, 2001; Cauli et al., 2005; Ferré et al., 2008) Adenosine produces hypnotic and anxiolytic effects as well as a reduction of locomotion (Deckert et al, 1998; Correa and Font, 2008), while caffeine blocks adenosine's sedative, anxiolytic and sleep-inducing effects. Ethanol increases adenosine levels by potentiating adenosine release (Clark and Dar, 1989; Fredholm and Wallman-Johansson, 1996) and by decreasing adenosine uptake (Diamond and Gordon, 1997). Secondarily, ethanol increases adenosine levels because acetate generated by ethanol metabolism promotes adenosine synthesis (Carmichael et al., 1991; Pardo et al., 2013a). Furthermore, adenosine seems to mediate alcohol-induced motor incoordination, hypnotic effects, and anxiolysis (Dar et al, 1994; Israel et al, 1994; Correa and Font, 2008; Batista et al, 2005). Thus, caffeine and ethanol seem to have opposite actions on the same neuromodulator. Alterations in adenosinergic signaling mediate many of the effects of acute ethanol administration, particularly with regard to motor function and sedation (Israel et al., 1994; Pardo et al., 2013a).

Research on the role of adenosine receptor subtypes in ethanol intake has mainly focused on A_{2A} receptors. Ethanol intake increased in A_{2A} KO mice compared to their WT counterparts in a free choice task. (Ruby et al., 2010) Similarly, acute and subchronic administration of A_{2A} receptor antagonists increased ethanol intake in alcohol-preferring rats in a free choice paradigm. (Kozlowski et al., 1993) In operant chambers, in which animals have to exert effort to have access to ethanol (e.g. lever

pressing), the pattern of effects produced by different A_{2A} receptor antagonists was more complex. While some increased, others reduced the number of ethanol-reinforced responses and ethanol consumption (Register et al., 1972; Kozlowski et al., 1993; Gilbert, 1976, 1979). No effect was observed with an adenosine A_1 antagonist (Gilbert, 1976; Register et al., 1972).

Taken together, it appears that the results from animal studies so far are not conclusive. The specific effects of adenosine antagonism on ethanol self-administration may depend on factors such as food restriction, sex, ethanol-intake or reinforcement paradigms, or other factors. For instance, it has been suggested that the suppressive effects of caffeine on ethanol intake seen in some studies could be due to the use of high toxic doses of caffeine. (Vendruscolo et al., 2012; Itsvan and Matarazzo, 1984).

Effect of caffeine on alcohol withdrawal

Withdrawal is a defining characteristic of drug dependence and is often characterized by impaired physiological function and enhanced negative affect, symptoms strongly associated with relapse (Potthoff et al., 1983). Some symptoms of ethanol withdrawal appear starting as soon as 12 hrs after the time when ethanol levels in blood are no longer detectable. For instance, acute withdrawal appears several hours after a high dose of ethanol has been administered, and produces a mild set of symptoms (i.e., hangover). Beliefs about the effects of mixing caffeine and alcohol on hangover or sleep may play a role in the motivation to consume mixtures of the two substances. However, recent studies show that this mixture does not affect amount of sleep or sleep latency, hangover, or sleepiness the morning after drinking to intoxication levels (Penning et al., 2011; Rohsenow et al., 2014).

Among other effects, acute and chronic withdrawal from ethanol typically includes anxiety symptoms (Schechter, 1974). (For review see Dietze and Kulkosky, 1991). In addition, high doses of caffeine have been demonstrated to induce anxiety in humans and rodents (For a review see Correa and Font 2008). Recently, in humans it has been reported that consumption of energy drinks (100 mL/day) also was significantly associated with anxiety (but not depression or stress) in young adult males (Trapp et al., 2013). Thus, the popular believe that a cup of strong coffee can antagonize some of the symptoms of ethanol-withdrawal, seems to be counterintuitive in the case of anxiety. Chronic ethanol exposure and withdrawal affect mainly A_1 receptor density in rodents (for a review see Butler and Prendergast, 2012) and although the impact of selective adenosine antagonists on anxiety induced by ethanol withdrawal has been investigated in a handful of studies (for a recent review see López-Cruz et al., 2013), leading to the conclusion that A_1 agonists attenuate and A_1 antagonists exacerbate the anxiogenic effect of ethanol withdrawal (Butler and Prendergast, 2012), there are no data so far directly assessing the impact of caffeine on ethanol withdrawal.

In mice, we recently demonstrated that an acute dose of caffeine (25.0 or 50.0 mg/kg) induces anxiogenic responses in an elevated plus maze (López-Cruz et al., 2011). Using the same testing parameters, we evaluated the impact of previous exposure to caffeine in mice that, after drinking ethanol for a long period of time, went through repeated episodes of withdrawal. Adult C57BL/6JRccHsd male mice (Harlan Labs. Spain) had 24 hours access to two different bottles of tap water (control group) or one of tap water and the other of ethanol 10% w/v (withdrawal group) during 10 weeks. In the last 6 weeks of this period, both groups received a dose of caffeine (0, 2.5, 5.0, 10.0, 20.0 and 40.0 mg/kg, intraperitoneally) once a week. Every mouse received all doses in a random order. After these 10 weeks the ethanol solution was removed for 4 days, after

which it was reintroduced for another 4 days. This cycle of removal and reintroduction of ethanol in the withdrawal group was repeated 3 times. Both groups had continuous access to water. Four days after the last ethanol removal animals in control and experimental groups received either saline or a dose of 40.0 mg/kg of caffeine, and were evaluated in the elevated plus maze (see figures 1A-D) for measures of anxiety (latency to enter an open arm, time spent in the open arms, and ratio between entries in the open arms and total entries) and a measure of locomotion (total number of entries). The two-way factorial ANOVA (intake solution x dose of caffeine) for the four dependent variables lead to the following results: latency (no effect of the intake solution, caffeine dose [$F(1,39)=3.82$, $p<0.05$], and interaction [$F(1,39)=12.53$, $p<0.01$]), time in open arms (no effect of caffeine dose, intake factor [$F(1,39)=8.23$, $p<0.001$], and interaction [$F(1,39)=6.49$, $p<0.01$]), ratio (caffeine dose [$F(1,39)=4.03$, $p<0.05$], intake factor [$F(1,39)=8.95$, $p<0.01$], and interaction [$F(1,39)=9.93$, $p<0.01$]) and for total arm entries (no effect of caffeine dose, the intake factor [$F(1,39)=8.95$, $p<0.01$], and interaction [$F(1,39)=12.53$, $p<0.01$]). Because all the interaction were significant LSD post hoc test were conducted to compare groups (results are shown in the graphs).

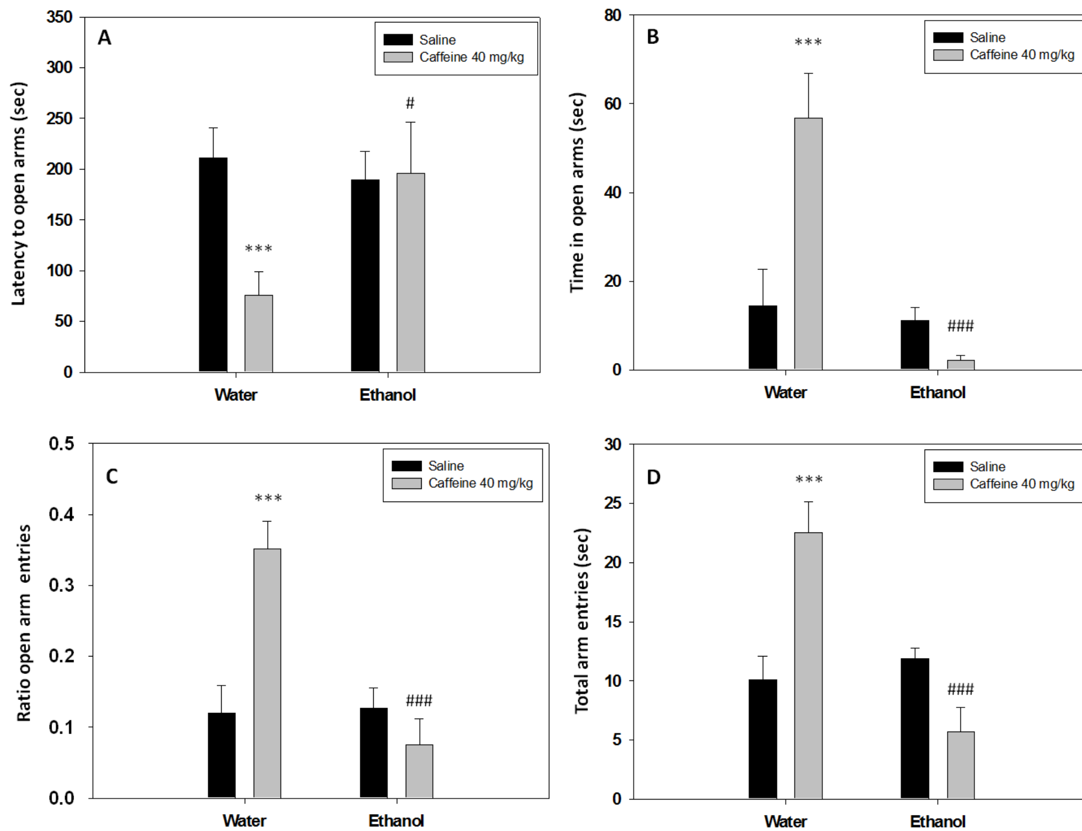

Fig. 1. Effect of acute administration of caffeine (0 or 40 mg/kg, IP) in mice pre-exposed to water or to several cycles of ethanol withdrawal on the elevated PM (N=11-13 per group). All mice had also previous repeated experience with caffeine (2.5 - 40 mg/kg, IP). Data are expressed as mean (± SEM) of **A)** latency (sec) to enter an open arm, **B)** time (sec) spent in the open arms, **C)** ratio of open arm entries, and **D)** total arm entries during 5 minutes. ***p<0.001 significant differences between doses of caffeine in the same intake group. #p<0.05, ###p<0.001 significant differences between the same dose of caffeine in different intake groups.

Thus, the water (control) group did not show an anxiogenic response to a challenge of 40.0 mg/kg of caffeine, as do animals exposed for the first time to this drug at high doses (López-Cruz et al., 2011). This result seems to be caused by the repeated administration of caffeine once a week for 5 weeks at doses ranging from 2.5-40.0 mg/kg that all the mice in the present experiment had received. In fact, 40.0 mg/kg

caffeine induced an anxiolytic response in the control mice, as well as increased locomotion. It seems then that repeated caffeine administration at a younger age inoculates an animal from the anxiogenic impact of acute effects of caffeine later in life. However, the group that had consumed ethanol did not show the anxiolytic effect of this dose of caffeine. The two groups that received saline instead of caffeine were no different from each other. Thus, ethanol pre-exposed animals did not show enhanced anxiety as compared to water-exposed mice 4 days after ethanol removal, but this previous treatment was able to block the anxiolytic effect of previous experience with caffeine.

Although there are no other animal studies focusing on the impact of caffeine on anxiety induced by ethanol withdrawal, our results generally agree with a study in male rats chronically exposed during adolescence to caffeine and ethanol in the drinking water (Hughes et al., 2011). After interruption of the treatment, animals were tested in adulthood for anxiety in an open field and in a dark-light box. The group that during adolescence consumed both caffeine plus ethanol showed a significantly higher anxiolytic behavior compared to animals exposed only to ethanol (Hughes et al., 2011).

Caffeine and Alcohol Interaction: subjective, cognitive and psychomotor effects.

In addition to the impact of caffeine on alcohol consumption and abuse, public health concerns also arise from reports of increased risk of alcohol-related negative consequences (Patrick and Maggs 2013; O'Brien et al., 2008; Arria et al., 2011; Berger et al., 2011; Howland et al., 2011). A significant number of consumers of caffeine-alcohol mixes use them before or during work, which increases the frequency of accidents in manual occupations (Cheng et al., 2012). Moreover, the consumption of

alcohol mixed with energy drinks has been strongly associated with a higher prevalence of serious alcohol-related consequences; including being sufficiently sick or injured as a result of drinking to seek medical attention, being taken advantage of sexually, having unprotected sex, riding with a drunken driver, or driving while intoxicated (O'Brien et al., 2008; Berger et al., 2013). Drinkers who reported mixing alcohol with energy drinks had a threefold higher risk of being legally intoxicated and a fourfold increase in the probability of reporting the intention to drive a motor vehicle, compared with drinkers who reported consuming alcohol alone (Thombs et al, 2010). It is this last harmful consequence that appears to have a higher impact among young people (Fudin and Nicastro, 1988).

The combination of high doses of caffeine and alcohol induces the so called 'wide-awake drunk' (Attwood, 2012). This can lead to a person underestimating the level of intoxication, which can lead to drinking for longer periods of time, increasing the risk of reaching higher blood alcohol levels that exceed the legal limits for driving. Risks associated with alcohol consumption increase after consuming 5 or more drinks for men and 4 for women in a short period of time (O'Brien et al., 2008). This pattern is considered as hazardous or "binge drinking", and is very typical among young alcohol consumers (NIAAA 2011). In the U.S., 51 % of drivers aged 18-24 years who died in traffic accidents in recent years had alcohol levels above the permissible blood levels, and although there is not a relation between blood alcohol levels and subjective intoxication among energy drink consumers, higher blood levels were associated with a greater number of negative consequences (Patrick and Maggs 2013).

The ability to evaluate our own and others' level of alcohol intoxication is a very important component of risk assessment (O'Brien et al., 2008), and combining alcohol with energy drinks can mask the signs of alcohol intoxication. Energy drinks with high

caffeine content have been demonstrated to improve subjective measures of mood, concentration, and feelings of alertness (Alford et al., 2001; Seidl et al., 2000), and the majority of consumers of energy drinks mixed with alcohol do so to reduce the sedative effects and lack of coordination that alcohol produces at high doses, and also to increase the stimulant effects that alcohol has at low doses.

However, the sedative and incoordination effects that can be an important part of the intoxication feeling after consuming ethanol, do not seem to be clearly improved by caffeine. Some studies in humans tested under laboratory conditions show that participants report feeling less intoxicated or impaired when caffeine and alcohol are co-administered (Ferreira et al., 2006; Marczinski and Fillmore, 2006). However, there are also data showing that alcohol-related impairment of cognitive and motor function seems to remain largely unaffected by consuming caffeine (Weldy, 2010; Ulbrich et al., 2013). In this regard, several studies show no significant changes in subjective feelings of depression, anxiety, drunkenness or subjective intoxication, subjective impairment, and sedation (Arria et al., 2011; Azcona et al., 1995; Peacock et al., 2013).

It has been suggested that with the addition of caffeine to alcohol, the qualitative change in intoxication is due mainly to an increase in self-reported stimulation, although not necessarily a quantitative reduction in intoxication per se (Attwood et al., 2011). Moderate increases in subjective stimulation ratings are observed after consuming both substances (Peacock et al., 2013), and laboratory studies in humans suggest that there appear to be mild stimulant-like effects on performance of objective tasks such as reaction time, digit symbol substitution, rapid information processing tasks, and memory recall (Azcona et al., 1995; Drake et al., 2003; Hasenfratz et al., 1993; Howland et al., 2010, Mackay et al., 2002). Caffeine attenuates ethanol-induced changes in psychological parameters such as information processing, memory, and

psychomotor performance (Ferré and O'Brien 2011). In contrast, in a classic study, the combination of alcohol plus caffeine produced no impact on reaction time compared to the alcohol alone group (Azcona et al., 1995). Also, in inhibitory control tasks such as go-no-go, or stop-signal, reports show mixed findings (Ferré and O'Brien 2011). Thus, caffeine improves alcohol-related detriment on some tasks, while having no effect, or even worsening performance, on others (Fillmore and Vogel-Sprott, 1999; Marczinski and Fillmore, 2003; Attwood et al., 2011).

It is possible that these discrepant findings are a result of the use of different methodologies. There seems to be a lack of consensus on the required dose of caffeine to reduce the psychomotor depressant effects of alcohol in humans. For example, a concentration of caffeine of 80.0 mg given in an energy drink may not be sufficient to antagonize the effects of medium doses of alcohol (0.6 and 1.0 g / kg) (Ferreira et al., 2004). Although this combination of doses reduced the subjective feeling of intoxication characterized as decrease in headache, reduced feelings of fatigue, less dizziness, fewer problems when walking, or less dry mouth (Ferreira et al, 2004, 2006), in a test of visual motor coordination physiological parameters and biochemical and behavioral measures assessed objectively, such as alcohol concentration in exhaled air or performance and reaction time, were not changed (Ferreira et al., 2004, 2006). Again, it appears that the subjective feeling of alcohol intoxication is reduced by caffeine, but not the intoxication itself (Riesselmann et al., 1996). In another study, a higher concentration of caffeine (400 mg) plus a low dose of alcohol (0.6 g/kg) improved psychomotor performance in relation to individuals who consumed only alcohol. However, no improvement on parameters such as the ability to drive a car in a simulator was observed (Liguori and Robinson, 2001). Results indicate that legally intoxicated individuals cannot antagonize alcohol-induced, driving-related decrements with caffeine

prior to driving an automobile (Fudin and Nicastro, 1988), thought to be the major behavior for which caffeine is used in attempts to antagonize alcohol-induced decrements, confirming the idea that consumers of energy drinks and alcohol may have a reduced subjective sense of intoxication (Riesselmann et al., 1996), thereby increasing the likelihood of accidents when combining both substances.

Animal studies, show that both drugs are able to stimulate locomotor activity in rodents at some dose (Arizzi-LaFrance et al., 2006; Himmel, 2008; Correa et al., 2003; 2009; López-Cruz et al., 2011), and low doses of caffeine can increase motor stimulant properties of moderate doses of alcohol, (Waldeck, 1974; Kuribara et al., 1992; López-Cruz et al, 2012). However, high doses of caffeine, such as those contained in energy drinks, have been shown to increase the motor incoordination produced by high doses of alcohol (López- Cruz et al., 2012). Pilcher (1911) concluded years ago that “when small doses of caffeine and alcohol are combined, the result is generally a qualitative algebraic summation of both actions, i.e. each drug produces, qualitatively, its ordinary effects. However, when large doses of the two drugs are combined, the effects of the stimulant drug tend to be reversed, resulting in a greater suppression than the suppressant drug alone” (Zhang, 2011).

Conclusions

Caffeine has been shown to have beneficial and therapeutic effects in motor performance. For example, low doses of caffeine and its metabolite theophylline have been shown to improve motor symptoms in humans and in animal models of Parkinson disease (Fredholm et al., 1999; Salamone et al., 2008; Pardo et al., 2013b). In addition, low doses of caffeine plus ethanol, equivalent to no more than 2 to 3 cups of strong coffee and 1 cocktail, reduce stroke damage in experimental models, and this effect is

now in clinical evaluation for treatment of ischemic stroke, with promising effects (Martin-Schild et al., 2009). In spite of this beneficial effects of low doses of both substances, concentrations of caffeine in energy drinks are so high than if a person consumes several of these energy drinks on a single episode (i.e., a binge over a few hours), he or she can end up self-administering a high dose of caffeine that is unlikely to have therapeutic effects. The European Commission and the American Food and Drug Administration (FDA) report that, in humans, when blood alcohol concentrations are low, low doses of caffeine can produce a "modest" effect on motor parameters. But this does not occur when doses and concentrations of both substances are high (European Commission Health & Consumer Protection Directorate- General, 2007), as is the case during many instances of recreational drug use.

Young people who consume energy drinks also drink alcohol more frequently and in higher quantities. Alcohol facilitates exposure to anxiogenic or risky situations, produces psychomotor disinhibition, and promotes impulsive behavior. In young people, perceived risk associated with alcohol consumption is determined by variables such as perceived control over the situation. A factor that increases this sense of control is the use of substances to "reduce the effects of alcohol." Combining alcohol with energy drinks can lead to a sense of control over the intoxicating effects of alcohol by virtue of masking signs of alcohol intoxication, which then results in greater levels of alcohol intake, dehydration, more severe and prolonged hangovers, and ultimately, alcohol poisoning.

Numerous brands of alcohol/caffeine combination drinks have been produced. Positive effects of caffeine/alcohol combination drinks are readily and aggressively relayed in marketing campaigns. But negative effects, if relayed at all, appear as ‘small print’ on labels that consumers often fail to read. Current European legislation

(European Directive 2002/67/EC on the labeling of food containing caffeine) rules that beverages containing up to 150 mg/l must be marked as ‘high caffeine content’ and that this statement should be in the same field of vision as the product name. In 2010, the US Food and Drug Administration issued warnings to several manufacturers of combination drinks identifying caffeine as an ‘unsafe food additive’ mixed with alcohol, and stated that their sale violated federal law. There are also restrictions on the production and sale of caffeinated alcohol beverages in some countries, including Canada, where caffeine can only be mixed with alcohol if it comes from a natural source (e.g. guarana), and Mexico, where caffeinated alcohol beverage sales are prohibited in bar rooms and night clubs. In the UK, alcohol-related harm and binge drinking are high on the political agenda, and there have been calls for a legal restrictions on the amount of caffeine that can be added to alcohol products.

Thus, different countries have already adopted strategies to avoid mixing alcohol with energy drinks. A number of countries have a requirement on manufacturers to label drinks, indicating the possibility that the mixture affects the perceived levels of intoxication. However, the debate over caffeinated alcohol beverages is somewhat undermined by the fact that caffeinated energy drinks are widely available. Although they have been banned in various countries in the past (e.g. France, Denmark, Norway), many of these bans have since been revoked. And individuals are still free to mix their own caffeine/alcohol beverages. In conclusion, public health concern over caffeinated alcohol drinks is justified, although the nature of the caffeine/alcohol relationship is yet to be fully elucidated.

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CHAPTER 2.

CAFFEINE ENHANCES ETHANOL-INDUCE IMPAIRMENTS IN VOLUNTARY LOCOMOTION, COORDINATION AND SEDATION IN MICE: INVOLVEMENT OF DARPP32 IN ACCUMBENS CORE

Abstract

Energy drinks are highly consumed beverages rich in caffeine. In humans, energy drinks are very frequently consumed with alcohol in order to reduce sedation and ataxia induced by high doses of this drug of abuse. Caffeine stimulates locomotion but can also produce motor impairments at high doses. In the present study the impact of a broad range of caffeine doses (3.75-30.0 mg/kg, IP) were used to assess their potential impairing effect on running wheel (RW) voluntary locomotion and incoordination on the rotarod in adult male CD1 mice. In addition, high doses of caffeine (15.0 and 30.0 mg/kg, IP) were administered in combination with ethanol (0.5-3.5 g/kg, IP) to assess if they can actually reverse the motor stimulating, ataxic and loss of righting reflex (LORR) induced by ethanol. The interaction between caffeine (30.0 mg/kg) and ethanol (1.5 g/kg) on DARPP-32 phosphorylation patterns in different subregions of striatum was also evaluated by immunohistochemistry. Caffeine dose-dependently reduced RW activity during 60 minutes, and was not able to reverse ethanol-induced suppression of locomotion. Although caffeine on its own did not impair rotarod performance, it exacerbated ethanol-induced ataxia. Caffeine also dose dependently potentiated ethanol induced LORR. In Nacb Core pDARPP32-Thr34 immunoreactivity increased significantly after administration of 30.0 mg/kg caffeine compared to vehicle, and ethanol 1.5 g/kg significantly reduced this effect of caffeine to vehicle levels. In Nacb Core the group that had received caffeine plus ethanol had significantly higher levels of pDARPP32-Thr75 expression compared to the group that had received caffeine alone. Thus, caffeine not only failed to act as a palliative treatment for the disruptive effects of ethanol on motor and sedative parameters, but in fact it further impaired them. These effects could be mediated by actions on accumbens neurons containing adenosine A1 receptors.

Key words: methylxantines, adenosine, alcohol, energy drinks, coordination, narcosis, DARPP-32, running wheel.

Introduction

Caffeine is the most widely used psychoactive substance in the world, and is one of the main active ingredients of energy drinks responsible for the behavioral and cognitive effects associated with these beverages (Giles et al., 2012). Consumers of energy drinks sometimes mix them with alcohol to reduce the sedation and lack of coordination that alcohol produces at high doses, and also to increase the stimulant effects that alcohol has at low doses. However, sedative and incoordinating effects of ethanol do not seem to be clearly improved by caffeine in humans (Ferreira et al., 2006; Marczinski and Fillmore, 2006). Control studies suggest that when consuming caffeine in combination with alcohol, the subjective feelings of alcohol intoxication are reduced, but not the intoxication-related impairments themselves (Riesselmann et al., 1996; Attwood et al., 2012; Peacock et al., 2013).

In rodents, ethanol has biphasic effects on locomotion, increasing motor activity under conditions of low baseline activity at low doses but suppressing locomotion and impairing motor coordination at moderate to high doses (Correa et al., 1999, 2003; Dar et al., 1990). Even higher doses can lead to narcosis and loss of righting reflex (LORR) (Correa et al., 2001). The stimulant effects of caffeine at low doses are well known (Zhang et al., 2011; López-Cruz et al., 2013), and in addition, low doses have been demonstrated to decrease motor incoordination induced by ethanol (Dar, 1988). However, caffeine at high doses on its own can also impair motor coordination (López-Cruz et al., 2013), and even at low doses, caffeine can block the development of rapid tolerance to ethanol-induced motor incoordination (Batista et al., 2005).

Caffeine acts as a nonselective adenosine A_1 and A_{2A} receptor antagonist (Ferré et al., 2008). Ethanol also affects the adenosine system, but in an opposite direction. Ethanol can increase extracellular adenosine levels by increasing adenosine release

(Clark and Dar, 1989), and by decreasing adenosine uptake (Diamond and Gordon, 1994) that takes place via a facilitative nucleoside transporter (Nagy et al., 1990). Adenosine receptors through their coupling to Golf or Gi proteins can stimulate or inhibit adenylyl-cyclase activity and activate or silence the cAMP-PKA signaling pathway, with leads to changes in phosphorylation of several signaling proteins such as dopamine- and cAMP-regulated phosphoprotein of molecular weight 32 kDa (DARPP-32), and the consequent increase in the expression of different genes and neuronal plasticity (Gould and Manji, 2005). DARPP-32-dependent signaling mediates the actions of multiple drugs of abuse including caffeine and ethanol (Svenningsson et al., 2005).

In the present study, we systematically explored the effect of caffeine and ethanol coadministration in mice on motor parameters such as voluntary locomotion that is performed at high levels in a running wheel (RW), equilibrium and coordination in the rotarod, and LORR induced by ethanol. The expression of DARPP-32 and its phosphorylation patterns on the threonine 34 and 75 amino acid loci was also studied in different areas of the striatum in order to determine what type of adenosine receptor and what brain area could be more affected by the combination of these drugs.

Materials and methods

Animals

CD1 adult male mice (N=438) purchased from Harlan-Interfauna Iberica S.A. (Barcelona, Spain) were 9 weeks old at the beginning of the study. Mice were housed in groups of three per cage, with standard laboratory rodent chow and tap water available ad libitum. Subjects were maintained at 22 ± 2º C with 12-h light/dark cycles. Animals

were handled and received a single saline injection the day before experimental procedures started. Different groups of animals were used in each experiment. All animals were covered 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.

Drugs

Ethanol solutions (20% v/v) were prepared from 96% ethanol (Panreac Química, Spain) dissolved in saline (0.9% w/v) and administered 10 minutes before testing except for the experiment in which LORR was evaluated. Caffeine (Sigma-Aldrich, Spain) was dissolved in saline and administered 30 minutes before behavioral testing started. The range of caffeine doses (3.75-30.0 mg/kg) and ethanol doses (0.5-3.5 g/kg) was selected based on previous studies (Correa et al., 2000, 2001, 2008; López-Cruz et al., 2014). Saline was used as the vehicle control. All solutions were administered intraperitoneally (IP).

Apparatus and Behavioral Procedures

Voluntary locomotion in a Running Wheel (RW). The RW consists of a stainless steel activity wheel (circumference = 24 cm) situated in a Plexiglas box (35 x 20 cm) with a magnetic switch attached to a LCD counter for recording number of wheel turns. Animals were exposed to the RW during 30 min in two consecutive days previous to the test. On the test day (3rd day), counts on the RW were register for one hour and data were analyzed in two periods: during the first 30 min and for the second 30 min.

Motor coordination in the rotarod. The rotarod apparatus (UGO Basile, 7650) consisted of an elevated rotating rod that requires coordinated movements in order to avoid falling. Each mouse was placed in the rotating rod accelerating from 4 rpm to 20 rpm in increments of 4 rpm every 30 s. Animals were trained during five trials for two consecutive days, and tested for five more trials the next day. A 390 s maximum cut-off on the rod was used. Time spent on the rotating rod was measured (in sec) at the moment in which the animal fell off the rod.

Loss of the righting reflex (LORR). In this test latency and duration of LORR were recorded consecutively. Ethanol (3.5 g/kg) was injected IP, and mice were immediately placed in a plexiglas cage individually. Latency was defined as the time elapsed between placement in the cage and the first time the mice lost the righting reflex. Mice were then put on their back in a V-shape bed. The duration was defined as the time elapsed from LORR to the time that righting reflex was regained. Recovery was determined when mice could right themselves twice in 1 minute after being placed on their backs. All the animals recovered the righting reflex. The behavioral room was illuminated with a soft light and external noise was attenuated.

DARPP-32 immunohistochemistry. Mice were deeply anesthesized and perfused 30 minutes after receiving treatments. Brains were collected and stored in 3.7% formaldehyde solution during 24 h and refrigerated in sucrose (30%), sodium azide (2%) and PB 0.1M solution prior to slicing. Free floating coronal sections (40 µm) were serially cut using a microtome cryostat (Weymouth, MA, USA), rinsed in 0.01 M PBS (pH 7.4) and incubated in 0.3% hydrogen peroxide for 30 minutes to block endogenous staining. Sections were then rinsed in 0.01 M phosphate buffer (PBS) (3 × for 5 minutes). To measure the immunoreactivity to phosphorylated DA and c-AMP-regulated phosphoprotein 32 kDa (pDARPP-32), nonspecific binding sites were

blocked, and cells were permeabilized in a solution containing 0.1% Triton X-100 (T.X), 1% Bovine Albumin serum (BSA) in PBS for 30 min 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 34 residue (pDARPP32-Thr34, 1:1000; Santa Cruz Biotechnology), or polyclonal rabbit antibody for pDARPP-32 phosphorylated at the threonine 75 residue (pDARPP32-Thr75, 1:500; Santa Cruz Biotechnology). These antibodies were dissolved in solutions that also contained 1% BSA and 0.1% T.X in PBS for 24 h (pDARPP32-Thr34) or 48 hours (pDARPP32-Thr75) 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 3,3diaminobenzidine chromagen (DAKO) Processed sections were then mounted to microscope slides (Menzel-Gläser, Superfrost ® Plus, Thermo scientific), air dried, and cover-slipped using Eukitt® (Sigma Aldrich) 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.

Statistics

Experiments used a between-groups design. Normally distributed and homogenous data were evaluated by one-way or factorial analyses of variance (ANOVA). Further analyses were conducted by nonorthogonal planned comparisons using the overall error term to assess differences between each dose and the control condition (Keppel, 1991). All data were expressed as mean ± SEM, and significance was set at $p<0.05$. STATISTICA 7 software was used.

Results

Experiment 1.1. Effect of caffeine on voluntary locomotion in the RW.

ANOVA showed a significant effect of caffeine on RW performance in the first 30 minutes ($F(4,43)=9.03$; $p<0.01$) (see figure 1.A). Planned comparisons revealed that all caffeine doses significantly decreased locomotion compared to vehicle (caffeine 3.75 mg/kg, $p<0.05$; caffeine 7.5, 15 and 30 mg/kg, $p<0.01$). The one-way ANOVA for the RW counts during the second 30 minutes also showed a significant effect of caffeine ($F(4,43)=7.02$; $p<0.01$). Planned comparisons revealed that caffeine doses of 15.0 mg/kg ($p<0.05$) and 30.0 mg/kg ($p<0.01$) significantly decreased the number of counts in this period compared to vehicle (see figure 1B). Thus, the two highest doses of caffeine seem to have had longer lasting impairing effects on the RW

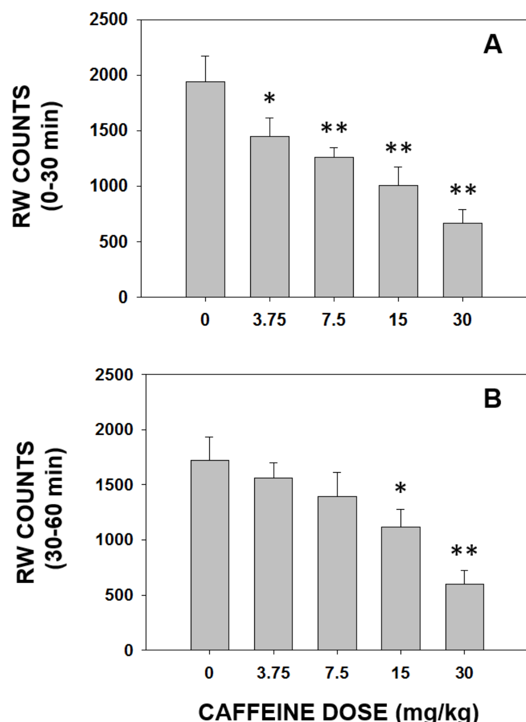


Fig. 1. Effect of caffeine on voluntary locomotion in the RW (N=9-10 per group) in the first (0-30 min, A) and second period (30-60 min, B) of the test session. Data are expressed as mean ± S.E.M. number of turning counts. *p<0.05; **p<0.01 significantly different from vehicle.

Experiment 1.2. Effect of caffeine-ethanol co-administration on RW performance.

The two-way ANOVA (dose of ethanol x dose of caffeine) demonstrated a statistically significant effect of caffeine ($F(2,79)=20.82$; $p<0.01$), a significant effect of ethanol ($F(3,79)=18.65$; $p<0.01$), and a significant effect of the interaction ($F(6,79)=2.33$; $p<0.05$) on the number of counts in the first 30 minutes (figure 2.A). Planned comparisons revealed that when compared with the vehicle-vehicle group all doses of ethanol (0.5, 1.5 and 2.5 g/kg) plus vehicle decreased number of counts (0.5 g/kg, $p<0.05$; and 1.5 and 2.5 g/kg, $p<0.01$). As seen in the previous experiment, both doses of caffeine (15.0 and 30.0 mg/kg) plus vehicle also reduced the number of counts ($p<0.01$) when compared to the vehicle-vehicle group. In terms of the interactions, the highest dose of caffeine (30.0 mg/kg) in combination with the two lowest doses of ethanol (0.5 and 1.5 g/kg) produced significant differences ($p<0.05$ and $p<0.01$ respectively) from their corresponding ethanol plus vehicle group. The lowest dose of caffeine (15.0 mg/kg) used in this experiment in combination with the middle dose of

ethanol (1.5 g/kg) also decreased the number of counts compared with the group that had received that dose of ethanol plus vehicle ($p<0.01$). However, none of the caffeine doses in combination with the highest dose of ethanol (2.5 g/kg) produced greater impairments compared to that dose of ethanol plus vehicle, which is probably due to a floor effect.

The two-way ANOVA for the number of counts during the second 30 minutes showed an effect of caffeine treatment ($F(2,79)=7.56$; $p<0.01$), a significant effect of ethanol treatment ($F(3,79)=12.92$; $p<0.01$), but not a significant effect of the interaction ($F(6,79)=1.72$, n.s.). Those data are shown in figure 2.B.

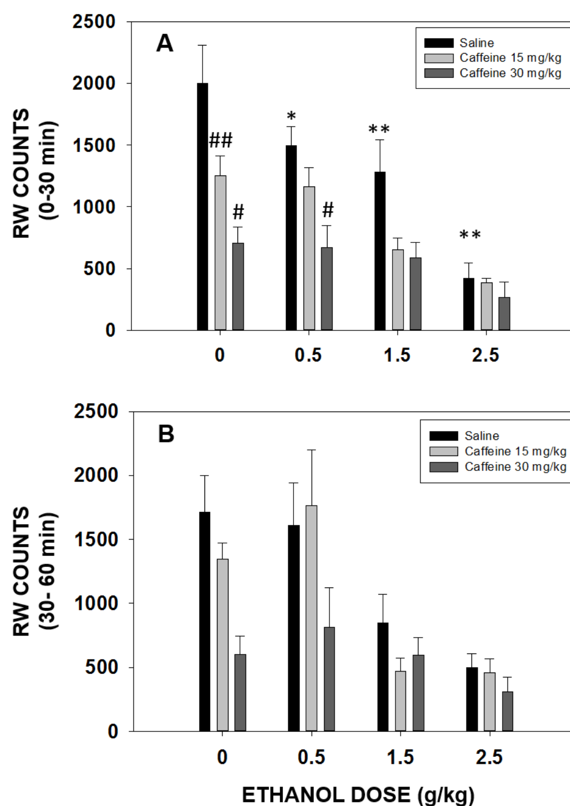


Fig. 2. Effect of ethanol and caffeine combination on locomotion in the RW (N=6-8 per group) in the first (0-30 min, A), and second period (30-60 min, B) of the test session. Data are expressed as mean ± S.E.M. number of turning counts. *$p<0.05$; **$p<0.01$ significantly different from saline-saline group. #$p<0.05$; ##$p<0.01$ significantly different from the saline group in the same ethanol dose.

Experiment 2.1. Effect of acute administration of caffeine on motor incoordination in the rotarod.

The effect of caffeine on rotarod performance is shown in figure 3. ANOVA revealed that there was not a significant overall effect of caffeine treatment on rotarod performance (F(4,47)=1.93; n.s.).

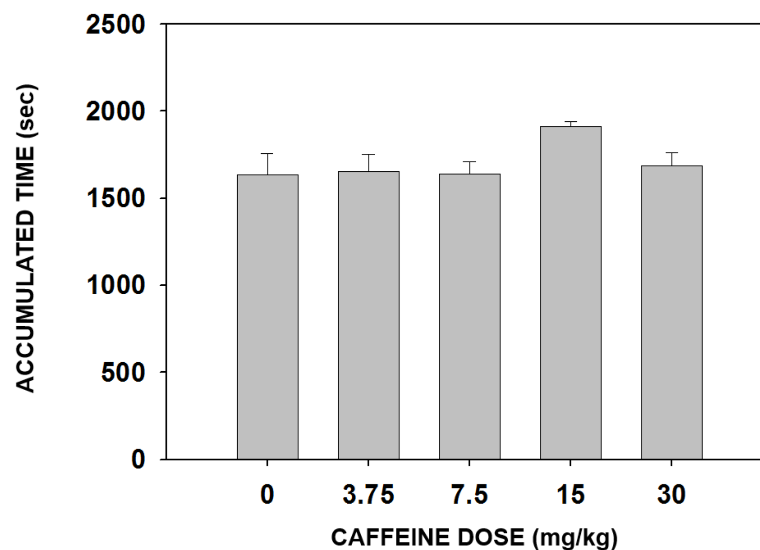


Fig. 3. Effect of caffeine on performance in the rotarod (N=10-12 per group). Data are expressed as mean ± S.E.M. accumulated time in seconds.

Experiment 2.2. Effect of caffeine-ethanol co-administration on motor incoordination in the rotarod.

Figure 4 depicts the results of the co-administration of caffeine and ethanol on motor incoordination. Factorial ANOVA did not reveal a significant effect of caffeine treatment (F(2,185)=2.10; n.s.). However, it did show a significant effect of ethanol treatment (F(4,185)=40.55; p<0.01), and a significant caffeine x ethanol interaction (F(8,185)=2.64; p<0.01). Planned comparisons showed that vehicle plus ethanol at high

doses (1.5 and 2.0 g/kg) reduced time spent on the rotating rod (p<0.01) when compared to the vehicle-vehicle group. Moreover, both caffeine doses (15.0 and 30.0 mg/kg) significantly potentiated the suppressive effects of this dose of ethanol (1.5 g/kg, p<0.01).

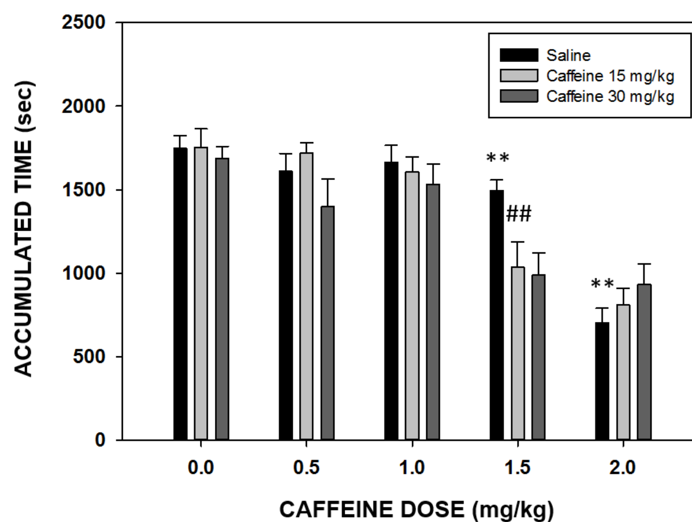


Fig. 4. Effect of coadministration of ethanol and caffeine on rotarod performance (N=11-23 per group). Data are expressed as mean ± S.E.M. accumulated time (seconds). **p<0.01 significantly different from the saline-saline group. ##p<0.01 significantly different from the saline group in the same ethanol dose.

Experiment 3. Effect of caffeine on ethanol induced LORR.

In this experiment (see figure 5) mice received caffeine 30 min before a single dose of ethanol (3.5 g/kg) was administered. The one-way ANOVA for the latency to reach LORR did not show a significant effect (F(2,31)=0.33; n.s.). However, the one-way ANOVA for the duration of LORR was statistically significant (F(2,31)=3.71; p<0.05). Planned comparisons revealed that the highest dose of caffeine (30.0 mg/kg) increased duration of LORR (p<0.05) in comparison with the vehicle group.

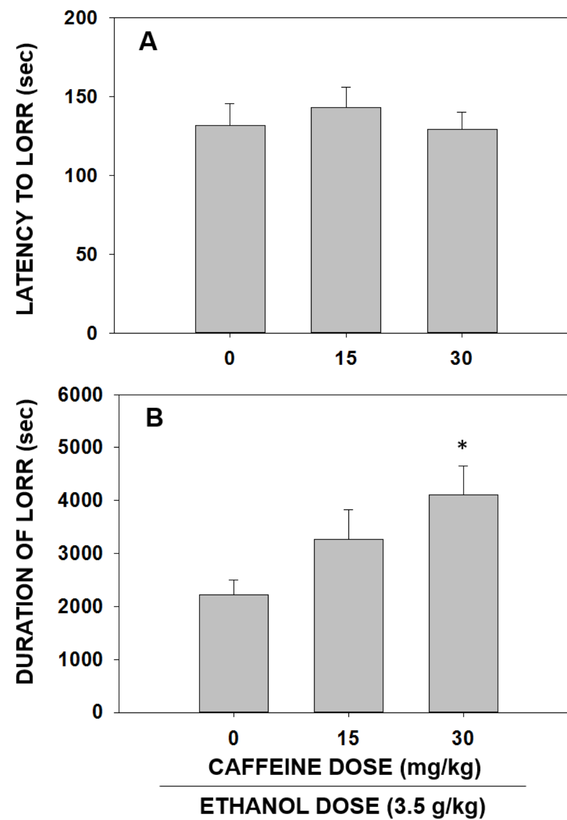


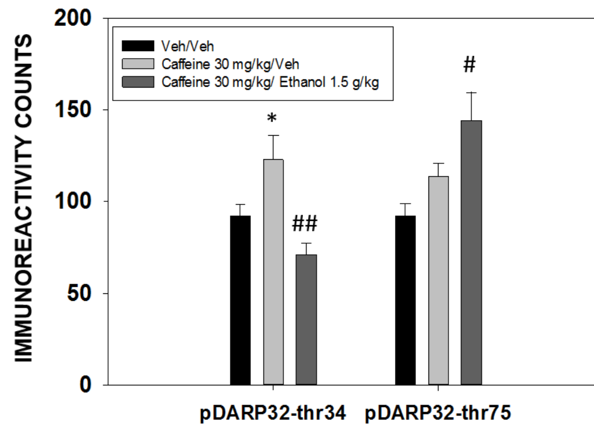
Fig. 5. Effect of an acute IP injection of caffeine on the latency to (A) and duration of (B) LORR induced by ethanol (3.5 g/kg) (N=11-12 per group). Data are expressed as mean ± S.E.M. time in seconds. *p<0.05 significantly different from vehicle.

Experiment 4. Effect of caffeine and ethanol on pDARPP-32(Thr34) and pDARPP-32(Thr75) expression in different areas of the striatum.

The effect of vehicle, caffeine (30.0 mg/kg), and caffeine (30.0 mg/kg) plus ethanol (1.5 g/kg) on pDARPP-32(Thr34) or pDARPP-32(Thr75) immunoreactivity levels were analyzed separately for every striatal area: nucleus accumbens core (NAcb Core), nucleus accumbens shell (NAcb Shell), and dorsal striatum (DST). Figure 6 and Table 1 depict the results of the co-administration of caffeine and ethanol on pDARPP-32(Thr34) and pDARPP-32(Thr75) levels.

Experiment 4.1. pDARPP32-(Thr34). One-way ANOVA conducted on the pDARP32-(Thr34) expression data in NAcb Core was significant ($F(2,10)=9.78$; $p<0.01$), and planned comparisons revealed that 30.0 mg/kg caffeine plus vehicle was significantly different from vehicle/vehicle ($p<0.05$), and from 30.0 mg/kg caffeine plus ethanol 1.5 g/kg ($p<0.01$). No statistically significant effects were observed in NAcb Shell ($F(2,10)=0.01$; n.s.), nor in DST ($F(2,7)=2.21$; n.s.).

Experiment 4.2. pDARPP32-(Thr75). The different one-way ANOVAs for the immunoreactivity data for every structure revealed a significant effect of treatment in pDARP32-thr75 levels in NAcb Core ($F(2,12)=7.55$; $p<0.01$). Planned comparisons revealed that the group that received 30.0 mg/kg caffeine plus vehicle was significantly different from 30.0 mg/kg caffeine plus 1.5 g/kg ethanol ($p<0.05$). The one-way ANOVAs for the other two structures did not yield any significant effects in NAcb Shell ($F(2,14)=1.92$; n.s.) nor in DST ($F(2,10)=2.15$; n.s.).


IMMUNOREACTIVITY COUNTS
200
150
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50
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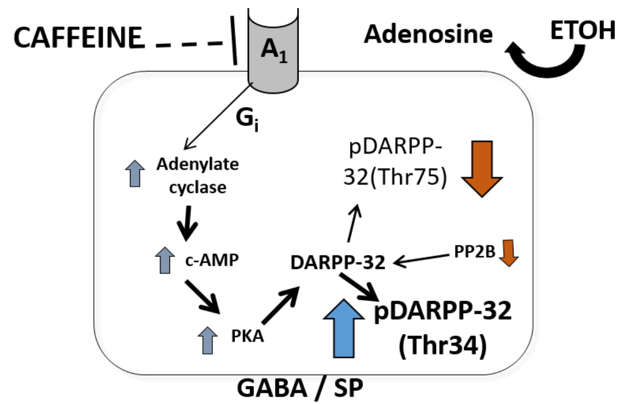
CAFFEINE
A1
Adenosine
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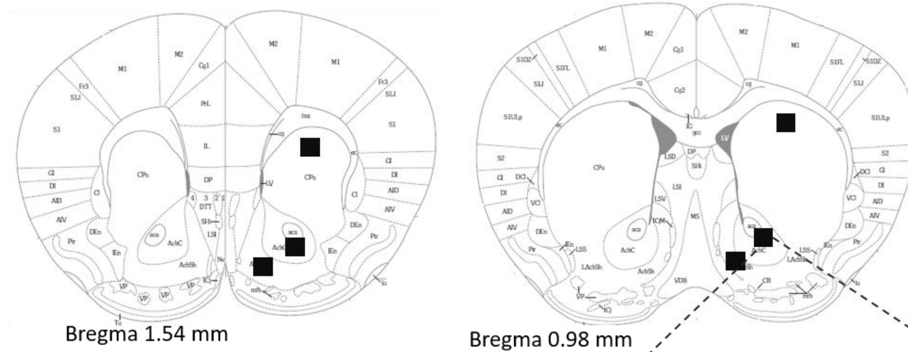
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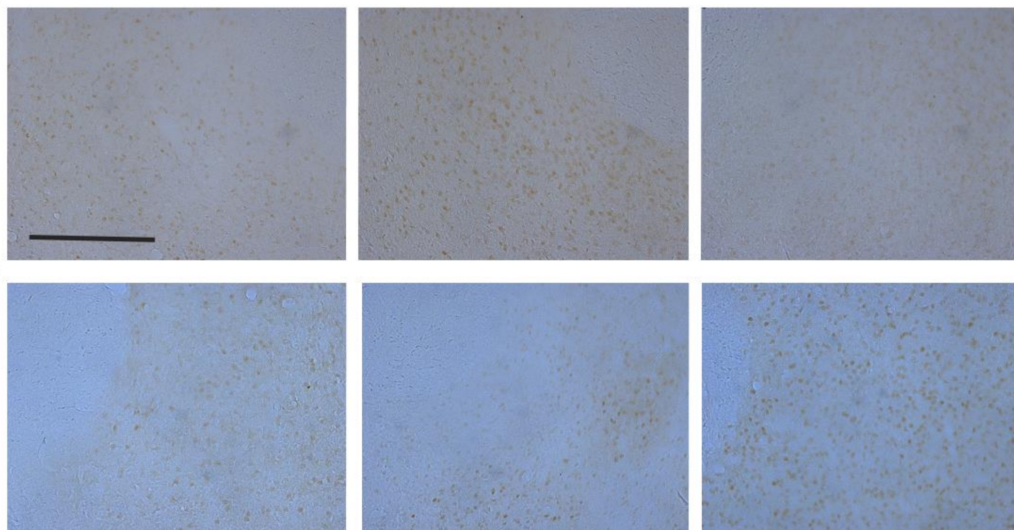
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Fig. 6. Upper part: Effect of vehicle, caffeine (30 mg/kg) or caffeine (30 mg/kg) plus ethanol (1.5 g/kg) administration on pDARPP-32(Thr34) and pDARPP-32(Thr75) immunoreactivity staining in Nacb Core. (N=4-6 per group). Mean (±SEM) number of positive cells per mm^2. *$p<0.05$ significantly different from vehicle/vehicle (Veh/Veh); #$p<0.05$, ##$p<0.01$ significantly different from caffeine 30 mg/kg/vehicle (Caf30/Veh). Middle part: Diagram of coronal sections with bregma coordinates from Franklin and Paxinos 2007, showing location of the brain areas for DARPP32 immunoreactivity counting. Lower part: Photomicrographs of pDARPP32-Thr34 staining in Nacb Core from representative animals in each treatment group. Low power images (20x). Scale bar=250 um.

Brain area	DARPP32 antibody	
	pDARPP32-(Thr34)	**pDARPP32-(Thr75)**
Nacb Shell		
Veh/Veh	82.86 ± 11.39	96.36 ± 12.27
Caf 30/Veh	84.77 ± 5.00	89.50 ± 7.21
Caf 30/Etoh 1.5	81.96 ± 13.34	116.11 ± 8.76
Dorsal Striatum		
Veh/Veh	65.66 ± 10.17	100.93 ± 18.32
Caf 30/Veh	27.33 ± 11.14	122.41 ± 17.01
Caf 30/Etoh 1.5	51.50 ± 13.57	147.12 ± 8.20

Table 1. Effect of vehicle, caffeine (30.0 mg/kg) or caffeine (30.0 mg/kg) plus ethanol (1.5 g/kg) on pDARPP-32(Thr34) and pDARPP-32(Thr75) immunoreactivity staining in different subregions of the striatum (N=5-6 per group). Mean (± S.E.M.) number of positive cells per mm2.

Discussion

Previous animal studies have shown that caffeine and ethanol are able to stimulate locomotor activity in rodents at some dose (Arizzi-LaFrance et al., 2006; Himmel, 2008; Correa et al., 2003, 2009; López-Cruz et al., 2014). Moreover, low doses of caffeine can increase the motor stimulant properties of moderate doses of alcohol (Waldeck, 1974; Kuribara et al., 1992). However, contradicting the popular believe among energy drinks users, it was postulated many years ago that when large doses of the two drugs are combined, the effects of the stimulant drug tend to be reversed, resulting in a greater suppression than the either drug alone (Pilcher, 1911). The present set of results using different animal models of motor stimulation, coordination, and sedation, demonstrated that high doses of caffeine (analogous to doses that can be reached by consuming energy drinks in a short period of time), not only fail to reverse the impairing effects of ethanol, but they can actually exacerbate them. Thus, in a rodent model of high levels of voluntary locomotion (the RW), caffeine on its own can suppress locomotion at all doses tested, and caffeine plus ethanol interacted to further suppress locomotion. Although caffeine does not impair coordination and does not induce LORR, when given in combination with ethanol it did potentiate ethanol-induced incoordination and LORR.

In the present work, the impact of the highest dose of caffeine (30.0 mg/kg) on its own is not only seen in terms of spontaneous locomotion, but it also was observed in terms of alterations of metabotropic signaling in a brain structure that has been demonstrated to regulate voluntary locomotion, the Nacb Core (Solinas et al., 2002; Retzbach et al., 2014). Previous studies have reported that large doses of caffeine increase c-Fos immunoreactivity in the Nacb in rats, while lower acute doses had no effect (Bennett and Semba, 1998). Unlike other psychostimulants, the effects of caffeine

on dopamine release in Nacb are not very conclusive. Using microdyalisis it has been shown that caffeine can induce dopamine release in the shell of the nucleus accumbens (Solinas et al., 2002), and in the medial prefontal cortex (Acquas et al., 2002), although not in Nacb Core (Acquas et al., 2002). Caffeine is an adenosine antagonist, and adenosine receptors are colocalized with dopamine receptors in striatal medium spiny neurons. Both sets of receptors interact in an antagonistic direction; 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 actions on the mesolimbic circuit to drugs that act on the dopaminergic systems. In the present studies, caffeine increased phosphorylation of DARPP-32 at Thr34 in this brain area, pointing to an antagonistic effect over adenosine A_1 receptors, which are typically located in Substance P containing medium spiny neurons that also contain dopamine D_1 receptors (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), and reduced phosphorylation at Thr34 in enkephalin containing striatal output neurons (Nunes et al., 2013). In the present results, an increase in pDARPP32-(Thr75) expression was not observed after the administration of caffeine alone, but it was seen after co-administration of caffeine plus ethanol. Ethanol can increase adenosine levels (Diamond and Gordon, 1994; Carmichael et al., 1991; Correa et al., 2012), and this purine is the endogenous agonist for adenosine receptors. Thus, in the group that received caffeine plus ethanol, it is possible that ethanol-induced potentiation of adenosine synthesis predominated, leading to a stimulation of A_1 receptor activity by adenosine, which increases pDARPP32-(Thr75) but decreases caffeine-induced increase in pDARPP32-

(Thr34) in the same type of medium spiny neurons. In previous studies ethanol (1.5 g/kg) was shown to increase phosphorylation of DARPP-32 at Thr34 in striatum of rats (Nuutinen et al., 2011). However, based upon the knowledge of how different receptors lead to different phosphorylation patterns (e.g. Svenningsson et al., 1999), we can suggest that those effects were mediated by A_{2A} receptor activation. Pilot studies in our laboratory did not demonstrate a significant effect of this dose of ethanol on its own on any of the markers of phosphorylated DARPP32.

In summary, despite the popular assumptions about the ability of caffeine in energy drings 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 alcohol, and have identified at least one potential brain area in which caffeine and ethanol interact to modulate behavior, i.e., the nucleus accumbens core. 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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CHAPTER 3.

CAFFEINE AFFECTS VOLUNTARY ALCOHOL INTAKE IN MICE DEPENDING ON THE ACCESS CONDITIONS: ROLE OF INDIVIDUAL DIFFERENCES.

Abstract

The most consumed psychoactive stimulant and the main active ingredient of energy drinks is caffeine. Epidemiological studies have shown a positive correlation between the consumption of energy drinks and that of ethanol. One of the reasons for combining caffeine with ethanol may stem from the popular belief that caffeine antagonizes the intoxicating effects of alcohol. Both drugs act on the adenosine system but have opposite effects on adenosine receptor function. Caffeine is a methylxanthine that acts as a nonselective adenosine antagonist, binding to A_1 and A_{2A} receptor subtypes. In contrast, ethanol increases the extracellular adenosinergic tone. The scientific literature on the impact of caffeine or selective adenosine receptor antagonists on alcohol consumption is very limited, and does not provide a consistent pattern of results. The purpose of this study was to examine the impact of a broad range of doses of caffeine and of selective adenosine A_1 and A_{2A} receptor antagonists on voluntary ethanol intake under different ethanol access conditions. C57BL/6J male mice had access to ethanol (10% w/v) under different conditions: restricted (2 hours in the dark), unrestricted (24 hours access), or after 4 days of alcohol deprivation following a period of unrestricted access. Mice reduced ethanol intake in the restricted access condition after receiving the highest dose of caffeine (20.0 mg/kg). Similarly, theophylline (20.0 mg/kg), another methylxantine, reduced ethanol intake. Selective A_1 and A_{2A} adenosine receptor antagonists, or their combination, did not have an effect. However, under unrestricted access conditions caffeine increased ethanol intake, and adenosine A_{2A} antagonist had the same effect. After splitting animals into high, moderate and low ethanol consumers, caffeine (2.5-20.0 mg/kg) significantly increased ethanol consumption in moderate consumers with no effect on low or high consumers. In addition, after reintroducing ethanol access, caffeine (5.0 mg/kg) decreased ethanol consumption among low to

moderate consumers. Caffeine produced different effects on ethanol intake depending on the access condition and the baseline consumption of ethanol. Overall, it seems that adenosine A_{2A} receptors play a role in modulating ethanol consumption under some conditions. Furthermore, it seems that a moderate level of ethanol intake is required for caffeine to induce consumption. In addition, caffeine may be useful for the prevention of intake reinstatement in moderate to low consumers.

Keywords: ethanol, adenosine antagonists, caffeine, energy drinks, methylxantines, selfadministration, reinstatement.

Introcution

Caffeine and alcohol are the two most consumed substances in the world. Interest in caffeine abuse has grown ever since the introduction to the market of so-called "energy drinks". Although energy drinks contain several components with clear psychoactive effects, caffeine is the main active ingredient responsible for the behavioral and cognitive effects associated with these beverages (Giles et al., 2012). The concentration of caffeine in these drinks may range from modest to relatively high levels (50–500 mg caffeine per serving; Reissig et al., 2009; Arria et al., 2011).

A common pattern of alcohol consumption in young people is characterized by repeated bouts of heavy drinking followed by abstinence for hours and days. Early onset of binge drinking has been linked to increased risk of binging in adulthood (Weitzman et al., 2003). Throughout intervals of binge drinking, blood alcohol levels are high and behavioral effects of alcohol intoxication such as drowsiness, sleepiness, impairment of motor coordination or fatigue are typical (Ferreira et al., 2004). The combined intake of alcohol and "energy drinks" is a relatively new phenomenon that is rising among young people. Combining caffeine with ethanol during binge drinking may stem from the popular belief that caffeine antagonizes those intoxicating effects of alcohol (Reissig et al., 2009). For example, college students report consuming energy drinks combined with alcohol to quicken the onset of intoxication, and to reduce fatigue after drinking (Marczinski, 2011). Moreover, epidemiologic studies have shown that energy-drink users tend to show increased levels of alcohol consumption. The consumption of alcohol mixed with energy drinks in students is strongly associated with high-risk drinking behavior, including increased binge drinking, and more frequent episodes of weekly drunkenness (O'Brien et al., 2008; Patrick and Maggs, 2013).

Pharmacological actions of caffeine are attributable to its activity as a non-selective A1 and A2A adenosine receptor antagonist (Fredholm et al., 1999). On the other hand, ethanol increases the concentration of extracellular adenosine by facilitating adenosine release (Clark and Dar, 1989), and inhibiting adenosine re-uptake (Nagy, 1990; Butler and Prendergast, 2012). Secondarily, ethanol increases adenosine levels because acetate generated by ethanol metabolism promotes adenosine synthesis (Carmichael et al., 1991; Lopez-Cruz et al., 2013; Pardo et al., 2013a). Several studies in rodents have provided information about the antagonistic behavioral interaction between caffeine and ethanol (for a review see López-Cruz, 2013). However, a limited number of studies have been performed to elucidate the impact of caffeine on alcohol consumption in experimental animal models. Very few studies have been done about the impact of acute doses of caffeine on ethanol intake (Dietze and Kulkosky, 1991; Kunin et al., 2000). Most studies have been performed in rats using very different methodologies. Based upon those studies, it seems that acute caffeine administration at a low dose (5.0 mg/kg, IP) increased ethanol drinking in animals assessed using a limited-access two-bottle choice paradigm (Kunin et al., 2000), while a high dose of caffeine (50.0 mg/kg, IP) decreased voluntary alcohol consumption, as well as food intake (Dietze and Kulkosky, 1991). In addition, caffeine reduced the alcohol deprivation effect, blocking the typical increase in ethanol intake seen after a period of abstinence (Carvalho et al., 2012).

Research on the role of adenosine receptor subtypes in modulating ethanol intake has mainly focused on A2A receptors. Acute and subchronic administration of an adenosine A2A receptor antagonist increased ethanol intake in alcohol-preferring rats (Micioni Di Bonaventura et al., 2012), and A2A KO mice consumed more ethanol than their WT counterparts in animals tested on two-bottle choice tasks (Naassila et al.,

2002). With operant conditioning tasks, the pattern of effects produced by different A2A receptor antagonists was more complex. While the A2A antagonist ANR94 produced a mild increase in ethanol-reinforced responding in ethanol-preferring rats (Micioni Di Bonaventura et al., 2012), SCH58261 reduced ethanol-reinforced responding in alcohol-preferring rats (Adams et al., 2008), and DMPX reduced responding for ethanol in Wistar rats (Thorsell et al., 2007). Adenosine A1 antagonists reduced ethanol intake in alcohol preferring rats (Adams et al., 2008), and in non-preferring rats produced a nonsignificant trend towards reduced ethanol self-administration (Arolfo et al., 2004).

The aim of the present study was to examine the effect of a broad range of acute doses of caffeine on voluntary ethanol intake under different non-operant access conditions in C57BL/6J mice, a strain that has a high baseline level of alcohol consumption. The effect of caffeine was studied under free choice unlimited access conditions, a paradigm that allows self-regulation of alcohol intake and allows the appearance of individual differences. This access condition was also used to explore the effect of caffeine on alcohol reinstatement after deprivation. In addition, we assessed the impact of caffeine in a rodent model of binge drinking (i.e., "drinking in the dark") in a restricted access condition. In order to explore the effects of adenosine receptor antagonists on ethanol intake, a broad range of doses of the selective adenosine receptor antagonists MSX-3 (A2A antagonist) and CPT (A1 antagonist), separately or in combination, were acutely administered. Theophylline, another methylxanthine that also is a metabolite of caffeine and has an analogous mechanism of action, was also assessed. Moreover, to account for possible non-specific effects of caffeine on palatability or calorie intake, sucrose consumption was also evaluated.

Materials and Methods

Subjects

Male C57BL/6JRccHsd mice (15-20 g) were purchased from Harlan Laboratories (Barcelona, Spain). Mice (N=148) were 4 weeks old upon arrival to the laboratory. They were group housed until the ethanol drinking procedures started, and then they were individually housed for the rest of the experiment with standard lab chow *ad libitum*. After 7 days of acclimatization to the colony, animals started the drinking procedures. The colony was maintained at 22 ± 1ºC, with humidity control and 12-h light/dark cycles.

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.

Pharmacological agents

Ethanol (Panreac Quimica S.A., Spain) was diluted to 10% (v/v) in tap water and sucrose (Sigma-Aldrich, S.A., Spain) was diluted to 5% (w/v) in tap water. The non-selective adenosine receptor antagonists caffeine and theophylline (Sigma-Aldrich, S.A., Spain) were dissolved in 0.9% w/v saline (final pH 7.4) and administered 30 minutes before testing. A saline solution was used as its vehicle control. The adenosine A_1 selective receptor antagonist CPT (8-cyclopentyltheophylline) (Sigma-Aldrich, S.A., Spain) was dissolved in distilled water (final pH 8.0) and administered 20 minutes before testing. The adenosine A_{2A} selective receptor antagonist MSX-3 ((*E*)-phosphoric

acid mono-[3-[8-[2-(3-methoxphenyl)vinyl]-7-methyl-2,6-dioxo-1-prop-2-ynyl-1,2,6,7-tetrahydropurin-3-yl]propyl] ester disodium salt) was dissolved in saline (final pH 5.5) and administered 20 minutes before testing. MSX-3 was synthesized at the laboratory of Dr. Christa Müller (Universität Bonn, in Bonn, Germany). Doses were selected based on previous studies (López-Cruz et al., 2014; Pardo et al., 2012; 2013b).

Apparatus and testing procedures

Baseline ethanol intake prior to the adenosine-related pharmacological manipulations lasted 6 weeks. Ethanol (10% v/v) was available in 10.0 ml graduated cylinders with sipper tubes. After baseline was established and two weeks before the test phase started, animals were habituated to receiving intraperitoneally (IP) saline injections once a week. During the test phase, once a week, each subject received all doses, including vehicle, in a randomly varied order.

Restricted access to one bottle in the dark cycle. We used a “drinking in the dark” (DID) procedure modified from the original (Rhodes et al., 2005). Beginning 3 h into the dark cycle, singly housed mice had their water bottles replaced by a 10 ml graduated cylinder with sipper tube containing alcohol (or sucrose depending on the experiment) for 2 h. During this period, the only fluid available was the test fluid. Animals were habituated to ethanol solutions during 4 days with free access (24 hours) to two drinking bottles; one with tap water and the other one with ethanol (two days with 2% v/v and two days with 5% v/v). After this habituation period, and for the rest of the experiment, during the 2 hours test animals had access to ethanol only, 5 days a week, starting 3 hours after beginning of the dark cycle.

Unrestricted access condition to two bottles. Animals were habituated to ethanol solutions during 4 days with free access (24 hours) to two drinking bottles; one with tap water and the other one with ethanol (two days with 2% v/v and two days with 5% v/v). After these 4 habituation days and for the rest of the experiment animals had 24 hours access to both solutions; water and ethanol (10% v/v). The position of the two bottles was alternated to prevent a placement effect. Ethanol intake data were collected after drug injection for the first 2 hours of the dark cycle and also after 24 hours. The same basic procedure was performed for the sucrose experiment although no habituation period was required.

Intermittent alcohol deprivation after unlimited access condition. For this procedure, an independent group of animals was asigned to the unlimited access condition. The last 3 days before ethanol was removed, baseline intake was registered for the first 2 hours of the dark cycle and also for 24 hours. After the last day of baseline, the first cycle of 4 deprivation days was introduced. The 5th day, caffeine was administered 30 minutes before mice had access again to ethanol for 4 consecutive days. These cycles of intake and deprivation were repeated two more times.

Western blotting. Mice from experiment 5 were used for the western blotting experiment. Saline or caffeine (10.0 mg/kg) were administered to mice and animals had access to ethanol and water during 2 hours, after which mice were deeply anaesthetized with CO2, and when the absence of reflexes was observed the animals were exposed to cervical dislocation and brains were removed. Striatal tissue samples were homogenized in ice cold lysis buffer [137mM NaCl, 20mM Tris-HCl (pH 8.8), 1% NP40, 10μg/ml of aprotinin, leupetin, 0.5mM orto sodium vanadate and 0.1mM PMSF, protease inhibitors]. Homogenates were centrifuged at 13.000 rpm for 15 minutes at 4°C.

Aliquots of supernatants were collected and used for Bradford quantification of total protein and others stored at -80ºC until analyses. Every sample was boiled for 5 minutes. Equal amounts (15.0 µg) of striatal protein samples were separated by 12.5% SDS-PAGE and transferred to nitrocellulose membrane for 90 minutes at 30 volts. Membranes were block with 5.0 % Bovine Serum Albumin (BSA) in TBS-Tween 0.1% for one hour and later incubated with polyclonal rabbit anti- DARPP32 (1:1000, Cell Signalling) and DARPP32-Thr75 (1:500, Cell Signalling) overnight at 4°C. After rinses with TBST 0.1%, membranes reacted with goat anti-rabbit peroxidase conjugated secondary antibody and developed by enhanced chemiluminescence (1:40 ThermoScientific). Filters were probed with anti-Actin monoclonal antibody (1:500; Abcam) as an internal standard for protein quantification. The membranes were scanned (ImageQuant LAS400) and levels of the band density were blind processed and quantified by densitometry with ImageJ software.

Data analyses

Experiments used a within-groups design, in which each animal received all drug doses in a randomly varied order. Normally distributed and homogenous data were evaluated by repeated measures analysis of variance (ANOVA). Further analyses were conducted by nonorthogonal planned comparisons using the overall error term to assess differences between each dose and the control condition (Keppel, 1991). All data were expressed as mean ± SEM, and significance was set at $p<0.05$. STATISTICA 7 software was used.

Results

Experiment 1. Effect of caffeine on sucrose or ethanol intake under restricted access conditions. This experiment (Fig 1C-D) studied the impact of caffeine on the volume of voluntary ethanol or sucrose intake during 2 hours in the dark cycle, an experimental setting that generates high levels of ethanol consumption. A group of animals consumed sucrose (n=11) and a different group consumed ethanol (n=15). Repeated measures ANOVA revealed a significant effect of caffeine on ethanol intake (in ml) ($F(4,56)=9.34$; $p<0.01$) as well as on ethanol intake (g/kg) ($F(4,56)=8.03$; $p<0.01$) (Fig 1A-B). Planned comparisons revealed a significant difference between vehicle and the highest dose of caffeine (20.0 mg/kg) ($p<0.01$) on both variables. However repeated measures ANOVA did not demonstrate a significant effect of caffeine on volume of sucrose intake (ml) ($F(4,40) = 1.73$; n.s.) nor on grams of sucrose consumed (g/kg)(($F(4,40)=1.73$; n.s.).

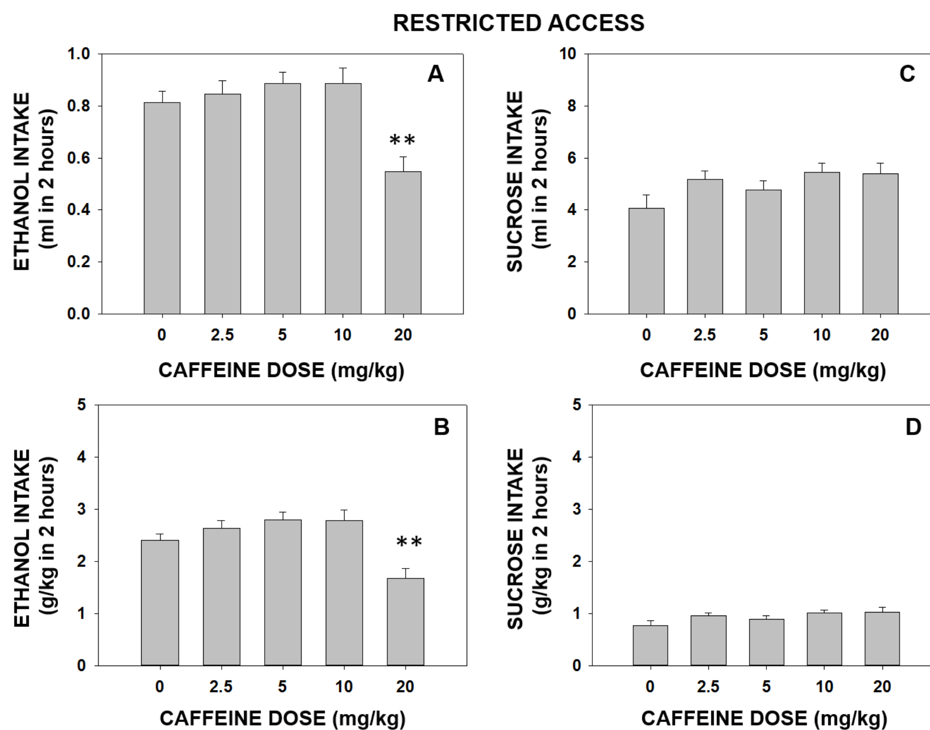

Fig 1. Effect of caffeine on ethanol (A-B) or on sucrose intake (C-D) in a 2 hours restricted access paradigm. Mean ± S.E.M. milliliters or grams of ethanol consumed per kilogram of body weight. **p<0.01 significantly different from vehicle.

Experiment 2. Effect of caffeine and other adenosine antagonists on ethanol intake under restricted access conditions. This study was conducted to determine if ethanol intake would be reduced after the administration of another methylxanthine, theophylline (0, 10.0 and 20.0 mg/kg) (n=15), or after the administration of the selective A_1 adenosine receptor antagonist CPT (0, 3.0, 6.0 and 9.0 mg/kg) (n=7), or the A_{2A} adenosine antagonist MSX-3 (0, 3.0, 6.0 and 9.0 mg/kg) (n=8), or finally the combination of both CPT (0, 3.0, 6.0 or 9.0 mg/kg) plus the same dose of MSX-3 (0, 3.0, 6.0 or 9.0 mg/kg) (n=15). Repeated measures ANOVA showed that there was a significant effect of theophylline treatment on ethanol intake ($F(2,28)=4.20$; $p<0.05$) (Fig 2A), and planned comparisons revealed that, as it was the case with caffeine, the highest dose of theophylline (20.0 mg/kg) showed a significant decline on ethanol intake ($p<0.05$) compared to vehicle. However, repeated measures ANOVA for the A_1 antagonist CPT ($F(3,18)=0.18$; n.s.)(Fig 2B), and for the A_{2A} antagonist MSX-3 ($F(3,21)=1.03$; n.s.)(Fig 2C) alone, did not show a significant effect. The combination of both selective adenosine antagonists (CPT + MSX3) showed no significant effect either ($F(3,11)=0.27$; n.s.) (Fig. 2D).

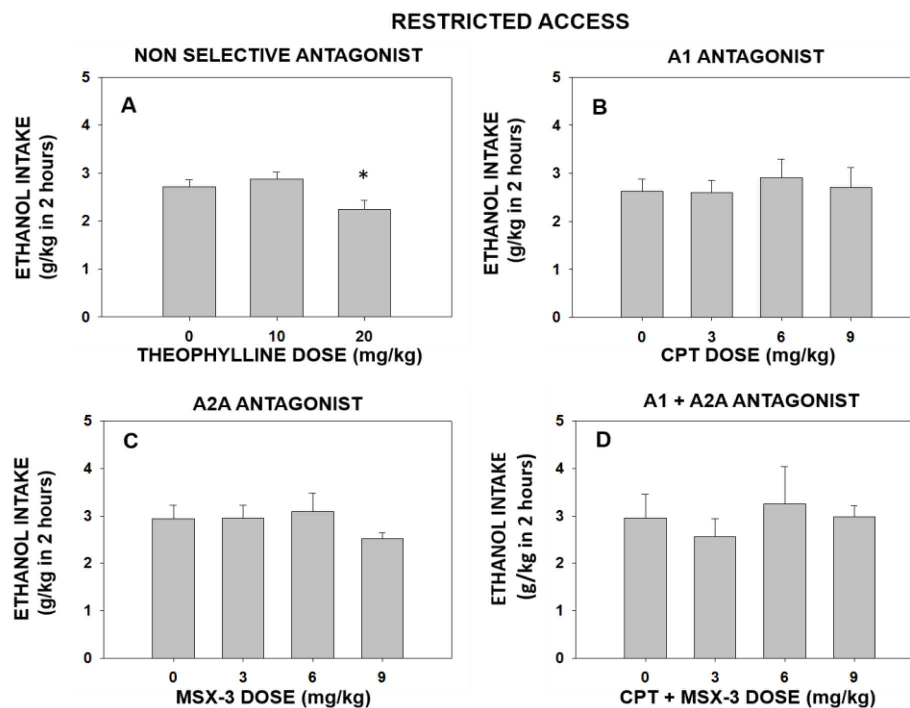

Fig 2. Effect of theophylline (A), CPT (B), MSX-3 (C) and CPT+MSX-3 (D) on ethanol intake in a 2 h restricted access paradigm. Mean ± S.E.M. g/kg of ethanol consumed. *p<0.05 significantly different from vehicle.

Experiment 3. Effect of caffeine on ethanol, sucrose or water intake during the first two hours of the unrestricted access condition. Repeated measures ANOVA showed that there was a significant effect of caffeine on ethanol intake (ml) during the first 2 hours of the dark cycle ($F(4,56)=2.82$; $p<0.05$). Planned comparisons revealed a significant difference between vehicle and the three higher doses of caffeine (5.0 mg/kg, $p<0.05$; 10 and 20.0 mg/kg, $p<0.01$). However, there was not a significant effect of caffeine on water intake during these two hours ($F(4,56)=1.44$; n.s.). The effect of caffeine on ethanol or water intake is shown in figures 3A and 3B (n=15). Repeated measures ANOVA revealed a significant effect of caffeine on sucrose intake (ml) during the first 2 hours of the dark cycle ($F(4,48)=7.95$; $p<0.01$). Planned comparisons

revealed a significant difference between vehicle and the three higher doses of caffeine (5.0 and 10.0 mg/kg, $p<0.05$; 20.0 mg/kg, $p<0.01$). Repeated measures ANOVA did not demonstrate a significant effect of caffeine on water intake ($F(4,48)=0.31$; n.s.). The effect of caffeine on sucrose or water intake is shown in figures 3C and 3D (n=13).

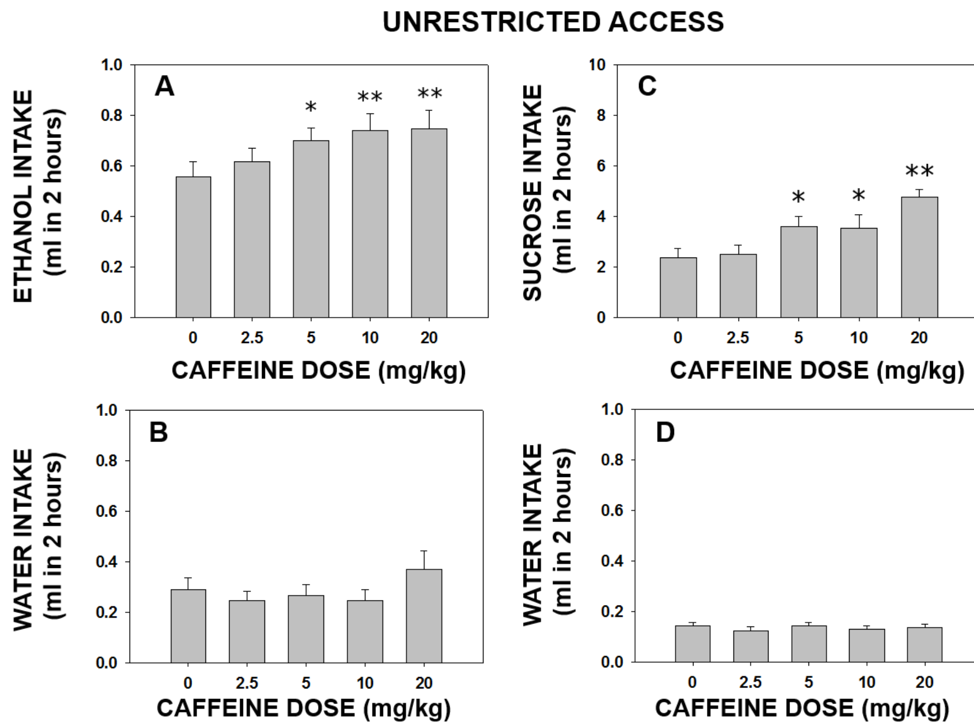

Fig. 3. Effect of caffeine on ethanol or water intake (A-B), or on sucrose or water intake (C-D), during the first 2 hours of the dark cycle under non-restricted 24 hours access conditions. Mean ± S.E.M. ml of fluid consumed. *$p<0.05$, **$p<0.01$ significantly different from vehicle.

Experiment 4. Effect of different adenosine antagonists on ethanol intake under unrestricted access condition: Analyses of the first 2 hours. Repeated measures ANOVA showed that there was a significant effect of caffeine on ethanol intake (g/kg) ($F(4,56)=2.75$; $p<0.05$) (Fig 4A, n=15). Planned comparisons revealed a significant difference between vehicle and 5.0, 10.0 and 20.0 mg/kg of caffeine ($p<0.05$ for the first one, and $p<0.01$ for the two last doses) on ethanol intake. Because caffeine acts as a non-selective antagonist of A_1 and A_{2A} receptors, we studied the impact of another non-selective adenosine antagonist; theophylline, and of CPT, a selective A_1 antagonist, and of MSX3, a selective A_{2A} adenosine receptor antagonist on animals with unlimited access to ethanol and water during 24 hours. The results for theophylline (n=10) analyzed with a repeated measures ANOVA showed no significant effect in the two first hours of ethanol access ($F(3,27)=0.17$; n.s.) (Fig. 4B). The repeated measures ANOVA for the CPT results (n=9) showed no significant effect ($F(3,24)=0.43$; n.s.) of this drug (Fig. 4C). However, the repeated measures ANOVA for the selective A_{2A} adenosine receptor antagonist MSX-3 (n=10) showed a significant effect ($F(3,27)=3.60$; $p<0.05$) (Fig 4D). The higher dose of MSX-3 (9.0 mg/kg) was significantly different from vehicle ($p<0.01$).

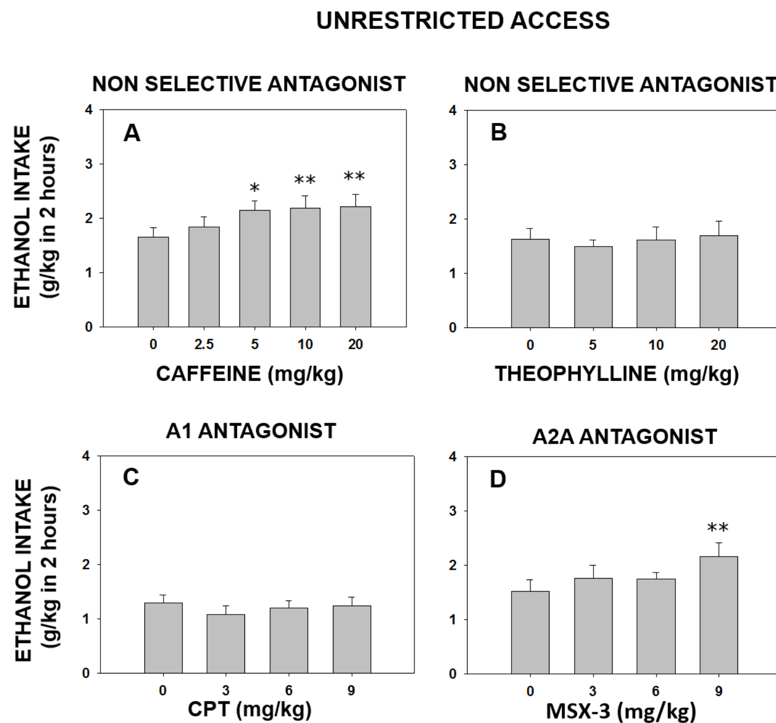

Fig. 4. Effect of caffeine (A), theophylline (B), CPT (C) and MSX-3 (D) on ethanol intake during the first 2 hours of the dark cycle in a 24 hours access condition. Mean ± S.E.M. g/kg of ethanol consumed. *p<0.05, **p<0.01 significantly different from vehicle.

Experiment 5. Impact of caffeine on ethanol intake under unlimited access conditions: role of individual differences during 24 hours of access.

Because the lapse of time in the unrestricted access condition is wider than the other time access conditions, it produced high variability on baseline ethanol intake among different mice. In order to see if caffeine modulated ethanol intake depending on the level of consumption, data were reanalyzed taking baseline levels of ethanol intake into account. Animals (n=30) were divided in quartiles (see figure 5) based on baseline ethanol intake, and 3 groups were stablished: low consumers (Q1 mean=5.66 ± 0.68 g/kg), moderate consumers (Q2+3 mean=12.40± 1.50 g/kg), and high consumers (Q4

mean=16.32 $\pm$ 0.41 g/kg). The one way ANOVA revealed an overall significant difference between groups on ethanol baseline intake levels ($F(2,27)=96.504$; $p<0.05$).

The repeated measures ANOVA showed a significant effect of caffeine on ethanol intake (g/kg) under unlimited access conditions ($F(4,116)=3.18$; $p<0.01$) (Fig. 6A). Caffeine, at several doses, was significantly different from vehicle (2.5 and 10.0 mg/kg, $p<0.05$; and 5 mg/kg, $p<0.01$). When analyzing the 3 baseline groups the repeated measures ANOVA for the low consumers did not show a significant effect of caffeine on ethanol intake ($F(4,28)=0.60$; n.s), and the same was true for the high consumers group ($F(4,28)=1.29$; n.s.). However, a very different pattern of results was revealed for the moderate group. Repeated measures ANOVA showed that there was a significant effect of caffeine ($F(4,52)=3.00$; $p<0.05$), and planned comparisons revealed that all doses increased significantly ethanol intake (2.5 and 20.0 mg/kg $p<0.05$; 5.0 and 10.0 mg/kg, $p<0.01$) (Fig 6B-D).

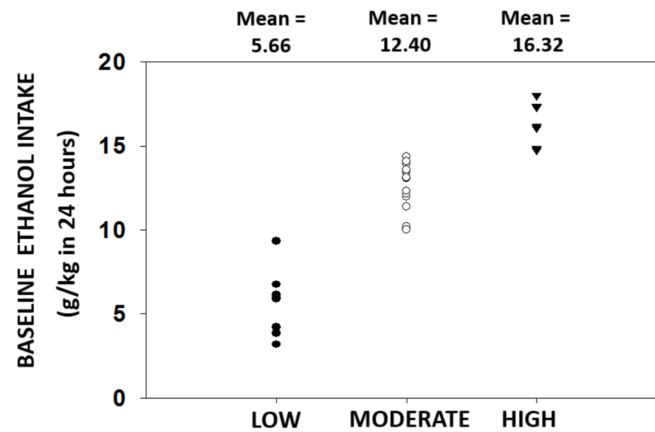

Fig. 5. Individual average scores during 3 days of baseline for ethanol intake (grams of ethanol consumed per kilogram of body weight) under unrestricted access conditions. Mice were divided in three groups (low, moderate and high ethanol consumers) using quartiles.

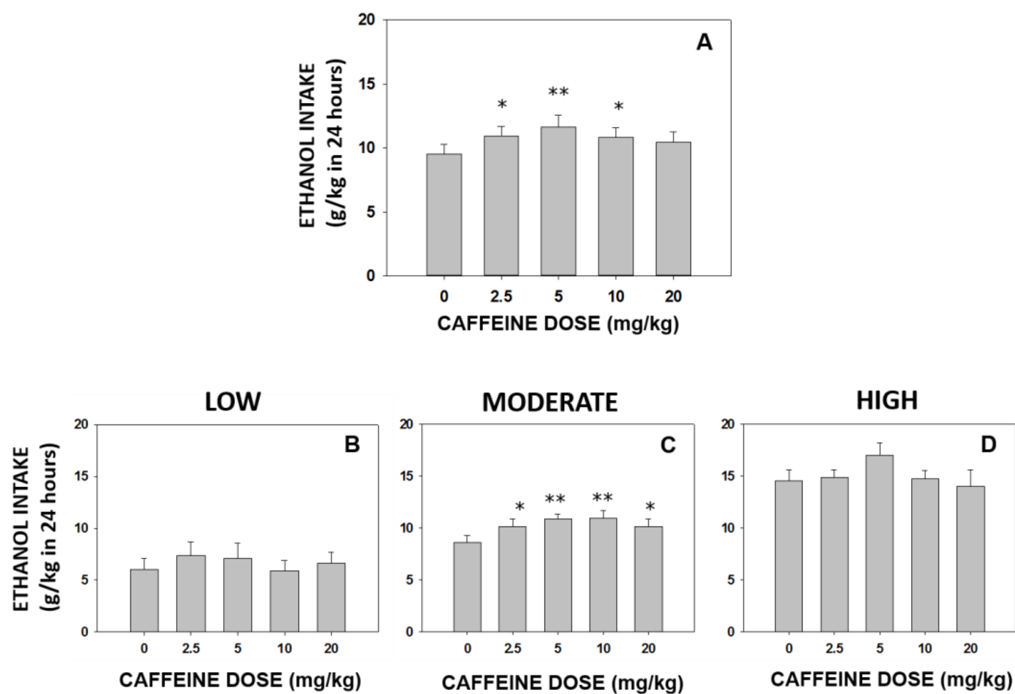

Fig. 6. Effect of caffeine on voluntary ethanol intake in C57BL/6J mice under unrestricted access conditions. (A) Ethanol intake in the entire group, (B) low, (C) moderate, and (D) high subgroups of ethanol drinkers. Mean ±S.E.M. grams of ethanol consumed per kilogram of body weight. *p<0.05, **p<0.01 significantly different from vehicle.

Experiment 6. Effect of caffeine on DARPP-32 and pDARPP-32(Thr75) levels in striatum of animals drinking ethanol during 24 hours. For this study we collected the striatal tissue samples from animals in the middle and high groups (n=13). Independent t-test for treatment groups did not show statistical differences between vehicle and caffeine 10.0 mg/kg treatment on DA/adenosine receptor metabotropic activity: DARPP-32 ($t(11)=0.30$; n.s.), and pDARPP-Thr75 ($t(10)=-0.32$; n.s.), (Fig. 7).

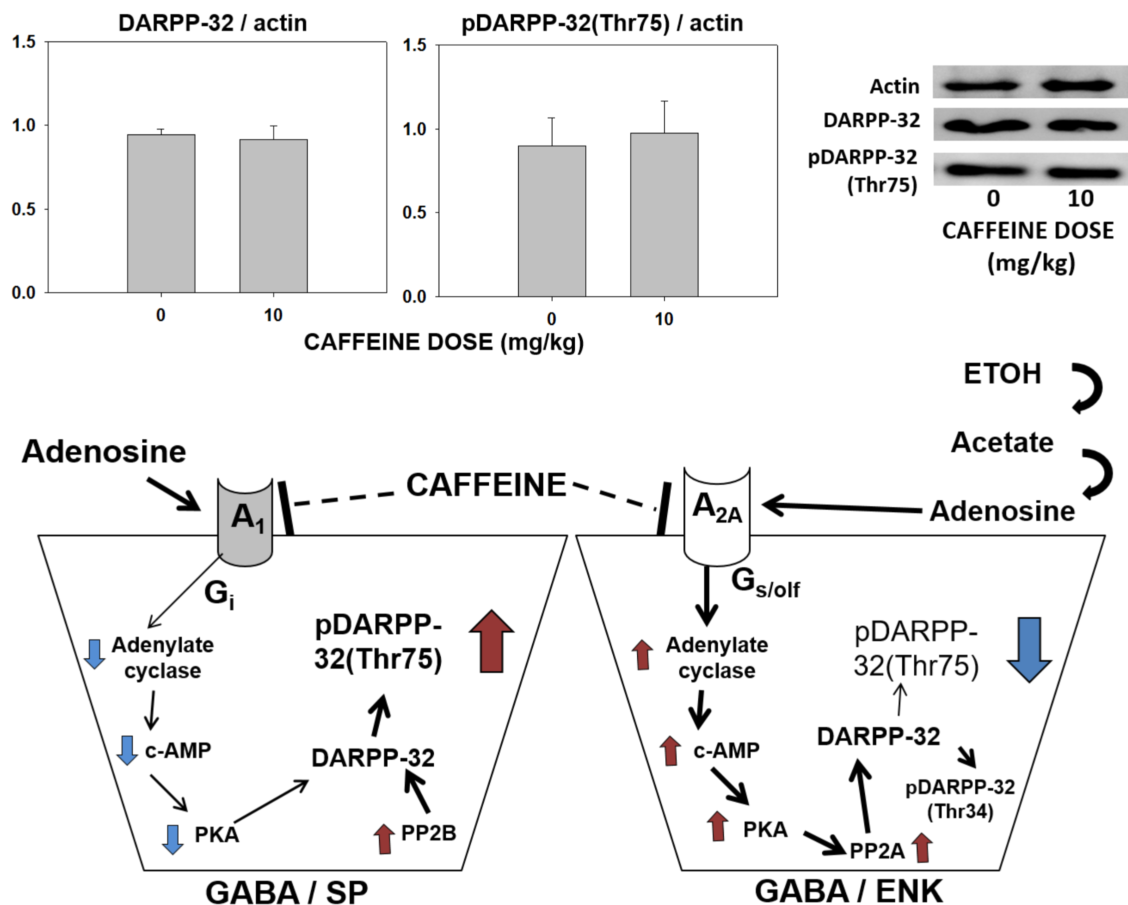

Fig. 7. Left upper part: effect of an acute administration of saline or caffeine (10.0 mg/kg) to mice drinking ethanol under unrestricted access conditions. Data are expressed as mean (±SEM) of density units of DARPP-32 (A) and pDARPP-32(Thr75) (B). Right upper part: representative western immunoblots of DARPP-32, pDARPP-32(Thr75) or the actine control, in the striatum of one saline- or one caffeine-treated mice. Lower part: Diagram showing effect of adenosine

synthesis and actions on adenosine A_1 and A_{2A} receptors in different populations of striatal output neurons on DARPP-32 phosphorylation patterns. Caffeine would produce opposite effects on this signal transduction markers.

Experiment 7. Effect of caffeine on ethanol reinstatement. In order to see if caffeine had any impact on ethanol intake during repeated episodes of withdrawal and reintroduction (see figure 8), saline or caffeine (5.0 mg/kg) were administered in different groups of mice (n=20) on the first day of every reintroduction cycle, and ethanol consumption was evaluated during 24 hours. Animals with moderate levels of ethanol consumption were selected. Average baseline (BL) intake was similar for both groups. The two-way ANOVA (time x treatment) for ethanol intake during first two hours revealed a significant effect of time ($F(12,216)=2.76$; $p<0.01$), but no significant effect of caffeine treatment ($F(1,18)=0.47$; n.s.), and no interaction ($F(4,72)=1.216$; n.s.) (Fig. 9A).

However, the two-way factorial ANOVA (time vs caffeine dose) for ethanol intake during 24 hours after the injection showed a significant effect of time ($F(12,216)=2.43$; $p<0.01$), no significant effect of treatment ($F(1,18)=0.02$; n.s.), but a significant interaction ($F(12,216)=2.48$; $p<0.01$). Planned comparisons between the corresponding BL group and the reintroduction day showed a significant change in ethanol intake in the group that received caffeine. The group treated with caffeine showed significant differences from its own baseline on the first and second day of the second ethanol reinstatement ($p<0.01$, $p<0.05$ respectively) and on the first, second, third and fourth days of the third ethanol reinstatement cycle ($p<0.01$ for the first two days; and $p<0.05$ for the second first days). Within the saline group there was a significant difference

between baseline levels of ethanol intake and the first day of the second ethanol reinstatement cycle ($p<0.05$) (Fig. 9B).

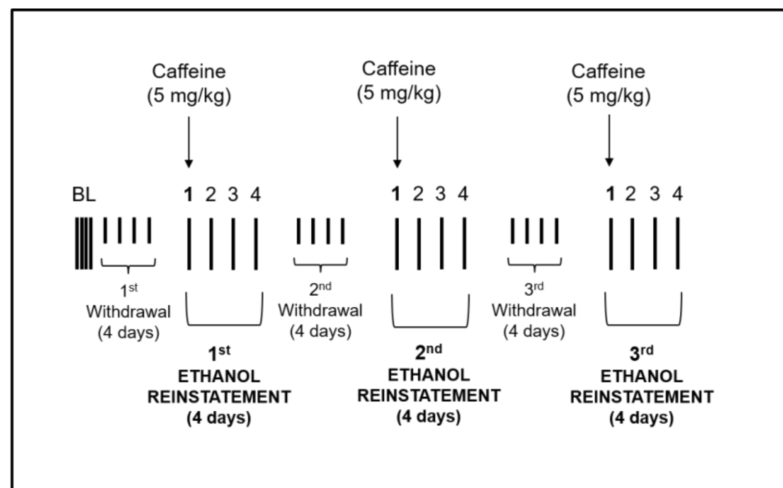

Fig. 8. Schematic diagram of the ethanol reinstatement procedure.

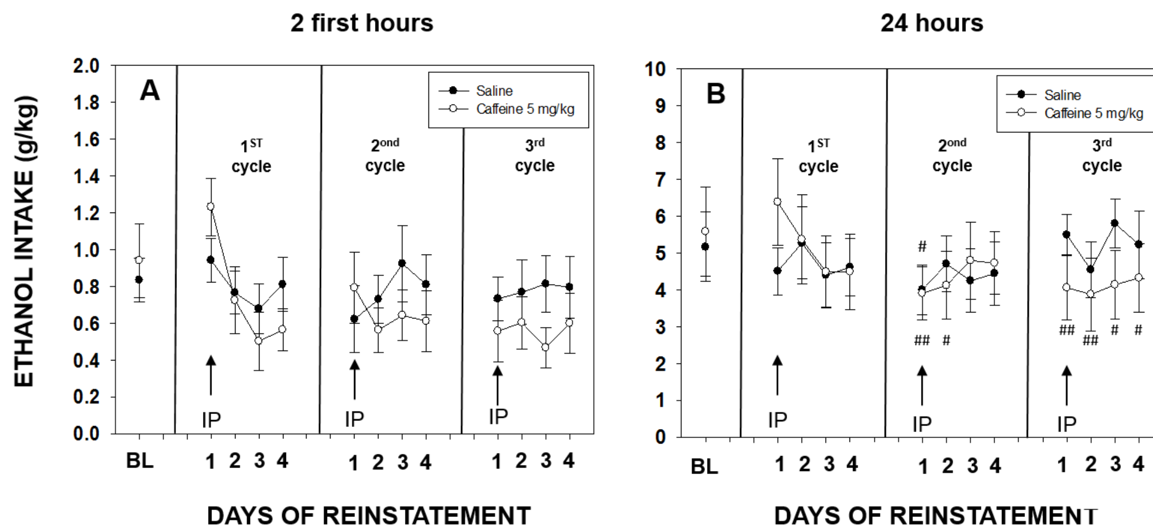

Fig. 9. Effect of caffeine on voluntary ethanol intake in the first 2 hours of the dark cycle (A), or after 24 hours (B) during repeated cycles of 4 days of ethanol removal and reintroduction. Mean ± S.E.M. grams of ethanol consumed per kilogram of body weight. #$p<0.05$, ##$p<0.01$ significantly different from baseline (BL) in each treatment group.

Discussion

In the present study, we investigated the effect of caffeine on voluntary ethanol intake under different parameters of access: restricted, unlimited, or unlimited after intermittent deprivation, in the alcohol preferring C57BL/6J strain of mice. The restricted access procedure facilitates pharmacologically relevant drinking, taking advantage of the innate tendency of rodents to consume higher levels of food and drinking solutions during the dark phase of the circadian cycle. Hence, it is thought to represent a model of binge drinking in rodents (Rhodes et al., 2005). Consistent with previous research (Dietze and Kulkosky, 1991), the highest dose of caffeine administered in the present study (20.0 mg/kg) decreased voluntary alcohol consumption. In our study, 20.0 mg/kg of caffeine did not affect sucrose intake, although in a pilot study with 40.0 mg/kg, caffeine did reduced both ethanol as well as sucrose intake. This non-specific reduction of sucrose intake was seen also with a dose of 50.0 mg/kg in rats (Dietze and Kulkosky, 1991).

While the DID paradigm explores high levels of ethanol consumption over a short period of time, when trying to model a binge-drinking situation, other procedures such as the unlimited 24 hours access can give information about how animals regulate ethanol intake when other solutions are present, and can give information about individual differences in this regulation. Thus, the effect of caffeine was also studied when animals had no time restriction. Caffeine dose dependently (5.0-20.0 mg/kg) increased ethanol intake during the first two hours of unlimited access, and this effect was also seen in sucrose intake. Thus, it seems that caffeine can have opposite effects on ethanol and sucrose intake depending on the access conditions; when animals drink high volumes of ethanol (around 0.8 ml) or sucrose (around 4.0 ml) because it is the only time and the only solution available (in the DID procedure), caffeine did not

change or tended to reduce consumption. However, when ethanol (or in other groups sucrose) is constantly present and they have water as an alternative drinking solution, they drink less in two hours (around 0.5 ml of ethanol and 2 ml of sucrose) and caffeine can increase the amount of volume that animals drink.

The studies involving additional adenosine receptor antagonists indicate that theophylline (another methylxanthine, with A1 and A2A adenosine receptor activity) also decreased voluntary ethanol intake at the highest dose used (20.0 mg/kg) in the DID procedure, but had no impact under unrestricted access conditions. The high dose of theophylline also did not produce any effect on sucrose consumption in the DID test (data not shown). Previous research has shown that subchronic administration of low doses of theophylline to rats increased ethanol consumption delivered in a liquid diet that was constantly accessible concurrently with water (Gatch and Selvig, 2002). The seeming lack of consistency among those results and the present study is probably due to the use of very different methodologies. Additionally, because pharmacological actions of caffeine are attributable to its activity as a non-selective A1 and A2A adenosine receptor antagonist (Fredholm, et al., 1999), the effect of selective adenosine antagonists on voluntary ethanol intake under restricted access conditions was also evaluated. However, in the DID experiment, administration of CPT, an A1 adenosine receptor antagonist, or MSX-3, an A2A adenosine receptor antagonist, did not exhibit any effect on ethanol intake, either separately nor in combination. The lack of effect of MSX-3 in the DID paradigm is consistent with a recent study (Fritz and Boehm, 2015). However, in the unrestricted access condition the A2A antagonist increased ethanol intake at the highest dose. A2A antagonists have been clearly implicated in modulating ethanol intake. Thus, data from free (non operant) access to ethanol indicate that A2AKO mice tested in a 48 hours access two-bottle choice task display higher ethanol

intake than WT mice (Naassila et al., 2002). Similarly, acute and subchronic administration of the A2A receptor antagonist ANR94 increased ethanol intake in alcohol-preferring rats in a 2 hours two-bottle choice paradigm (Micioni Di Bonaventura et al., 2012), and C57BL/6J mice can be considered an ethanol preferring strain. Selective adenosine A1 receptor antagonists such as DPCPX have been demonstrated to reduce ethanol intake in animals tested on a DID procedure (Fritz and Boehm, 2015). It is possible that we did not find an effect of CPT in the present results because CPT is not as selective for A1 as DPCPX. In an operant study, subthreshold doses of DPCPX given in combination with the adenosine A2A receptor antagonist SCH 58261 had no effect on alcohol responding in alcohol-preferring rats trained to self-administer alcohol under operant conditions FR3 (Adams et al., 2008). The present experiment used a range of combined doses that varied from low to quite high (e.g. 9.0 mg/kg of CPT plus 9.0 mg/kg of MSX-3) and still we did not see a change on ethanol consumption. In summary, the effect of selective A1 and A2A antagonists on voluntary ethanol intake seems to indicate that the increase observed after caffeine administration could be mediated by A2A receptors under unrestricted access conditions.

Additionally, we decided to analyze our results taking into account differences in basal ethanol intake. When taking into account all the animals, caffeine had a biphasic effect on ethanol intake. Using the two extreme quartiles as the statistical criteria to divide animals into high, moderate and low ethanol consumers, we did not find any impact of caffeine among the low or high consumers, but there was a significant increase in ethanol consumption after all doses of caffeine administration (2.5, 5.0, 10.0 and 20.0 mg/kg) in the intermediate intake group. In summary, animals that consume moderate levels of ethanol seem to be more affected by caffeine. It is possible that low consuming animals do not increase ethanol consumption because of some side effect

such as anxiogenic actions of caffeine (further studies should address that possibility), and probably caffeine could not increase intake among the high consumers due to a ceiling effect.

Adenosine neurotransmission is a direct mechanistic link between caffeine and alcohol (Ferrè and O'Brien, 2011; Ruby et al., 2010; López-Cruz et al., 2014). For instance, acute caffeine administration can reverse some of the biphasic effects of ethanol on locomotion (Waldeck, 1974; Koo, 1999). Because adenosine receptors, especially A2A receptors, are highly expressed in Nacb where they modulate DA-related signal transduction, we analyzed the impact of caffeine on DARPP32, an intracellular marker of DA/adenosine receptor activation (Nunes et al., 2013). Compared to saline-treated mice, caffeine did not produce a change in DARPP32 or pDARPP-Thr75, among mice consuming moderate to high levels of ethanol in experiment 5. Previous studies have shown that caffeine increases pDARPP-Thr75 (Lindskog et al., 2002; Hsu et al., 2009). However, the lack of effect on DARPP expression in the present studies could be due to the fact that in our study all animals were drinking ethanol. Ethanol metabolism has been shown to increase adenosine levels (for a review see López-Cruz et al., 2014), which is the endogenous agonist for adenosine receptors, thus having opposite effects on the intracellular cascade to caffeine (see figure 5).

A predominant feature in human alcohol abuse is the reported desire or "craving" to consume ethanol along with frequent episodes of drinking after periods of abstinence. These and other factors may be responsible for relapse to uncontrolled ethanol drinking (Heyser et al., 1997). When relapse occurs after a period of abstinence, ethanol drinking temporarily increases, a phenomenon known as the "alcohol deprivation effect" (Sinclair 1979; Heyser et al., 1997). In the present study we examine the impact of an

acute caffeine injection after several cycles of forced time-off on ethanol reinstatement in an unrestricted access paradigm. In a previous study, chronic caffeine did not increase ethanol consumption in rats exposed to a free-choice procedure, however, it did prevent the alcohol deprivation effect (Carvalho et al., 2012). The present results show that after the second time-off cycle, caffeine (5.0 mg/kg) reduced ethanol consumption compared to BL, even during days when caffeine was not administered. Thus, the impact of caffeine emerged after repeated cycles. Our results do not show the alcohol deprivation effect after 4 days of ethanol removal, probably due to the fact that our animals did not show signs of dependence during withdrawal because their levels of intake were low to moderate. Thus, consistent with previous data (Carvalho et al., 2012), our data show that caffeine administered after ethanol removal can prevent ethanol reinstatement. Chronic ethanol exposure and withdrawal has been shown to change adenosine A1 receptor density (Concas et al., 1996). Further research should address if reduction of ethanol intake in caffeine treated mice may be mediated by these receptors.

In summary, the present results show that when ethanol consumption is very high (in the binge drinking paradigm and among the high consumers in the free access paradigm) caffeine does not increase ethanol consumption, and even tends to reduce it. In addition, among low consumers, caffeine does not increase ethanol intake either even after repeated cycles of removal and reintroduction. Moreover caffeine reduces baseline ethanol consumption. Thus, only among moderate consumers that have free access to ethanol and have other sources of fluid (water), caffeine does increase ethanol consumption, and that effect seems to be mediated by adenosine A2A receptors. This result may be particularly important because this last group can be considered to represent the majority of consumers.

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CHAPTER 4.

INVOLVEMENT OF ADENOSINE AND DOPAMINE ON ETHANOL CONSUMPTION IN RATS: FREE VERSUS WORK-MEDIATED ACCESS.

Abstract

Nucleus accumbens dopamine (DA) plays a critical role in behavioral activation and effort-based decision-making. DA depletions reduce the level of effort that an animal is willing to make to obtain a natural reinforcer, but it does not change preference between freely available reinforcers. Adenosine A_1/A_{2A} receptors are colocalized and modulate the same intracellular cascade that DA D_1/D_2 receptors but in an opposite way. Caffeine (A_1/A_{2A} antagonist) has been demonstrated to reverse the impact of DA antagonists on effort related tasks. In the present studies, Tetrabenazine (TBZ), a VMAT-2 inhibitor that produces a reversible DA depletion, bupropion (which increases DA levels by blocking DAT), and caffeine were administered to male Wistar rats self-administering ethanol under conditions of low effort (freely available ethanol solution) or high effort demands (lever pressing on a fixed ratio 5, FR5). TBZ decreased lever pressing and ethanol consumption in the FR5 task. However, TBZ-treated animals approached and consumed the same amount of freely available ethanol as controls. Caffeine did not change free ethanol consumption either, but increased FR5-mediated alcohol consumption. Bupropion did not change ethanol consumption in either access condition. Bupropion and caffeine reversed TBZ-suppresion of operant responding for ethanol. A marker of DA/adenosine receptor activation pDARPP32-Thr75 immunoreactivity increased significantly in NAcb Core and Shell, DMS and DLS after administration of TBZ, and bupropion or caffeine significantly reversed this TBZ-effect in all striatal regions. These results indicate that DA modulates activational aspects of motivation related to instrumental responding for ethanol, but not consumption itself, and ventral as well as dorsal areas of striatum seem to be involved in this actions.

Keywords: antidepressant, alcohol intake, effort, bupropion, caffeine.

Introduction

Effort-based processes in animals (Salamone et al., 1997, 2007; Walton et al., 2003; Cagniard et al., 2006; Floresco and Ghods-Sharifi, 2007; Mingote et al., 2008; Hauber and Sommer, 2009; Salamone and Correa, 2012; Nunes et al., 2013; Pasquereau and Turner, 2013) and humans (Croxson et al., 2009; Kurniawan et al., 2010; Wardle et al., 2011; Treadway et al., 2012) have been extensively charaterized. Nucleus accumbens (Nacb) dopamine (DA) is a critical component of the brain circuitry involved in behavioral activation and effort-related behavioral processes. DA antagonism or depletion reduces effort-based decission making for food and other natural reinforcers (Hauber and Sommer, 2009; Salamone and Correa, 2012; Nunes et al., 2013; Pereira et al., 2011).

Effort-based decision-making is studied with tasks offering choices between high effort options leading to highly valued reinforcers versus low effort/low reward options. It has been shown that in operant tasks, animals under control conditions tend to press the lever to obtain preferred food and that correlates with DA release and metabolism in Nacb (Church et al., 1987; Salamone et al., 1989; McCullough et al., 1993; Segovia et al., 2011). On the contrary, rats with Nacb DA depletions or DA receptor blockade reduce lever pressing for food and show alterations in response allocation (Salamone et al., 1991, 1997, 2003, 2005, 2006, 2007). Tetrabenazine (TBZ) a selective and reversible inhibitor of vesicular monoamine transporter-2 (VMAT-2) blocks storage and depletes monoamines, but its greatest impact is upon striatal DA (Pettibone et al., 1984; Tanra et al., 1995). TBZ is used to treat Huntington's disease, but depressive symptoms including fatigue are major side effects (Frank, 2009, 2010). Moreover, TBZ has frequently been used in studies involving animal models of depression (Preskorn et al.,

1984; Kent et al., 1986; Wang et al., 2010).

Striatal areas are rich not only in DA receptors, but also in adenosine A_1 and A_{2A} receptors (DeMet and Chicz-Demet, 2002; Ferré et al., 2004, 2008; Jarvis and Williams, 1989, Schiffmann et al., 1991). Adenosine A_{2A} receptors are colocalized with D_2 receptors and adenosine A_1 with D_1 receptors, and there is a functional and inverse effect between them; striatal dopamine D_2 and adenosine A_{2A} receptors (Ferré, 1997, 2008; Ferré et al., 1997, 2005; Fuxe et al., 2003). Several studies have focused in its relation in aspects of behavioral activation and effort-related processes (Font et al., 2008; Mingote et al., 2008; Mott et al., 2009; Wolden et al., 2009; Pereira et al., 2011; Pardo et al., 2012, 2015).

Epidemiology studies have shown that a positive correlation may exist between the consumption of caffeine and that of ethanol. Studies in animal models have not yield a consistent pattern of results. Acute caffeine administration has demonstrated to increase ethanol consumption in male rats using a limited access paradigm. A_{2A} adenosine receptors have been proposed as the target to modulate ethanol intake, although conclusions have been opposite in different studies. While ANR94, an A_{2A} antagonist, increases ethanol intake in a FR1 operant schedule (Micioni Di Bonaventura et al., 2012), other A_{2A} antagonist, SCH58261, decreased ethanol intake in a FR3 operant schedule (Adams et al., 2008). DMPX have also shown different results in the same study (Arolfo et al., 2004) decreasing ethanol intake in a FR1 operant schedule and showing a bimodal effect in a FR3 operant schedule.

The present set of experiments studied the effect of several pharmacological manipulations on ethanol self-administration under two different access conditions; free access (low effort), or operant fixed ratio 5 (FR5) task (high effort demand): the effect of the selective and reversible inhibitor of vesicular monoamine transporter-2 TBZ was

evaluated, as well as the non-selective adenosine antagonist caffeine and the antidepressant bupropion. Finally, the ability of these drugs to reverse the effects of TBZ was asssed. In addition, two validation experiments were conducted: the reinforcer devaluation provided by pre-feeding, and the effects of increasing palatability of the drinking solution. As a measure of the impact of these manipulation on metabotropic markers related to the functional interaction of DA and adenosine receptors, we also evaluated the phosphorilation of the DARPP-32 protein at the Thr75. Thus, immunohistochemistry was utilized to further investigate signal transduction activity in 4 specific regions of interest: NAcb Core, NAcb Shell, dorsomedial (DMS) and dorsolateral (DLS) striatum.

Materials and Methods

Animals

Adult male Wistar rats (Charles River, France) were housed in pairs in a colony maintained at 23+2 °C with 12-h light/dark cycles (lights on at 8:00 h). Rats (N=46) weighed 130 – 170 g at the beginning of the study. They were handle during three days and initially water restricted to 15 ml/day/rat before the first day of training. After that, they had access to 20 ml/day/rat of water to maintain a moderate level of water restriction throughout the study. Food was available ad libitum in the home cages. Despite water restriction, rats gained weight normally throughout the experiment. 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.

Pharmacological agents.

Drinking solutions. Ethanol (Panreac Quimica S.A., Spain) was diluted to 10% (v/v) in tap water. Sucrose (Sigma-Aldrich, S.A., Spain) was diluted to 2% (w/v) in tap water.

Tetrabenazine (Tocris Bioscience), the VMAT-2 inhibitor, was dissolved and sonicated in a vehicle solution of 0.9% saline (80%) and dimethylsulfoxide (DMSO; 20%) (pH=4.5). DMSO was used as its vehicle control. All doses were administered 120 minutes before testing. Caffeine (1,3,7-trimethylxanthine) (Sigma-Aldrich, S.A., Spain), a non-selective adenosine receptor antagonists, and bupropion (Alfa Aesar, Germany), a norepinephrine-dopamine uptake blocker, were dissolved in 0.9% saline. Caffeine and bupropion were administered 30 minutes before testing and saline solution was used as their vehicle control. All drugs were administered intraperitoneally (IP). Doses of tetrabenazine were based upon previous research (Nunes et al. 2013, Pardo, et al. 2015). The range of caffeine doses (2.5, 5.0, 10.0, and 20.0 mg/kg) was selected based on previous studies (Salamon et al., 2009) and pilot studies. Bupropion doses were selected based on previous papers (Nunes et al., 2013).

Apparatus and testing procedures.

Operant chambers (28 cm × 23 cm × 23 cm; Med Associates Inc., St. Albans, VT) were used for ethanol self-administration. The chambers were equipped with a lever that was located on the central side of one wall (2 cm above the floor). Lever pressing activated a liquid dispenser delivering a 0.1 ml of fluid to the drinking spout. The opposite wall had a stainless steel cubicle with a graduated tube which spout protruded 1.5 cm. Bottle approach was measured as the number of times that an animal closed the lickometer

circuit. All chambers were housed in sound-attenuated enclosure with exhaust fans that masked external noise. Electrical inputs/outputs of each chamber were controlled by an IBM compatible PC (Med-Associates software).

Ethanol self-administration procedure. After seven days in the laboratory, rats were subjected to 22 hours water restriction (15 ml/water for each rat) and to overnight access to the training solution to avoid neophobia for the first day of training. Ethanol self-administration procedure was acquired using a modification of the "sucrose-fading procedure" (Samson, 1986). The fixed ratio (FR) schedule of reinforcement increased progressively across days (FR1, FR2, FR3 with a 5.0 % sucrose combined with 5.0 % ethanol solution; FR4, FR5 with a 2.5% sucrose plus 7.5% ethanol solution). Finally, rats were trained in 30 min sessions to self-administer 0.1 ml of 10.0 % ethanol three days per week in alternating days (Monday, Wednesday and Friday). Training lasted six weeks more on this schedule (FR5) until stable baseline levels of lever pressing until drug testing began. Lever pressing is the contingent variable of the self-administration procedure and bottle approach, the non-contingent variable. Daily intake and body weight measurements were registered, and grams of ethanol per kilogram/body weight were calculated. Rats received supplemental water (20 ml/day/rat) in the home cage. For the pre-exposure experiment, animals had *ad libitum* access to 10.0 % ethanol and water for 24 hours in their home cage before the test session. For the sucrose experiment, animals received 2% sucrose solution instead of 10% ethanol during the test session.

Ethanol free-access procedure. Different group of rats were used in these experiments. Experiment procedures started after seven days of quarantine. Rats were subjected to 22 hours of water restriction and overnight access to the initial solution. Introduction to the different sucrose plus ethanol solutions was done in parallel to the lever pressing experiments (starting with a 5.0 % sucrose combined with 5.0 % ethanol

solution for 9 sessions 5 days/week; a 2.5% sucrose plus 7.5% ethanol solution for 5 sessions 5 days/week; and a 2.5% sucrose plus 7.5% ethanol solution for 12 sessions in alternating days). Finally, rats had access to 10.0 % ethanol, three days per week in alternating days (Monday, Wednesday and Friday) and training lasted six weeks more on these conditions until stable intake levels until drug testing began. At the end of the session, rats were immediately removed from the chamber, and ethanol intake was determined by measuring the remaining fluid in the tube. Daily intake and body weight measurements were taken, and grams of ethanol per kilogram body weight were calculated. Approach behavior to the spout was recorded also as the number of times that the circuit was closed while the animal was drinking. Lever pressing was the non-contingent variable of the free-access procedure and is measured as an unspecific measure.

DARPP-32 immunohistochemistry. Rats were anesthesized and perfused 30 minutes after receiving treatments. Brains were collected and stored in 3.7% formaldehyde solution during 24 h and refrigerated in sucrose (30%), sodiumazide (2%) and PB 0.1M solution prior to slicing. Free floating coronal sections (40 μm) were serially cut using a microtome cryostat (Weymouth, MA, USA), rinsed in 0.01 M PBS (pH 7.4) and incubated in 0.3% hydrogen peroxide for 30 minutes to block endogenous staining. Sections were then rinsed in 0.01 M phosphate buffer (PBS) (3 times × for 5 minutes). To measure the immunoreactivity to phosphorylated DA and c-AMP-regulated phosphoprotein 32 kDa (pDARPP-32), nonspecific binding sites were blocked, and cells were permeabilized in a solution containing 0.1% Triton X-100 (T.X), 1% Bovine Albumin serum (BSA) in PBS for 30 min 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 34 residue (pDARPP32-Thr34, 1:1000; Santa Cruz Biotechnology), or polyclonal rabbit antibody for pDARPP-32 phosphorylated at the threonine 75 residue (pDARPP32-Thr75, 1:500; Santa Cruz Biotechnology). These antibodies were dissolved in solutions that also contained 1% BSA and 0.1% T.X in PBS for 24 h (pDARPP32-Thr34) or 48 hours (pDARPP32-Thr75) 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 3,3diaminobenzidine chromagen (DAKO) Processed sections were then mounted to microscope slides (Menzel-Gläser, Superfrost ® Plus, Thermo scientific), air dried, and cover-slipped using Eukitt® (Sigma Aldrich) 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.

Statistics.

Within-groups design was used in all the experiments, in which each rat received all drug doses in their particular experiment in a randomly varied order (one treatment per week, with none of the treatment sequences repeated across different animals in the same experiment). Baseline (i.e., nondrug) sessions were conducted two additional days per week. The specific treatments and testing times for each experiment are listed

below. STATISTICA 7 software was used for statistical analysis of the data. All data were expressed as mean ±SEM, and significance was set at $p<0.05$.

RESULTS

Experiment 1. Effect of the DA depleting agent tetrabenazine on ethanol consumption under operant or free access conditions.

1.1. Effects of tetrabenazine on operant ethanol self-administration. On the test day, trained rats (N=7) received the following tetrabenazine doses: 0.0, 0.25, 0.5, 0.75 and 1.0 mg/kg (120 min before testing) (Fig 1A-C). The ANOVA for repeated measures indicated that tetrabenazine significantly reduced lever pressing ($F(4,20)=4.62$, $p<0.01$), as well as ethanol intake (g/kg) ($F(4, 20)=4.60$, $p<0.01$). As expected, non-contingent bottle approaches ($F(4,20)=0.10$, n.s.) were not affected. Planned comparisons showed that tetrabenazine significantly reduced lever pressing at the highest doses, 0.75 and 1.0 mg/kg ($p<0.01$) compared to vehicle, as well as reducing ethanol intake (0.75 mg/kg, $p<0.05$, and 1 mg/kg, $p<0.01$).

1.2. Effect of tetrabenazine on free-access ethanol intake (Fig 1D-F). These rats (N=8) received the same range of doses as in the operantt experiment. Repeated measures ANOVA yielded no effects on non-contingent lever pressing ($F(4,28)=0.98$, n.s.). More importantly, neither free ethanol intake (g/kg) ($F(4,28)=0.48$, n.s.) nor bottle approach ($F(4,28)=1.40$ n.s.) were affected by tetrabenazine.

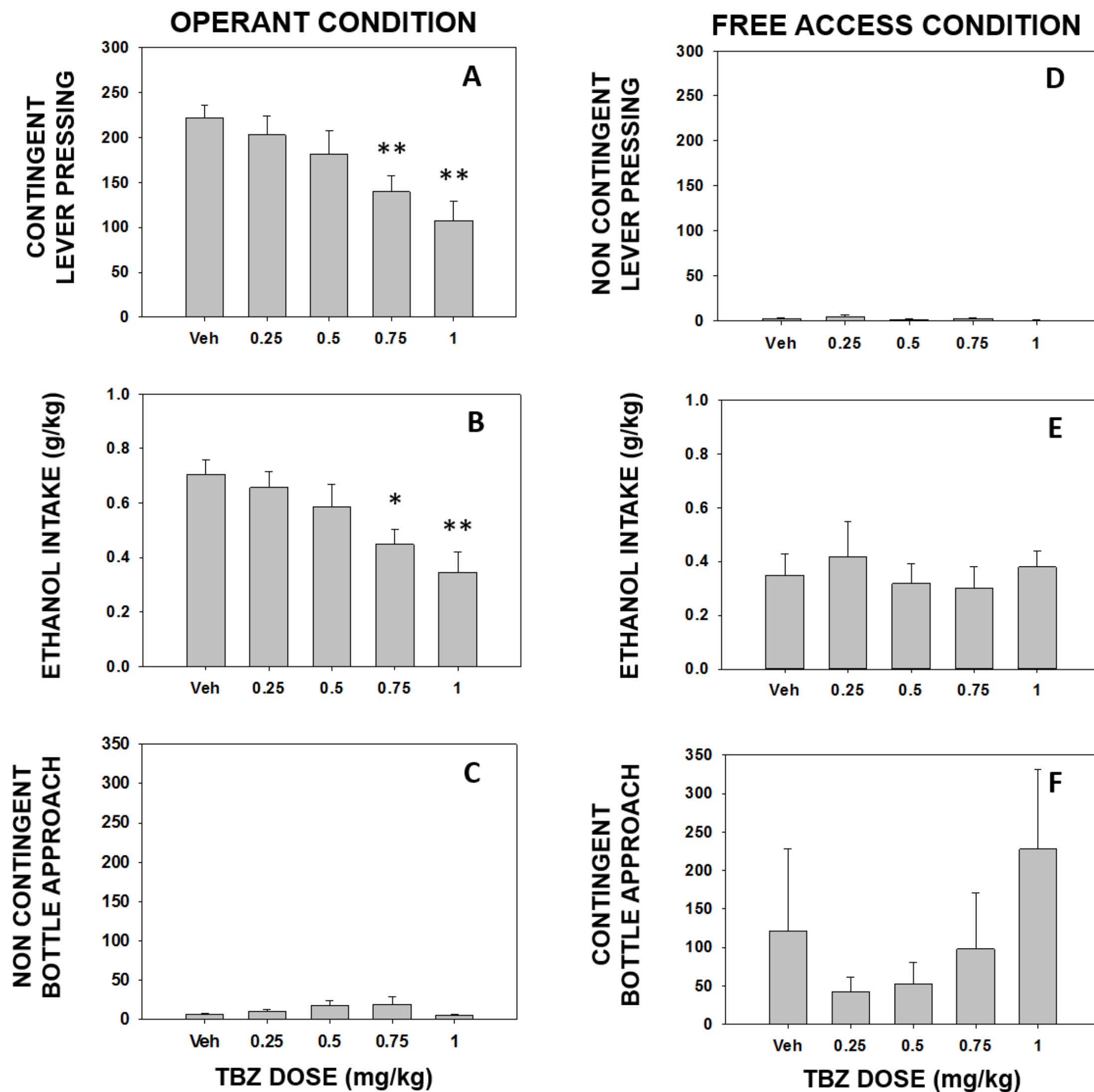

Fig. 1. Effects of tetrabenazine on ethanol consumption under operant or free access conditions. Mean (±SEM) number of lever presses after treatment with vehicle and various doses of tetrabenazine (0.25, 0.5, 0.75 and 1.0 mg/kg) under operant as a contingent variable (A) or free access condition as a non-contingent variable (D). Mean (±SEM) intake of ethanol (g/kg) after treatment with vehicle and various doses of tetrabenazine under operant (B) or free access condition (E). Mean (±SEM) number of bottle approaches after treatment with vehicle and various doses of tetrabenazine under operant as a non-contingent variable (C) or free access condition as a contingent variable (F). *p<0.05, **p<0.01 significantly different from vehicle, planned comparison.

Experiment 2. Effect of the non-selective adenosine antagonist caffeine on ethanol consumption under operant or free access conditions.

2.1. Effect of caffeine on operant ethanol self–administration (Fig 2A-C). On the test day, trained rats (N=7) received the following caffeine doses: 0.0, 2.5, 5.0 and 10.0 mg/kg (30 min before testing). The ANOVA for repeated measures indicated that caffeine significantly increased lever pressing ($F(3,18)=3.20$, $p<0.05$), and ethanol intake (g/kg) ($F(3,18)=3.89$, $p<0.05$), but not non-contingent bottle approach ($F(3,18)=0.35$; n.s.). Planned comparisons showed that caffeine significantly increased lever pressing at all doses (2.5, 5.0 and 10.0 mg/kg; $p<0.01$) compared to vehicle, as well as increasing ethanol intake (2.5 and 10.0 mg/kg, $p<0.05$; 5.0 mg/kg, $p<0.01$).

2.2. Effect of caffeine on the ethanol free-access group (Fig 2D-F). On the test day, rats (N=8) received the same doses of caffeine as in the operant experiment. Repeated measures ANOVA yielded no effects on non-contingent lever pressing ($F(3,21)=0.72$, n.s.), neither on ethanol intake (g/kg) ($F(3,21)=0.59$, n.s.), nor on contingent bottle approach ($F(3,21)=1.17$; n.s.).

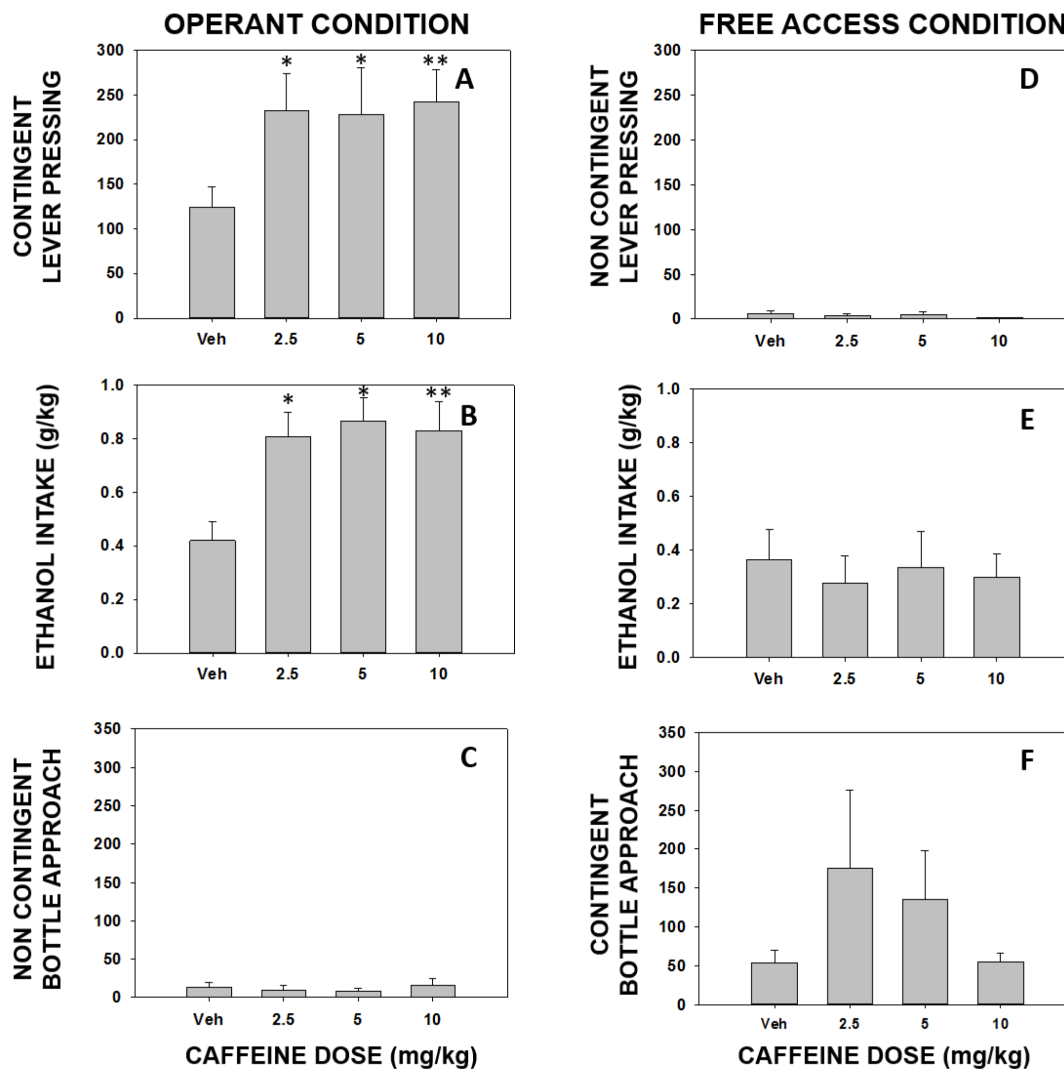

Fig. 2. Effects of the non-selective adenosine antagonist caffeine on ethanol consumption under operant or free access conditions. Mean (+SEM) number of lever presses in 30 minutes under operant (A) or free access condition (D). Mean (+SEM) intake of ethanol (g/kg) under operant (B) or free access condition (E). Mean (+SEM) number of bottle approaches under operant (C) or free access condition (F). *p<0.05, **p<0.01 significantly different from vehicle, planned comparison.

Experiment 3. Effect of the catecholamine uptake inhibitor bupropion on ethanol consumption under operant or free access conditions.

3.1. Effect of bupropion on ethanol self-administration in the operant group. Trained rats (N=7) received the following bupropion doses: 0.0, 5.0, 10.0 and 15.0 mg/kg (30 min before testing) (Fig 3A-C). The ANOVA for repeated measures did not show a significant effect of bupropion on lever pressing ($F(3,18)=0.54$, n.s.), or on ethanol intake (g/kg) ($F(3,18)=0.75$, n.s.). Non-contingent bottle approach ($F(3,18)=2.68$, n.s.) was not significant either.

3.2. Effect of bupropion on free-access ethanol intake (Fig 3D-F). On the test day, rats (N=8) received the same doses as in the previous experiment. Repeated measures ANOVA showed no effects on any of the dependent variables: non-contingent lever pressing ($F(3,21)=1.79$, n.s.), ethanol intake (g/kg) ($F(3,21)=0.31$, n.s.), and contingent bottle approach ($F(3,21)= 1.99$ n.s.).

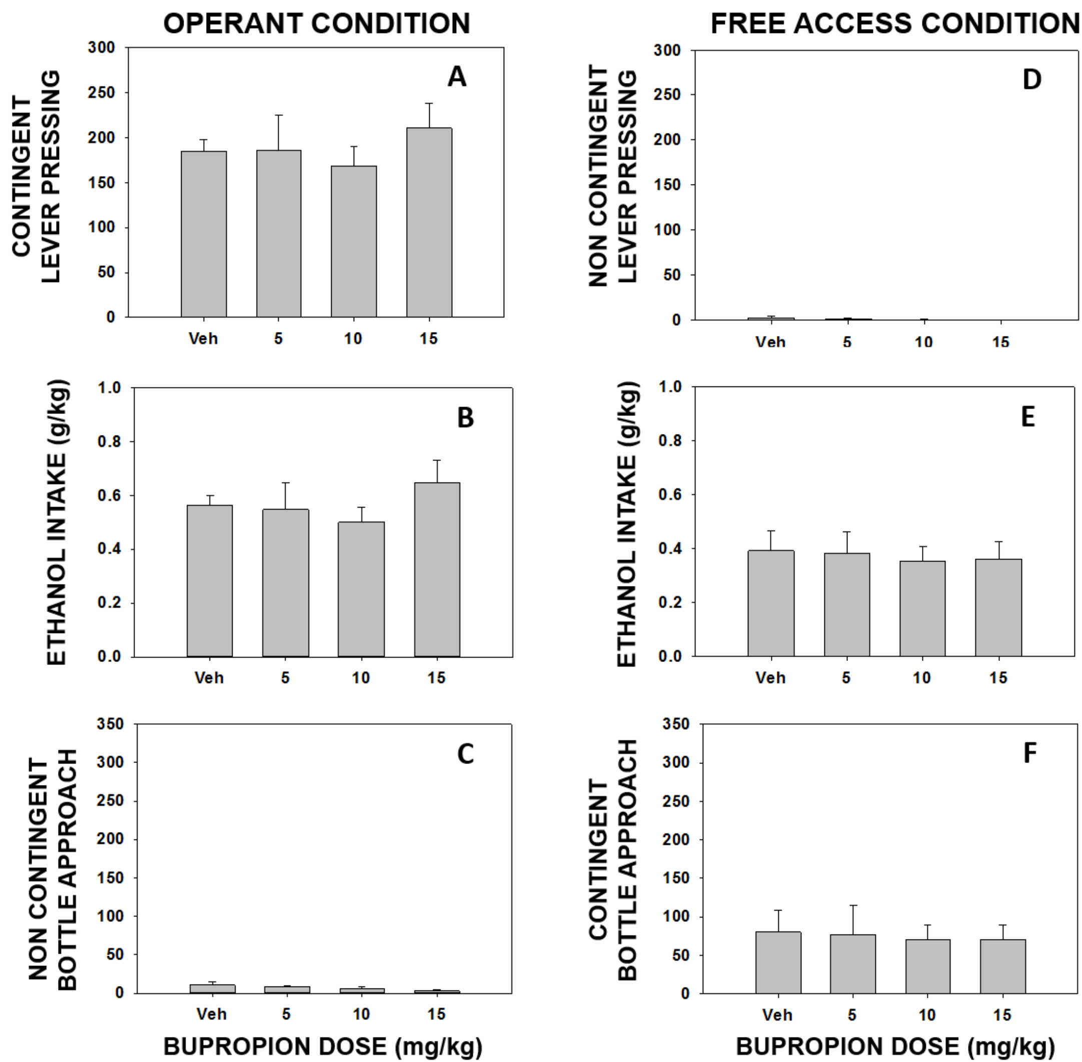

Fig. 3. Effects of the catecholamine uptake inhibitor bupropion on ethanol consumption under operant or free access conditions. Mean (+SEM) number of lever presses in 30 minutes under operant (A) or free access condition (D). Mean (+SEM) intake of ethanol (g/kg) under operant (B) or free access condition (E). Mean (+SEM) number of bottle approaches under operant (C) or free access condition (F).

Experiment 4. Ability of caffeine and bupropion to reverse the effects of tetrabenazine in the self-administration procedure.

All animals (N=8) received the following combined treatments in different weeks: DMSO (120 min before testing) plus saline (30 min before testing), or 1.0 mg/kg TBZ plus saline, or 1.0 mg/kg TBZ plus 10.0 mg/kg caffeine, or 1.0 mg/kg TBZ plus 15.0 mg/kg bupropion. Repeated measures ANOVA showed a significant effect of drug treatment on lever pressing ($F(5,35)=3.68$; $p<0.01$). Planned comparisons showed that TBZ suppressed lever pressing ($p<0.05$). Both caffeine and bupropion were able to attenuate the effects of TBZ on lever pressing ($p<0.05$). Repeated measures ANOVA revealed an overall significant effect of drug treatment on ethanol intake ($F(5,35)=2.76$; $p<0.05$). Planned comparisons demonstrated that tetrabenazine decreased ethanol intake ($p<0.01$). Figure 4.A and 4.B depict the effects of caffeine and bupropion on tetrabenazine actions in the self-administration procedure.

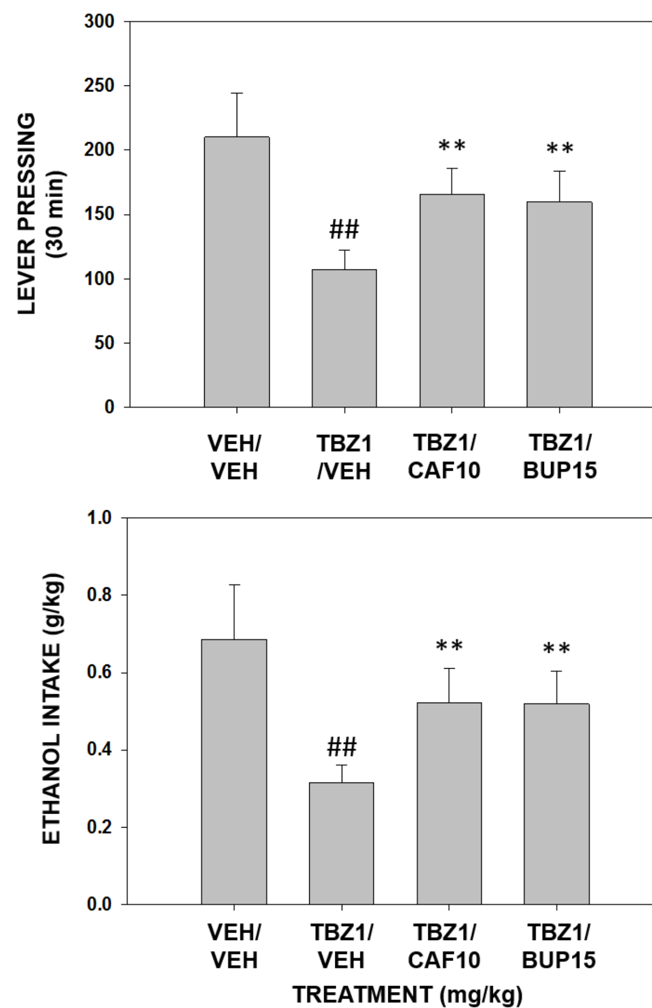

Fig 4. Ability of caffeine and bupropion to attenuate TBZ in effort-related behavior. Mean (± SEM) number of lever presses (A) and ethanol intake (g/kg; B) during the 30 min session after treatment with vehicle/vehicle (VEH/VEH), vehicle/tetrabenazine 1 mg/kg (VEH/TBZ1), tetrabenazine 1 mg/kg/caffeine 10 mg/kg (TBZ 1/CAF 10) and tetrabenazine 1 mg/kg/bupropion 15 mg/kg (TBZ 1/BUP 15). ## $p < 0.01$; significant differences from vehicle treatment. **$p<0.01$ significant differences from TBZ 1/BUP 15 treatment.

Experiment 5. Ability of caffeine and bupropion to reverse the effects of tetrabenazine on pDARPP-32(Thr75).

pDARPP-32(Thr75) immunoreactivity levels were analyzed separately for every striatal area. There was an overall effect of drug treatment on the number of pDARPP-32(Thr75) – positive cells ($F(3,16)=44.75$; $p<0.01$) on NAcb Core. Planned comparisons analysis showed a significant increase on pDARPP-32(Thr75) after VEH/TBZ 1 mg/kg treatment relative to vehicle control ($p<0.01$). Coadministration of TBZ 1 mg/kg/ CAF 10 mg/kg and TBZ 1 mg/kg BUP 15 mg/kg were significantly different from VEH/TBZ 1 mg/kg ($p<0.05$, in both cases). One-way ANOVA also showed a significant effect of treatment on the number of pDARPP-32(Thr75) – positive cells ($F(3,16)=11.76$; $p<0.01$) on NAcb Shell. VEH/TBZ 1 mg/kg treatment significantly increased pDARPP-32(Thr75) relative to vehicle control (planned comparison, $p<0.01$). Coadministration of either TBZ 1 mg/kg/ CAF 10 mg/kg and TBZ 1 mg/kg BUP 15 mg/kg were significantly different from VEH/TBZ 1 mg/kg ($p<0.01$, in both cases). One-way ANOVA demonstrated a significant effect of treatment on the number of pDARPP-32(Thr75) – positive cells on DMS ($F(3,15)=6.32$; $p<0.01$). Planned comparisons also revealed a significant increase on pDARPP-32(Thr75) after VEH/TBZ 1 mg/kg treatment relative to vehicle control ($p<0.05$). Coadministration of TBZ 1 mg/kg/ CAF 10 mg/kg and TBZ 1 mg/kg BUP 15 mg/kg were significantly different from VEH/TBZ 1 mg/kg ($p<0.01$, in both cases). There was an overall effect of drug treatment on the number of pDARPP-32(Thr75) – positive cells on DLS ($F(3,16)=5.78$; $p<0.01$). VEH/TBZ 1 mg/kg treatment significantly increased pDARPP-32(Thr75) relative to vehicle control (planned comparison, $p<0.05$). Coadministration of TBZ 1 mg/kg/ CAF 10 mg/kg and TBZ 1 mg/kg BUP 15 mg/kg were significantly different from VEH/TBZ 1 mg/kg ($p<0.01$ and $p<0.05$, respectively). Figure 5 depicts

the results of the co-administration of caffeine and bupropion with ethanol on pDARPP-32(Thr75) levels.

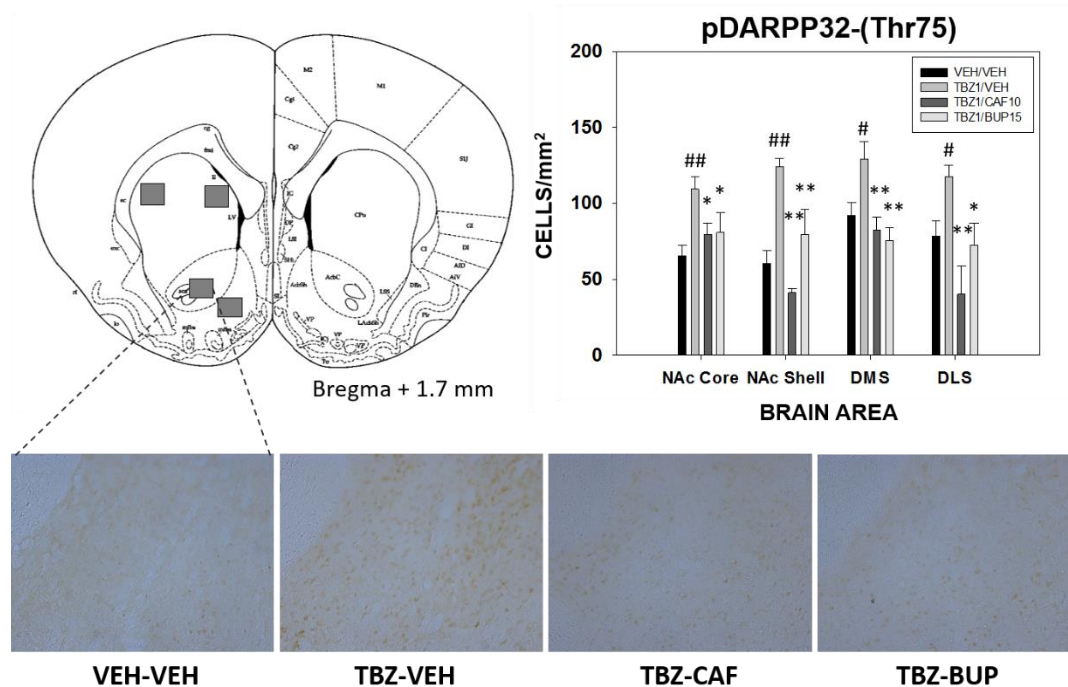

Fig. 5. Left upper part: diagram of a coronal section with bregma coordinates from Franklin and Paxinos, 2007, showing location of the brain areas for pDARPP32(Thr75) immunoreactivity counting. Right upper part: effects of TBZ 1 mg/kg/Veh, TBZ 1 mg/kg/CAF 10 mg/kg, and TBZ 1 mg/kg/ BUP 15mg/kg on pDARPP-32(Thr75) levels on nucleus Accumbens Core (NAcb Core), nucleus Accumbens Shell (NAcb Shell), dorsomedial (DMS) and dorsolateral striatum (DLS). Mean (±SEM) of number of pDARPP-32(Thr75) staining in 300 µm2 ROI. # $p<0.05$, ## $p <0.01$; significant differences from vehicle treatment. *$p<0.05$, **$p<0.01$ significant differences from TBZ 1 mg/kg/Veh treatment. Lower part: Photomicrographs of pDARPP32-Thr34 staining in Nacb Core from representative animals in each treatment group. Low power images (20x). Scale bar=250 um.

Experiment 6. Effect of pre-exposure to ethanol solution on the operant and on the free access ethanol intake tests.

6.1. The paired t-test for the operant experiment demonstrated that pre-exposing animals to the ethanol solution the night before the test produced a significant decrease on contingent lever pressing ($t(6)=4.86$, $p<0.01$), and on ethanol intake (g/kg) ($t(6)=4.95$, $p<0.01$), but no effect on non-contingent bottle approach ($t(6)=0.30$; n.s.). These data are shown in figures 6.A-C.

6.2. In the free access experiment, the paired t-test demonstrated also that pre-exposing animals to the ethanol solution before the test session produced a significant decrease on ethanol intake (g/kg) ($t(7)=3.32$, $p<0.05$) but not on non.contingent lever pressing ($t(7)=0.91$, n.s.), nor on bottle approach ($t(7)=1.91$; n.s.). Effects of pre-exposure to ethanol on the free-access procedure are shown in figures 6.D-F.

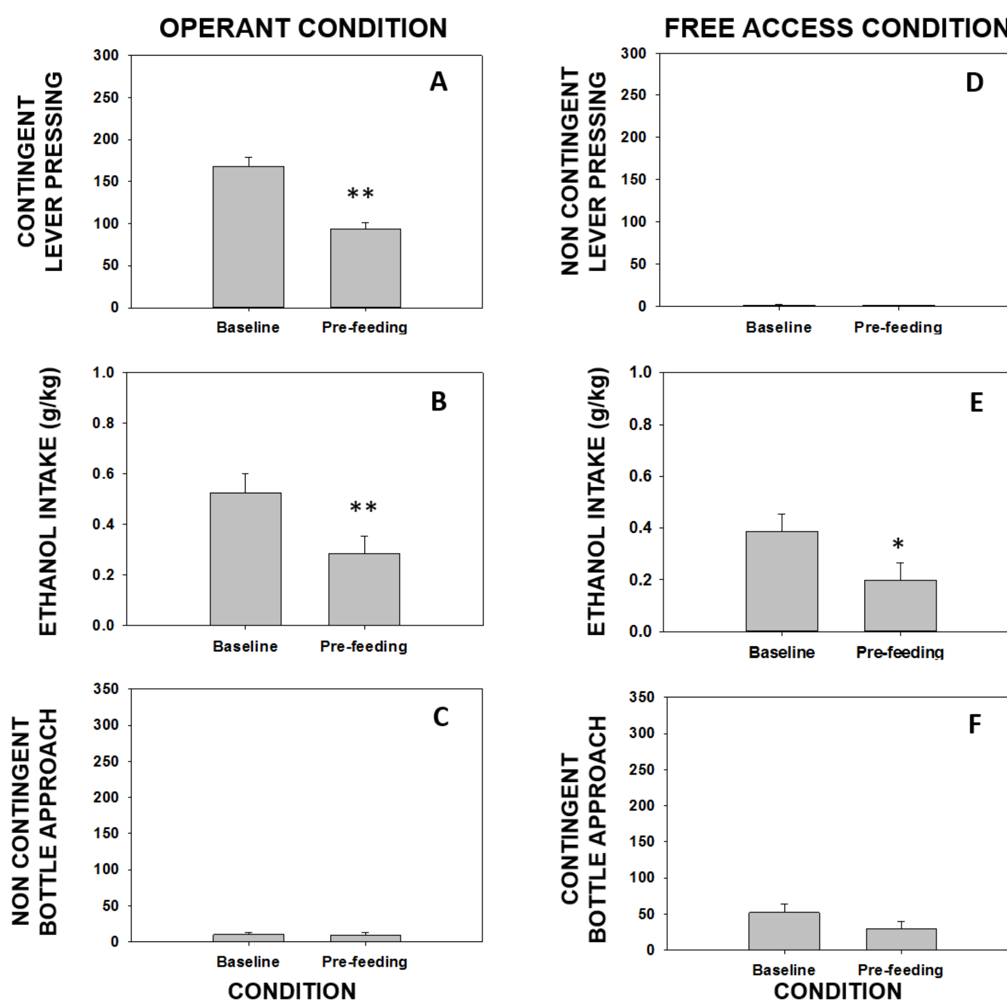

Fig. 6. Effect of pre-feeding on ethanol consumption under operant or free access conditions. Mean (±SEM) number of lever presses in 30 minutes under operant (A) or free access condition (D). Mean (±SEM) intake of ethanol (g/kg) under operant (B) or free access condition (E). Mean (±SEM) number of bottle approaches under operant (C) or free access condition (F). *$p<0.05$, **$p<0.01$ significantly different from vehicle, planned comparison.

Experiment 7. Effects of increasing palatability of the drinking solution on the operant and on the free access intake tests.

7.1. Changing the drinking solution to 2% sucrose instead of 10% ethanol significantly increased contingent lever pressing ($t(6)=-11.45$, $p<0.01$), and solution intake (ml) ($t(6)=-8.45$, $p<0.01$). Non-contingent bottle approach was not significant either ($t(6)=1.17$; n.s.). Effects of sucrose intake on operant procedure are shown in figure 7.A-C.

7.2. In the free access experiment, changing the drinking solution to sucrose 2% significantly increased solution intake (ml) ($t(7)=-3.46$, $p<0.05$). Neither lever pressing ($t(7)=1.23$; n.s.) nor bottle approach ($t(7)=0.48$; n.s.) were modified by changing the liquid solution . Effects of sucrose intake on free access condition are shown in figure 7.D-F.

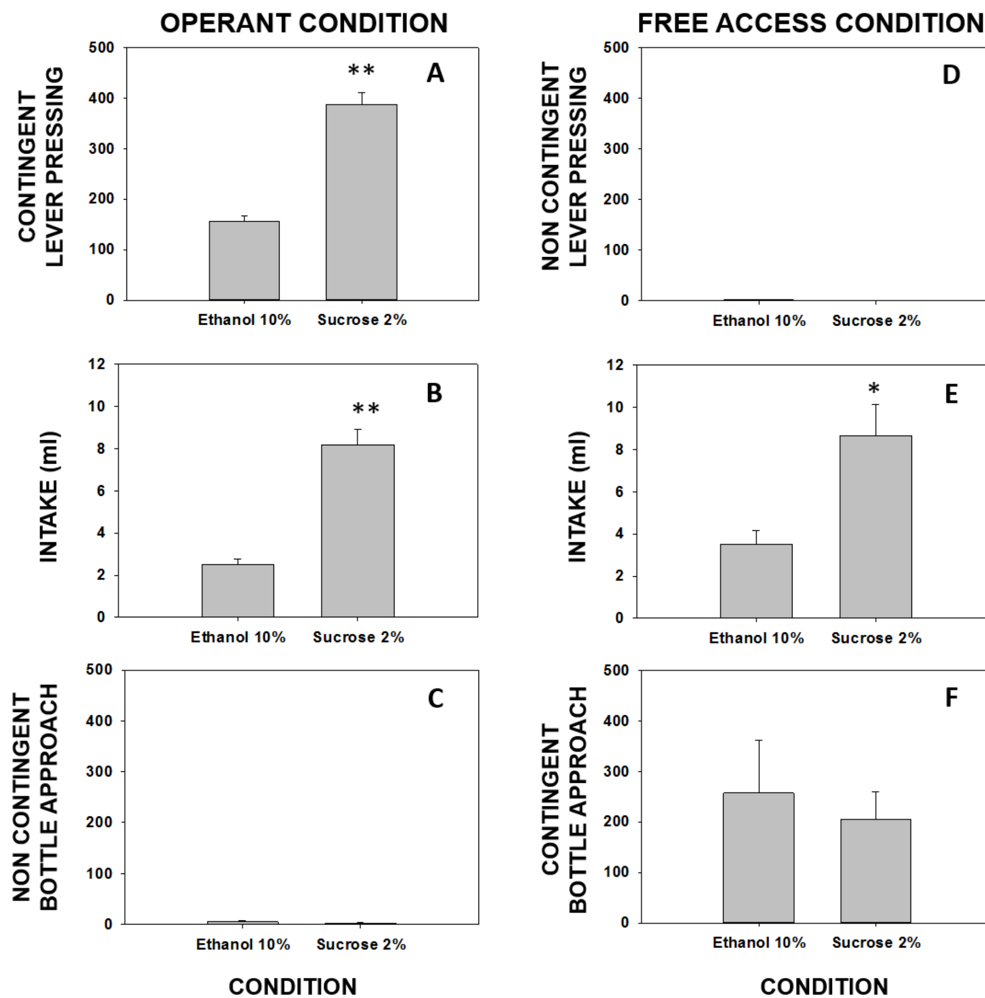

Fig. 7. Effect of sucrose (2% w/v) pre-exposure in the operant choice paradigm, (A) lever presses, (B) 5% sucrose intake, and (C) 0.3% sucrose intake, and in the free choice paradigm. (D) lever presses, (E) 5% free sucrose intake and (F) 0.3% free sucrose intake. Mean (±SEM) number of lever presses or ml consumed in 15 min. *$p<0.05$, **$p<0.01$ significantly different from control condition.

Discussion

The present experiments evaluated the involvement of DA in the regulation of motivated responses for ethanol intake in two groups of rats differentiated by work output: operant (FR5) or free condition. Contingent and non-contingent variables were assessed in each condition. Lever pressing is the contingent variable of the self-administration procedure, and bottle approach is the non-contingent variable, whereas, for the free access condition, lever pressing is the non-contingent and bottle approach the contingent variable. In experiment 1, TBZ dose dependently decreased the contingent variable lever pressing and ethanol intake (g/kg) in the operant condition but the non-contingent variable bottle approach remained stable. Moreover, no dose of TBZ modify any of the variables measured, even bottle approach. These results are consistent with other studies in which DA depletion or antagonism of Nacb decreased the tendency to work for a reinforcer when lever pressing is required (Salamone et al., 1991, 2002; Koch et al., 2000, Nowend et al., 2001; Ishiwari et al., 2004; Sink et al., 2008, Farrar et al., 2010; Pardo et al., 2012, 2015). This drug is commonly used in humans to treat hyperkinectic disorders, but common side effects include fatigue and anergia (Astin and Gumpert, 1974; Kingston, 1979; Jankovic and Beach, 1997; Kenney et al., 2007).

Caffeine at all doses used in experiment 2, significantly increased lever pressing in the operant condition, and also significantly increased ethanol intake, but did not modify any variable of the free access condition. Previous data have also shown that caffeine (5 mg/kg) produced a dose-related facilitation in ethanol drinking (Kunin et al., 2005). In our study, due to the fact that caffeine effect only modified contingent variable for the operant condition, appeared to be specific to ethanol and not to unspecific behavioral activation. Adenosine antagonists have shown opposite results. Ethanol intake modulation in operant conditioning tasks has been focused on A_{2A} adenosine

receptors. Thus, in ethanol-preferring rats, ANR94, an A_{2A} adenosine antagonist, produced a mild increase in ethanol-reinforced responding (Micioni Di Bonaventura et al., 2012), whereas SCH58261 reduced ethanol-reinforced responding in alcohol-preferring rats (Adams et al., 2008), and DMPX reduced responding for ethanol in Wistar rats (Thorsell et al., 2007). On the other hand, adenosine A1 antagonists reduced ethanol intake in alcohol preferring rats (Adams et al., 2008), and in non-preferring rats produced bimodal effects on self-administration (Arolfo et al., 2004).

Bupropion is a catecholamine uptake inhibitor that that has been used for many years as an antidepressant (Dwoskin et al., 2006). Although several studies have demonstrated that bupropion could increase food-reinforced responding on a conventional PROG schedule (Bruijnzeel and Markou, 2003; Randall et al., 2014) and increased the tendency to work for food reinforcement, as marked by increases in all measures of PROG lever pressing, in our studies, bupropion did not increased lever pressing in the operant condition neither increased ethanol intake. However, co-administration of caffeine 10 mg/kg or bupropion 15 mg/kg with TBZ reversed the effect of this DA depletor. As shown in previous studies, bupropion is capable of reversing the effort-related impairments induced by TBZ (Nunes et al., 2013b; Randall et al., 2014). However, in our study, bupropion did not modify any of the FR5 variables by itself.

Experiment 4 employed pDARPP-32-(Thr75) immunohistochemistry to determine if there were neurochemical differences between treatments in four subregions of striatum: NAcb Core and Shell, DMS and DLS. TBZ significantly increased pDARPP-32-(Thr75) levels in the four regions measured. Furthermore, co-administration of caffeine 10 mg/kg and bupropion 15 mg/kg also was able to decreased pDARPP-32-(Thr75) immunoreactivity in all subregions, Previous results suggested

that TBZ-induced increases in pDARPP-32(Thr75) would reflect reduced transmission at DA D1 receptors (Svenningsson et al., 2004, 1999; Bateup et al., 2008; Yger and Girault, 2011; Nunes et al., 2013). Thus, probably administration of the non-selective adenosine caffeine reversed the increase of pDARPP-32(Thr75) induced by TBZ (Nunes et al., 2013) due to its action on A_1 adenosine receptors which are colocaliced witg D_1 receptors on substance-P positive neurons (Svenningsson et al., 1999).

Finally, pharmacological manipulations differ substantially from those produced by motivational manipulations such as pre-feeding (Salamone et al., 2009) or increasing palatability of the drinking solution. These appetite-related manipulations showed a different pattern of response, devaluating the reinforcer produce a decrease in the contingent lever pressing for the operant condition whereas it did not affect the contingent bottle approach for the free access condition. When animals had access to a preferred solution like 2% sucrose, animals increased significantly intake independent of the access condition.

These results indicate that caffeine and bupropion may be relatively effective for treating anergia, fatigue, or psychomotor symptoms observed in many depressed patients (Rampello et al., 1991; Stahl, 2002; Demyttenaere et al., 2005; Papakostas et al., 2006; Pae et al., 2007). Methylxanthines and new antidepressants acting on the DA system can help to increase work output when the requirement of the task is high or when performance has been impaired by dopaminergic manipulations that affect the activational component of behavior (Salamone et al., 2009; Pardo et al., 2012).

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CHAPTER 5.

IMPACT OF CAFFEINE ON CONSUMPTION OF HIGHLY PALATABLE FOOD IN MICE UNDER BINGE EATING, ANXIOGENIC OR EFFORTFUL CONDITIONS

Abstract

Caffeine is a methylxanthine present in many beverages and some foods. Its effects have been well studied on parameters such as sleep, vigilance, and anxiety and it has been also used as an appetite suppressant. Because it is consumed in many different contexts and among normal as well as groups with psychopathologies in which appetite is pathologically altered, is important to understand its effects at different doses and under different access conditions. CD1 male adult mice had access to palatable food under binge eating inducing, anxiety and effortful conditions. Caffeine (20.0 mg/kg) increased even further sweet food intake in animals with a binge eating pattern. This dose produce the opposite effect on food intake under anxiogenic conditions in a modified dark and light paradigm. In the T-maze with barrier task to evaluate consumption under effort-requiring conditions, caffeine (10.0 and 15.0 mg/kg) decreased latency to reach the food, but not affecting selection neither arm selection nor the total pellets consumed. Caffeine (5.0 mg/kg) reversed behavior to control levels in animals that had received a dopamine-depleting agent. These results suggest that caffeine can potentiate binge eating, but it can lead to reductions of food consumption if the context is prone to increase anxiety. Finally, caffeine does not change appetite and it does not impair orientation towards food under effortful conditions, but it rather helps to achieve the goal by improving speed and by reversing performance to normal levels when fatigue was induced by dopamine depletion.

Keywords: sucrose, tetrabenazine, anxiety, appetite, decision-making.

Introduction

Caffeine is the most widely psychostimulant substance consumed in the world, and it is found in several types of food and beverages (Mitchell et al., 2014). Psychostimulants are characterized by stimulation of locomotion, although at high doses they can induce stereotypies and anxiety (Berthold et al., 1992; Drouin et al., 2000, 2002; Wellman et al., 2002). This category of drugs is known for its anorectic effects, and in fact caffeine as a minor psychostimulant, is a common constituent in over-the-counter weight-loss supplements (Blanck et al., 2007). However, caffeine in humans does not seem to have a consistent pattern of effects on appetite and energy intake. While some studies report that it exerts a slight anorectic effect (Tremblay, 1988), others do not report significant changes (Judice et al., 2013; Gavrieli et al., 2013).

In animal studies, the literature also shows a complicated type of results. In food restricted rats acute doses of caffeine produce seemingly contradictory results. High doses of caffeine (≥ 50 mg/kg), reduce lever pressing for chow in a variable interval schedule, while lower doses, had no effect (Carney, 1982). However, in a fixed interval schedule, caffeine (up to 20 mg/kg) increased lever pressing with no net increase in access to highly palatable food (Randall et al., 2011). In addition, in this last study, doses up to 40 mg/kg of caffeine reduced lever pressing in a fixed ratio 20 schedule that requires a high level of performance in order to increase the amount of food (Randall et al., 2011). Contrary to those results, caffeine (up to 25 mg/kg) improves performance in a progressive ratio schedule, also a highly demanding operant task, thus increasing fluid sucrose consumption (Sheppard et al. 2012; Retzbach et al. 2014). In mice, a recent study demonstrated that acute administration of caffeine (6-24 mg/kg) increases standard free chow intake in non-deprived mice at least for 2 hours, and animals are not

more activated or anxious after a relatively high dose of caffeine (20 mg/kg) (Sweeney et al., 2016).

Caffeine is a non-selective adenosine receptor antagonist, and there is a functional interaction between striatal dopamine and adenosine receptors (Ferré, 1997, 2008; Ferré et al., 1997, 2005; Fuxe et al., 2003). Previous studies have focused on this functional interaction in studies of effort-related processes leading to consumption of different types of food (Mott et al., 2009; Wolden et al., 2009; Pardo et al., 2012). For example in a T-maze procedure developed to assess the effects of dopamine manipulations on effort-related decision-making (Salamone et al., 1994; Pardo et al. 2012), co-administration of theophylline, another methylxantine, reversed the anergia inducing effects of a dopamine D_2 receptor antagonist haloperidol, restoring normal levels of palatable food access in food restricted mice (Pardo et al., 2012). However, the impact of methylxantines on their own, and more specifically caffeine, have never been assessed in this paradigm that requires effort in order to get access to higher amounts of palatable food.

Clearly, additional research is required to fully understand the impact of caffeine on food consumption in different contexts. Therefore, the present work was undertaken to evaluate the impact of different doses of caffeine on highly palatable food consumption under different access conditions. Experiment 1 and 2 studied the effects of different doses of caffeine on palatable food consumption under restricted access conditions: in the first experiment animals had unrestricted access in an habitual context, and in the second one, mice had access to the same type of food under anxiogenic conditions in a modified dark and light box. The impact of caffeine was also evaluated in relation to anxiety parameters as well as locomotor activity in an open field and on a running wheel. In the last group of experiments, we studied the impact of

caffeine in the T-maze procedure that imposes an effort restriction in order to get access to higher quantities of food. In addition, we also evaluated the ability of caffeine to reverse the effects of a dopamine-depleting agent that reduces willingness to work for food but not food consumption itself.

Materials and methods

Subjects

Male CD1 male mice (24-28 g) were 4 weeks old upon arrival to the laboratory (N=54). All mice were purchase from Harlan Laboratories (Barcelona, Spain). Mice were housed in groups of 3 animals per cage with tap water and standard chow food available *ad libitum.* In experiments 4 and 5, mice were food-restricted to reach 85% free feeding body weight throughout the study. The colony was maintained at 22 ± 1ºC, with humidity control and 12-h light/dark cycles. 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.

Pharmacological agents

Caffeine (1,3,7-trimethylxanthine) (Sigma-Aldrich, Spain) was dissolved in 0.9% w/v saline and was administered 30 min before testing. Saline solution was used as its vehicle control. The range of caffeine doses (2.5, 5.0, 10.0, 15.0 and 20.0 mg/kg) was selected based on previous and pilot studies (López-Cruz et al., 2014). All solutions

were administered intraperitoneally (IP). Tetrabenazine (Tocris Bioscience), administered 120 minutes before testing, was dissolved and sonicated in a vehicle solution of 0.9% saline (80%) and dimethylsulfoxide (DMSO; 20%) (pH=4.5). DMSO was used as its vehicle control.

Apparatus and testing procedures

The same type of food was used in all the experiments; 45 mg (experiments 1 and 2) or 20 mg (experiment 4) precision pellets for rodents (TestDietTM) with a balanced nutrient composition and a 50% sucrose content that gave it a palatable property.

Palatable food consumption during one hour under habitual conditions. During 6 weeks (5 days per week) mice were placed individually in standard home cages where they had free access to highly palatable pellets. Sessions lasted 60 minutes (data were registered every 30 minutes), starting 3 hours prior to the start of the dark cycle. After these weeks of baseline, animals were habituated to receive an IP saline injection once a week for two more weeks. Test phase lasted five weeks more during which each subject received all caffeine doses in a randomly varied order, once a week.

Palatable food consumption under anxiogenic conditions. After completing experiment 1, the same mice had access to the highly palatable food for three additional weeks of baseline with no treatment, and shorter sessions (15 minutes). On the single test day, animals were divided in two treatment groups (saline or 20.0 mg/kg of caffeine, the dose that had increase food consumption in the previous experiment) and placed for 15 minutes in a modified dark and light (DL) paradigm. In the DL box, one chamber was enclosed and dark, and the open chamber was divided in two areas (see figure 2); one dimly lit, and the further one, where the food dish was placed, intensely illuminated. Test started when each subject was placed in the dark chamber. Classical

anxiety measures (evaluated during 5 minutes), as well as consumption measures (15 minutes) were registered: first latency (sec) to the intermediate compartment from the dark compartment, total number of crosses between dark and intermediate compartments, time (sec) spent in dark compartment, time (sec) spent in the food compartment, and amount of food (mg) consumed during 15 minutes, were registered manually.

Open field exploratory locomotion. Independent groups of mice were used in this experiment. The open-field apparatus consisted of a clear glass cylinder 25 cm in diameter and 30 cm high. The floor of the cylinder was divided into four equal quadrants by two intersecting lines drawn on the floor, and a horizontal locomotion score (count) was assigned each time an animal crossed over 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 (central rearings), or rested them on the wall (lateral rearing). The behavioral test room was illuminated with a soft light, and external noise was attenuated. Animals were placed in the open field during 30 minutes for habituation to the apparatus. Then, mice received an IP injection of saline or 20.0 mg/kg of caffeine and were placed back in the open field for an additional 30 minutes during which behavior was recorded for later evaluation.

Voluntary locomotion in running wheel (RW). Independent groups of mice were used for the RW experiment. The automated RW (Ugo Basile) consisted of a cage (32 x 15 x 13 cm) with a wheel (11 cm in diameter) inserted on top. Turns of the wheel were registered by a LCD counter. A completed turn of the wheel was registered as 4 counts. Animals placed in the cage had free access to the wheel. Mice were trained during 3 weeks to achieve a stable baseline of locomotion in 30 min sessions per day (5 days per

week). One week previous to the test session, animals where habituated to the saline injection.

Effort-based decision-making for palatable food in a T-maze with barrier. A new group of animals was used for the present experiments. This procedure is based on previous published procedures (Pardo et al., 2012). The T-maze apparatus consisted of a central corridor with two opposed arms (see figure 4). Each arm provided a different density of food: 2 pellets (20 mg each) were in the high density (HD) arm and 1 pellet was in the low density (LD) arm. The HD arm contained a vertical barrier that provided the effort-related challenge. Pellets were located in dishes placed near the far walls of the maze arms. Half the mice had the HD arm with the barrier consistently located on the left side, while half the mice had the HD arm and barrier on the right side. During the first training phase no barrier was present, and for the first 2 days of the initial training, mice had free access to both arms of the T-maze upon exiting the start arm, and were allowed to consume all pellets in both HD and LD arms of the maze before being returned to the start arm. Upon completion of this initial training, mice were only allowed to choose one arm of the maze; after the initial arm choice, the other arm was blocked. During 2 weeks mice choose between the two arms with no barrier in place. The last day of those 2 weeks mice received an acute dose of caffeine (10.0 mg/kg). In the second training phase a small barrier (6 cm high) was introduced in the HD arm for one week, and the last day mice received a single dose of caffeine (15.0 mg/kg). Animals were then trained with a 14 cm barrier in the HD arm for the rest of the sessions. A training phase lasting three weeks was allowed before the pharmacological interactions between caffeine and tetrabenazine were studied.

Data analyses

Experiment 1 and 4 used a within-groups design, and normally distributed and homogenous data were evaluated by repeated measures analysis of variance (ANOVA). Further analyses were conducted by nonorthogonal planned comparisons using the overall error term to assess differences between each dose and the control condition (Keppel, 1991; the number of comparisons was restricted to the number of treatments minus one). T-test for dependent samples analysis was used in experiment 4.A. Experiments 2 and 3 used a between groups design and data were analized by T test for independent samples. All data were expressed as mean ± SEM, and significance was set at $p<0.05$. STATISTICA 7 software was used.

Results

Experiment 1. Effect of caffeine on highly palatable food intake under habitual conditions.

Mice (n=16) were used to evaluate the effect of caffeine (0, 2.5, 5.0, 10.0 and 20.0 mg/kg) on pellets intake during one hour (in two 30 minutes periods). Repeated measures ANOVA for pellets intake during the first 30 minutes revealed a significant effect of caffeine ($F(4,60)=10.52$; $p<0.01$). Planned comparisons showed that the highest dose of caffeine (20.0 mg/kg) significantly increased the amount of palatable food consumption ($p<0.01$) (Fig. 1A). However, the repeated measures ANOVA did not show a significant effect of caffeine on pellets intake during the second 30 minutes ($F(4,60)=2.30$; n.s.), (Fig. 1B).

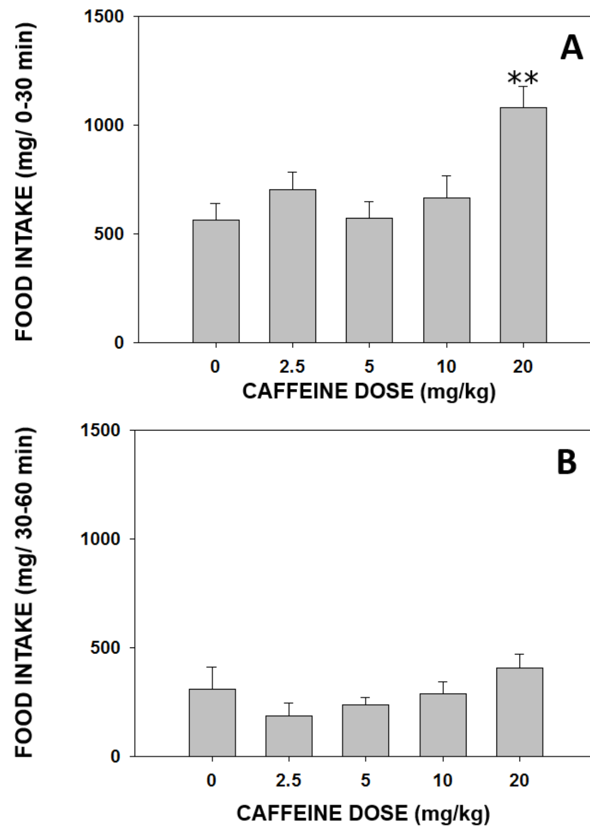

Fig. 1. Effect of caffeine (0, 2.5, 5.0, 10.0 and 20.0 mg/kg) on palatable food intake under habitual conditions during the first 30 minutes (A), and the second 30 minutes (B) of the one-hour session. Mean ±S.E.M. milligrams consumed. **p<0.01 significantly different from vehicle.

Experiment 2. Effect of caffeine on highly palatable food intake under anxiogenic conditions.

The same group of mice used in the previous experiment, was used to evaluate the effect of caffeine on pellets consumption under anxiogenic conditions. Animals were split in two groups and one group received the dose of caffeine that increased pellets intake (20.0 mg/kg) during the first 30 minutes in experiment 1 and the other group received saline. The t-test revealed significant differences on total food intake (mg in 15

minutes) ($t(14)=1.97$, $p<0.05$) (Fig. 2.A), as well as on latency to the lit compartment ($t(14)=2.36$, $p<0.05$) (Fig. 2.B) after the administration of the highest dose of caffeine and vehicle (Fig. 2A). However, the t-test for independent samples did not reveal significant differences on total crosses ($t(14)=1.63$, n.s.) (Fig. 2.C), time in food compartment ($t(14)=-0.10$, n.s.) (Fig. 2.D), or time in dark compartment ($t(14)=-0.58$, n.s.) (Fig. 2.E).

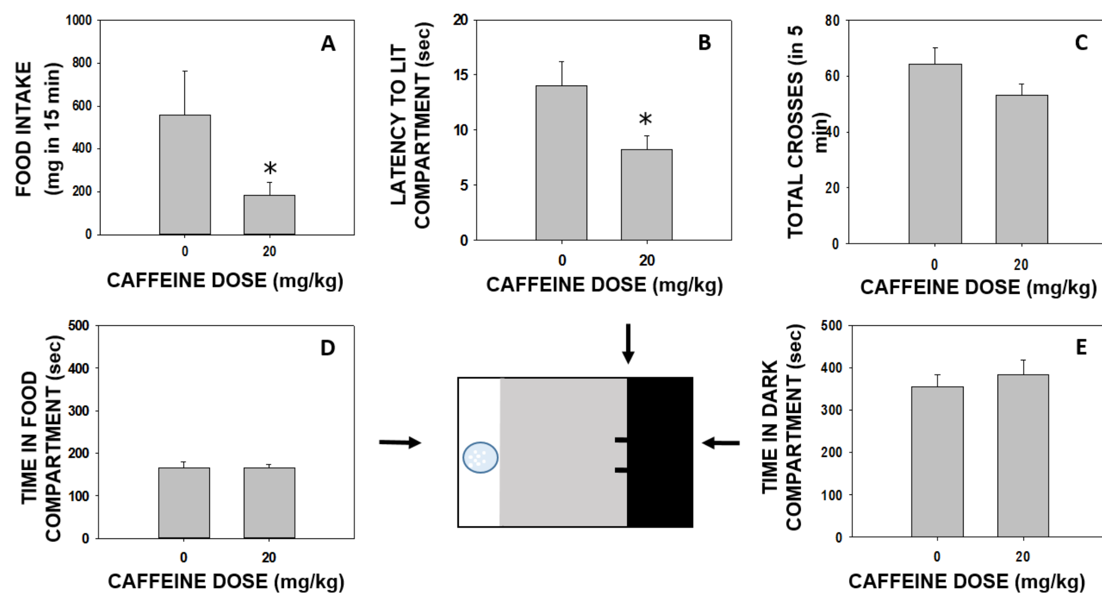

Fig. 2. Effect of caffeine (0 and 20.0 mg/kg) evaluated on a dark and light box on measures of consumption and anxiety: food intake (A), latency to lit compartment (B), total number of crosses between dark and medium compartments (C), time in food compartment (D) and time in dark compartment (E). Data are expressed as mean ± S.E.M. milligrams, number of crosses or seconds. *$p<0.05$ significantly different from vehicle.

Experiment 3. Effect of the highest dose of caffeine (20.0 mg/kg) on exploration in the open field, and on RW activity.

Different and naïve groups of animals received either saline or 20.0 mg/kg of caffeine: Open field n= 9, and RW n=10. The t-test for independent samples revealed a significant suppressant effect of caffeine on voluntary running in the RW ($t(9)=6.83$; $p<0.01$), (Fig. 3.A). However, caffeine did not show any effect on exploration measures in the open field (Figs. 3.B-D): horizontal crosses ($t(8)=-0.30$; n.s.), lateral rearings ($t(8)=-0.89$; n.s.), and central rearings ($t(8)=1.83$; n.s.).

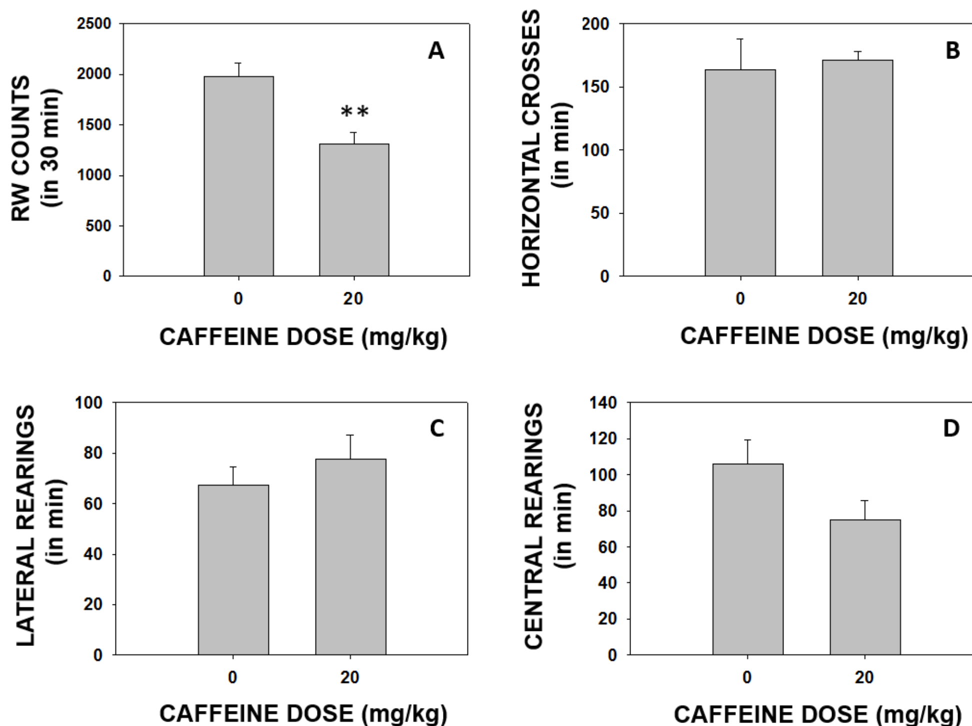

Fig. 3. Effect of caffeine (0 and 20.0 mg/kg) on measures of exploration in an open field and voluntary running in a RW: Number of turns (A), horizontal crosses (B), lateral rearings (C), and central rearings (D). Data are expressed as mean ± S.E.M. number of turns in 30 minutes and number of counts during 30 minutes. **p<0.01 significantly different from vehicle.

Experiment 4. Effect of caffeine on highly palatable food consumption under effortful conditions.

The schematic of the procedure and the T-maze used in experiments 4.A and 4.B is depicted in figure 4. Figures 5 and 6 represent data from experiment 4.A.

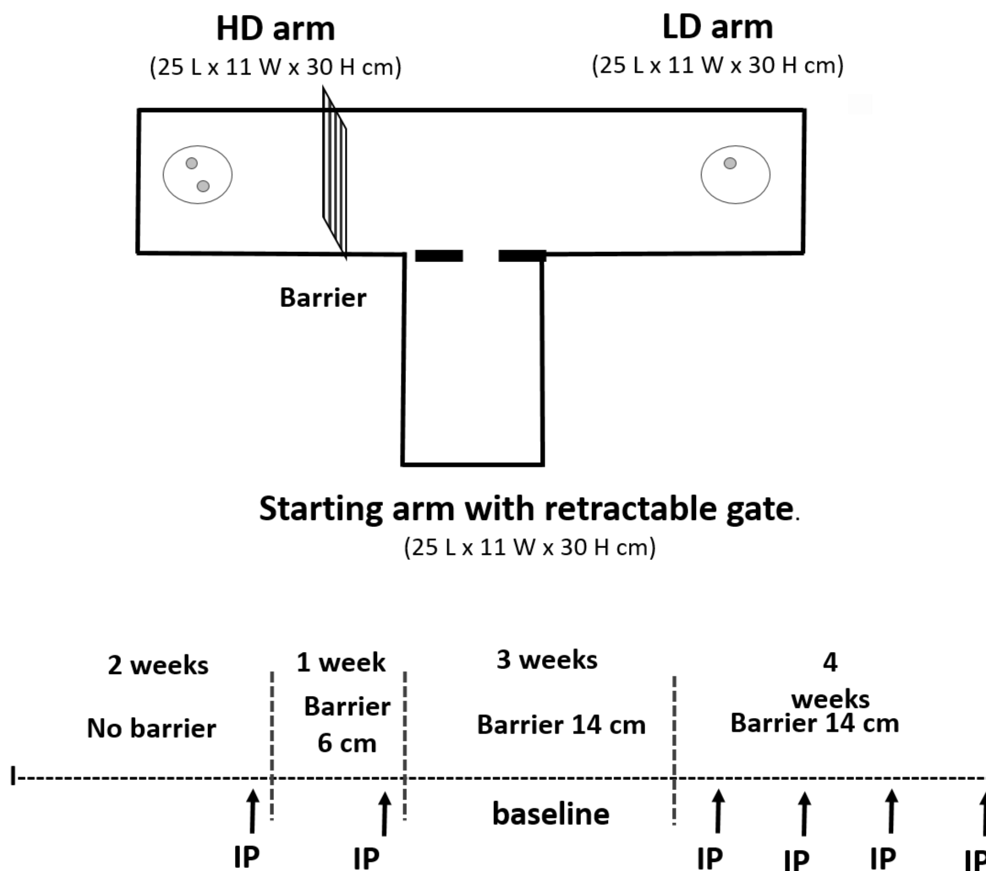

Fig. 4. Schematic representation of the T-maze apparatus used in the present studies, and experimental procedure. All the surfaces and the doorway were constructed out of Plexiglas, and the barrier (depicted in the high density arm, to the left) was constructed of wire mesh. The high density (HD) arm contained 2 food pellets, and the low density (LD) arm contained 1 food pellet.

Experiment 4.A. Effect of caffeine (10.0 or 15.0 mg/kg) alone on the two first phases of the T-maze procedure.

In the T-maze with no barrier (N=9), we evaluated the effect of caffeine (10.0 mg/kg) on performance comparing it to baseline performance the day before. Average latency to food in the 10 first trials was analyzed with a t-test for dependent samples. The results showed a significant effect ($t(9)=2.22$; $p<0.05$) (Fig. 5.A). However, this caffeine effect on latency to food disappeared after analyzing the 30 total trials ($t(9)=0.79$, n.s.) (Fig. 5.B). Neither the number of trials in which mice chose the HD arm ($t(9)=2.15$; n.s.) (Fig. 5.C), nor the total pellets consumed ($t(9)=0.41$, n.s.) (Fig. 5.D) were significantly modified after caffeine treatment.

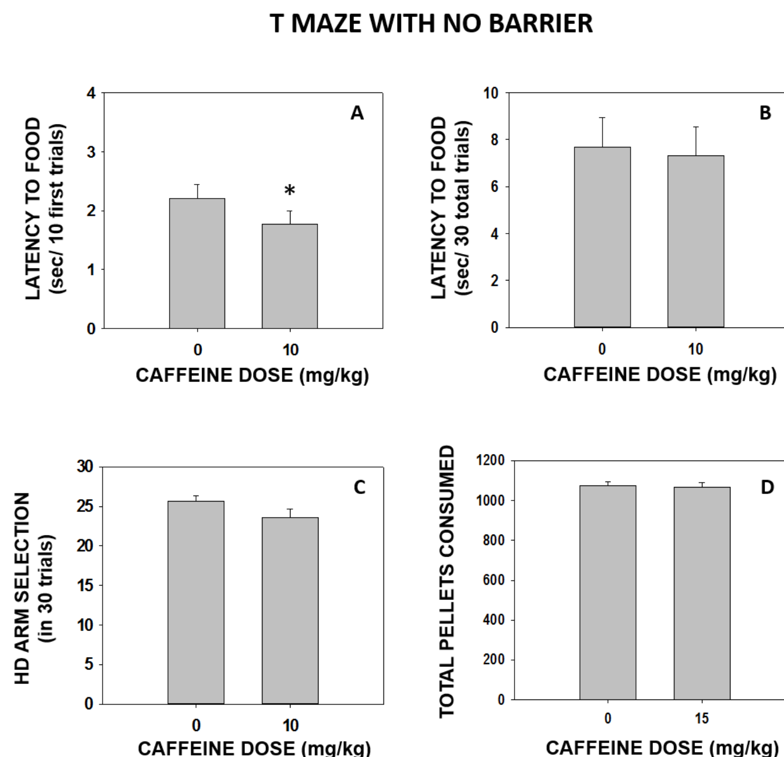

Fig. 5. Effect of caffeine (10.0 mg/kg) on average latency to reach the food during the first 10 trials (A), during the 30 total trials (B), on HD arm selection (C) and food consumption (D), in a T-maze with no barrier. Data are expressed as mean (± S.E.M.) of average time (seconds) to reach the food, total number of trials in which animals chose the HD arm, and number of 20 mg pellets consumed. *$p<0.05$ significantly different from control.

In the T-maze with a 6 cm barrier (N=7), caffeine (15.0 mg/kg), reduced average latency to food in the 10 first trials ($t(6)=3.96$; $p<0.01$) (Fig. 6.A), as well as in the 30 total trials ($t(6)=3.21$, $p<0.01$) (Fig. 6.B). No significant differences were found on the HD arm selection ($t(6)=1.82$; n.s.) (Fig. 6.C), or on the total pellets consumed ($t(6)=1.34$, n.s.) (Fig. 6.D).

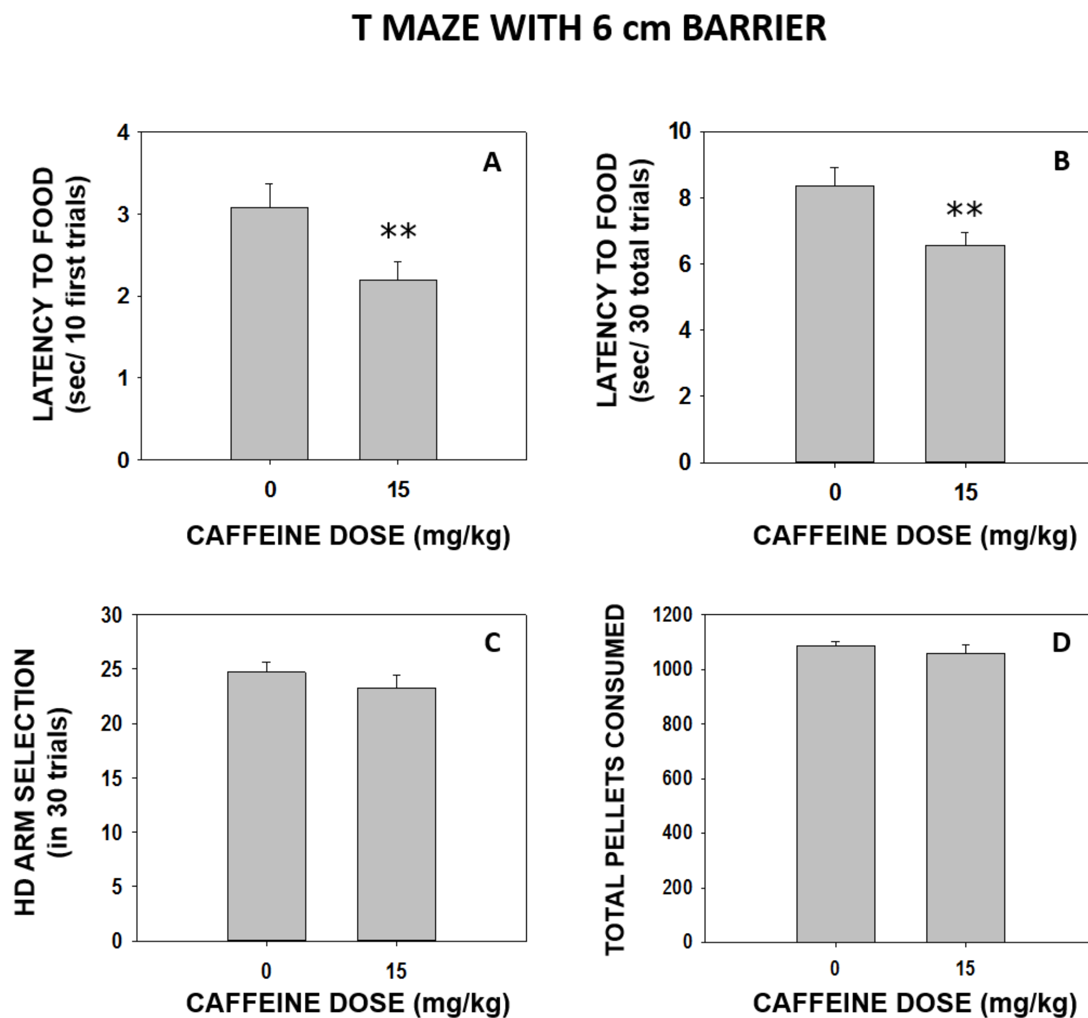

Fig. 6. Effect of caffeine (15.0 mg/kg) on average latency to reach the food during the first 10 trials (A), during the 30 total trials (B), on HD arm selection (C) and food consumption (D), in a T-maze with a 6 cm barrier in the HD arm. Data are expressed as mean (± S.E.M.) of average time (seconds) to reach the food, total number of trials in which animals chose the HD arm, and number of 20 mg pellets consumed. **$p<0.01$ significantly different from control.

Experiment 4.B. Ability of caffeine to reverse the effect of dopamine depletion on the T-maze with the higher barrier.

In the caffeine reversal study (Fig. 7.A-C) ($N=8$), repeated measures ANOVA yielded an overall effect of drug treatment ($F(3,21)=11.91$, $p<0.01$) on HD arm selection. Planned comparisons showed that the TBZ/VEH and TBZ/Caffeine 2.5 mg/kg condition were significantly different from VEH/VEH control condition ($p<0.01$). In addition, co-administration of TBZ with the highest dose of caffeine (5.0 mg/kg) significantly increased HD arm selection ($p<0.01$) compared to the TBZ/VEH condition, indicating an attenuation of the dopamine-depleting agent effects. LD arm selection was also modified in the reversal study. Repeated measures ANOVA indicated a significant effect of drug treatment ($F(3,21)=8.44$, $p<0.01$). Planned comparisons showed that the TBZ/VEH and TBZ/Caffeine 2.5 mg/kg condition were significantly different from VEH/VEH control condition ($p<0.01$), and TBZ plus caffeine (5.0 mg/kg) significantly decreased LD arm selection compared to TBZ/VEH condition ($p<0.01$). The same pattern of results was found for the dependent variable number of pellets earned during the T-maze performance: a significant effect of drug treatment ($F(3,21)=4.15$, $p<0.01$), and a significant difference between VEH/VEH and TBZ/VEH ($p<0.01$) on the one hand, as well as with TBZ/Caffeine 2.5 mg/kg on the other ($p<0.05$). Moreover, TBZ/Caffeine (5.0 mg/kg) significantly restored number of pellets earned compared to TBZ/VEH condition ($p<0.01$).

Further analysis of the difference between TBZ/VEH and TBZ/caffeine (5.0 mg/kg) treatments on average latency to reach the food performed with a two-way factorial ANOVA (treatment x trials). The results show a significant effect of drug treatment ($F(1,18)=5.17$; $p<0.05$), and trials ($F(2,18)=3.30$, $p<0.05$), but not of the interaction ($F(2,18)=0.66$, n.s.) (Fig. 8).

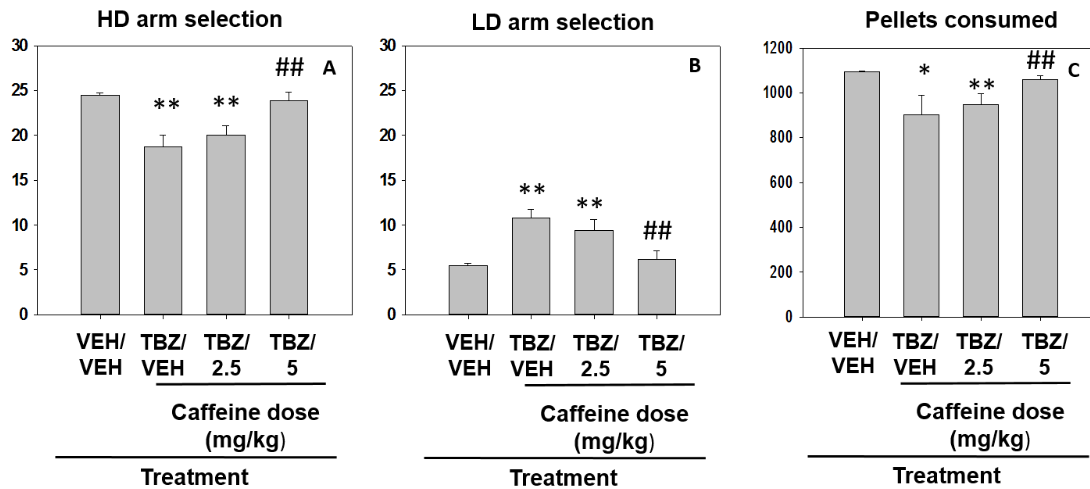

Fig. 7. Effects of caffeine (0, 2.5 and 5.0 mg/kg) in mice co-administered with TBZ (0 or 4.0 mg/kg) on HD (A), LD (B) arm selection, and total pellets earned (C) in the T-maze with a 14 cm barrier in the HD arm. Mean (± S.E.M.) number of arm choices in 30 trials and number of pellets consumed. *p<0.05; **p<0.01 significantly different from Veh/Veh; #p<0.05; ##p<0.01 significantly different from TBZ/Veh.

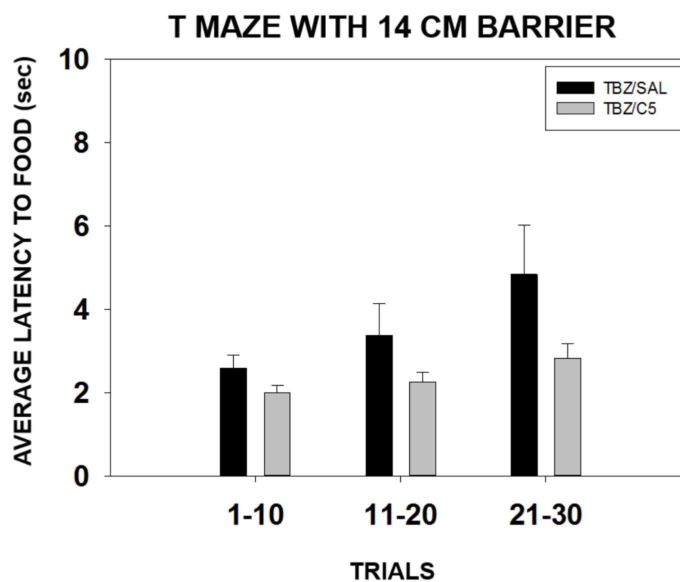

Fig. 8. Effect of caffeine (5.0 mg/kg) in TBZ (4.0 mg/kg) treated animals on latency to reach the food in any arm of the T-maze. Data are expressed as mean (± S.E.M.) of average time per ten trials blocks to reach food.

Discussion

The present experiments indicate that the non-selective adenosine antagonist caffeine produces a complex pattern of effects on sweet food consumption depending on the conditions in which the food is presented to mice. In experiment 1, we have used a paradigm of food consumption in which after several weeks of daily access during 1 hour, the higher dose of caffeine used (20.0 mg/kg) increased consumption of this palatable food mainly during the first 30 minutes of access. Considering that these animals are non-food deprived and in the first half hour they eat around 25 pellets (45 mg each, around 1.100 milligrams of palatable food), is not surprising that even the dose of caffeine that has an effect at the beginning, has no effect on the second part of the session, since the animals have already eaten very high amounts of rich food. The effect of caffeine is in agreement with a recent study in which acute caffeine (up to 26 mg/kg) increased standard chow consumption under habitual home conditions on the first 2 hours (Sweeney et al., 2016). In that study, animals did not reached such a high level of intake (the maximum consumption of chow was 400 mg in 2 hours). Thus, our results suggest that our procedure induces "binge eating" which is characterized by excessive food intake during a short period of time, and typically is induced by offering a highly palatable food or fluid on a limited, or intermittent schedule generally 2 hours per day (Wojnicki et al., 2007; Czyzy et al., 2010). Thus, caffeine under repeated but limited access conditions, does not have an anorectic effect, but it rather potentiates patterns of binge eating for palatable food.

Because caffeine can have anxiogenic effects as well (El Yacoubi et al., 2000; López-Cruz et al., 2014), and anxiety and stress can affect food consumption, we explored the impact of the highest dose of caffeine (20.0 mg/kg) that was effective in increasing food intake, to evaluate its effects under anxiogenic conditions in a modified

version of the classical DL box. Comparing animals that had received saline with animals that had received this dose of caffeine we observed the opposite effect than in the previous experiment; caffeine decreased the amount of food consumed in 15 min. However, this dose of caffeine did not modify any of the classical anxiety measures, and even decreased latency to enter the lit compartment for the first time. This lack of anxiety effects is in accordance with previous experiments in mice using similar doses (20-25 mg/kg) (López-Cruz et al., 2014; Sweeney et al., 2016). Thus, although caffeine at this dose is not very anxiogenic in terms of exploration in the DL box, and the animals that received saline were able to eat the same amount of food per minute than in experiment 1, the interaction between the context in which food was presented plus caffeine seems to potentiate those anxiogenic properties, leading to a reduced consumption of food.

As a psychostimulant, caffeine can also affect locomotion and exploration (El Yacoubi et al., 2000; López-Cruz et al., 2014; Sweeney et al., 2016). Increases in locomotion with moderate doses of caffeine are seen in studies that habituate mice to the open field, and thus reduce basal activity (Waldeck, 1975; Logan et al., 1986; El Yacoubi et al., 2000). In our study, 20.0 mg/kg did not change exploration (neither horizontal nor vertical) in the open field, but it reduced voluntary running in a RW that generates high levels of locomotion that are easier to decrease (Harri et al., 1999; de Visser et al., 2005). Thus, for the following T-maze effortful conditions in which exploration, voluntary running and barrier climbing are required, lower doses of caffeine were used.

It has been demonstrated that the T-maze is a good tool to measure effort-related decision-making (Salamone et al., 1994; Pardo et al, 2012; Yohn et al., 2015), and that this paradigm is sensitive to behavioral manipulations such as prefeeding that devalues

food reinforcement by reducing appetite and food motivation increasing omissions (Pardo et al., 2012). As we have seen in the present results, caffeine (10.0 or 15.0 mg/kg) decreased latency to reach the food independently of the presence or absence of the barrier. However, none of these doses affect preference for the arm that contained more pellets, and did not increased omissions, leading to no changes in pellets earned and consumed. Under different conditions, when anergia was induced by administering a dopamine-depleting agent, animals reduced selection of the HD arm but compensated by increasing the selection of the LD arm. This treatment did not change appetite since animals did not increase the number of omissions and eat all the pellets that they earned, although they were significantly fewer. Interestingly, coadministration of a relative low dose of caffeine (5 mg/kg), reversed this anergia inducing effect of tetrabenazine, and also decreased latency to food although data did not reach significance.

In summary, caffeine can potentiate binge eating when subjects have already stablish a pattern of excessive eating. However, it can lead to clear reductions of food consumption if the context is prone to increase anxiety levels. Finally, it does not change appetite and it does not impair orientation towards food under effortful conditions, but it rather helps to achieve the goal by improving speed and by reversing performance to normal levels when fatigue and psychomotor slowing was induced by dopamine-depleting agents.

The present work has potential clinical relevance, since appetite is impaired in many disorders such as anorexia and bulimia, anxiety and depression. In addition, effort-related motivational symptoms such as anergia, fatigue, and psychomotor slowing seen in depressed humans are very resistant to classical antidepressant treatments such as 5-HT uptake inhibitors (Stahl, 2002; Fava et al., 2014), and caffeine has

demonstrated to enhance the antidepressant-like activity of common antidepressant drugs (Szopa et al., 2016).

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CHAPTER 6.

INDIVIDUAL DIFFERENCES IN THE ENERGIZING EFFECTS OF CAFFEINE ON EFFORT-BASED DECISION-MAKING TESTS IN RATS.

Abstract

Normal motivated behavior is characterized by behavioral activation, persistence and high work output. Nucleus accumbens (Nacb) plays a critical role in the modulation of behavioral activation and effort-based decision-making. Caffeine, the most consumed stimulant in the world, acts as non-selective adenosine A_1/A_{2A} receptor antagonist. These receptors are highly concentrated in Nacb. Adenosine agonists in Nacb shift preference towards low effort alternatives in animals tested on effort-based choice tasks. The present studies characterized the effort-related effects of caffeine in a concurrent progressive ratio (PR)/free reinforcer choice procedure that requires high levels of work to obtain the preferred reinforcer and generates great variability among different animals. Different groups of male Sprague Dawley rats received an acute dose of caffeine (2.5-20.0 mg/kg, IP) and 30 minutes later were tested in operant boxes either for high carbohydrate pellets (in food deprived animals) or for a high sucrose solution (in non-deprived animals). Caffeine (2.5 and 5.0 mg/kg) increased performance for high carbohydrate pellets only among animals with high baseline rates of responding. Among animals lever pressing for a sucrose solution, caffeine (5.0 and 10.0 mg/kg) increased performance only in low responders, however, it only decreased performance in high responders (at 10.0 and 20.0 mg/kg). The highest dose of caffeine also suppressed high levels of performance in a concurrent fixed ratio 7/free sucrose paradigm, but did not modify sucrose preference under free access conditions. These results show that caffeine has a clearer effect when analyzing individual differences, potentiating or impairing performance depending on baseline performance.

Keywords: caffeine, adenosine antagonists, methylxanthines, effort, behavioral activation, appetite.

Introduction

Motivated behaviors have two major components; the directional aspect that guides behavior to specific ends (e.g. a reinforcer), and the activational aspect, which refers to the vigor or persistence of the reinforcer-seeking behavior (Cofer and Appley, 1964; Salamone, 1988, 1991, 1992; Salamone et al., 1997). In everyday life animals must make cost/benefit analyses in which they weigh the value of a stimulus (e.g. taste of a food, caloric value, etc.) relative to the cost of obtaining it (e.g. nature of the instrumental response to get access to the reinforcer) (Salamone and Correa, 2002, 2012; Salamone et al., 2007). Extensive animal data have demonstrated that mesolimbic dopamine (DA) is a key mediator of effort-based decision-making processes (for a review see Salamone and Correa, 2012). Interference with DA transmission biases behavior towards less valued rewards that involve less effort and less activity, while enhancing DA transmission increases selection of activities that require more effort (Salamone and Correa, 2002, 2012). However, DA seems not to be regulating the directional component under situations in which, to achieve the preferred stimuli, effort is not required (Nunes et al., 2013; Pardo et al., 2015).

In effort-related decision making tests, animals are given a choice between a more valued reinforcer that can only be obtained by engaging in a more demanding-higher effort activity vs. a low effort/low value option. Thus, in operant tasks animals are given a choice between lever pressing for the more preferred reward using fixed ratio (FR) or progressive ratio (PR) schedules vs. approaching and consuming a less preferred reinforcer that is concurrently freely available in the chamber (Salamone et al., 1991; Randall et al. 2012; Pardo et al., 2015). When tested on concurrent FR/free reward choice tasks, rats typically spend most of the time pressing the lever for the preferred reward and much less time consuming freely available food or fluids (Salamone et al.,

1991, 2002; Pardo et al., 2015). These schedules (FR5 or FR7) typically generate high rates of responding uniformly in all animals. Thus, they are not useful for assessing drugs or conditions that potentially can invigorate performance and bias animals even further towards the high-effort activity (i.e., lever pressing). For example, FR schedules are very sensitive to drugs that can deteriorate performance, produce anergia and make animals less active, inducing in that case a compensation towards the less effortful option, thus increasing free consumption of the less valued reward. In contrast, rats tested on the concurrent PR/free chow choice task show more individual variability in the effort component, and some animals tend to disengage more readily from PR lever pressing because of the increasing work requirement, shifting then to the less preferred source of food that is the less effort-demanding alternative (Randall et al., 2012, 2014, 2015). This individual variability in willingness to keep lever pressing in spite of the increasing work demands has been associated with DA-related signaling activity in the accumbens core (Randall et al., 2012). Furthermore, treatment with drugs that increase DA transmission by blocking DA uptake, such as GBR12909 and the antidepressant bupropion, increases selection of high-effort PR lever pressing (Randall et al., 2015; Yohn et al., 2016). Bupropion was shown to be more potent for improving performance of the “high workers” than the “low workers”, although at high doses it benefited both groups (Randall et al., 2015).

In addition to DA, adenosine also is involved in effort related decision-making processes (Farrar et al., 2007, 2010; Nunes et al., 2010; Salamone et al., 2007, 2009). Nucleus accumbens has a high concentration of adenosine A_{2A} receptors, and there is a functional interaction between DA D_2 and adenosine A_{2A} receptors and between A_1 and DA D_1 receptors in the same striatal neurons (Ferré, 1997, 2008; Ferré et al., 1997, 2005; Fuxe et al., 2003). Microinjections of the adenosine A_{2A} agonist CGS 21680 into

the nucleus accumbens core produced effects on the concurrent FR5/free chow procedure that resembled those produced by accumbens DA antagonism or depletion (Font et al., 2008). The selective A_{2A} antagonist MSX-3 had no effect on the FR5/free chow procedure on its own (Salamone et al., 2009), but increased PR/chow performance, decreasing also chow consumption (Randall et al., 2012). Caffeine is a naturally occurring methylxanthine that acts as a non-selective A_1 and A_{2A} adenosine receptor antagonist (Fredholm et al., 1999). This methylxantine is found in common beverages as well as a variety of medications (Barone and Roberts, 1996; Andrews et al., 2007), and is typically consumed in order to increase alertness, arousal and energy (Malinauskas et al., 2007; Smith et al., 2002). Its consumption has been related to changes in performance in normal population (Smith et al., 2002), as well as in people with fatigue (Childs and de Wit, 2008). In fact, caffeine enhances performance more in fatigued than well-rested subjects (Lorist et al., 1994).

In the present studies rats were tested in the PR/free reinforcer choice procedure in order to determine if caffeine at a broad range of doses can improve the willingness to work in a highly demanding effort-based decision-making task. Thus, in two independent experiments different reinforcers were used with different value: 1) high carbohydrate pellets versus chow in one experiment, and 2) water containing a high sucrose concentration versus a low concentration (5% versus 0.3% w/v). Sweet taste stimulation can act as a powerful natural reward (Levine et al., 2003; Yamamoto, 2003). In the first experiment the reinforcers were different in palatability, but in addition, because animals were food deprived, conditions were set so that motivation also had a homeostatic component. In the second experiment, however, animals had normal amounts of water in the home cage. Thus, sucrose solutions was used to evaluate the willingness to work for a stimulus with mainly a sensory component. These different

conditions can create differences in motivation that could be observed in baseline performance leading to potentially different results when caffeine is administered. The impact of caffeine was analyzed also in each experiment based on individual differences in lever pressing performance at baseline.

Materials and methods

Subjects

Adult male, Sprague-Dawley rats (Harlan Sprague-Dawley, Indianapolis, IN) were housed in pairs in a colony maintained at 23ºC with 12-h light/dark cycles (lights on at 8:00 h). Rats in experiment 1 (N=12) were food restricted to 85% of their free-feeding body weight for training and they were fed supplemental food to maintain weight throughout the study with water available *ad libitum* in the home cage. Rats in experiment 2 (N=18) had normal, but limited, amount of water in the home cage (20 ml/day/rat). Typically all rats finished the bottle content before the training session, but if any water remained in the hour before training, it was removed by the experimenter to avoid satiation. Food was available *ad libitum* in the home cages, and these rats gained weight normally throughout the experiment. Additional groups of rats were used for experiment 3 (N=8), experiment 4 (N=9), and experiment 5 (N=12). These animals were kept under the same housing conditions as animals in experiment 2. All animals were under a protocol approved by the Institutional Animal Care and Use Committee of Universitat Jaume I, and all experimental procedures complied with European Community Council directive (86/609/EEC), Connecticut Institutional Animal Care and Use Committee and followed NIH guidelines (DHEW Publications, NIH, 80-23). All

efforts were made to minimize animal suffering, and to reduce the number of animals used.

Pharmacological agents

Caffeine (1,3,7-trimethylxanthine Sigma-Aldrich, S.A., Spain) was dissolved in 0.9% w/v saline (final pH 7.4) and administered intraperitoneally (IP) 30 minutes before testing. The range of caffeine doses (2.5, 5.0, 10.0, and 20.0 mg/kg) was selected based on previous studies (Salamone et al., 2009; Randall et al., 2011). Sucrose (Sigma Quimica C.O) was dissolved in tap water for oral consumption (0.3% and 5% solutions).

Apparatus and testing procedures

PR/free chow feeding choice task. Behavioral sessions were conducted in operant conditioning chambers (28×23×23cm; Med Associates). Rats were initially trained to lever press on a FR1 schedule for high carbohydrate pellets (45-mg pellets, Bioserve, Frenchtown, NJ, USA) for 1 week, and then were shifted to the PR schedule (30-min sessions, 5 days/week) for 6 additional weeks. During the PR session, the ratio started at FR1 and was increased by one additional response every time 15 reinforcers were obtained (FR1×15, FR2×15, FR3×15,…). This schedule included a “time-out” feature that deactivated the response lever if 2 minutes elapsed without a ratio being completed. Upon reaching stable lever press baseline responding, free chow was then introduced. Weighed amounts of laboratory chow (Laboratory Diet, 5P00 Prolab RMH 3000, Purina Mills, St. Louis, MO, USA; typically 15–20 g) were concurrently available on the floor of the chamber during the PR sessions. At the end of the session, rats were removed from the chamber, and chow intake was determined by weighing the remaining food (including spillage). Rats were trained for an additional 5 weeks so that they could

attain relatively consistent levels of baseline lever pressing and chow intake, after which drug testing began.

PR/free sucrose drinking choice task. The operant conditioning chambers were basically the same as in experiment 1, but there was a free access bottle placed in the chamber. Rats were trained to lever press on a FR1 schedule for 100 microliters of sucrose fluid (5% w/v sucrose solution) for 2 weeks, and then were shifted to the PR schedule (30-min sessions, 5 days/week) for 6 additional weeks during which stable baseline performance was reached before the concurrent reinforcer was introduced. The PR program had the same parameters as in experiment 1. After those 6 weeks a bottle containing 0.3% w/v sucrose solution was introduced and was concurrently available on the opposite wall of the chamber during the rest of the PR sessions. At the end of the session, rats were removed from the chamber, and 0.3% sucrose fluid consumed was determined by measuring the remaining fluid. Rats were trained for an additional 5 weeks so that they could attain consistent levels of baseline lever pressing and 0.3% sucrose consumption after which drug testing began.

Concurrent FR7/free sucrose drinking choice task. This experiment was conducted to compare the impact of the highest dose of caffeine (20.0 mg/kg) used for the PR studies, which had been used also in a previous FR/ free chow choice task (Salamone et al., 2009) in a FR/free sucrose choice task. In this experiment we used liquid sucrose, following the same methodological parameters used in previous studies (Pardo et al., 2015). In the same operant boxes, animals were trained to lever press for access to a 5% sucrose solution. Rats were initially trained to lever press on a FR1 reinforcement schedule during 3 days and then shifted to FR7 for 2 weeks before the concurrent free sucrose 0.3% solution was introduced. At the end of the session, rats were immediately removed from the chamber, and 0.3% sucrose intake was determined by measuring the

remaining fluid. Rats were trained until they attained stable levels of baseline lever pressing and free 0.3% sucrose intake after which drug testing began.

Concurrent free access sucrose drinking task. In order to see if caffeine can be changing preferences for the different concentrations of sucrose, we choose the highest dose of caffeine (20 mg/kg) and evaluated sucrose consumption when the two sucrose solutions were concurrently present and both bottles were freely available. This method was based on previous studies (Pardo et al., 2015). Every day (5 days/week) animals were individually placed in new home cages (20 cm x 45 cm x 25 cm) where two graduated cylinder tubes containing 5% and 0.3% sucrose drinking solutions were placed separated 10 cm apart. Rats were initially exposed to the 5% sucrose concentration (30 min, for 3 days) after which 0.3% and 5% sucrose were concurrently present for 3 weeks before testing started. At the end of the session, rats were immediately removed from the chamber, and sucrose intake was determined by measuring the remaining fluids.

Locomotion in the open field. Because caffeine is considered a minor psychostimulant, locomotor parameters during open field exploration were assessed using the highest dose of caffeine (20.0 mg/kg) used in the present experiments. The open field chamber was 80 cm x 60 cm x 52 cm. The behavioral test room was illuminated with a soft light, and external noise was attenuated. Animals were habituated to the open field during 15 minutes 24 hours before the test session. Locomotor activity was registered manually. Number of crosses was registered each time the animal crossed from a quadrant to another with all four legs and number of rears was registered each time the animal raised its forepaws in the air higher than its back or rested them on the wall.

Statistical analyses

The dependent variables from experiments 1 and 2 were analyzed first with all the animals using repeated measures analysis of variance (ANOVA). When the overall ANOVA was significant, non-orthogonal planned comparisons using the overall error term were used to compare each dose with the vehicle control (Keppel, 1991). Then animals in experiment 1 and 2 were separated into high and low performance groups by a median split of the baseline lever presses, after which data were analyzed with a 2 (performance group) x 4 (drug treatment) factorial ANOVA with repeated measures on the drug treatment factor. Separate ANOVAs and nonorthogonal planned comparisons of each performance group were used to determine differences between each drug treatment vs vehicle. Experiments 3 and 4 were analyzed using repeated measures ANOVA and experiment 5 with between-groups simple ANOVA. Because latency to start lever pressing in experiment 3 did not follow a normal distribution data were analyzed with a non-parametric Wilcoxon matched pairs test. STATISTICA 7 software was used for statistical analysis of the data. All data were expressed as mean ±SEM, and significance was set at $p<0.05$.

Results

Experiment 1. Effect of caffeine on PR/ free chow feeding choice performance: analysis on high and low performers.

The effect of caffeine (0, 2.5, 5.0, 10.0 and 20.0 mg/kg) on PR/chow feeding choice performance is shown in figs. 1A-C. Repeated measures ANOVA revealed a significant effect of caffeine on total lever presses ($F(4,44)=2.47$; $p<0.05$). Planned comparisons showed that total lever presses were significantly increased at 5.0 mg/kg compared to vehicle ($p<0.05$). Repeated measures ANOVA did not reveal any significant effect of

caffeine on pellets consumed (gr) ($F(4,44)=2.10$; n.s.), or on chow consumed ($F(4,44)=0.66$; n.s.).

Additional analyses were performed separating by a median split (120 lever presses) of high and low performers based on baseline lever pressing (figure 2A). A one-way ANOVA yielded significant differences between both groups ($F(1,10)=37.12$; $p<0.01$). Using factorial ANOVAs (performance group x dose of caffeine) for every dependent variable (Fig. 2B-D), the results showed that there was a significant effect of performance group ($F(1,10)=14.52$; $p<0.01$), treatment ($F(4,40)=3.14$; $p<0.05$) and group x treatment interaction ($F(4,40)=3.98$; $p<0.01$) on total lever presses. Planned comparison analysis showed a significant difference between 5.0 mg/kg caffeine and vehicle ($p<0.01$) among high responders, and when comparing high with low responders there were significant differences among them also at 5.0 mg/kg of caffeine ($p<0.01$). For the dependent variable high carbohydrate pellets consumed, the factorial ANOVA showed a significant effect of performance group ($F(1,10)=26.20$; $p<0.01$), caffeine treatment ($F(4,40)=3.04$); $p<0.05$), and interaction ($F(4,40)=5.89$; $p<0.01$). Planned comparisons yielded a significant difference between vehicle and caffeine 5.0 mg/kg, ($p<0.01$) among the high responders. In addition, the two lowest doses of caffeine (2.5 and 5.0 mg/kg) produced significant differences between the low and the high responder groups ($p<0.01$). With chow intake, the factorial ANOVA did not revealed any significant main effects of performance group ($F(1,10)=3.02$; n.s.) and caffeine treatment ($F(4,40)=0.70$; n.s.), and no significant interaction ($F(4,40)=1.64$; n.s.).

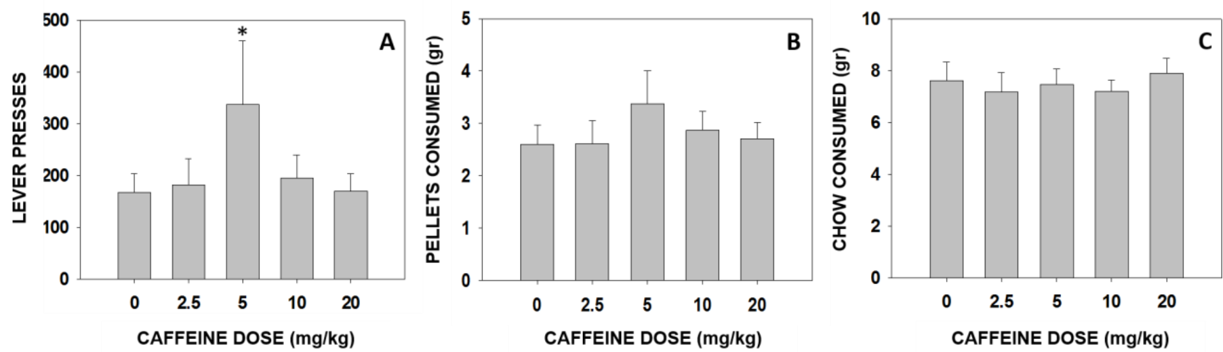

Figure 1. Effects of caffeine on PR/chow feeding choice performance in 30 minutes sessions. Mean (±SEM) total lever presses (A), amount of operant pellets consumed in grams (B), and grams of chow consumed C). *p<0.05, significantly different from vehicle.

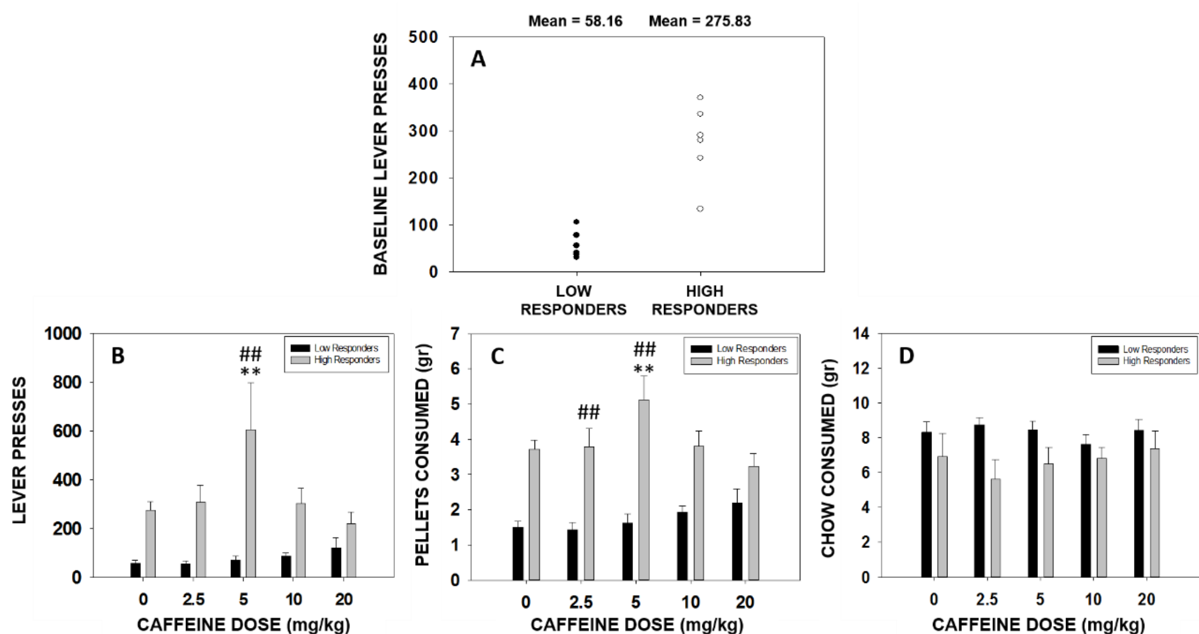

Figure 2. Upper part: Individual average scores on baseline lever presses on the PR/chow feeding choice task (A). Rats were divided in two groups using the median split (median = 120 lever presses). Lower part: effects of caffeine on the PR/chow feeding choice task in low and high responders. Mean (±SEM number of total lever presses, B), pellets consumed (in grams, C) and chow consumed (in grams, D). **p<0.01, significantly different from vehicle; ## p<0.01, high responders significantly different from low responders.

Experiment 2. Effect of caffeine on PR/ free sucrose choice performance: analysis on high and low performers.

The effect of caffeine on PR/ free sucrose choice performance in all animals is shown in figs. 3A-C. Repeated measures ANOVA did not show a significant effect of the caffeine dose (0, 5.0, 10.0 and 20.0 mg/kg) on total lever presses ($F(3,48)=0.69$; n.s.), on 5% sucrose consumed ($F(3,48)=1.56$; n.s.), or on 0.3% sucrose consumed ($F(3,48)=0.99$; n.s.).

Data were reanalyzed to study individual differences. Animals were separated using the median split (160 lever presses) into low responders and high responders based on baseline lever pressing, and a one-way ANOVA showed statistical differences between both groups ($F(1,15)=32.18$; $p<0.01$) (figure 4A). The factorial ANOVA (performance group x caffeine treatment) of lever pressing data did not show a significant effect of the main factors; neither performance group ($F(1,15)=0.27$; n.s.), nor caffeine treatment ($F(3,45)=0.90$; n.s.) were significant. However, there was a significant interaction ($F(3,45)=4.16$; $p<0.01$). Planned comparisons yielded a significant difference in lever presses between low and high responders in the vehicle condition ($p<0.05$). Moreover, there was a significant increase of lever pressing among the group of low responders after receiving 10.0 mg/kg of caffeine compared to vehicle ($p<0.05$). However, among the high performers there was a significant decrease on lever pressing after caffeine treatment at doses of 10.0 and 20.0 mg/kg compared to vehicle ($p<0.01$ and $p<0.05$, respectively). A very similar pattern was observed in the amount of 5% sucrose consumed (ml). The factorial ANOVA did not show a significant effect of treatment ($F(3,45)=2.12$; n.s.), or performance group ($F(1,15)=0.39$; n.s.), but it showed a significant interaction ($F(3,45)=5.27$; $p<0.01$). Planned comparisons revealed that caffeine at doses of 5.0 and 10.0 mg/kg increased the amount of 5% of sucrose

consumed ($p<0.05$) compared to the vehicle condition among the low responders. Moreover, caffeine at 10.0 and 20.0 mg/kg decreased the consumption of 5% sucrose compared to vehicle condition ($p<0.01$) among the high responders. As with lever pressing, all these changes were in opposite directions among low and high responders, which lead to a pattern in which these two groups of animals were only different in the vehicle condition ($p<0.05$). For the dependent variable 0.3% sucrose consumed, the factorial ANOVA did not show a significant effect of treatment ($F(3,45)=0.44$; n.s.), or of performance group ($F(1,15)=0.50$; n.s.), and there was no interaction effect ($F(3,45)=0.41$; n.s.).

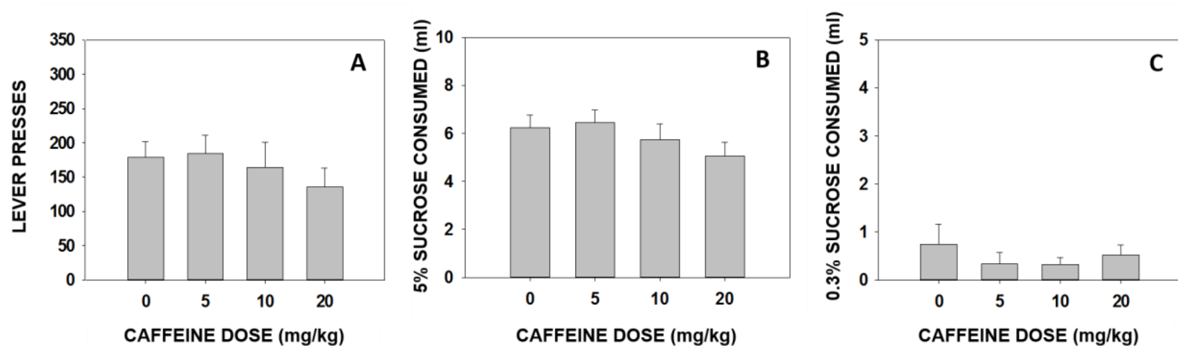

Figure 3. Effects of caffeine on PR/sucrose drinking choice task in 30 minutes sessions. Mean (±SEM) total lever presses (A), amount of operant pellets consumed in grams (B), and grams of chow consumed (C).

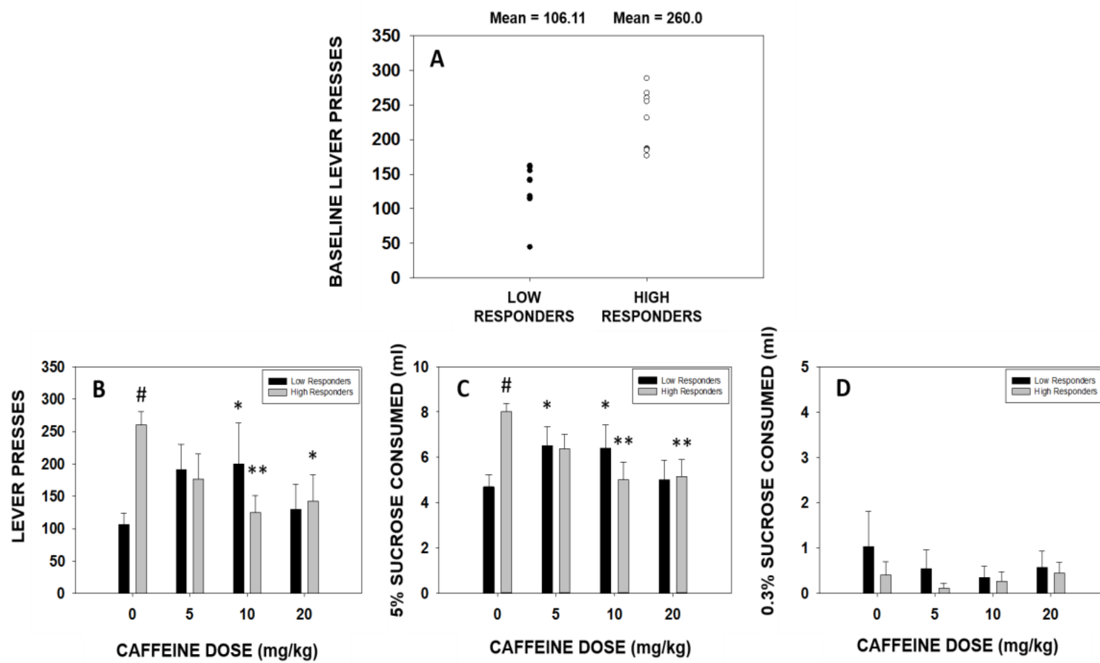

Figure 4. Upper part: individual average scores on baseline lever presses on the PR/sucrose drinking choice task (A). Rats were divided in two groups using the median split (median = 160 lever presses). Lower part: effects of caffeine on PR/sucrose drinking choice task in low and high responders. Mean (±SEM) number of total lever presses (B), milliliters of 5% sucrose consumed (C), and milliliters of 0.3% sucrose consumed (D). **$p<0.01$, *$p<0.05$ significantly different from vehicle; ## $p<0.01$, # $p<0.05$, high responders significantly different from low responders.

Experiment 3. Effect of the highest dose of caffeine on the FR7/free sucrose drinking choice task.

The effect of 20.0 mg/kg of caffeine on latency to start lever pressing in the operant chamber was analyzed using non-parametric Wilcoxon matched pairs test, ($z=0.77$; n.s.). Results did not show a significant effect of caffeine on latency to lever press in the operant chamber. However the one-way repeated measures ANOVA yielded a significant effect of caffeine on lever pressing ($F(1,8)=8.52$; $p<0.01$), on 5% sucrose consumed ($F(1,8)=6.56$; $p<0.05$), and on free 0.3% sucrose consumed ($F(1,8)=5.53$; $p<0.05$) (see Table 1).

			SUCROSE CONSUMED (ml)	
	Latency (sec)	**Lever presses**	**Operant dependent 5%**	**Free concurrent access 0.3%**
Vehicle	670.88 ± 279.78	253.33 ± 16.24	7.35 ± 0.33	0.68 ± 0.27
Caffeine 20 mg/kg	287.77 ± 55.34	191.11 ± 20.94**	5.62 ± 0.68*	0.04 ± 0.01*

Table 1. Effect of caffeine on FR7/free sucrose drinking choice task: latency to start lever pressing, total number of lever presses, and total volume of 5% sucrose and 0.3% sucrose consumed. Mean (±SEM) seconds, lever presses or milliliters. **$p<0.01$ and *$p<0.05$ significantly different from vehicle.

Experiment 4. Impact of a high dose of caffeine (20.0 mg/kg) on preference and level of sucrose intake under free access conditions.

The effect of caffeine on free access sucrose intake is shown in table 2. Repeated measures ANOVA did not show a significant effect of caffeine neither on free 5% sucrose consumed ($F(1,9)=4.44$; n.s.), or on intake of the 0.3% solution ($F(1,9)=0.18$; n.s.).

Experiment 5. Additional studies on potential effects of a high dose of caffeine (20.0 mg/kg) on locomotor activity.

When independent groups of animals were assessed for locomotion in the open field, animals treated with caffeine 20.0 mg/kg had significantly more locomotion in both measures compared to the vehicle group. The one-way ANOVAs for horizontal ($F(1, 12)=9.22$, $p<0.01$), and for vertical locomotion ($F(1, 12)=7.80$, $p<0.05$) were significant. These data are shown in table 2.

	SUCROSE CONSUMED (ml)		**OPEN FIELD**	**OPEN FIELD**
	Free access 5%	**Free access 0.3%**	**Horizontal crosses**	**Rearings**
Vehicle	16.30 ± 0.86	0.30 ± 0.04	115.9 ± 19.1	46.8 ± 8.2
Caffeine 20 mg/kg	13.80 ± 0.96	0.25 ± 0.86	202.3 ± 22.4**	76.2 ± 6.2*

Table 2. Effect of caffeine on preference for 5% or 0.3% sucrose concentration assessed on a concurrent free access paradigm, and locomotion in the open field. Mean (±SEM) milliliters, number of crosses and number of rearings. **$p<0.01$ and *$p<0.05$ significantly different from vehicle.

Discussion

The present results show how caffeine can act as a drug that helps to activate high levels of performance to achieve access to valued rewards. However, this property of caffeine is dependent on individual differences in baseline levels of performance. Moreover, these differences can explain also the ability of caffeine to impair performance under the same conditions in different groups of animals. Thus, when homeostatic demands are high (experiment 1), even the higher dose of caffeine did not impair performance. However, when the preferred reward did not have a homeostatic component (i.e., no food restriction; experiment 2), caffeine only increased lever pressing in this highly demanding task in the low responders, and had opposite effects on the high responders, significantly reducing lever pressing in these animals.

The highest dose of caffeine (20.0 mg/kg), which had no impact on the general population of animals under concurrent PR procedures, significantly reduced lever pressing (32% relative to vehicle) in animals tested on the concurrent FR7 procedure. This result is not surprising when considering that animals in this FR task operate at or near their ceiling level (the same amount of lever pressing in half the time), and as predicted, this schedule was very sensitive to the impairing effects of caffeine. Similar results were found in a previous study using the FR5/free chow procedure in which this same dose of caffeine (20.0 mg/kg) had a tendency to decrease responding (29% relative to vehicle) (Salamone et al., 2009). Thus, animals that have a high level of performance when tested under conditions of no food restriction, such as in the FR7 procedure, and also the high responders in the PR/free sucrose procedure, did not appear to get any beneficial effect of high doses of caffeine. In fact, caffeine tended to reduce performance in these animals.

Several studies in humans have shown that across various condition, caffeine can be facilitative, detrimental or ineffective (James, 1994, 1998; Kerr et al., 1991; Nehlig et al., 1992; Jarvis, 1993). The response to caffeine seems to be determined by multiple factors including dose and age. In terms of individual differences, older subjects appear to be more sensitive to the objective effects of caffeine than younger ones (Swift and Tiplady, 1988). In a study comparing younger (20-25 years old) and older (50-65 years old) subjects, it was showed that caffeine can induce small but significant improvements in vigilance and psychomotor performance, particularly in offsetting declining performance over time in the elderly (Rees et al., 1999). Dosage can also be a critical factor for certain tasks. Thus, moderate amounts of caffeine (250 mg) improved performance on a continuous attention task whereas high doses (500 mg) impaired performance (Frewer and Lader, 1993). Beneficial effects were restricted to a fairly narrow dose range up to about 4.0 mg/kg, and doses above 6.0 mg/kg were likely to degrade performance (James, 1994). Wardle et al. (2012) tested humans on an effort-based decision making task, and reported that 200 mg caffeine increased motor speed but did not enhance selection of the high effort alternative.

Tasks measuring behavioral activation and effort-based functions have been suggested as potential animal models for the motivational symptoms of depression such as fatigue, psychomotor retardation and anergia (Salamone et al., 2006, 2007, 2010). In operant tasks with different work demands, methylxanthines such as caffeine and theophylline produced rate-dependent effects on lever pressing reinforced by high-carbohydrate food pellets (Randall et al., 2011). Caffeine and theophylline increased responding on the low demanding task; a fixed interval 240 seconds (FI-240 sec) schedule, and decreased responding in rats tested on a FR20 schedule, which typically generates high rates of responding (Randall et al., 2011). The A2A antagonists MSX-3

and istradefylline increased lever pressing in the low effort-demanding task. However, A1 antagonists failed to increase lever-pressing rate, and decreased FR20 responding at higher doses (Randall et al., 2011), suggesting that the work potentiating effects of methylxanthines are mediated by their actions on adenosine A2A receptors.

Mental fatigue associated with high attentional demands can also be overcome by the use of psychostimulants such as caffeine (Peeling and Dawson, 2007; Silber et al., 2016). In cost/benefit decision-making tasks for the evaluation of the cost involved in high attention-demanding tasks, rats can choose between engaging in hard trials (difficult visuospatial discrimination) leading to more reward versus easy trials leading to less reward (Cocker et al., 2012). Similar to the results of experiment 2, caffeine administered in the same dose range as the one used in the present experiments reduced selection of the high cognitive effort task only in animals with higher preference for the difficult task, defined as “workers”, but had no effect on “slackers” choice (Cocker et al., 2012). Arousal and attention were components of these tasks not improved by caffeine (Cocker et al., 2012), which, on the other hand, seemed to benefit selection of responses that require behavioral activation, repetition and perseverance.

Behavioral variables such as preference between different types of food or between different drinking solutions and amount of food or fluid consumed can indicate if animals a drug affects directional aspects of food motivation. A characteristic of the concurrent PR/free chow paradigm used in experiment 1 is that animals, independently of their level of responding, tend to eat high amounts of free chow since they are food deprived (Randall et al., 2011; 2012; 2014). Major psychostimulants such as amphetamine and cocaine can suppress appetite (Vee et al., 1983; Sanghvi et al., 1975; White et al., 2010). However, in our PR/free reward choice studies caffeine did not have a suppressant effect on food or fluid intake. In experiment 2 animals were not water

deprived, thus the free reinforcer was consumed at a very low level and this did not change either after caffeine increased lever pressing in the low responders, or decreased lever pressing in the high responders. However, unlike previous studies using the FR5/free chow choice task in food restricted animals, in which a clear compensation towards more free chow intake is typically seen when drugs reduce lever pressing (Salamone et al., 2009), in the present results using the FR7/free sucrose drinking choice, animals did reduced lever pressing for the high concentrated sucrose but did not show increased intake of the free low concentration of sucrose, and even reduced consumption. However, when both concentrations of sucrose were freely available and the amount consumed was much higher than under operant conditions, the relative preference for the two fluids was not altered after caffeine administration at the highest dose and there was no significant reduction in consumption. Because this dose of caffeine increased general exploration in the open field, increasing horizontal locomotion and rearing, and also produced a tendency to reduce latency to start lever pressing in the concurrent FR7/ free sucrose drinking procedure, it seems plausible that lever pressing reduction was not caused by motor impairments but by an increase in non-specific exploration.

Thus from studies in humans and animals it seems that adenosine antagonists such as caffeine may be a potential pharmacological approach for treating the motivational impairments observed in some neurological and psychiatric diseases such as Parkinsonism or depression. High doses of caffeine have an impairing effect on performance if performance is already high, but methylxanthines can help to increase work output when the requirement of the task is high or when performance has been impaired by dopaminergic manipulations that affect the activational component of behavior (Salamone et al., 2009; Pardo et al., 2012).

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GENERAL CONCLUSIONS

The present dissertation presents different studies in rodent models to assess the involvement of adenosinergic and dopaminergic systems in the regulation of alcohol (Chapters 1-4) or sucrose and palatable food (Chapters 5-6) intake.

Chapter 1 reviewed epidemiological studies and laboratory human and rodent work that have assessed the impact of caffeine on alcohol consumption and other drugs of abuse. Special emphasis is made on the impact of this methylxanthine on alcohol withdrawal and psychomotor performance. Caffeine and alcohol consumption have increased lately due to the popular belief that caffeine could reduce the undesirable effects of ethanol. However, there seems to be a lack of consensus on the range of doses of caffeine that could reduce the psychomotor depressant effects of alcohol in humans. High doses of caffeine induce anxiety and ethanol withdrawal has the same effect, thus, we studied the impact of caffeine on anxiety induced by ethanol withdrawal. Interestingly, after several cycles of ethanol withdrawal and caffeine administration, our results showed an anxiolytic behavior in animals that had experienced repeated caffeine administration.

Chapter 2 evaluated the interaction of a broad range of ethanol and caffeine doses using animal models of motor stimulation, coordination and sedation. Caffeine not only failed to reverse the impairing effects of ethanol in this tasks but it exacerbated them. The interaction between caffeine and ethanol on DARPP-32 phosphorylation patterns was evaluated in different subregions of striatum. pDARPP32-Thr34 immunoreactivity increased significantly after administration of caffeine, and ethanol reduced this effect of caffeine to vehicle levels.

Due to the fact that epidemiology studies have shown a positive correlation between the consumption of energy drinks and that of ethanol, and the inconsistency in the results among the scientific literature on this topic we examined the impact of a broad range of doses of caffeine and of selective adenosine A_1 and A_{2A} receptor antagonists on voluntary ethanol intake under different ethanol access conditions. Thus, **Chapter 3** studied the effect of a broad range of doses of caffeine and selective adenosine antagonists on voluntary ethanol intake under different patterns of access condition; restricted (2 hours), unrestricted (24 hours), or after 4 days of deprivation in an unrestricted condition. Results showed that when ethanol consumption is very high (in the restricted condition) caffeine tends to reduce it. However, when ethanol is constantly available, caffeine can increase the amount of volume that animals drink. When taking into account all the animals, caffeine had a biphasic effect on ethanol consumption. However, when animals were separated in 3 different groups based on their baseline level of intake, the intermediate intake group was the only one that increased consumption after caffeine administration. Additionally, the effect of selective adenosine antagonists on voluntary ethanol intake demonstrated a similar effect of the A2A antagonist. In addition, acute caffeine administration after several cycles of forced ethanol time-off actually reduced ethanol reinstatement.

On **Chapter 4** we studied caffeine's action on effort-based decision-making. Caffeine was administered to Wistar rats self-administering ethanol under conditions of low effort (freely available ethanol solution) or high effort demands (lever pressing on a fixed ratio 5, FR5). In addition, the impact of tetrabenazine (TBZ), a VMAT-2 inhibitor that produces a reversible DA depletion, and bupropion (which increases DA by blocking DAT) were also studied. TBZ reduced ethanol compsumption by reducing lever pressing, but it did not reduced free ethanol consumption. Bupropion, on its own,

had no effect on any of the self-administration procedures. Caffeine increased ethanol self-administration only among the animals lever pressing for ethanol. Caffeine as well as bupropion, reversed the impact of DA depletion on lever pressing for ethanol. These results were parallel to phosphorilation patterns on DARPP32-Thr75.

Caffeine is consumed in many different contexts, and among normal as well as groups with psychopathologies in which appetite is pathologically altered. **Chapter 5** assessed caffeine effects at different doses on palatable food consumption in mice. Caffeine increased sweet food intake in animals with a binge eating pattern although it produced the opposite effect on food intake under anxiogenic conditions in a modified dark and light paradigm. On the other hand, consumption under effort-requiring conditions was evaluated. Caffeine did not change appetite and it did not impair orientation towards food under effortful conditions, but it rather helped to achieve the goal by improving speed, and it did reversed the effects of DA depletion on this effort-based task.

The last set of studies (**Chapter 6**) characterized the effort-related effects of caffeine in a concurrent progressive ratio (PR)/free reinforcer choice procedure that requires high levels of work to obtain the preferred reinforcer and generates great variability among different animals. These results show that caffeine has a clearer effect when analyzing individual differences, potentiating or impairing performance depending on baseline performance.

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