Facultat de Ciències de la Salut

Departament de Psicologia Bàsica, Clínica i Psicobiologia

The effects of adenosine antagonists on distinct aspects of motivated behavior: interaction with ethanol and dopamine depletion.

PhD Candidate

Laura López Cruz

Advisor

Dr. Mercè Correa Sanz

Co-advisor

Dr. John D.Salamone

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Als meus pares i germà

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ABSTRACT

Adenosine is a neuromodulator in the central nervous system (CNS) that interacts with other neurotransmitters and with some substances like alcohol, which elevates the adenosinergic tone. It has been shown that increases in adenosine levels produce sedation and fatigue, two behavioral effects that can have an impact on other more complex processes such as anxiety or motivation. Adenosine acts on different receptors, being adenosine A_1 and A_{2A} receptors the most relevant for their presence and mechanism of action in specific brain areas involved in the modulation of mood and motivational processes such as striatum, prefrontal cortex and amygdala. Caffeine is a worldwide consumed methylxanthine that acts as a non-selective adenosine A_1/A_{2A} receptor antagonist. This drug is generally consumed to reduce fatigue and increase alert. More recently, caffeine, at high concentrations, is combined with alcoholic beverages under the popular belief that can counteract some of the sedative and impairing effects of ethanol. The knowledge about how high levels of caffeine can affect complex motivated behaviors such alcohol abuse, or social interaction patterns is limited, and potential side effects such as increases in anxiety and motor impairments can modulate them. Thus, the first part of the present dissertation (Chapters 1-4), reviews the literature on caffeine-ethanol interaction, and addresses the impact of high doses of caffeine on anxiety and how they can modulate social interaction patterns in animal models. High doses of caffeine are studied also in interaction with moderate doses of ethanol that had demonstrated to produce anxiolysis. In order to provide evidence about a potentially selective mechanism of action on adenosine A_1 or on A_{2A} receptors, other selective and non-selective adenosine antagonists and A_{2A} receptor KO mice are also used.

On the other hand, A_1 and A_{2A} receptors have been proposed as therapeutical targets for the treatment of motivational impairments such as psychomotor retardation and fatigue observed in some psychopathologies such as depression. Mesolimbic dopamine (DA) is involved in the regulation of the activational component of motivation and DA depletion or antagonism has shown to impair this aspect of motivation in effort-based decision making tasks, shifting preferences from high effort/high reward options to low

effort/low reward options. Adenosine A_1 and A_{2A} are colocalized with DA D_1 and D_2 receptors respectively in striatal areas and they interact in antagonistic way at the cellular and also the behavioral level. Several selective adenosine antagonists have shown to revert the anergia-like effect induced by DA impairments. However, the therapeutical impact of caffeine on those impairments has not been widely explored. Thus, in Chapters 5-6 the impact of caffeine on depression is reviewed, and its potential on effort-based decision-making tasks in animal models is studied after the administration of a DA depleting agent. Cellular markers activated after adenosine and DA receptor interactions were analyzed in order to elucidate the mechanism of action.

Efecto de los antagonistas de adenosina en diferentes componentes de la conducta motivada: estudios de interacción con alcohol y con disminución en los niveles de dopamina.

RESUMEN

La adenosina es un neuromodulador del Sistema Nervioso Central (SNC) que interactúa con otros neurotransmisores y otras sustancias como el alcohol, el cual incrementa los niveles de adenosina. Se ha demostrado que estos incrementos adenosinérgicos producen sedación y fatiga, dos efectos conductuales que pueden tener impacto en procesos como la ansiedad o la motivación. La adenosina actúa en diferentes receptores, siendo los A_1 y los A_{2A} los más relevantes por su presencia y mecanismo de acción en áreas cerebrales involucradas en la modulación de procesos emocionales y motivacionales como el estriado, el córtex prefrontal y la amígdala. La cafeína es una metilxantina ampliamente consumida que actúa como antagonista no selectivo de los receptores A_1/A_{2A} de adenosina. Esta droga es generalmente consumida para reducir la fatiga e incrementar los niveles de alerta. Recientemente, la cafeína a dosis altas, se ingiere junto a bebidas alcohólicas bajo la creencia popular de que esta metilxantina puede compensar los efectos sedativos e intoxicantes del alcohol. El conocimiento acerca de cómo altas concentraciones de cafeína pueden afectar directamente aspectos complejos de conductas motivadas como el abuso de alcohol o la interacción social, es limitado, y además estas conductas pueden verse moduladas también por los efectos secundarios asociados a la cafeína, como es el incremento de ansiedad y las alteraciones motoras. En este sentido, la primera parte de esta tesis (Capítulos 1-4), revisa la literatura relacionada con la interacción cafeína-alcohol, y explora el impacto de dosis altas de cafeína en ansiedad y como estas sustancias pueden modular la interacción social en modelos animales. Con el objetivo de conocer el mecanismo de acción selectivo de la cafeína sobre los receptores de adenosina A_1 o A_{2A}, se estudian también otros antagonistas selectivos y no selectivos de los receptores de adenosina, así como animales KO para los receptores de adenosina A_{2A}.

Por otra parte, los receptores A_1 y A_{2A}, se han propuesto como dianas terapéuticas para el tratamiento de alteraciones motivacionales como el enlentecimiento motor y la fatiga observadas en algunas psicopatologías como la depresión. La dopamina (DA)

mesolímbica está involucrada en la regulación del componente activacional de la motivación. La disminución de los niveles de DA o el antagonismo dopaminérgico han demostrado alterar este aspecto de la motivación en tareas de toma de decisiones basadas en el esfuerzo, cambiando las preferencias del individuo desde la elección de reforzadores con gran valor que requieren un alto esfuerzo a reforzadores de menor valor que requieren menor esfuerzo. Los receptores de adenosina A_1 y A_{2A} están co-localizados con los receptores de DA D_1 y D_2 respectivamente en estructuras estriatales e interactúan de forma antagónica a nivel intracelular y conductual. Sin embargo, el impacto terapéutico de la cafeína en dichas alteraciones motivacionales no ha sido ampliamente estudiado. En este sentido, en los Capítulos 5-6, se revisa la literatura sobre el impacto de la cafeína en la depresión, y se estudia su efecto potencial en tareas de toma de decisiones basadas en el esfuerzo en modelos animales tras la administración de un agente farmacológico que reduce los niveles de DA de manera reversible. Con el objetivo de conocer los mecanismos intracelulares que regulan estos procesos, se estudia también el impacto de estas manipulaciones farmacológicas sobre marcadores de activación de los receptores de DA y de adenosina.

GENERAL INTRODUCTION

1. Adenosine as a neuromodulator in the CNS

Adenosine is considered a neuromodulator in the CNS, which 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). Unlike classical neurotransmitters that are synthesized, stored, and released into the synapse in response to electrochemical stimulation, adenosine 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). Stimulation of A_{2A} receptors has the opposite effect to A_1 receptor stimulation, and they 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). The role of A_{2B} and A_3 receptors has received considerably less attention, because they are present at very low levels in the CNS (Zhou et al., 1992; Daly et al., 1983; Dixon et al., 1996). The existence of pharmacological tools, such as selective adenosine receptor agonists and antagonists, 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 bradykinesia, catalepsy and tremor (Correa et al., 2004; Betz et al., 2009; Hauber et al., 2001; Morelli et al., 2012), psychomotor stimulation or sedation (El Yacoubi et al., 2003; Nagel et al., 2003; Pardo et al., 2014; Farrar et al., 2007; Font et al., 2008; Mingote et al., 2008), sensorimotor gating (Hauber and Koch, 1997; Koch and Hauber, 1998), memory (Hauber and Bareiss, 2001; Prediger et al., 2005), and in the regulation of affective (Correa and Font, 2008; Prediger et al., 2004; Kaster et al., 2007), and motivational processes (Salamone and Correa, 2009; Pereira et al., 2011; Pardo et al., 2012; Correa et al., 2016).

2. Caffeine and ethanol interaction: actions on the adenosine system.

Two commonly consumed drugs that have an impact on the adenosinergic system are caffeine and alcohol. 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). However, the differential concentration of A_1 and A_{2A} receptors in distinctive brain areas responsible for the modulation of different behaviors can lead to a predominant role of A_1 or of A_{2A} receptors in these behavioral effects of caffeine. Ethanol does not act directly on adenosine receptors, but 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). There is some evidence that adenosine may contribute to some of the sedative and motor incoordination effects of ethanol (Dar, 1990; Meng and Dar, 1995; Correa and Font, 2008; Correa et al., 2012).

Interest in this methylxanthine and some of its metabolites, such as theophylline, has grown since the introduction to the market of the so-called “energy drinks”. Caffeine, and to some extent theophylline, are the main psychoactive components of these drinks (a behavioral comparison between these two methylxantines is presented in **Chapter 2** of the present dissertation). 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 controversial results (as summarized in **Chapter 1**). 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 or social interaction. Thus, **chapters 3 and 4** present data on the impact of caffeine and selective adenosine receptor antagonism alone or in combination with ethanol, on social behavior and anxiety. 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. Social interaction and its modulation by anxiety.

Social behavior has been widely explored as a natural reinforcer in the overall context of the study of motivation (Martin et al., 2014; Pansskep and Lahvis, 2010; Martin and

Iceberg, 2015). Most of these studies used maternal behavior, or access to opposite sex conspecifics, as reinforcers (Matthews et al., 2005; Martín-Sánchez et al., 2015; Pereira and Ferreira, 2016), although rodents express a robust motivation to approach conspecifics in general (Brodkin et al. 2004; Moy et al. 2004, 2006; Terranova et al., 1993). Approach towards a conspecific, also referred to as social approach, is a basic behavioral component of all social interactions (Pankssep and Lahvis, 2010). This apparently simple behavior has reinforcing properties evaluated in classical paradigms such as social conditioned place preference, or operant tasks that use sex-matched conspecifics as reinforcers (Pankssep and Lahvis, 2010; Martin and Iceberg, 2015). Time spent approaching and exploring a conspecific as opposed to exploration of a non-social stimulus can offer information about the preference for social stimuli, and is a measure of appetitive social motivation.

In addition to the intrinsic motivational properties of social interaction, this behavior has shown to be sensitive to anxiolytic or anxiogenic effects of drugs and, in fact, social interaction tasks have been widely used as animal models of anxiety (File and Seth, 2003; File, 1980). Acutely administered caffeine and ethanol have been shown to have opposite effects on anxiety (Gulick and Gould, 2009; Correa et al., 2008). As reported in **Chapter 2**, caffeine (Jain et al., 2005; Prediger et al., 2004), as well as its metabolite theophylline, induce anxiogenic effects at moderate and high doses. The anxiolytic effects of ethanol have been widely explored in mice and rats (Correa et al., 2008; Prediger et al., 2004). Each of these drugs has shown to affect social interaction in a manner that is consistent with their anxiogenic or anxiolytic profile; anxiolytic drugs will enhance social interaction and anxiogenic drugs will decrease it (Prediger et al., 2004). However, a direct positive relationship between anxiety and social interaction is not always so clear, and contradictory results have been found depending on the animal model and parameters used (Baldwin et al., 1989; Baldwin and File, 1989; Hilakivi and Seth, 1989; Nadal et al., 1993; Guy and Gardner, 1985). **Chapter 4** characterizes social behaviors and anxiety in adenosine A_{2A} receptor KO mice.

It has been suggested that the opposite effects of ethanol and caffeine on anxiety are due to opposing actions on the adenosine system (Prediger et al., 2004; Correa and Font 2008): ethanol increases adenosine levels, while caffeine acts as a non-selective adenosine A_1/A_{2A} receptor antagonist. The region specific concentration of adenosine A_1 and A_{2A} receptors in the brain suggests that these receptors could play a differential

role in many behaviors. For instance, the broad distribution of A_1 receptors in the brain, with a relatively high concentration in the hipoccampus (Murphy and Snyder, 1982), suggests that they may play an important role in memory consolidation (Hauber and Bareiss, 2001) and possibly, social memories. On the other hand, adenosine A_{2A} receptors are highly concentrated in olfactory tubercle, and striatum (Fredholm et al., 2001; Schiffmann et al., 2007; Vontell et al., 2012), regions that are involved in social behavior, motivation and motor processes (Cabib et al., 2000; Salamone and Correa, 2002; Koch and Hauber, 1998). Thus, the ability of caffeine and selective A_1 and/or A_{2A} receptor antagonists, or genetic deletion, either alone or in combination with ethanol, to affect social motivation and long-term memory was studied in **chapters 3 and 4**. The use of a novel paradigm that allows free allocation of time to explore social versus non-social stimuli, a situation in which social contact is not possible, gives a measure of preference that is less affected by anxiety and social patterns like submission-dominance.

4. Functional co-localization of DA and Adenosine receptors: relevance for the activational component of motivation.

Mesolimbic DA 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). This activational aspect of motivation can be evaluated with tasks that offer the choice for distinct reinforcers that can be obtained by instrumental behaviors with different work requirements. 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), which are used in the present thesis in the experiment shown in **Chapter 5.** Effort discounting tasks (Floresco et al., 2008; Bardgett et al., 2009) also are described in the literature. Across these tasks, low doses of 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), 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 many pathologies including depression (Salamone et al., 2016). In fact, patients with depression also have shown impairments in an effort-based decision-making task adapted for humans (Treadway et al., 2012).

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., 2008, 2004; 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., 2014). 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 have 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), and the data about its impact on depression is anecdotal in humans. Thus, **chapter 5** summarizes the research about possible therapeutic actions of the non-selective A_1/A_{2A} receptor caffeine on depression, emphasizing its effects on motivational symptoms. Moreover, a T-maze barrier task for mice was used to study the impact of caffeine in effort based-decision making. In addition, in **chapter 6**, the effects of caffeine are evaluated in a recently developed task for mice (Correa et al., 2015), a 3-choice running wheel (RW) T-maze task (Correa et al., 2015), that evaluates preference for activity-based reinforcers, and has shown to be sensitive to the effects of DA D_2 antagonism. Caffeine is proposed as a therapeutic agent to reverse or attenuate the anergia-like effects induced by DA depletions. Moreover, intracellular markers of DA and adenosine receptor activation are quantified in order to explore the predominant role of D_1-A_1 or D_2-A_{2A} receptor interaction on caffeine-TBZ effects.

5. Functional co-localization of DA and Adenosine receptors: intracellular cascade.

Several lines of evidence indicate that 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).

5.1. DARPP-32 phosphorylation at threonine 34 and 75 as an index of DA receptor D_1 or D_2 activation.

As discussed above, 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 an 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, 2001). 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 et al. 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; 2004; 1999).

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)) (**Figure 1**). In this sense, DA D_1 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) (**Figure 1**).

In the present thesis (**Chapter 6**), in order to study the effects of the drugs used in the behavioral procedures (the DA depleting agent TBZ alone or in combination with caffeine as a non-selective A_1/A_{2A} receptor antagonist) on intracellular markers of D_1/A_1 and D_2/A_{2A} receptor activity, DARPP-32, pDARPP-32(Thr34) and pDARPP-32(Thr75) were quantified by western blot.

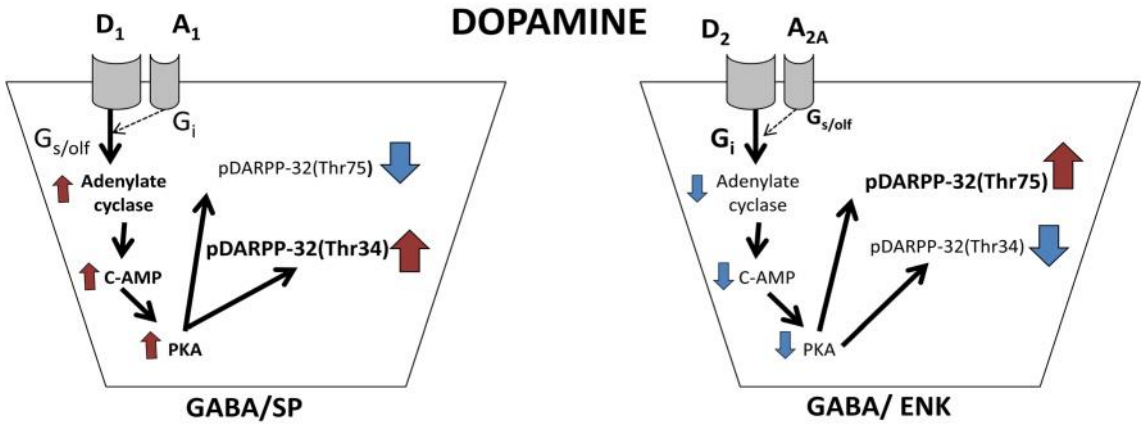

Figure 1. Diagram showing the intracellular cascade in the A_{2A}–D_2, A_1–D_1, and A_1–A_{2A} receptor heteromers and effect of DA on DARPP-32 phophorylation. D_1 receptor stimulation increases c-AMP production and PKA activity, which phosphorylates DARPP-32 to yield pDARPP-32(Thr34). D_2 receptor stimulation decreases c-AMP production and PKA activity, which decreases the dephosphorylation of pDARPP-32(Thr34) and therefore increases on pDARPP-32(Thr75) expression (for details, see Svenningsson et al., 2004; Bateup et al., 2008; Yger and Girault, 2011; Ferré 2008; Nunes et al., 2013).

5.2. TBZ as a tool to induce anergia-like effects in animal models: impact on DARPP-32 phosphorlation patterns.

The pharmacological tool used in **Chapter 6** to induced anergia-like effects is the monoamine depleting agent TBZ. This drug is a selective and reversible inhibitor of vesicular monoamine transporter-2 (VMAT-2). It blocks storage and depletes monoamines (**Figure 2**), but its greatest impact is upon striatal DA (Pettibone et al., 1984; Tanra et al., 1995; Nunes et al., 2014). TBZ is used to treat hyperkinetic movements in Huntington's disease, but depressive symptoms including fatigue are major side effects (Frank, 2009, 2010). TBZ has been used in studies involving animal models of depression (Kent et al., 1986; Wang et al., 2010), and also has been shown to induce anergia-like effects on effort-based decision-making tasks in rats producing decreases on selection of high effort/high reward options and a compensatory increase in selection of low effort/low reward choices (Yohn et al., 2014; Nunes et al., 2013; Pardo et al., 2015).

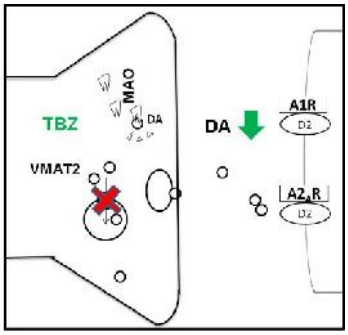

Figure 2. Tetrabenazine (TBZ) mechanism of action.

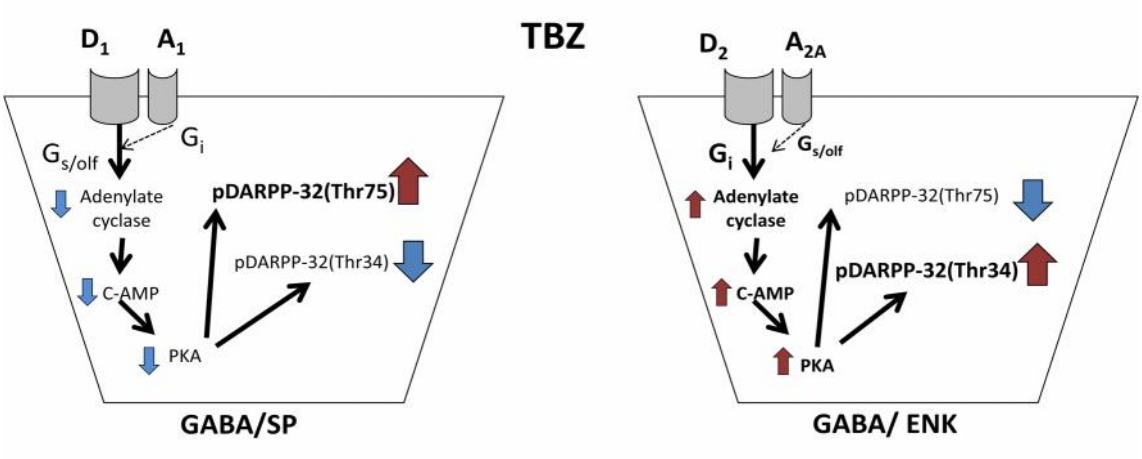

Figure 3. TBZ, which depletes DA, was hypothesized to have the opposite effect of DA, increasing pDARPP-32(Thr75) in substance-P-positive neurons and pDARPP-32(Thr34) in enkephalin-positive neurons (For more details: Nunes et al., 2013)

TBZ substantially reduced extracellular DA in NAcb core measured by microdialysis, and also affected DA-related signal transduction in a manner consistent with reduced NAcb D_1 and D_2 receptor transmission (Robertson et al., 1992; Santerre et al., 2012; Nunes et al., 2013). TBZ increased cFos immunoreactivity in NAcb core and shell, which is consistent with a reduction in D_2 transmission (Robertson et al., 1992; Santerre et al., 2012). In addition, immunocytochemical studies have evaluated the different forms of phosphorylated DARPP-32 after TBZ administration. This drug significantly increased NAcb expression of both pDARPP-32(Thr34) and pDARPP-32(Thr75), and previous results suggest that TBZ-induced increases in pDARPP-32(Thr75) would reflect reduced transmission at DA D_1 receptors, whereas the increase in pDARPP-32(Thr34) would mark reduced transmission at D_2 receptors (Svenningsson et al., 2004, 1999; Bateup et al., 2008; Yger and Girault, 2011; Nunes et al., 2013) (**see Figure 3**). Nunes et al., (2013) showed an increase pDARPP-32(Thr34) and pDARPP-32(Thr75) in NAcb after TBZ administration in rats. Administration of the selective adenosine A_{2A} antagonist MSX-3 reversed the increase of pDARPP-32(Thr34) but not the pDARPP-32(Thr75) increase induced by TBZ (Nunes et al., 2013). Again, this was consistent with studies showing that adenosine A_{2A} receptors are co-localized with D_2 receptors on enkephalin positive neurons, but not with D_1 receptors on substance-P positive neurons (Svenningsson et al., 1999), and that A_{2A} and D_2 receptors can form heteromers and interact via convergence onto c-AMP signal transduction cascades with opposite effects (Ferré et al., 2008; Svenningsson et al., 1999). Moreover, TBZ at the same doses used in

the immunochemical study, shifted response choice in rats, producing a decrease in lever pressing and a concomitant increase in chow intake in the concurrent fixed-ratio 5/chow feeding choice task (Nunes et al., 2013) and this effect was reversed by MSX-3 (Nunes et al., 2013). Thus, these markers can be studied as an index of DA-adenosine activity in the context of the study of motivation, and probably are involved in the behavioral effects observed after DA-adenosine manipulations, as suggested by previous studies showing that DARPP-32-KO mice are less sensitive to catalepsy induced by a DA D_2 antagonist (Fienberg et al., 1999).

References

- Agnati LF, Ferré S, Lluis C, Franco R, Fuxe K (2003) Molecular mechanisms and therapeutical implications of intramembrane receptor/receptor interactions among heptahelical receptors with examples from the striatopallidal GABA neurons. Pharmacol Rev. 55:509-550.

- Baldwin HA, File SE (1989). Caffeine-induced anxiogenesis: The role of adenosine, benzodiazepine and noradrenergic receptors. Caffeine-induced anxiogenesis: the role of adenosine, benzodiazepine and noradrenergic receptors. Pharmachol Biochem Behav. 32:181-186.

- Baldwin HA, Johnston AL, File SE (1989) Antagonistic effects of caffeine and yohimbine in animal tests of anxiety. Eur J Pharmacol 159(2):211-215.

- Bardgett ME, Depenbrock M, Downs M, Points M, Green L (2009) Dopamine modulates effort-based decision making in rats. Behavioral Neuroscience. ;123:242–251.

- Bateup HS, Svenningsson P, Kuroiwa M, Gong S, Nishi A, Heintz N, Greengard P (2008) Cell type-specific regulation of DARPP-32 phosphorylation by psychohostimulant and anti-psychotic drugs. Nat Neurosci. 11:932-939.

- Betz AJ, Vontell R, Valenta J, Worden L, Sink KS, Font L, Correa M, Sager TN, Salamone JD (2009). Effects of the adenosine A 2A antagonist KW 6002 (istradefylline) on pimozide-induced oral tremor and striatal c-Fos expression: comparisons with the muscarinic antagonist tropicamide. Neuroscience. 29;163(1):97-108.

- Bibb JA, et al. Phosphorylation of DARPP-32 by Cdk5 modulates dopamine signalling in neurons. Nature. 1999;402:669-671.

- Brodkin ES, Hagemann A, Nemetski SM, Silver LM (2004) Social approach avoidance behavior of inbred mouse strains towards DBA/2 mice. Brain Res. 1002: 151-157.

- Burnstock G (1972) Purinergic nerves. Pharmacolology. 24(3):509-581.

- Burnstock G (2006) Historical review: ATP as a neurotransmitter. Trends in Pharmacological Science. 27(3):166-176.

- Burnstock G (2008) Purinergic signalling and disorders of the central nervous system. Nature Review Drug Discovery. 7(7):575-590.

- Cabib S, D'Amato FR, Puglisi-Allegra S, Maestripieri D (2000). Behavioral and mesocorticolimbic dopamine responses to non aggressive social interactions depend on previous social experiences and on the opponent's sex. Behav Brain Res. 112(1-2):13-22

- Correa M, Font L (2008). Is there a major role for adenosine A2A receptors in anxiety? Front Biosci.13:4058-4070.

- Correa M, Manrique HM, Font L, Escrig MA, Aragon CM (2008) Reduction in the anxiolytic effects of ethanol by centrally formed acetaldehyde: the role of catalase inhibitors and acetaldehyde-sequestering agents. Psychopharmacology (Berl). 200(4):455-64.

- Correa M, Salamone JD, Segovia KN, Pardo M, Longoni R, Spina L, Peana AT, Vinci S, Acquas E (2012). Piecing together the puzzle of acetaldehyde as a neuroactive agent. Neurosci Biobehav Rev. 36(1):404-30.

- Correa M, San Miguel N, López-Cruz N, Bayarri P, Monferrer L, Salamone JD (2014). Caffeine Modulation of Alcohol Intake: Impact on its psychomotor effects and withdrawal. Pp: 89-111. In: Caffeine: Consumption, Side Effects and Impact on Performance and Mood. Tolley, A.S. (editor). NOVA Science Publishers, Inc.

- Correa M, Pardo M, Bayarri P, López-Cruz L, San Miguel N, Valverde O, Ledent C, Salamone JD (2015). Choosing voluntary exercise over sucrose consumption depends upon dopamine transmission: effects of haloperidol in wild type and adenosine A2AKO mice. Psychopharmacology (Berl). 233(3):393-404.

- Daly JW, Butts-Lamb P, Padgett W (1983). Subclasses of adenosine receptors in the central nervous system: interaction with caffeine and related methylxanthines.Cell Mol Neurobiol. 3(1):69-80.

- Dar MS (1990) Central adenosinergic system involvement in ethanol-induced motorincoordination in mice. J Pharmacol Exp Ther 255:1202-1209.

- Demet EM, Chicz-Demet A (2002). Localization of adenosine A2A-receptors in rat brain with [3H] ZM-241385. Naunyn Schmiedebergs Arch Pharmacol. 366:478-481.

- Diamond I, Gordon AS (1994) The role of adenosine in mediating cellular and molecular responses to ethanol. EXS. 71:175-83.

- Dixon AK, Gubitz AK, Sirinathsinghji DJ, Richardson PJ, Freeman TC (1996). Tissue distribution of adenosine receptor mRNAs in the rat. British Journal of Pharmacology, 118(6), 1461-1468.

- El Yacoubi M, Ledent C, Parmentier M, Costentin J, Vaugeois JM (2003). Caffeine reduces hypnotic effects of alcohol through adenosine A2A receptor blockade. Neuropharmacology. 45(7):977-985.

- Farrar AM, Pereira M, Velasco F, Hockemeyer J, Muller CE, Salamone JD (2007). Adenosine A(2A) receptor antagonism reverses the effects of dopamine receptor antagonism on instrumental output and effort-related choice in the rat: mplications for studies of psychomotor slowing. Psychopharmacology. 191:579-586

- Ferré S (1997). Adenosine-dopamine interactions in the ventral striatum. Implications for the treatment of schizophrenia. Psychopharmacology. 133:107-120

- Ferré S (2008). An update on the mechanisms of the psychostimulant effects of caffeine. J Neurochem. 4:1067-1079.

- Ferré S, Ciruela F, Canals M, Marcellino D, Burgueno J, Casado V, et al. (2004) Adenosine A2A-dopamine D2 receptor- receptor heteromers. Targets for neurço-psychiatric disorders. Parkinsonism Relat Disord.10:265-271.

- Ferré S, Quiroz C, Woods AS, Cunha R, Popoli P, Ciruela F, Lluis C, Franco R, Azdad K, Schiffmann SN (2008). An update on adenosine A2Adopamine D2 receptor interactions: implications for the function of G protein-coupled receptors. Curr Pharm Des. 14:1468-1474.

- Fienberg AA, Hiroi N, Mermelstein PG, Song W, Snyder GL, et al. (1998) DARPP-32: Regulator of the efficacy of dopaminergic neurotransmission. Science 281: 838–842.

- File SE (1980). The use of social interaction as a method for detecting anxiolytic activity of chlordiazepoxide-like drugs, Journal of Neuroscience Methods. 2(3):219-238.

- File SE, Seth P (2003). A review of 25 years of the social interaction test. Eur J. Pharmacol. 463(1-3):35-53.

- Floresco SB, St Onge JR, Ghods-Sharifi S, Winstanley CA (2008) Cortico-limbic-striatal circuits subserving different forms of costbenefit decision-making. Cogn Affect Behav Neurosci 8:375–389

- Fredholm BB, Abbracchio MP, Burnstock G, Daly JW, Harden TK, Jacobson KA, Leff P, Williams M (1994). Nomenclature and classification of purinoceptors. Pharmacol Rev. 46(2):143-156.

- Fredholm BB, IJzerman AP, Jacobson KA, Klotz KN, Linden J (2001). International Union of Pharmacology. XXV. Nomenclature and classification of adenosine receptors. Pharmacol Rev. 53(4):527-552

- Greengard P, Allen PB, Nairn AC (1999). Beyond the dopamine receptor: the DARPP-32/protein phosphatase-1 cascade. Neuron. 23(3):435-447.

- Gulick D, Gould TJ (2009). Effects of ethanol and caffeine on behavior in C57BL/6 mice in the plus-maze discriminative avoidance task. Behav Neurosci.123(6):1271-1278.

- Guy AP, Gardner CR (1985). Pharmacological characterisation of a modified social interaction model of anxiety in the rat. Neuropsychobiology. 13(4):194-200.

- Hauber W, Neuscheler P, Nagel J, Müller CE (2001). Catalepsy induced by a blockade of dopamine D1 or D2 receptors was reversed by a concomitant blockade of adenosine A2A receptors in the caudate putamen of rats. Eur J Neurosci. 14:1287-1293.

- Hauber W1, Bareiss A (2001). Facilitative effects of an adenosine A1/A2 receptor blockade on spatial memory performance of rats: selective enhancement of reference memory retention during the light period. Behav Brain Res. 118(1):43-52.

- Jain NS, Hirani K, Chopde CT (2005).Reversal of caffeine-induced anxiety by neurosteroid 3-alpha-hydroxy-5-alpha-pregnane-20-one in rats. Neuropharmacology. 48(5):627-638.

- Kaster MP, Machado NJ, Silva HB, Nunes A, Ardais AP, Santana M, Baqi Y, Müller CE, Rodrigues AL, Porciúncula LO, Chen JF, Tomé ÂR, Agostinho P, Canas PM, Cunha RA (2015). Caffeine acts through neuronal adenosine A2A receptors to prevent mood and memory dysfunction triggered by chronic stress. Proc Natl Acad Sci U S A. 112(25):7833-7838.

- Koch M, Hauber W. (1998) Regulation of sensorimotor gating by interactions of dopamine and adenosine in the rat. Behav Pharmacol. 9(1):23-29.

- Martin LJ, Tuttle AH, Mogil JS (2014). The interaction between pain and social behavior in humans and rodents. Curr. Top. Behav. Neurosci. 1-18.

- Martin L, Iceberg E (2015) Quantifying Social Motivation in Mice Using Operant Conditioning. J Vis Exp. (102):e53009.

- Martín-Sánchez A, Valera-Marín G, Hernández-Martínez A, Lanuza E, Martínez-García F, Agustín-Pavón C (2015). Wired for motherhood: induction of maternal care but not maternal aggression in virgin female CD1 mice. Front Behav Neurosci. 9:197.

- Matthews TJ, Abdelbaky P, Pfaff DW (2005). Social and sexual motivation in the mouse.Behav Neurosci. 119(6):1628-1639.

- Meng ZH, Dar MS (1995). Possible role of striatal adenosine in the modulation of acute ethanol-induced motor incoordination in rats. Alcohol Clin Exp Res. 19:892-901.

- Mott AM, Nunes EJ, Collins LE, Port RG, Sink KS, Hockemeyer J, Müller CE, Salamone JD (2009). The adenosine A2A antagonist MSX-3 reverses the effects of the dopamine antagonist haloperidol on effort-related decision making in a T-maze cost/benefit procedure. Psychopharmacology (Berl). 204:103-112.

- Moy SS, Nadler JJ, Perez A, Barbaro RP, Johns JM, Magnuson TR, Piven J, Crawley JN (2004). Sociability and preference for social novelty in five inbred strains: an approach to assess autistic-like behavior in mice. Genes Brain Behav. 3:287–302

- Moy SS, Nadler JJ, Young NB, Perez A, Holloway LP, Barbaro RP, Barbaro JR, Wilson LM, Threadgill DW, Lauder JM, Magnuson TR, Crawley JN (2006). Mouse behavioral tasks relevant to autism: phenotypes of 10 inbred strains. Behav Brain Res. Epub ahead of print, doi: 10.1016/j.bbr.2006.07.030

- Murphy KM, Snyder SH (1982). Heterogenity of adenosine A1 receptor binding in brain tissue. Mol Pharmacol. 22:250-257.

- Nadal RA, Pallares MA, Ferré NS (1993). The effects of caffeine in the social interaction test and on exploration in rats: comparison with ethanol and clorazepate. Behav Pharmacol. 4(5):501-508.

- Nowend KL, Arizzi M, Carlson BB, Salamone JD (2001). D_1 or D_2 antagonism in nucleus accumbens core or dorsomedial shell suppresses lever pressing for food but leads to compensatory increases in chow consumption. Pharmacology Biochemistry and Behavior. 69:373-382.

- Ouimet C. C., Langley-Gullion K. C., Greengard P. (1998). Quantitative immunocytochemistry of DARPP-32-expressing neurons in the rat caudatoputamen. Brain Res. 808, 8–1210.1016/S0006-8993(98)00724-0 - Panksepp JB, Lahvis GP (2007) Social reward among juvenile mice. Genes, Brain, and Behavior. 6(7):661-671.

- Pardo M, López-Cruz L, San Miguel N, Salamone JD, Correa M (2015). Selection of sucrose concentration depends on the effort required to obtain it: studies using tetrabenazine, D1, D2, and D3 receptor antagonists. Psychopharmacology (Berl). 232(13):2377-91.

- Pardo M, Lopez-Cruz L, Valverde O, Ledent C, Baqi Y. Müller CE. Salamone JD, Correa M (2012). Adenosine A2A receptor antagonism and genetic deletion attenuate

the effects of dopamine D2 antagonism on effort-based decision making in mice. Neuropharmacology. 62:2068-2077.

- Pereira M, Ferreira A (2016). Neuroanatomical and neurochemical basis of parenting: Dynamic coordination of motivational, affective and cognitive processes. Horm Behav. 77:72-85.

- Pettibone DJ, Totaro JA, Pflueger AB (1984) Tetrabenazine-induced depletion of brain monoamines: characterization and interaction with selected antidepressants. Eur J Pharmacol 102:425-430.

- Prediger RD, Batista LC, Takahashi RN (2004) Adenosine A1 receptors modulate the anxiolytic-like effect of ethanol in the elevated plus-maze in mice. Eur J Pharmacol.499(1-2):147-154.

- Proctor WR, Baker RC, Dunwiddie TV (1985). Differential CNS sensitivity to PIA and theophylline in long-sleep and short-sleep mice. Alcohol. 2(2):387-391.

- Randall PA, Nunes EJ, Janniere SL, Stopper CM, Farrar AM, Sager TN, Salamone J D (2011). Stimulant effects of adenosine antagonists on operant behavior: differential actions of selective A_{2A} and A_1 antagonists. Psychopharmacology, 216(2):173–186.

- Robbins TW, Everitt BJ (2007) A role for mesencephalic dopamine in activation: commentary on Berridge (2006). Psychopharmacology. 191:433-437.

- Robertson GS, Vincent SR, Fibiger HC (1992). D1 and D2 dopamine receptors differentially regulate c-fos expression in striatonigral and striatopallidal neurons. Neuroscience 49:285-296.

- Salamone JD, CorreaM (2002) Motivational views of reinforcement: implications for understanding the behavioral functions of nucleus accumbens dopamine. Behav Brain Res 137(1–2):3–25.

- Salamone JD, Cousins MS, Snyder BJ (1997) Behavioral functions of nucleus accumbens (DA): empirical and conceptual problems with the anhedonia hypothesis. Neurosci Biobehav Rev 21:341–359.

- Salamone JD, Correa M, Farrar A, Mingote SM (2007) Effort-related functionsof nucleus accumbens and associated forebrain circuits. Psychopharmacology(Berl) 191:461– 482.

- Salamone JD, Correa M, Farrar AM, Nunes EJ, Collins LE (2010) Role of dopamine-adenosine interactions in the brain circuitry regulating effortrelateddecision making: insights into pathological aspects of motivation. Future Neurol 5:377–392.

- Salamone JD, Nunes EJ, Randall PA, Huizenga M, Nowak V, Pardo M, Correa M (2012) Development of rodent models of the motivational symptoms of depression: Comparison of the effects of the monoamine depleting agents reserpine and tetrabenazine on effort-related decision making. Program No. 923.05. 2012 Neuroscience Meeting Planner, New Orleans, LA: Society for Neuroscience, Online.
- Salamone JD, Arizzi MN, Sandoval MD, Cervone KM, Aberman JE (2002) Dopamine antagonists alter response allocation but do not suppress appetite for food in rats: contrast between the effects of SKF 83566, raclopride, and fenfluramine on a concurrent choice task. Psychopharmacology (Berl).160:371-380.
- Salamone JD, Correa M (2009). Dopamine/adenosine interactions involved in effort-related aspects of food motivation. Appetite. 53(3):422.
- Salamone JD, Steinpreis RE, McCullough LD, Smith P, Grebel D, Mahan K (1991) Haloperidol and nucleus accumbens (DA) depletion suppress lever pressing for food but increase free food consumption in a novel food choice procedure. Psychopharmacology 104:515-521.
- Santerre JL, Nunes EJ, Kovner R, Leser CE, Randall PA, Collins-Praino LE, Lopez Cruz L, Correa M, Baqi Y, Mu¨ller CE, Salamone JD (2012). Behavioral studies with the novel adenosine A2A antagonist MSX-4: reversal of the effects of D2 antagonism. Pharmacol Biochem Behav 102:477-487.
- Schiffmann SN, Fisone G, Moresco R, Cunha RA, Ferré S (2007). Adenosine A2A receptors and basal ganglia physiology. Prog Neurobiol. 83(5):277-292.
- Svenningsson P, Lindskog M, Ledent C, Parmentier M, Greengard P, Fredholm BB, Fisone G (1999). Regulation of the phosphorylation of the dopamine- and cAMP-regulated phosphoprotein of 32 kDa *in vivo* by dopamine D_1, dopamine D_2, and adenosine A_{2A} receptors. *Proceedings of the National Academy of Sciences of the United States of America*. 97(4):1856-1860.
- Svenningsson P, Hall H, Sedvall G, Fredholm BB (1997). Distribution of adenosine receptors in the postmortem human brain: an extended autoradiographic study.Synapse. 27(4):322-335.
- Svenningsson P, Lindskog M, Rognoni F, Fredholm BB, Greengard P, Fisone G (1998). Activation of adenosine A2A and dopamine D1 receptors stimulates cyclic AMP-dependent phosphorylation of DARPP-32 in distinct populations of striatal projection neurons. Neuroscience. 84(1):223-228.

- Svenningsson P, Nishi A, Fisone G, Girault JA, Nairn AC, Greengard P (2004). DARPP-32: an integrator of neurotransmission. Annu Rev Pharmacol Toxicol. 44:269-296.

- Tanra AJ, Kagaya A, Okamoto Y, Muraoka M, Motohashi N, Yamawaki S (1995). TJS-010, a new prescription of oriental medicine, antagonizes tetrabenazine-induced suppression of spontaneous locomotor activity in rats. Prog Neuropsychopharmacol Biol Psychiatry 19:963–971.

- Terranova ML, Laviola G, Alleva E (1993) Ontogeny of amicable social behavior in the mouse: gender differences and ongoing isolation outcomes. Dev Psychobiol. 26: 467-481.

- Urry E, Landolt HP (2015). Adenosine, caffeine, and performance: from cognitive neuroscience of sleep to sleep pharmacogenetics. Curr Top Behav Neurosci. 25:331-366.

- Vontell R, Segovia KN, Betz AJ, Mingote S, Goldring K, Cartun RW, Salamone JD (2010). Immunocytochemistry Studies of Basal Ganglia Adenosine A2A Receptors in Rat and Human Tissue. 33(1):41-47.

- Walaas SI, Greengard P. DARPP-32, a dopamine- and adenosine 3':5'-monophosphate-regulated phosphoprotein enriched in dopamine-innervated brain regions. I. Regional and cellular Distribution in the rat brain. J Neurosci. 1984 Jan;4(1):84-98.

- Yger M, Girault JA (2011) DARPP-32, jack of all trades… master of which? Front Behav Neurosci. 5:56.

- Yohn SE, Thompson C, Randall PA, Lee CA, Müller CE, Baqi Y, Correa M, Salamone JD (2015).The VMAT-2 inhibitor tetrabenazine alters effort-related decision making as measured by the T-maze barrier choice task: reversal with the adenosine A2A antagonist MSX-3 and the catecholamine uptake blocker bupropion. Psychopharmacology (Berl). 232(7):1313-1323.

- Zhou QY, Li C, Olah ME, Johnson RA, Stiles GL, Civelli O (1992). Molecular cloning and characterization of an adenosine receptor: the A3 adenosine receptor. Proceedings of the National Academy of Sciences of the United States of America. 89(16):7432-7436-Treadway MT, Bossaller N, Shelton RC, Zald DH (2012). Effort-Based Decision-Making in Major Depressive Disorder: A Translational Model of Motivational Anhedonia. Journal of Abnormal Psychology. 121(3):553-558.

OBJECTIVES

The present dissertation reviews and studies the role of caffeine as a non-selective adenosine A_1 and A_{2A} receptor antagonist in different mood and motivational processes. The effects of caffeine will be compared with other selective and non-selective adenosine antagonists.

Because ethanol is often consumed in combination with high doses of caffeine, and since ethanol increases adenosine levels, several studies will address the interacting effects of both substances on a motivated behavior that is also modulated by anxiety, i.e., social interaction.

Adenosine receptors are co-localized with DA receptors and their activation leads to functionally opposite effects. Thus, a second group of studies will evaluate the interaction between caffeine and a DA depleting agent on the activational component of motivation and its implications for depression.

C**hapter 1** reviews human and animal studies that have focused so far on the behavioral interaction between caffeine and ethanol, explaining the potential mechanism of action for the interaction.

Chapter 2 compares the effect of high doses of caffeine and its active metabolite theophylline (also present in some "energy drinks"), on behavioral activation, coordination, anxiety and endocrine parameters.

C**hapters 3** studies the effect of a broad range of doses of caffeine and selective adenosine antagonists on their own or in combination with ethanol in order to explore the adenosinergic substrate underlying the effect of caffeine-ethanol combinations, focusing on their effects on social motivation and long-term social memory.

C**hapter 4** evaluates the impact of A_{2A} receptor deletion on social interaction and its interaction with ethanol. The impact of the A_{2A} deletion on social interaction will be compared to its effect on anxiety.

C**hapter 5** reviews the literature about the possible therapeutic effects on of caffeine on depression in humans. In addition it reviews animal data on the impact of caffeine and selective adenosine antagonists on animal models of depression, including a study about the impact of caffeine on anergia-like impairments induced by a DA depleting agent

(tetrabenazine (TBZ), which is known to induce symptoms of depression in humans), using a T-maze barrier choice task for mice.

C**hapter 6** explores the effect of caffeine on motivational impairments induced in mice by a DA depleting agent (TBZ), using a novel 3-choice T-maze task for the assessment of preferences between reinforcers with different effort demands. Intracellular markers of DA receptor activity are also evaluated in order to assess the interaction between D_2-A_{2A} or D_1-A_1 receptors after TBZ-caffeine administration.

CHAPTER 1:

The Impact of Caffeine on the Behavioral Effects of Ethanol Related to Abuse and Addiction: A Review of Animal Studies

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The impact of caffeine on the behavioral effects of ethanol related to abuse and addiction: a review of animal studies.

López-Cruz L[1], Salamone JD[2] and Correa M[1,2,*.]

[1]Àrea de Psicobiologia, Campus de Riu Sec, Universitat Jaume I, 12071 Castelló, Spain

[2]Department of Psychology, University of Connecticut, Storrs, CT, USA 06269-1020

Running title: Caffeine and ethanol interaction: animal studies

*Corresponding author: Mercè Correa, Ph.D.

Àrea de Psicobiologia, Universitat Jaume I,

Campus Riu Sec, 12071 Castelló, Spain.

Tel: +34-964-729841.

Fax: +34-964-729267.

E-mail address: correa@psb.uji.es

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ABSTRACT:

The impact of caffeine on the behavioral effects of ethanol, including ethanol consumption and abuse, has become a topic of great interest due to the rise in popularity of so-called “energy drinks”. Energy drinks high in caffeine are frequently taken in combination with ethanol under the popular 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 effects related to alcohol consumption and the development of ethanol addiction. In the present review we discuss the relatively small number of animal studies that have assessed the interactions between caffeine and ethanol, as well as the interactions between ethanol and subtype selective adenosine receptor antagonists, in order to understand the basic findings and determine the possible mechanisms of action underlying caffeine-ethanol interactions.

KEYWORDS: ethanol intake, energy drink, adenosine, methylxanthine, anxiety, locomotion, withdrawal.

1. **Caffeine as a modulator of ethanol abuse**

Caffeine and ethanol are widely consumed recreational drugs.[1,2] Alcohol abuse is a worldwide health problem, with serious medical, economic, and social consequences.[3,4] On the other hand, caffeine intake, even in excess, appears to be relatively well accepted because methylxanthines have activating and attention preserving properties that can help productivity and enhance performance. Interest in caffeine has grown ever since the introduction to the market of the so-called "energy drinks", which contain caffeine and related substances in quite high concentrations. These drinks are being increasingly consumed, often in combination with substances that have abuse potential.[5] In addition, research with animals has demonstrated the ability of methylxanthines, and in particular caffeine, to modulate the psychopharmacological effects of drugs of abuse such as methamphetamine[6], amphetamine[7], nicotine[8,9], cocaine[10], and ethanol.[11] The reasons for combining caffeine with ethanol may stem from the popular belief that caffeine can antagonize the intoxicating effects of alcohol.[12] Some studies have supported this hypothesis, demonstrating that caffeine attenuates ethanol-induced changes in psychological parameters in humans such as information processing, memory, psychomotor performance, and others (for a review[13]).

Caffeine has been shown to indirectly modulate the activity of many neurotransmitters and neuromodulators, including dopamine, acetylcholine or glutamate[14-17] in various brain areas. But in terms of direct actions, caffeine is most widely described as an adenosine receptor antagonist that is nonselective for A_1 and A_{2A} subtypes of adenosine receptors in the central nervous system (CSN). [1,17-19] Several papers have demonstrated that there are interactions between adenosine and ethanol. Ethanol can increase extracellular adenosine levels by increasing adenosine release[20,21], and by decreasing adenosine uptake[22] that takes place via a facilitative nucleoside transporter.[23,24] Inhibition of this transporter in the

presence of ethanol would lead to an increase in extracellular adenosine and could thereby modulate some of the effects of ethanol.[21] Secondarily, ethanol increases adenosine levels because acetate generated by ethanol metabolism promotes adenosine synthesis[25] (see Figure 1).

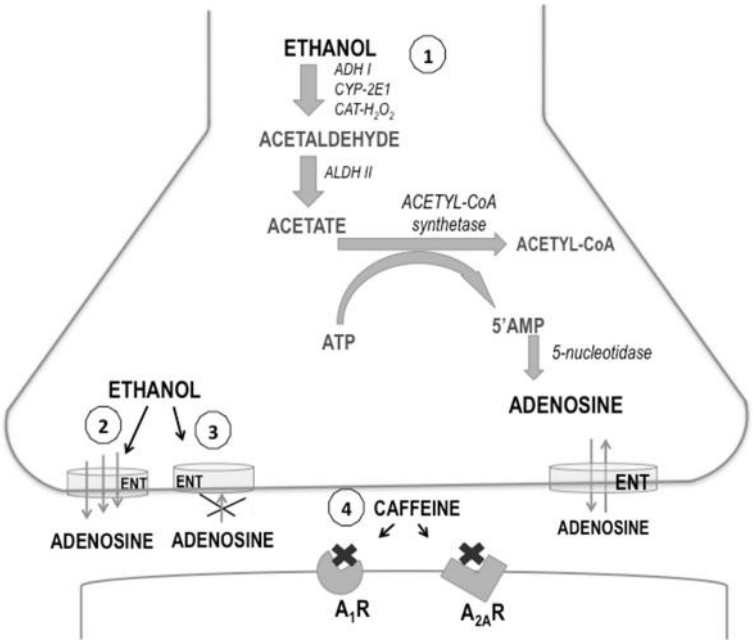

Fig. 1. Schematic showing ethanol regulation of adenosine production (1) release (2), and uptake (3), as well as caffeine blockade of adenosine receptors (4) in the CNS. Abbreviations: A_1R and $A_{2A}R$, adenosine A_1 and A_{2A} receptors; ADH, alcohol dehydrogenase; ALDH, aldehyde dehydrogenase; ATP, adenosine triphosphate; AMP, adenosine monpohospahte; CAT-H_2O_2, catalase; CYP-2E1, cytochrome P4502E1; ENT, equilibrative nucleoside transporters.

In contrast to the studies showing that caffeine can blunt the effects of ethanol, there also is evidence that fails to support the idea of an antagonistic behavioral interaction between caffeine and ethanol, either in humans[26,27] (for review[13]), or in rodents.[28,29,30] A considerable number of studies employing experimental animal models have been performed to elucidate the impact of caffeine on the effects of ethanol and on ethanol consumption. In the present review we have emphasized those studies addressing behaviors that can be relevant for the development of alcohol consumption, abuse, and

addiction as a compulsive habit, as well as studies that evaluate signs of dependence after withdrawal, such as physical abstinence and craving, which are factors that can lead to relapse.

Drug addictions, including alcoholism, can be conceptualized as disorders of motivation characterized by an excessive control of the drug over behavior.[31-33] This disorder involves a reorganization of the preference structure of the person, dramatic changes in the allocation of behavioral resources towards the addictive substance[34,35], and alterations in the elasticity of demand for the drug.[36] Typically, there is a heightened tendency to engage in drug-reinforced instrumental behavior and drug consumption, often at the expense of other behavioral activities. Addicts will go to great lengths to obtain the drug, overcoming numerous obstacles and constraints. In addition, the development of addiction is attributed to a profound sensitization in the neural processes that mediate drug-seeking behavior, which can facilitate the incentive properties of drugs and drug-related stimuli as the addiction process proceeds.[37,38] Thus, as addiction progresses, the drug itself, as well as drug-associated stimuli, trigger an automatic seeking response that ultimately resolves in the consumption of the drug. This automatism has compulsive characteristics that are devoid of instrumental feedback, leading to the formation of drug-related habits.[39,40] Thus, addiction is a very complex set of behavioral and physiological processes that range all the way from drug consumption, to tolerance for some effects, sensitization of motor activity, establishment of implicit and explicit learning, initial sensitivity to reward and punishment, attention shifts, responsivity to Pavlovian cues, and other processes.

In the present review, studies addressing the impact of caffeine on some of those behaviors modulated by ethanol will be summarized. Because the opposing actions of ethanol and caffeine on the adenosine system, studies focusing on the effects of selective adenosine receptor agonists and antagonists and their interaction with ethanol will be also presented in an attempt to shed light upon potential receptor mechanisms involved.

2. Caffeine-ethanol interactions: effects on locomotion

Evaluation of the behavioral stimulant or suppressant actions of drugs is frequently conducted by analyzing the locomotor activity of animals.[41,42] Although ethanol is generally classed as a sedative-hypnotic and caffeine is considered to be a minor stimulant, both drugs are able to stimulate locomotor activity in rodents at some dose[43-48], typically with bell-shaped (or inverted-u) dose response functions. Rodents (more in mice than rats) show a time- and dose-dependent locomotor response to acute ethanol administration, with low doses stimulating and high doses reducing locomotion.[46,49-52] Methylxanthines such as caffeine also can affect locomotor activity in a biphasic way.[53-56] However, few studies have evaluated caffeine-ethanol interactions using locomotion as a measure.[51,53,57,**58**] Waldeck (1974) evaluated the effect of ethanol (1, 3 or 4 g/kg, intraperitoneal; IP) and caffeine (25, 50 or 100 mg/kg, IP) on locomotor activity in female mice, and observed that a moderate dose of caffeine (25 mg/kg) that stimulated locomotion also potentiated the stimulation induced by ethanol administered at the lowest dose (1 g/kg), although it abolished the stimulant effect of a higher dose of ethanol (3 g/kg). On the other hand, a motor suppressant dose of caffeine (100 mg/kg) totally blocked the stimulant effect of ethanol (1 g/kg). Moreover, the motor suppressant effect of the higher dose of ethanol (4 g/kg) was potentiated by all doses of caffeine employed[51]. These results with female mice are in close agreement with the observations obtained from cats reported by Pilcher (1911). This author concluded 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".[57]

Oral administration of both drugs in mice could be a useful tool for studying the effects of ethanol-caffeine interactions, since both drugs are consumed orally in humans. Indeed, as mentioned above, energy drinks contain high concentrations of caffeine, and their consumption in combination with alcoholic beverages is a common practice among young people. The popular belief suggests that, in humans, energy drinks could reduce the intensity of the motor suppressant effects of ethanol.[26] However, only one study has explored the effects of ethanol on the stimulant effects of energy drinks in animal models.[59] In this study done in mice, the oral administration of energy drinks did not significantly alter the effects of moderate oral doses of ethanol (0.5, 1.0 or 1.5 g/kg), but was able to reduce the suppressant effects of a higher dose of ethanol (2.5 g/kg). It is possible that in this study some effects could be attributed to other stimulant components of the energy drinks, such as taurine, which has been shown to interact with ethanol on locomotion.[60,61] However, acute oral co-administration of caffeine at a low dose (10 mg/kg) combined with ethanol (1.6, 2.4 and 3.2 g/kg) was demonstrated to increase locomotor activity compared with the effect observed after separate administration of each individual drug.[53]

It is also relevant to consider the effects of acute administration of caffeine or ethanol on the chronic actions of these substances.[58,62-64] Chronic caffeine intake reduces spontaneous locomotion in mice[62], and rats.[58] However, chronic caffeine consumption (0.1% during 30 days) increased sensitivity (relative to water consumption) to the activating effects of an acute dose of ethanol (1.5 g/kg, IP) in rats.[58] In contrast, in mice exposed to chronic caffeine (1 g/L during 7 days), acute doses of ethanol (1.5 and 2.5 g/kg, IP) significantly induced locomotion, but never to the level of animals in the water control group.[62-64] Furthermore, acute caffeine administration (10-35 mg/kg) increased locomotion to a similar extent in mice chronically consuming ethanol (5%, v/v) and those in the water control group (in this case ethanol did not affect spontaneous locomotion). Thus, chronic

consumption of ethanol did not change the acute stimulant effects of caffeine.[62] The same pattern of results was found after acute administration of 5'-*N*-ethylcarboxamidoadenosine (NECA), an adenosine agonist with high affinity for both A_1 and A_{2A} adenosine receptors. In this case, NECA suppressed locomotion in a similar manner in mice chronically consuming either water or ethanol.[62]

Adolescence is a vulnerable time for organisms exposed to drugs of abuse such as ethanol.[65] It is widely acknowledged that the human adolescent brain is not fully mature[66,67], and there is evidence from animal studies that exposure to alcohol during adolescence can affect subsequent brain/behavior development.[68,69] Voluntary consumption of ethanol (at a concentration of 8.5 g/L that led to a dose of 1.0-1.5 g/kg), caffeine (at a concentration of 170 mg/L that led to a dose of 20-30 mg/kg), or an ethanol-caffeine combination during late adolescence in male and female rats had effects on subsequent adult behavior that were dependent on the sex of the rats.[70] Males showed more ambulation following exposure to the alcohol-caffeine mixture, while females exposed to the mixture showed the opposite effects, i.e., suppressed ambulation.[70] This pattern of results could be related to sex differences in the sensitivity to the neurotoxic effects of caffeine.[71] In hippocampal cultures pre-exposed to 5 mM ethanol for 10 days, caffeine (5 or 20 µM) produced greater neurotoxicity in cultures from female tissues than from male ones, specifically in the dentate gyrus and CA1 region.[71] These results demonstrate the importance of including both sexes in investigations of this sort.

In summary, the interacting effects of caffeine and ethanol on locomotor activity are quite complex. It seems that at low doses, acute caffeine administration can increase the stimulant effects of acute doses of ethanol. However, when caffeine or ethanol doses are higher, a potentiation of the suppressant effects of both substances is most evident. On the other hand, chronic administration of either substance does not appear to change the acute doses at which locomotion can be stimulated.

3. Caffeine-ethanol interactions: effects on motor coordination

At medium to high doses, a typical action of ethanol is to impair motor coordination.[72-76] This effect generally shows tolerance with repeated ethanol exposure.[77,78] The development of tolerance appears to be relevant for the emergence of ethanol abuse and dependence, because it can attenuate the performance impairing effect of the drug, which promotes the use of escalating doses.[79] Several studies have investigated the ability of caffeine to modulate ethanol-induced motor incoordination and have explored the possible involvement of adenosine receptors.[28,29,76,80-82]

A single injection of a broad range of doses of caffeine (5 - 75 µg) administered in the brain ventricles (ICV) or peripherally (2.5 - 62.5 mg/kg, IP) did not alter motor coordination in mice evaluated in the rotarod test.[80,81] However, pretreatment with low doses of caffeine (2.5-25.0 µg ICV, or 2.5-5.0 mg/kg IP) was effective in decreasing the degree and duration of motor incoordination produced by a single dose of ethanol (2 g/kg, IP). The antagonism by caffeine of ethanol-induced motor incoordination was dose related, since higher doses of caffeine (75 µg ICV, or 62.5 mg/kg IP) enhanced ethanol-induced motor incoordination.[80,81] The methylxanthine (and caffeine metabolite) theophylline was less potent, but dose-dependently attenuated (100-150 µg, ICV, 50 mg/kg IP) the motor incoordinating effect of acute ethanol (1.5-2 g/kg, IP).[73,74] On the other hand, potentiation of ethanol-induced ataxia was also observed after pretreatment with another methylxanthine, 3-isobutyl-1-*methylxanthine* (IBMX).[81]

Chronic oral administration of caffeine for 10 days (45 and 90 mg/kg/day) and IBMX (30 and 60 mg/kg/day), potentiate acute ethanol-induced motor incoordination (1.5 g/kg, IP), an effect that was associated with increased adenosine A_1 receptor binding compared to tap water controls.[28] However, no interaction with ethanol-induced motor incoordination (1.5

g/kg, IP) was observed after chronic theophylline (75 and 150 mg/kg/day) consumption.[28] This lack of effect of chronic theophylline on motor incoordination induced by ethanol was paralleled with the lack of changes in A_1 receptor density.[28]

More recently, it has been demonstrated that acute oral co-administration of caffeine (20 mg/kg) and ethanol (2.5 g/kg) attenuated the ethanol-induced motor impairment in rats evaluated in the accelerating rotarod.[29] This effect was also observed after acute IP administration of an A_1 selective receptor antagonist (8-cyclopentyl-1,3-dipropylxanthine; DPCPX) injected after oral ethanol administration, but not with an A_{2A} selective receptor antagonist 2-(2-Furanyl)-7-(2-phenylethyl)-7*H*- pyrazolo[4,3-*e*][1,2,4]triazolo[1,5-*c*]pyrimidin-5-a mine (SCH 58261), suggesting again that A_1 adenosine receptors are involved in motor incoordination induced by ethanol.[29] However, microinfusions of both the A_1 receptor-selective agonist cyclohexyladenosine (CHA) and the A_{2A} selective agonist 5'-Nethylcarboxamido-2-[2-(4-phenyl-(3-propanoic acid)] (CGS21680) into the rat motor cortex significantly accentuated motor incoordination induced by ethanol (1.5 g/kg IP) in a dose-related manner.[76] CHA was more potent than CGS21680 in producing this effect. However, the potentiation induced by A_1 and A_{2A} agonists was attenuated by the A_1-selective antagonist DPCPX but not by the A_{2A} receptor-selective antagonist 8-(3-chlorostyryl)caffeine (CSC), further emphasizing the involvement of the adenosine A_1 receptor subtype in these effects.[76]

The involvement of different adenosine receptors in the development of rapid tolerance to ethanol-induced motor incoordination in mice has also been evaluated.[82] A single administration of caffeine (3, 10 or 30 mg/kg, IP) or selective antagonists of A_1 or A_{2A} receptors did not change the performance of animals treated with ethanol (2.5 g/kg) on the first day of testing. However, caffeine administered on the first day was able to block the development of tolerance to ethanol that was manifested on the second day. Moreover,

caffeine's blockade of the rapid tolerance to ethanol-induced incoordination appears to be mediated by A_1 rather than A_{2A} receptors, because DPCPX but not 4-(2-[7-Amino-2-(2-furyl)[1,2,4]tri azolo[2,3-*a*][1,3,5]triazin-5-ylamino]ethyl)phenol (ZM241385) also blocked rapid tolerance. These data are in agreement with previous studies[29,76], and it is reasonable to suggest that this effect may be due to the high number of A_1 receptors in areas controlling motor coordination, such as the cortex and cerebellum.[83]

To summarize, acute low doses of caffeine can reduce the incoordination effects of ethanol, but high doses of caffeine can potentiated them. Moreover, adenosine A_1 receptors appear to be more important for these effects than A_{2A} receptors. The ability of caffeine to attenuate the rapid tolerance to ethanol-induced incoordination effects also has been attributed more to A_1 than A_{2A} receptors.

4. Caffeine-ethanol interactions: sedation and narcosis

Ethanol intoxication produces sedative and, at high doses, even hypnotic effects.[72,84-86] In contrast, caffeine enhances wakefulness and alertness, effects that are associated with its ability to block adenosine receptors.[87-91] Although the effects of ethanol or caffeine on sedation and alertness have been widely described, their interaction is much less well characterized, and only a few studies have explored the impact of caffeine on the narcosis or loss of the righting reflex (LORR) induced by ethanol in rodents.[28,92-95]

For example, it has been demonstrated in mice that when coffee (15 mg/ml) or caffeine (0.5 mg/ml) were orally administered before ethanol (75% v/v), the latency to LORR increased.[92] However, this effect was not observed when caffeine was administered after ethanol. Moreover, this effect was not due to pharmacokinetic interference, since no decrease in plasma ethanol levels was detected in mice pretreated with coffee or caffeine.[92] In another study in mice, an intermediate dose of caffeine (25 mg/kg, IP) administered

before an IP injection of narcotic doses of ethanol also blunted the effect of ethanol, in this case by reducing the duration of LORR.[93] This effect was not seen with higher doses of caffeine (40-100 mg/kg).[81,93] Theophylline (50 mg/kg, IP), produced the same pattern of effects, prolonging the onset and shortening the duration of ethanol-induced LORR[81,73], however IBMX (12.5 mg/kg IP) did not altered LORR induced by ethanol.[81]

Comparisons between caffeine and theophylline have also been conducted in long-sleep (LS) and short-sleep (SS) mice, which are selectively bred for differences in sensitivity to the LORR induced by ethanol but also have differential sensitivity to purinergic agonists and antagonists.[94] LS and SS mice showed differences in sensitivity to the non-selective adenosine antagonists, theophylline and caffeine.[95] These drugs also produced a distinct pattern of effects in the two strains of mice; while theophylline reduced the duration of LORR induced by ethanol in both strains of animals (at a broader range of doses in LS mice), caffeine only did so in LS mice. Moreover, caffeine at doses of 10 and 20 mg/kg increased LORR in SS mice. Theophylline did not change blood or brain ethanol elimination rate, but the effects of caffeine on blood ethanol levels were affected.[95] The A_1 receptor-selective agonists CHA and l-phenylisopropyladenosine (PIA), as well as the non-selective A_1-A_{2A} agonists, 2-chloroadenosine (CAD) and N-ethylcarboxamidoadenosine (NEC), increased LORR in both LS and SS mice.[95] In general, LS mice were more affected than SS mice by purinergic drugs, suggesting that there may be differences in the adenosine systems of these lines of mice; this observation may aid in understanding how they differ in ethanol sensitivity as well.

As discussed above, adenosine is involved in mediating many of ethanol's intoxicating effects, such as ataxia [74,96,97] and sedation (for review[98,99]). However, in rodents, adenosine analogues seem to increase LORR only during interactions with hypnotic drugs, rather than causing a direct deep hypnotic effect or unconsciousness.[100] Thus, dipyrimadole (30-

40 mg/kg IP), an inhibitor of adenosine uptake, increased duration of LORR in mice only following the administration of hypnotic doses of ethanol (3.5-4.0 g/kg, IP).[73,93] In regard to the specific adenosine receptors implicated in the modulation of the hypnotic effects of alcohol, more recent studies using novel selective A_{2A} antagonists suggest that A_{2A} rather than A_1 receptors seem to mediate this effect. The A_{2A} antagonist SCH58261 but not the A_1 antagonist DPCPX blocked LORR induced by ethanol.[93] In addition, female and male mice lacking the adenosine A_{2A} receptor (i.e., A_{2A} KO mice) showed a reduced duration of LORR compared to their wild-type (WT) siblings after ethanol administration.[93,101]

In summary, adenosine agonists seem to potentiate the duration of LORR, while adenosine antagonists reduce LORR induced by high doses of ethanol. In general, non-selective adenosine receptor antagonists, as well as selective A_{2A} antagonism or genetic deletion, reduce ethanol induced LORR.

5. Caffeine-ethanol interactions: effects on learning and memory.

High doses of ethanol can also cause learning impairments, amnesia, or impaired retrieval of information, effects that can persist long after the drug wears off.[102-104] Complete or partial memory impairment occurs commonly from episodes of binge drinking in both alcoholics and nonalcoholics.[105] This memory impairment may reflect a disruption of encoding, storage, consolidation, and/or retrieval capability.[106,107] Other studies have shown that moderate doses of ethanol delivered after learning generally enhance or have little effect on memory examined the next day[108,109], and caffeine at moderate doses has been shown to facilitate memory acquisition and retention in animals assessed on various learning tasks.[110-113]

A few papers have focused on the interaction between caffeine and ethanol on memory in rodents.[114,115] Ethanol and caffeine co-administration has demonstrated to be neuroprotective in different models of ischemia.[114,116,117] Thus, an acute administration of

caffeinol (combination of 10 mg/kg caffeine plus 0.65 g/kg alcohol, IP) 15 minutes after traumatic brain injury in rats, produced an improvement in working memory tasks in the Morris water maze, compared to vehicle treated animals.[114] This protection was not due to effects on motor performance.

Retrograde amnesic effects of ethanol, caffeine or a combination of both agents have been evaluated in rats with an olfactory memory test that uses social odors.[115] A high dose of ethanol (3.0 g/kg, IP) administered after exposure to a novel odor produced memory recall or retrograde memory impairments the following day, and caffeine (5 mg/kg, IP), either 20 min before or 1 h after exposure to the novel odor prevented this ethanol disruption in recognition memory.[115]

In humans, ethanol and caffeine can also produce state-dependent memory effects.[118,119] State dependent learning or memory is the term applied to the condition in which a behavior that is learned in a drug state is most readily recalled when the organism is in the same drug state.[120] In rodents, administration of ethanol before training can impair the retrieval of tasks learned in a state- dependent manner, which is reversible by re-administering ethanol before the retrieval test.[121,122] This type of study also reflects the ability of ethanol to serve as an interoceptive cue that can aid learning and performance of a specific operant response.[123] Defined in this way, acute ethanol administration can exert state-dependent effects on conditioned avoidance responding.[124,125] However, caffeine (100 mg/kg, IP) does not change the performance of rats already trained to discriminate the interoceptive cue produced by ethanol administration (1.5 g/kg, IP) in an active avoidance task performed in a typical 3-chamber apparatus.[126]

The interaction between caffeine and ethanol also has been evaluated using the acquisition of an avoidance task performed in a plus-maze discrimination apparatus.[127] This apparatus uses an elevated plus-maze consisting of two opposing open arms and two opposing

enclosed arms. During training, animals are free to explore all four arms but are conditioned to avoid one of the enclosed arms (the aversive arm) by the presentation of both light and white noise stimuli when they enter that arm. During the testing session (24h after the training session), animals are free to explore all four arms again, but no cues are presented. Time in the aversive arm was used as an index of memory. Ethanol alone (1.0 and 1.4 g/kg, IP) or in combination with caffeine (20 and 40 mg/kg, IP) administered before the training session produced a learning deficit manifested during the test session. Only the highest dose of caffeine alone (40 mg/kg) produced that effect. However, that was not due to a state-dependent effect since the administration of this dose of caffeine before the test did not reverse the learning deficit.[127]

Caffeine also does not change the conditioned avoidance of a sweet solution produced by ethanol. This conditioned taste avoidance (CTA) is produced by administering an acute dose of ethanol following voluntary consumption of sacharine, and is observed as a reduction in saccharine consumption the following day.[128] Caffeine (2.5-10 mg/kg, IP) did not block the association between taste and ethanol effects (1.0-1.5 g/kg, IP), thus saccharine consumption was not restored. However, caffeine by itself was able to produce CTA at a moderate dose (20 mg/kg, IP).[128]

Taken together, these studies indicate that caffeine appears to prevent explicit memory deficits induced by high doses of ethanol, but does not affect the perception of the interoceptive cue generated by ethanol, and it does not prevent the disruptive effects of ethanol on avoindance learning in discriminative procedures, suggesting a lack of effect of caffeine on implicit learning processes regulated by ethanol.

6. Caffeine-ethanol interactions: effects on anxiety and stress

Considerable evidence indicates that ethanol is capable of reducing anxiety levels in humans and other animals[129-131], and adenosine has been proposed as a mediator of this anxiolytic effect.[132-134] In this regard, adenosine itself, as well as adenosine receptor agonists, have anxiolytic effects as assessed by a number of ethological tests in rodent models.[135,136] On the other hand, methylxantines such as caffeine and theophylline have been demonstrated to increase anxiety in humans[137-140] and in rodents in different anxiety paradigms.[127,141-144]

Caffeine modulation of the effects of ethanol on anxiety has been explored in a handful of studies[70,127,132], which also assessed the role of adenosine receptor subtypes in this interaction. Thus, caffeine, across a broad range of doses that extended into the anxiogenic range (10-40 mg/kg) was shown to reduce the anxiolytic-like effect of ethanol (1.0-1.4 g/kg, IP) in the elevated plus-maze in mice.[127,132] The effects of caffeine on acutely administered ethanol appeared to be mediated by A_1 adenosine receptors, since the selective adenosine A_1 receptor antagonist DPCPX but not the A_{2A} receptor antagonist ZM241385 significantly reduced the anxiolytic-like effect of ethanol (1.2 g/kg).[132] Moreover, an anxiolytic response was observed after co-administration of non-anxiolytic doses of the A_1 adenosine agonist 2-Chloro-N6-cyclopentyladenosine (CCPA) and ethanol.[132]

A different pattern emerges when these substances are administered chronically. The anxiety-related effects of chronic oral consumption of alcohol (1.0-1.5 g/kg) combined with oral consumption of caffeine (20-30 mg/kg) during adolescence was evaluated in male and female rats when they reached mid-adulthood.[70] Males that had previously consumed alcohol plus caffeine showed anxiolysis in the light and dark box and in the open field. However, females exposed to the drug mixture showed an anxiogenic-like effect.[70] Thus, as described above, results in females and males seem to be opposite.

Caffeine and ethanol not only regulate anxiety-like behavior, but also regulate stress responses involving activation of the hypothalamo–pituitary–adrenal (HPA) axis.[144-151] HPA axis activation ultimately leads to increases in the biosynthesis and systemic secretion of adrenocorticosteroids. The effects of alcohol and other drugs of abuse on this axis are relevant because a link between the stress response and drug abuse and addiction has been observed. Stress is one of the main factors stimulating drug consumption and the relapse to drug taking in abstinent addicts.[152,153] Furthermore, chronic drug exposure affects the brain stress response systems. Thus, drug abuse is often accompanied by enhanced brain stress responses, which in turn may contribute to the addiction process.[153]

In regard to ethanol and caffeine, moderate acute doses of ethanol[145-148] or caffeine[144,149-151] have been shown to increase plasma corticosterone levels in rodents and cortisol in humans. But only one study so far has explored the interaction of caffeine and ethanol on corticosterone release.[154] In this study, a low dose of caffeine (5 mg/kg IP) delivered before a low dose of ethanol (0.8 g/kg IP) elevated plasma corticosterone levels. This increase was not observed after ethanol or caffeine were administered alone.[154]

In summary, more studies need to evaluate this complex interaction, but so far, the evidence suggests that caffeine and ethanol can counteract each other's effects on acute anxiety levels in rodents, and some of this evidence points to A_1 adenosine receptors as being responsible for the anxiolytic effects of ethanol as well as of the reversal of this effect by caffeine. It would be very important to have a clearer view of the interaction between these substances after chronic consumption, because tension reduction theories suggest that the anxiolytic effects of alcohol facilitate alcohol use by anxious individuals.[155,156] Moreover, a growing body of evidence shows that corticosterone may directly modulate alcohol drinking.[157-160]

7. Effect of caffeine on alcohol self-administration

Epidemiology studies have shown that a positive correlation may exist between the consumption of caffeine and that of ethanol.[161,162] Moreover, it has been demonstrated that people who use energy drinks consume alcohol more frequently than people who do not (for review[13]). Studies in rodents have shown a complex relationship between caffeine and ethanol intake.[11,163-165] Caffeine administered in the diet of malnourished female rats has been shown to facilitate voluntary ethanol drinking in a free access two-bottle paradigm[163-164], and removal of caffeine from the diet restored alcohol consumption to baseline levels. This effect was not taste-related, because quinine did not produce the same pattern as caffeine.[164] However, slow-release caffeine pellets (200 mg/day during 21 days) failed to alter ethanol intake in an un-limited free choice paradigm in female rats.[166]. This lack of effect was specific to caffeine, since slow-release pellets containing other stimulants did increase ethanol consumption.[166] Caffeine administered acutely did not produce a consistent pattern of effects; a low dose of caffeine (5 mg/kg, IP) promoted ethanol drinking in male rats using a limited-access two-bottle choice paradigm.[11] However, a high acute dose of caffeine (50 mg/kg, IP) decreased ethanol as well as food intake in deprived male and female rats.[167] The lack of caffeine effects on ethanol intake has been also demonstrated in a recent study.[168] The presence of caffeine (1g/L) in alcoholic solutions (10% v/v) did not increase the ethanol consumption of male rats exposed to a free-choice procedure during 50 days. Interestingly, it did prevent the alcohol deprivation effect (ADE), blocking an increase of ethanol intake after an abstinent period of 7 days.[168] Because ADE has been suggested as an animal model of human alcohol craving and relapse[169], the effect of caffeine on such effect is a very relevant finding.

Research on the role of adenosine receptor subtypes in ethanol intake has mainly focused on A_{2A} receptors. Ethanol intake and preference was increased in male and female KOA_{2A} mice compared to their WT counterparts in a free choice task.[101] Results in the same

direction have been observed in studies employing pharmacological manipulation of adenosine transmission. Both acute and subchronic (7 days) IP administration of the A_{2A} receptor antagonist 8-Ethoxy-9-ethyl-9*H*-purin-6-amine (ANR94) increased levels of ethanol intake in alcohol-preferring rats assessed in a free choice task.[170] Conversely, a reduction of ethanol intake was observed after acute IP administration of the A_{2A} receptor agonists CGS21680 and 5′-N-ethylcarboxamido-2-(2-phenethylthio (VT7).[170]

The involvement of adenosine A_{2A} receptors in ethanol seeking and intake also has been evaluated in operant chambers in which animals have to exert various levels of effort to have access to ethanol (e.g. lever pressing on fixed ratio (FR) schedules ranging from FR1 to FR3.[170-173] In this case, the pattern of effects produced by different A_{2A} receptor antagonists was more complex. While SCH58261 reduced the number of ethanol-reinforced responses and ethanol consumption[173], ANR94 increased responding.[170] Moreover, DMPX had a multiphasic effect on the number of lever presses and amount of ethanol consumed during operant self-administration.[171,172] The A_{2A} agonists CGS21680 and VT7 decreased lever pressing and alcohol consumption in alcohol-preferring rats tested on a FR1 schedule.[170] Using the same behavioral procedure, no effect was observed with an adenosine A_1 antagonist DPCPX.[171,173]

Taken together, it appears that the results 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.[166,167] However, the fact that chronic caffeine blocked the ADE effect[168] suggests that caffeine could be promising as a treatment for protrective abstinence, although more studies should assess this point.

Free intake					
Drug	**Mechanism of action**	**Sex/Species**	**Ethanol concentration**	**Ethanol Intake**	**Refs.**
Caffeine	Non selective antagonist A_1/A_{2A}	Male and female rats	10% (v/v)	Increase	11, 163, 164
		Male and female rats	5% (w/v) 10% (v/v)	Decrease	166, 167
		Male rats	10%(v/v)	No effect	168
ANR94	A_{2A} antagonist	Male alcohol-preferring rats	10% (v/v)	Increase	170
A_{2A} genetic deletion		Male and female mice	3%-20% (v/v)	Increase	101
CGS 21680	A_{2A} agonist	Male alcohol-preferring rats	10% (v/v)	Decrease	170
VT7	A_{2A} agonist	Male alcohol-preferring rats	10% (v/v)	Decrease	170
Operant self-administration					
Drug	**Mechanism of action**	**Sex/Species**	**Ethanol concentration/ schedule**	**Ethanol Intake**	**Refs.**
ANR94	A_{2A} antagonist	Male alcohol-preferring rats	10 % (v/v), FR1	Increase	170
SCH58261	A_{2A} antagonist	Male alcohol-preferring rats	10 % (v/v), FR3	Decrease	173
DMPX	A_{2A} antagonist	Male rats	10% (w/v) FR1	Decrease	172
		Male rats	10 % (v/v), FR3	Bimodal effect	171
DPCPX	A_1 antagonist	Male Alcohol-preferring rats	10 % (v/v), FR3	No effect	171, 173
CGS21680	A_{2A} agonist	Male alcohol-preferring rats	10 % (v/v), FR1	Decrease	170
VT7	A_{2A} agonist	Male alcohol-preferring rats	10 % (v/v), FR1	Decrease	161

Table 1. Summary of the effects of pharmacological and genetic manipulations of adenosine receptors on free ethanol intake and operant self-administration.

8. Effect of caffeine on ethanol 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.[174] Symptoms of ethanol withdrawal appear between 12 and 24 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) that, among other effects, can include increased anxiety.[133] Moreover, the withdrawal syndrome after chronic administration or chronic consumption of significant amounts of ethanol is also characterized by an increased anxiety response (for review[175]). Other common symptoms of this syndrome in rodents are marked hyperalgesia[176], tremors, piloerection[177,178], changes in cardiovascular[179] and gastrointestinal functions[177], seizures or convulsions[180,181], which corresponds to the withdrawal symptoms observed in humans (for review see [175,177]).

Although there are no animal studies focusing on the impact of caffeine on anxiety induced by ethanol withdrawal, other adenosine receptor modulators have been shown to regulate signs of ethanol withdrawal. The administration of adenosine 18 h after an acute ethanol injection in mice, which is at the onset of the peak of withdrawal as characterized by high levels of anxiety, reduced increases in anxiety observed in an elevated plus-maze.[133] This reversal effect was also observed after the administration of a selective adenosine A_1 receptor agonist CCPA, but not after a selective adenosine A_{2A} receptor agonist N^6-[2-(3,5-dimethoxyphenyl)-2-(2methylphenyl)ethyl]adenosine (DPMA).[133] Moreover, the anxiolytic effect of CCPA on ethanol withdrawal-induced anxiety was reversed by the selective adenosine A_1 antagonist DPCPX.[133] The results from studies involving chronic ethanol administration appear to be different from those observed after acute ethanol administration. In this case the A_1 receptor antagonist CPT reduced the anxiogenic effect

produced by ethanol withdrawal in the elevated plus-maze and in the dark/light test in rats.[176]

Removal of a liquid diet containing ethanol (6.7%, v/v) after chronic exposure led to handling-induced hyperexcitability, a less frequently used behavioral measure of withdrawal.[182] Administration of an adenosine A_1 receptor agonist R-PIA and the adenosine A_{2A} receptor agonist CGS21680 significantly reduced this withdrawal sign, suggesting the involvement of both A_1 and A_{2A} receptors.[182] In this study there were no changes in adenosine A_1 and A_{2A} receptors or in adenosine transporter binding sites in the frontal cortex and cerebellum. However, a reduction in adenosine transporter binding sites was observed in the striatum of ethanol-withdrawn mice.[182]

The administration of adenosine, adenosine analogs, or dipyridamole (an inhibitor of adenosine reuptake) has been shown to reduce the number of rats in which audiogenic convulsions appeared during ethanol withdrawal.[180] The adenosine A_1 receptor agonist CCPA also produced a dose-dependent reduction of the convulsions induced by an intense audiogenic stimulus, as well as tremors, which were apparent 24 h after repeated high doses of oral ethanol administration (12-18 g/kg per day) in rats.[183] Moreover, administration of the adenosine A_1 antagonist DPCPX completely abolished the antagonistic effects of the adenosine A_1 agonist CCPA on both tremors and audiogenic seizures during ethanol withdrawal.[183] The A_{2A} adenosine receptor also has been implicated in withdrawal-induced convulsions.[184,185] In fact, these receptors are expressed in areas of the brain involved in epileptogenesis, including the striatum, neocortex and hippocampus.[186] A_{2A}R KO mice are less susceptible to seizures caused by ethanol withdrawal that was induced by the cessation after 10 consecutive days of ethanol intake (up to 6.3% v/v). This effect has also been observed when the A_{2A} adenosine receptor antagonist ZM 241385 was administered during the last 5 of 10 days of ethanol intake.[181]

Similarly, subchronic coadministration of theophylline (1 g/kg, IP; twice daily) during chronic ethanol intake (6.5% w/v) was demonstrated to decrease hyperalgesia and withdrawal scores in rats during ethanol withdrawal.[187] However, the "protective" effect of A_{2A} receptor antagonism or repeated theophylline administration was not observed after the acute administration of caffeine or theophylline (5-25 mg/kg, IP); in this case, there was no effect on the audiogenic seizures observed during ethanol withdrawal in rats.[180] However, caffeine and theophylline did antagonize the suppressive effects of adenosine analogs on these withdrawal symptoms.[180]

In summary, adenosine seems to play an important role in the regulation of ethanol withdrawal. Agonism of the adenosinergic system, especially via stimulation of A_1 adenosine receptors, reduces some of the withdrawal symptoms that occur after acute or chronic ethanol administration. More importantly, pharmacological antagonism or genetic deletion of adenosine A_1 and/or A_{2A} receptors could have a role in prevention of withdrawal during ethanol intake.[181,187] Nevertheless, most of these studies have employed manipulations affecting specific adenosine receptor subtypes rather than caffeine itself, and therefore have not directly assessed the popular believe that a cup of strong coffee can antagonize some of the symptoms of ethanol-withdrawal, especially after an acute episode of alcohol consumption in non alcoholic individuals.

9. Future directions

After reviewing the literature on caffeine-ethanol interactions one can see that a significant body of work has been performed. However, a clear pattern of results does not easily emerge. Further experiments are needed to establish the specific range of doses, patterns of administration, sex differences, and other factors that could clarify some of the apparent contradictions in the results observed in many of the studies presented above.

More importantly, there is a dearth of studies about the interactions of both agents on processes that are particularly relevant for addiction, such as Pavlovian conditioning, habit formation, or motor sensitization, which seem to contribute to the acquisition and intensification of compulsive drug-seeking behavior.[38,40] Although sensitization of locomotor activity by caffeine as well as cross-sensitization with other drugs such as amphetamine[188] and nicotine[189] has been observed, so far there are no studies of possible cross-sensitization between ethanol and caffeine. In fact, preliminary studies from our laboratory show that caffeine reduces locomotion in animals repeatedly exposed to a sensitizing dose of ethanol.[190] Furthermore, the effects of caffeine-ethanol interactions on learning processes are not well understood, in part due to the complexity of learning processes *per se*. Caffeine has been demonstrated to induce conditioned place preference[191-193], and also to modulate conditioned place preference induced by methamphetamine or cocaine[6] It also would be important to study the effects of caffeine on the acquisition of Pavlovian cues associated with ethanol in this paradigm.

In summary, despite the fact that this area of inquiry has grown increasingly important due to the potential dangers of combining high-caffeine "energy" drinks with ethanol, animal researchers have only scratched the surface of this complex and multifaceted field. Additional investigations will be required to identify how caffeine and ethanol interact to modulate the behavioral processes related to ethanol consumption, dependence, abuse and addiction.

10. References

1. Fredholm BB, Bätting K, Holmén J, et al. Actions of caffeine in the brain with special reference to factors that contribute to its widespread use. Pharmacol Rev. 1999;51:83-133.

2. DeWit H, Pierri J, Johanson CE. Assessing individual differences in ethanol preference using a cumulative dosing procedure. Psychopharmachology. 1988;1:113-119.

3. Rehm JT, Bondy SJ, Sempos CT, Vuong CV. Alcohol consumption and coronary heart disease morbidity and mortality. Am J Epidemiol. 1997;146:495-501.

4. Hingson RA, Howland J. Alcohol and non traffic unintended injuries. Addiction. 1993; 88:877-883.

5. Morelli M, Simola N. Methylxanthines and Drug Dependence: A focus on interactions with substances of abuse. In: Methylxanthines. Fredholm BB (Ed). Springer; 2011: pp.484-501.

6. Tuazon DB, Suzuki T, Misawa M, et al. Methylxanthines (caffeine and theophylline) blocked methamphetamine-induced conditioned place preference in mice but enhaned that induced by cocaine. Ann N Y Acad Sci. 1992;654:531-533.

7. Simola N, Tronci E, Pinna A, Morelli M. Subchronic-intermittent caffeine amplifies the motor effects of amphetamine in rats. Amino Acids. 2006;31:359-363.

8. Shoaib M, Swanner LS, Yasar S, Golberg SR. Chronic caffeine exposure potentiates nicotine self-administration in rats. Psychopharmacology (Berl). 1999;142:327-333.

9. Gasior M, Jaszyna M, Munzar P, Witkin JM, Goldberg SR. Caffeine potentiates the discriminative-stimulus effects of nicotine in rats. Psychopharmacology. 2002;162:385-395.

10. Green TA, Schenk S. Dopaminergic mechanism for caffeine-produced cocaine seeking in rats. Neuropsychopharmacology. 2002;26:422-430.

11. Kunin D, Gaskin S, Rogan F, Smith BR, Amit Z. Caffeine promotes ethanol drinking in rats. Examination using a limited-access free choice paradigm. Alcohol. 2000;21:271-277.

12. Hasenfratz M, Bunge A, Dal Prá G, Bättig K. Antagonistic effects of caffeine and alcohol on mental performance parameters. Pharmacol Biochem Behav. 1993;46:463-465.

13. Ferré S, O'Brien MC. Alcohol and Caffeine: The Perfect Storm. J Caffeine Res. 2011;1:153-162.

14. Ferré S. Role of the central ascending neurotransmitter systems in the psychostimulant effects of caffeine. J Alzheimers Dis. 2010;20 (Suppl 1):S35-S49.

15. Acquas E, Tanda G, Di Chiara G. Differential effects of caffeine on dopamine and acetylcholine transmission in brain areas of drug-naive and caffeine-pretreated rats. Neuropsychopharmacology. 2002;27:182-193.

16. Acquas E, Vinci S, Ibba F, Spiga S, De Luca MA, Di Chiara G. Role of dopamine D(1) receptors in caffeine-mediated ERK phosphorylation in the rat brain. Synapse. 2010;64:341-349.

17. Cauli O, Morelli M. Caffeine and the dopaminergic system. Behav Pharmacol. 2005;16:63-77.

18. Fredholm BB, Ijzerman AP, Jacobson KA, Klotz KN, Linden J. International Union of Pharmacology: XXV. Nomenclature and classification of adenosine receptors. Pharmacol Rev. 2001;53:527-552.

19. Ferré S, Ciruela F, Borycz J, et al. Adenosine A1-A2A receptor heteromers: new targets for caffeine in the brain. Front Biosci. 2008;13:2391-2399.

20. Clark M, Dar MS. Effect of acute ethanol on release of endogenous adenosine from rat cerebellar synaptosomes. J Neurochem. 1989;52:1859-1865.

21. Fredholm BB, Wallman-Johansson A. Effects of ethanol and acetate on adenosine production in rat hippocampal slices. Pharmacol Toxicol. 1996;79:120-123.

22. Diamond I, Gordon AS. Cellular and molecular neuroscience of alcoholism. Physiol Rev. 1997;77:1-20.

23. Nagy LE, Diamond I, Casso DJ, Franklin C, Gordon AS. Ethanol increases extracellular adenosine by inhibiting adenosine uptake via the nucleoside transporter. J Biol Chem. 1990;265:1946-1951.

24. Nagy LE. Ethanol metabolism and inhibition of nucleoside uptake lead to increased extracellular adenosine in hepatocytes. Am J Physiol. 1992;262:1175-1180.

25. Carmichael FJ, Israel Y, Crawford M, et al. Central nervous system effects of acetate: Contribution to the central effects of ethanol. Journal of Pharmacology and Experimental Therapeutics. 1991;259:403-408.

26. Ferreira SE, de Mello MT, Pompéia S, de Souza-Formigoni ML. Effects of energy drink ingestion on alcohol intoxication. Alcohol Clin Exp Res. 2006;30:598-605.

27. Liguori A, Robinson JH. Caffeine antagonism of alcohol-induced driving impairment. Drug Alcohol Depend. 2001;63:123-129.

28. Dar MS, Wooles WR. Effect of chronically administered methylxanthines on ethanol-induced motor incoordination. Life Sci. 1986;39:1429-1437.

29. Connole L, Harkin A, Maginn M. Adenosine A1 receptor blockade mimics caffeine's attenuation of ethanol-induced motor incoordination. Basic Clin Pharmacol Toxicol. 2004;95:299-304.

30. Kunin D, Bloch RT, Terada Y, Rogan F, Smith BR, Amit Z. Caffeine promotes an ethanol-induced conditioned taste aversion: a dose-dependent interaction. Exp Clin Psychopharmacol. 2001;9:326-33.

31. Di Chiara G. A motivational learning hypothesis of the role of dopamine in compulsive drug use. J Psychopharmacol. 1998;12:54-67.

32. O'Brien CP, Childress AR, McLellan AT, Ehrman R. Classical conditioning in drug-dependent humans. Ann N Y Acad Sci. 1992;654:400-415.

33. Robinson TE, Berridge KC. The neural basis of drug craving: an incentive-sensitization theory of addiction. Brain Res Brain Res Rev. 1993;18:247-291.

34. Heyman GM. Addiction: A disorder of choice. Cambridge, MA: Harvard University Press; 2009.

35. Vezina P, Lorrain DS, Arnold GM, Austin JD, Suto N. Sensitization of Midbrain Dopamine Neuron Reactivity Promotes the Pursuit of Amphetamine. J Neurosci. 2002;22:4654-4662.

36. Heyman GM. An economic approach to animal models of alcoholism. Alcohol Res Health. 2000;24:132-139.

37. Stewart J, De Wit H, Eikelboom R. Role of unconditioned and conditioned drug effects in the self-administration of opiates and stimulants. Psychol Rev. 1984;91:251-268.

38. Robinson TE, Berridge KC. The psychology and neurobiology of addiction: an incentive-sensitization view. Addiction. 2000;95 (Suppl 2):91-117.

39. Everitt BJ, Robbins TW. Neural systems of reinforcement for drug addiction: from actions to habits to compulsion. Nat Neurosci. 2005;8:1981-1989.

40. Belin D, Jonkman S, Dickinson A, Robbins TW, Everitt BJ. Parallel and interactive learning processes within the basal ganglia: relevance for the understanding of addiction. 2009;199:89-102.

41. Dunne F, O'Halloran A, Kelly JP. Development of a home cage locomotor tracking system capable of detecting the stimulant and sedative properties of drugs in rats. Prog Neuropsychopharmacol Biol Psychiatry. 2007;31:1456-1463.

42. Himmel HM. Safety pharmacology assessment of central nervous system function in juvenile and adult rats: effects of pharmacological reference compounds. J Pharmacol Toxicol Methods. 2008;58:129-146.
43. Read GW, Cutting W, Furst A. Comparison of excited phases after sedativesand tranquilizers. Psyhopharmacologia (Berl).1960;1:346-350.
44. Carlsson A, Engel J, Svensson TH. Inhibition of ethanol-induced excitation in mice and rats by a-methyl-p-tyrosine. Psychopharmacology (Berl). 1972;26:307-312.
45. Correa M, Miquel M, Sanchis-Segura C, Aragon, CMG. Acute lead acetate administration potentiates ethanol-induced locomotor activity in mice: the role of brain catalase. Alcohol Clin Exp Res. 1999:23;799-805.
46. Correa M, Sanchis-Segura C, Pastor R, Aragon CMG. Ethanol intake and motor sensitization: the role of brain catalase activity in mice with different genotypes. Physiol Behav. 2004:82;231-240.
47. Correa M, Viaggi C, Escrig MA, Pascual M, Guerri C, Vaglini F, Aragon CMG, Corsini GU. Ethanol intake and ethanol-induced locomotion and locomotor sensitization in Cyp2e1 knockout mice. Pharmacogenet Genomics. 2009;19:217-225.
48. Sanchis-Segura C, Correa M, Aragon CM. Lesion on the hypothalamic arcuate nucleus by estradiol valerate results in a blockade of ethanol-induced locomotion. Behav Brain Res. 2000;114:57-63.
49. Phillips TJ, Shen EH. Neurochemical bases of locomotion and ethanol stimulant effects. In: International Review of Neurobiology. R.J. Bradley, R.A. Harris, P.Jenner (Eds). San Diego, Inc: Academic Press; 1996: pp 243-282.
50. Pohorecky LA. Biphasic action of ethanol. Biobehav Rev. 1977;1:231-240.
51. Waldeck B. Ethanol and Caffeine: A Complex Interaction with Respect to Locomotor Activity and Central Catecholamines. Psychopharmacologia (Berl). 1974;36:209-220.

52. Correa M, Arizzi MN, Betz A, Mingote S, Salamone JD. Open field locomotor effects in rats after intraventricular injections of ethanol and the ethanol metabolites acetaldehyde and acetate. Brain Res Bull. 2003;197-202

53. Kuribara H, Asahi T, Tadokoro S. Ethanol enhances, but diazepam and pentobarbital reduce the ambulation-increasing effect of caffeine in mice. Arukoru Kenkyuto Yakubutsu Ison. 1992;27:528-539.

54. Haghgoo S, Hasegawa T, Nadai M, et al. Brain distribution characteristics of xanthine derivatives and relation to their locomotor activity in mice. J Pharm Pharmacol. 1995;47:412-419.

55. El Yacoubi M, Ledent C, Menard JF, et al. The stimulant effects of caffeine on locomotor behaviour in mice are mediated through its blockade of adenosine A(2A) receptors. British Journal of Pharmacology. 2000;129:1465-1473.

56. Zhang Q, Yu YP, Ye YL, Zhang JT, Zhang WP, Wei WQ. Spatiotemporal properties of locomotor activity after administration of central nervous stimulants and sedatives in mice. Pharmacol biochem Behav. 2011;97:577-585.

57. Pilcher, JD. Alcohol and caffeine: A study of antagonism and synergism. J Pharmacol exp Ther. 1911: 8; 267-298.

58. Sudakov SK, Rusakova IV, Medvedeva OF. Effect of chronic caffeine consumption on changes in locomotor activity of WAG/G and Fischer-344 rats induced by nicotine, ethanol, and morphine. Bull Exp Biol Med. 2003;136:563-565.

59. Ferreira SE, Hartmann Quadros IM, Trindade AA, Takahashi S, Koyama RG, Souza-Formigoni ML. Can energy drinks reduce the depressor effect of ethanol? An experimental study in mice. Physiol Behav. 2004;82:841-847.

60. Aragon CM, Trudeau LE, Amit Z. Effect of taurine on ethanol induced changes in open-field locomotor activity. Psychopharmacology (Berl). 1992;107:337-340.

61. Miquel M, Correa M, Sanchis-Segura C, Aragon CM. The ethanol-induced open-field activity in rodents treated with isethionic acid, a central metabolite of taurine. Life Sci. 1999:64;1613-1621.
62. Daly JW, Shi D, Wong V, Nikodijevic O. Chronic effects of ethanol on central adenosine function of mice. Brain Res. 1994;650:153-156.
63. Nikodijević O, Jacobson KA, Daly JW. Effects of combinations of methylxanthines and adenosine analogs on locomotor activity in control and chronic caffeine-treated mice. Drug Dev Res. 1993;30:104-110.
64. Nikodijević O, Jacobson KA, Daly JW. Locomotor activity in mice during chronic treatment with caffeine and withdrawal. Pharmacol Biochem Behav. 1993;44:199-216.
65. Arnett, J. Reckless behavior in adolescence: a developmental perspective. Dev Rev. 1992;12:339-373.
66. Casey BJ, Getz S, Galvan A. The adolescent brain. Dev Rev. 2008;28:62-77.
67. Smith RF. Animal models of periadolescent substance abuse. Neurotoxicol Teratol. 2003;25:291-301.
68. Gilpin NW, Karanikas CA, Richardson HN. Adolescent Binge Drinking Leads to Changes in Alcohol Drinking, Anxiety, and Amygdalar Corticotropin Releasing Factor Cells in Adulthood in Male Rats. PLoS One. 2012;7:e31466.
69. Huang C, Titus JA, Bell RL, Kapros T, Chen J, Huang R. A mouse model for adolescent alcohol abuse: stunted growth and effects in brain. Alcohol Clin Exp Res. 2012;36:1728-1737.
70. Hughes RN. Adult anxiety-related behavior of rats following consumption during late adolescence of alcohol alone and in combination with caffeine. Alcohol. 2011;45:365-372.

71. Butler TR, Smith KJ, Berry JN, Sharrett-Field LJ, Prendergast MA. Sex differences in caffeine neurotoxicity following chronic ethanol exposure and withdrawal. Alcohol Alcohol. 2009;44:567-574.
72. Chuck TL, McLaughlin PJ, Arizzi-LaFrance MN, Salamone JD, Correa M. Comparison between multiple behavioral effects of peripheral ethanol administration in rats: Sedation, ataxia, and bradykinesia. Life Sci. 2006:79;154-161.
73. Dar MS, Mustafa, S. J., Wooles, W. R. Possible role of adenosine in the CNS effects of ethanol. Life Sci. 1983;33:1363-1374.
74. Dar MS. Central adenosinergic system involvement in ethanol-induced motor incoordination in mice. J Pharmacol Exp Ther. 1990;255(3):1202-1209.
75. Dar MS. Mouse cerebellar adenosinergic modulation of ethanol-induced motor incoordination: possible involvement of cAMP. Brain Res. 1997;792: 263-274.
76. Barwick VS, Dar MS. Adenosinergic modulation of ethanol-induced motor incoordination in the rat motor cortex. Prog Neuropsychopharmacol Biol Psychiatry. 1998;22:587-607.
77. Crabbe JC, Rigter H, Uijlen J, Strijbos C. Rapid development of tolerance to the hypothermic effect of ethanol in mice. J Pharmacol Exp Ther. 1979;208:128-133.
78. Khanna JM, Morato GS, Kalant H. Effect of NMDA antagonists, an NMDA agonist, and serotonin depletion on acute tolerance to ethanol. Pharmacol Biochem Behav. 2002;72:291-298.
79. American Psychiatric Association. Diagnostic and Statistical Manual of Mental Disorders, 4th edn. DSM IV. Washington:DC; 1994.
80. Dar, MS. The biphasic effects of centrally and peripherally administered caffeine on acute ethanol-induced MI in mice. J Pharm Pharmacol. 1988;40:482-487.
81. Dar MS, Jones M, Close G, Mustafa SJ, Wooles WR. Behavioral interactions of ethanol and methylxanthines. Psychopharmacology. 1987;91:1-4

82. Batista LC, Prediger RD, Morato GS, Takahashi RN. Blockade of adenosine and dopamine receptors inhibits the development of rapid tolerance to ethanol in mice. Psychopharmacology (Berl). 2005;181:714-721.

83. Ribeiro JA, Sebastiao AM, de Mendoça A. Adenosine receptors in the nervous system: pathophysiological implications. Prog Neurobiol. 2002;68:377-392.

84. Correa M, Sanchis-Segura C, Aragon, CMG. Influence of brain catalase on ethanol-induced loss of righting reflex in mice. Drug Alcohol Depend. 2001;65:9-15.

85. Correa M, Pascual M, Sanchis-Segura C, Guerri C, Aragon CM. Lead-induced catalase activity diferentially modulates behaviors induced by short-chain alcohols. Pharmacol Biochem Behav. 2005;82:443-452.

86. Miquel M, Correa M, Aragon CMG. Methionine enhances alcohol-induced narcosis in mice. Pharmacol Biochem Behav. 1999;64:89-93.

87. Fredholm BB. Are methylxanthine's effects due to antagonism of endogenous adenosine? Trends Pharmacol Sci. 1980;1:129-132.

88. Snyder SH, Katims JJ, Annau Z, Bruns RF, Daly JW. Adenosine receptors and behavioral actions of methylxanthines. Proc Natl Acad Sci U S A. 1981;78:3260-3264.

89. Daly JW, Butts-Lamb P, Padjett W. Subclasses of adenosine receptors in the central nervous system: interaction with caffeine and related methylxanthines. Cell Moll Neurobiol. 1983;3:69-80.

90. Yanik G, Glaum S, Radulovacki M. The dose–response effects of caffeine on sleep in rats. Brain Res. 1987;403:177-180.

91. Ledent C, Vaugeois JM., Schiffmann, SN, et al. Aggressiveness, hypoalgesia and high blood pressure in mice lacking the adenosine A2a receptor. Nature. 1997;388: 674-678.

92. Koo MW. Effects of ginseng on ethanol induced sedation in mice. Life Sci. 1999;64:153-160.

93. El Yacoubi M, Ledent C, M Parmentier M, Costentin J, Vaugeois JM. Caffeine reduces hypnotic effects of alcohol through adenosine A_2A receptor blockade. Neuropharmacology. 2003:45;977-985.

94. Proctor WR, Dunwiddie TV. Behavioral sensitivity to purinergic drugs parallels ethanol sensitivity in selectively bred mice. Science. 1984;224:519-521.

95. Smolen TN, Smolen A. Purinergic modulation of ethanol-induced sleep time in long-sleep and short-sleep mice. Alcohol. 1991;8:123-130.

96. Dar MS. Involvement of kappa-opioids in the mouse cerebellar adenosinergic modulation of ethanol-induced motor incoordination. Alcohol Clin Exp Res. 1998;22:444-454.

97. Dar MS, Mustafa SJ. Acute ethanol/cannabinoid-induced ataxia and its antagonism by oral/systemic/ intracerebellar A1 adenosine receptor antisense in mice. Brain Res. 2002; 957:53-60.

98. Ruby CL, Adams CA, Knight EJ, Nam HW, Choi DS. An essential role for adenosine signaling in alcohol abuse. Curr Drug Abuse Rev. 2010;3:163-74.

99. Asatryan L, Nam HW, Lee MR, Thakkar MM, et al. Implication of the purinergic system in alcohol use disorders. Alcohol Clin Exp Res. 2011;35:584-94.

100. Dunwiddie TV. The physiological role of adenosine in the central nervous system. International Review of Neurobiology. 1985;27:63-139.

101. Naassila M, Ledent C, Daoust M. Low ethanol sensitivity and increased ethanol consumption in mice lacking adenosine A_2A receptors. J Neurosci. 2002;22:10487-10493.

102. Goodwin DW. Alcohol amnesia. Addiction. 1995:90;315-317.

103. Hartzler B, Fromme K. Fragmentary and en bloc blackouts: similarity and distinction among episodes of alcohol-induced memory loss. J Stud Alcohol. 2003;64:547-550.

104. Wixted JT. A theory about why we forget what we once knew. Curr Dir Psychol Sci. 2005;14:6-9.

105. White AM. What happened? Alcohol, memory blackouts, and the brain. Alcohol Res Health. 2003;27:186-196.

106. Gold PE. The many faces of amnesia. Learn Memory. 2006;13:506-514.

107. Weitemier AZ, Ryabinin AE. Alcohol-induced memory impairment in trace fear conditioning: a hippocampus-specific effect. Hippocampus. 2003;13:305-315.

108. Alkana RL, Parker ES. Memory facilitation by post-training injection of ethanol. Psychopharmacology. 1979;66:117-119.

109. Manrique HM, Miquel M, Aragon CM. Brain catalase mediates potentiation of social recognition memory produced by ethanol in mice. Drug Alcohol Depend. 2005;79:343-350.

110. Angelucci ME, Cesário C, Hiroi RH, Rosalen PL, Da Cunha C. Effects of caffeine on learning and memory in rats tested in the Morris water maze. Braz J Med Biol Res. 2002;35:1201-1208.

111. Izquierdo JA, Costas SM, Justel EA, Rabiller G. Effect of caffeine on the memory of the mouse. Psychopharmacology. 1979;61:29-30.

112. Furusawa K. Drug effects on cognitive function in mice determined by the non-matching to sample task using a 4-arm maze. Jpn J Pharmacol. 1991;56:483-493.

113. Molinengo L, Scordo I, Pastorello B. Action of caffeine, L-PIA and their combination on memory retention in the rat. Life Sci. 1994;54:1247-1250.

114. Dash PK, Moore AN, Moody MR, Treadwell R, Felix JL, Clifton GL. Post-trauma administration of caffeine plus ethanol reduces contusion volume and improves working memory in rats. J Neurotrauma. 2004;21:1573-1583.

115. Spinetta MJ, Woodlee MT, Feinberg LM, et al. Alcohol-induced retrograde memory impairment in rats: prevention by caffeine. Psychopharmacology. 2008;201:361-371.

116. Strong R, Grotta JC, Aronowski J. Combination of low dose ethanol and caffeine protects brain from damage produced by focal ischemia in rats. Neuropharmacology. 2000;39:515-522.

117. Piriyawat P, Labiche LA, Burgin WS, Aronowski JA, Grotta JC. Pilot dose-escalation study of caffeine plus ethanol (caffeinol) in acute ischemic stroke. Stroke. 2003 May;34(5):1242-5

118. Kelemen WL, Creely CE. State-Dependent Memory Effects Using Caffeine and Placebo Do Not Extend to Metamemory. J Gen Psychol. 2003;130:70-86.

119. Duka T, Weissenborn R, Dienes Z. State-dependent effects of alcohol on recollective experience, familiarity and awareness of memories. Psychopharmacology. 2011;153:295-306.

120. Overton DA. State-dependent learning produced by alcohol and its relevance to alcoholism. In: The Biology of Alcoholism, Vol. II: Physiology and Behavior. B. Kissen and H. Begleiter (Eds). New York ,Plenum Press; 1974: pp.368-373.

121. Nakagawa Y, Iwasaki T. EtOH-induced state-dependent learning is mediated by 5-hydroxytryptamine3 receptors but not by N-methyl-D-aspartate receptor complex. Brain Res. 1996;706:227-232.

122. Rezayof A, Motevasseli T, Rassouli Y, Zarrindast MR. Dorsal hippocampal dopamine receptors are involved in mediating ethanol state-dependent memory. Life Sci. 2007;80:285-292.

123. Solinas M, Panlilio LV, Justinova Z,Yasar S, Goldberg SR. Using drug-discrimination techniques to study the abuse-related effects of psychoactive drugs in rats. Nat Protoc. 2006;1:1194-206.

124. Crow, Lowell T. Effects of alcohol on conditioned avoidance responding. Physiol Behav. 1966;1:89-91.

125. Overton D. State-dependent learning produced by depressant and atropine-like drugs. Psychopharmacology(Berl). 1966;10:6-31.

126. Schechter MD. Effect of propranolol, d-amphetamine and caffeine on ethanol as a discriminative cue. Eur J Pharmacol. 1974;29:52-57.

127. Gulick D, Gould TJ. Effects of Ethanol and Caffeine on Behavior in C57BL/6 Mice in the Plus-Maze Discriminative Avoidance Task. Behav Neurosci. 2009:123;1271-1278.

128. Kunin D, Bloch RT, Terada Y, Rogan F, Smith BR, Amit Z. Caffeine promotes an ethanol-induced conditioned taste aversion: a dose-dependent interaction. Exp Clin Psychopharmacol. 2001;9:326-333.

129. Correa M, Manrique HM, Font L, Escrig MA, Aragon CM. Reduction in the anxiolytic effects of ethanol by centrally formed acetaldehyde: the role of catalase inhibitors and acetaldehyde-sequestering agents. Psychopharmacology. 2008;200;455-464.

130. Kushner M, Sher K, Beitman B. The relation between alcohol problems and the anxiety disorders. Am J Psychiatry. 1990;147:685-695.

131. Cappell H, Greeley J. Alcohol and tension reduction: an update on research and theory. In: Psychological theories of drinking. H. Blaine and K. Leonard (Eds). Guilford, New York; 1987: pp.15-51.

132. Prediger RD, Batista LC, Takahashi RN. Adenosine A1 receptors modulate the anxiolytic-like effect of ethanol in the elevated plus-maze in mice. Eur J Pharmacol. 2004;499:147-154.

133. Prediger RD, Da Silva GE, Batista LC. Bittencourt AL, Takahashi RN. Activation of adenosine A1 receptors reduces anxiety-like behavior during acute ethanol withdrawal (hangover) in mice. Neuropsychopharmacology. 2006;31:2210-2220.

134. Correa M, Font L. Is there a major role for adenosine A_{2A} receptors in anxiety? Front Biosci. 2008;1:4058-4070.

135. Okuyama E, Ebihara H, Takeuchi H, Yamazaki M. Adenosine, the anxiolytic-like principle of the Arillus of Euphoria longana. Plant Med. 1992:65;115-119.

136. Florio C, Prezioso A, Papaioannou A, Vertua R.Adenosine A1 receptors modulate anxiety in CD1 mice. Psychopharmacology. 1998;136:311-319.

137. Uhde TW, Boulenger JP, Jimerson DC, Post RM. Caffeine: relationship to human anxiety, plasma MHPG and cortisol. Psychopharmacol Bull. 1984;20:426-430.

138. Bruce MS. The anxiogenic effects of caffeine. Postgrad Med J. 1990;(Suppl 2)66:18-24.

139. Boulenger JP, Uhde TW, Wolf EA 3rd, Post RM. Increased sensitivity to caffeine in patients with panic disorders: preliminary evidence. Arch Gen Psychiatry.1984;41:1067-1071.

140. Gea J, de Pablo J, Marti J, Picado C, Pujol A, Agustí-Vidal A. Anxiety crisis (panic attack) secondary to the administration of theophylline in an oral sustained-release preparation. Rev Clin Esp. 1988;183-280.

141. El Yacoubi M, Ledent C, Parmentier M, Costentin J, Vaugeois JM. The anxiogenic-like effect of caffeine in two experimental procedures measuring anxiety in the mouse is not shared by selective A(2A) adenosine receptor antagonists. Psychopharmacology (Berl). 2000;148:153-163.

142. Bhattacharya SK, Satyan KS, Chakrabarti A. Anxiogenic action of caffeine: an experimental study in rats. J Psychopharmacol. 1997;11:219-224

143. Correa M, López-Cruz L, Pardo M, Valverde O, Ledent C, Salamone JD. Effects of the nonselective adenosine antagonists caffeine and theophylline on measures of motor activity and anxiety in mice: studies of acute and chronic administration and comparison with adenosine A2A receptor KO mice. Society for Neuroscience; 2011. Online. 103.09/XX6.

144. López-Cruz, Pardo M, Dosda A, Salamone JD, Correa M. Comparison Between high doses of caffeine and theophylline on motor and anxiogenic effects in CD1 mice: studies of acute and chronic administration. Behavioural Pharmacology 2011;eS22: 71-72.

145. Ellis FW. Effect of ethanol on plasma corticosterone levels. J Pharmacol Exp. Ther 1966;153:121-127.

146. Kakihana R, Butte JC, Noble EP. Corticosterone response to ethanol in inbred strains of mice. Nature. 1968; 218:360-336.

147. Roberts AJ, Crabbe JC, Keith LD. Genetic differences in hypothalamic–pituitary–adrenal axis responsiveness to acute ethanol and acute ethanol withdrawal. Brain Res. 1992;579:296-302.

148. Escrig MA, Pardo M, Aragon CM, Correa A M. Anxiogenic and stress-inducing effects of peripherally administered acetaldehyde in mice: similarities with the disulfiram-ethanol reaction. Pharmacology Biochemistry and Behavior. 2012;100:404-412.

149. Nicholson SA (1989) Stimulatory effect of caffeine on plasma corticosterone and pituitary-adrenocortical axis in the rat. J Endocrinol. 1989:122;535-543.

150. Patz MD, Day HE, Burow A, Campeau S. Modulation of the hypothalamo-pituitary adrenocortical axis by caffeine. Psychoneuroendocrinology. 2005;31:493-500.

151. Lin AS, Uhde TW, Slate SO, McCann UD. Effects of intravenous caffeine administered to healthy males during sleep. Depress Anxiety. 1997;5:21-28.

152. Delaney WP, Grube JW, Greiner B, Fisher JM, Ragland DR. Job stress, unwinding and drinking in transit operators. J Stud Alcohol. 2002;63:420-429.

153. Koob GF, Volkow ND. Neurocircuitry of addiction. Neuropsychopharmacology. 2010;35:217-238.

154. Kunin D, Gaskin S, Rogan F, Smith BR, Amit Z. Augmentation of corticosterone release by means of a caffeine-ethanol interaction in rats. Alcohol. 2000;22:53-56.

155. Kalodner CR, Delucia JL, Ursprung, AW. An examination of the tension reduction hypothesis: the relationship between anxiety and alcohol in college students. Addict Behav. 1989;14; 649-654.

156. Spencer RL, Hutchison KE. Alcohol, aging and the stress response. Alcohol Res Health. 1999;23:272-283.

157. Fahlke C, Hard E, Hansen, S. Facilitation of ethanol consumption by intracerebroventricular infusions of corticosterone. Psychopharmacology (Berlin). 1996;127:133-139.

158. Lamblin, F, De Witte P. Adrenalectomy prevents the development of alcohol preference in male rats. Alcohol. 1996;13:233-238.

159. Pastor R, Reed C, Burkhart-Kasch S, et al. Ethanol concentration-dependent effects and the role of stress on ethanol drinking in corticotropin-releasing factor type 1 and double type 1 and 2 receptor knockout mice. Psychopharmacology (Berl). 2011;218:169-177.

160. Vendruscolo LF, Barbier E, Schlosburg JE. Corticosteroid-dependent plasticity mediates compulsive alcohol drinking in rats. J Neurosci. 2012;32:7563-7571.

161. Itsvan J, Matarazzo JD. Tobacco, alcohol, and caffeine use: a review of their interrelationships. Psycol Bull. 1984;95:301-326.

162. Kozlowski LT, Henningfield JE, Keenan RM, et al. Patterns of alcohol, cigarette, and caffeine and other durg use in tow drug abusing populations. J Subs Abuse Treat. 1993;10:171-179.

163. Gilbert, RM. Dietary caffeine and EtOH consumption by rats. J Stud Alcohol. 1976;37:11-18.

164. Gilbert RM. Augmentation of EtOH-consumption by caffeine in malnourished rats. J Stud Alcohol. 1979:40;19-27.

165. Register, UD, MArhs SR, Thurston CT, et al. Influence of nutrients on the intake of alcohol. J Amer Diet Assoc. 1972;61:159-162.

166. Potthoff AD, Ellison G, Nelson L. Ethanol intake increases during continuous administration of amphetamine and nicotine, but not several other drugs. Pharmacol Biochem Behav. 1983;18:489-493.

167. Dietze MA, Kulkosky PJ. Effects of caffeine and bombesin on ethanol and food intake. Life Sci. 1991;48:1837-1844.

168. Carvalho CR, Cruz JS, Takahashi RN. Prolonged Exposure to Caffeinated Alcoholic Solutions Prevents the Alcohol Deprivation Effect in Rats. J Caffeine Res. 2012;2;83-89.

169. Heyser CJ, Schulteis G, Koob GF. Increased ethanol self-administration after a period of imposed ethanol deprivation in rats trained in a limited access paradigm. Alcohol Clin Exp Res. 1997;21:784-791.

170. Micioni Di Bonaventura MV, Cifani C, Lambertucci C, et al. Effects of A_2A adenosine receptor blockade or stimulation on alcohol intake in alcohol-preferring rats. Psychopharmacology (Berl). 2012;219:945-957.

171. Arolfo MP, Yao L, Gordon AS, Diamond I, Janak PH. Ethanol operant self-administration in rats is regulated by adenosine A_2 receptors. Alcohol Clin Exp Res. 2004;28:1308-1316.

172. Thorsell A, Johnson J, Heilig M. Effect of the adenosine . Alcohol Clin Exp Res. 2007;31:1302-1307.

173. Adams CL, Cowen MS, Short JL, Lawrence AJ. Combined antagonism of glutamate mGlu5 and adenosine A2A receptors interact to regulate alcohol-seeking in rats. Int J Neuropsychopharmacoly. 2008;11:229-241.

174. Cloninger CR. Neurogenetic adaptive mechanisms in alcoholism. Science. 1987;236:410-416.

175. Finn DA, Crabbe JC. Exploring alcohol withdrawal syndrome. Alcohol Health Res World. 1997;21;149-156.

176. Gatch MB, Lal H. Effects of ethanol and ethanol withdrawal on nociception in rats. Alcohol Clin Exp Res.1999;23:328-333.

177. Friedman HJ. Assessment of physical dependence on and withdrawal from ethanol in animals. In: Alcohol Tolerance and Dependence. H. Rigter and J.C. Crabbe (Eds). Amsterdam: Elsevier/North-Holland Biomedical Press; 1980: pp. 93-121.

178. Frye GD, McCown TJ, Breese GR. Differential sensitivity of ethanol withdrawal signs in the rat to gamma-aminobutyric acid (GABA) mimetics: Blockade of audiogenic seizures but not forelimb tremors. Journal of Pharmacology and Experimental Therapeutics.1983;226:720-725.

179. Crandall DL, Ferraro GD, Lozito RJ, Cervoni P, Clark LT. Cardiovascular effects of intermittent drinking: Assessment of a novel animal model of human alcoholism. J Hypertens. 1989;7:683-687.

180. Malec D, Michalska E, Pikulicka J. Influence of adenosinergic drugs on ethanol withdrawal syndrome in rats. Pol J Pharmacol. 1996;48:583-588.

181. El Yacoubi M, Ledent C, Parmentier M, Daoust M, Costentin J, Vaugeois J. Absence of the adenosine A(2A) receptor or its chronic blockade decrease ethanol withdrawal-induced seizures in mice. Neuropharmacology. 2001;40:424-432.

182. Kaplan GB, Bharmal NH, Leite-Morris KA, Adams WR. Role of adenosine A1 and A2A receptors in the alcohol withdrawal syndrome. Alcohol. 1999;19:157-162.

183. Concas A, Mascia MP, Cuccheddu T, et al. Chronic ethanol intoxication enhances [3H]CCPA binding and does not reduce A1 adenosine receptor function in rat cerebellum. Pharmacol Biochem Behav. 1996;53:249-255.

184. Morgan PF, Durcan, M.J. Caffeine-induced seizures: apparent proconvulsant action of N-ethyl carboxamidoadenosine (NECA). Life Sci.1990;47:1-8.

185. De Sarro G, De Sarro A, Di Paola ED, Bertorelli R. Effects of adenosine receptor agonists and antagonists on audiogenic seizure-sensible DBA/2 mice. Eur J Pharmacol. 1999;371:137-145.

186. Johansson B, Georgiev V, Parkinson FE, Fredholm BB. The binding of the adenosine A2A receptor selective agonist [3H]CGS 21680 to rat cortex differs from its binding to rat striatum. Eur J Pharmacol. 1999;247:103-110.

187. Gatch MB, Selvig M. Theophylline blocks ethanol withdrawal-induced hyperalgesia Alcohol Alcohol. 2002;37:313-317.

188. Simola N, Cauli O, Morelli M. Sensitization to caffeine and cross-sensitization to amphetamine: influence of individual response to caffeine. Behav Brain Res. 2006;172:72-79.

189. Celik E, Uzbay IT, Karakas S. Caffeine and amphetamine produce cross-sensitization to nicotine-induced locomotor activity in mice. Prog Neuropsychopharmacol Biol Psychiatry. 2006;30:50-55.

190. López-Cruz L, Pardo M, San Miguel N, Salamone JD, Correa M. Impact of high doses of caffeine on acute and sensitized motor activity induced by ethanol in mice. Presented at: Federation of European Neuroscience Societies. Barcelona, 2012.

191. Hsu CW, Chen CY, Wang CS, Chiu TH. Caffeine and a selective adenosine A2A receptor antagonist induce reward and sensitization behavior associated with increased phospho-Thr75-DARPP-32 in mice. Psychopharmacology (Berl) 2009;204:313-325.

192. Bedingfield JB, King DA, Holloway FA. Cocaine and caffeine: conditioned place preference, locomotor activity, and additivity. Pharmacol Biochem Behav. 1998; 61:291-296.

193. Patkina NA, Zvartau EE. Caffeine place conditioning in rats: comparison with cocaine and ethanol. Eur Neuropsychopharmacol. 1998;8:287-291.

CHAPTER 2:

Differences between the nonselective adenosine receptor antagonists caffeine and theophylline in motor and mood effects: Studies using medium to high doses in animal models.

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Differences between the nonselective adenosine receptor antagonists caffeine and theophylline in motor and mood effects: studies using medium to high doses in animal models.

Laura López-Cruz[a], Marta Pardo[a], John D. Salamone[b], Mercè Correa [a,b]

[a]Àrea de Psicobiologia, Campus de Riu Sec, Universitat Jaume I, 12071 Castelló, Spain.
[b]Dept. of Psychology, University of Connecticut, Storrs, CT, USA 06269-1020.

Corresponding Author: Dr. Mercè Correa, Àrea de Psicobiologia, Campus de Riu Sec, Universitat Jaume I, 12071 Castelló, Spain (email: correa@psb.uji.es). Tel: +34 964729841. Fax: +34 964729267

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Abstract

Rationale. Caffeine and theophylline are methylxanthines that are broadly consumed, sometimes at high doses, and act as minor psychostimulants. Both are nonselective adenosine antagonists for A_1 and A_{2A} receptors, which are colocalized with dopamine D_1 and D_2 receptors in striatal areas. Adenosine antagonists generally have opposite actions to those of dopamine antagonists. Although the effects of caffeine are widely known, theophylline has been much less well characterized, especially at high doses.

Methods. Adult male CD1 mice were used to study the effect of a broad range of doses (25.0, 50.0 or 100.0 mg/kg) of caffeine and theophylline on measures of spontaneous locomotion and coordination, as well as the pattern of c-Fos immunoreactivity in brain areas rich in adenosine and dopamine receptors. In addition, we evaluated possible anxiety and stress effects of these doses.

Results. Caffeine, at these doses, impaired or suppressed locomotion in several paradigms. However, theophylline was less potent than caffeine at suppressing motor parameters, and even stimulated locomotion. Both drugs induced corticosterone release, however caffeine was more efficacious at intermediate doses. While caffeine showed an anxiogenic profile at all doses, theophylline only did so at the highest dose used (50 mg/kg). Only theophylline increased c-Fos immunoreactivity in cortical areas.

Conclusion. Theophylline has fewer disruptive effects than caffeine on motor parameters and produces less stress and anxiety effects. These results are relevant for understanding the potential side effects of methylxanthines when consumed at high doses.

Key Words: methylxantines; incoordination; behavioral activation; anxiety; striatum; prefrontal.

1. Introduction

Caffeine is the most widely used psychoactive substance worldwide [1,2]. Average consumption ranges from 100 to 400 mg per day, but consumption increased in some groups of consumers with the introduction in the market of energy drinks [2]. Theophylline is a metabolite of caffeine that is also present in teas, as well as some common dietary products [3].

Both methylxanthines exert their psychostimulant effects mainly through adenosine receptor blockade [4,5]. Adenosine is a neuromodulator that is involved in multiple functions such as sleep, attention, locomotion, and anxiety [6-8]. Adenosine acts on four G-protein-coupled receptors: A_1, A_{2A}, A_{2B} and A_3 [4]. A_1 and A_{2A} receptors are the main target for both caffeine and theophylline [4,5]. Whereas A_1 receptors are widely expressed in the brain, A_{2A} receptors are mainly concentrated in the striatal complex [4,9]. On striatal medium spiny neurons, A_1 receptors are colocalized with dopamine D_1 receptors while A_{2A} receptors are colocalized and interact with D_2 receptors; adenosine and dopamine receptors can interact by forming heteromeric complexes, and also by convergence onto the same signal transduction pathways [10,11]. Moreover, there is a substantial amount of behavioral and neurochemical data showing that antagonism of adenosine receptors, either with nonselective or A_{2A} selective drugs, can reverse the effects of dopamine D_2 receptor antagonists on motor and motivational functions that involve nucleus accumbens (Acb) and neostriatum [12-15]. Caffeine is being considered as a possible therapeutic agent because of its ability to interact with dopamine receptors and affect signal transduction in striatal neurons. In addition, caffeine has been proposed as a neuroprotective agent to counteract the effects of dopaminergic neural loss [16,17]. Thus, caffeine is potentially useful for the pharmacological treatment of some symptoms of Parkinson disease [18-20], depression [21] and other disorders that involve dopamine transmission or basal ganglia circuitry.

However, although low doses of caffeine stimulate locomotion and do not impair motor coordination in rodents [6,22,23], high doses can suppress locomotion [6,24,25]. High doses of caffeine that are able to suppress locomotion also increase *c-fos* markers throughout the striatum [26,27]. In addition, high doses of caffeine have been shown to increase physiological parameters of stress such as plasma cortisol levels in humans [28], and corticosterone levels in rats [8,29], and also to promote anxiety in humans (for a review see [7]), and anxiogenic-like behaviors in animal models [30,31].

Theophylline, despite its similar therapeutical potential [13,32] has been much less explored, but it has been demonstrated that theophylline can suppress parkinsonian symptoms in humans [33,34]. As is the case with caffeine, low doses of theophylline can induce motor stimulant effects in rodents [32,35]. Nevertheless, there is a general lack of information about the effects of theophylline, especially at higher doses.

Thus, the present experiments were undertaken to explore and compare systematically the effects of moderate to high doses of caffeine and theophylline on measures of motor activity, anxiety and neuroendocrine parameters, as well as their effect on c-Fos immunoreactivity (to provide a marker of neuronal activation in dopamine and adenosine-receptor rich brain areas). The effects of both drugs on different aspects of exploration, vigorous exercise, and motor coordination, as well as the knowledge of their impact on mood and stress responses, could be useful information for understanding their potential side effects at high doses.

2. Materials and methods

2.1 Animals

CD1 adult male mice (N=406) purchased from Harlan-Interfauna Ibérica S.A. (Barcelona, Spain) were 9 weeks old (30-45 g) at the beginning of the study. Mice were housed in groups of three or four per cage, with standard laboratory rodent chow and tap water available *ad libitum*. Subjects were maintained at 22 $\pm$ 2 ºC with 12-h light/dark cycles (lights on at 13:00 hours). To habituate the animals to the procedures, they were handled and received a single saline injection the day before experimental procedures started. Different groups of animals were used in each experiment, except for the anxiety tests in which the same animals were serially tested in both paradigms. 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/ECC).

2.2. Drugs

Caffeine and Theophylline (Sigma-Aldrich, Spain) were dissolved in 0.9% w/v saline. Saline solution was used as the vehicle control. All solutions were administered intraperitoneally (IP) 30 minutes before behavioral testing, 90 minutes before brain

extraction in the immunohistochemical study and 60 minutes before blood samples were collected.

2.3. Behavioral apparatus and testing procedures

2.3.1. Locomotion in the open field arena (OF). The OF 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. The behavioral test room was illuminated with a soft light, and external noise was attenuated. Tests were videotaped and locomotor activity was registered manually during 10 minutes. An activity count was registered as horizontal locomotion each time the animal crossed one quadrant with four legs. Animals were not pre-exposed to the OF in order to study novelty-induced exploration and locomotion.

2.3.2. Locomotion in the 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 minutes in two consecutive days previous to the test. The test day, counts on the wheel were registered during 30 minutes. The RW generates stable basal high levels of activity when the animals are trained, and thus is useful for evaluating conditions that suppress voluntary self-induced locomotion.

2.3.3. Motor coordination in the rotarod. The rotarod apparatus (UGO Basile, 7650) consisted of an elevated rotating rod that requires coordinated movement 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 seconds. Animals were trained during 5 trials, and tested for 5 more trials. A 390 seconds maximum cut-off on the rod was used. The apparatus automatically recorded the time (in seconds) at the moment in which the animal fell off the rod.

2.3.4. Anxiety in the elevated plus maze (EPM). The EPM consists of two open and two enclosed arms arranged in a plus configuration. This anxiety paradigm measures the avoidance that rodents show to elevated open spaces. The behavioral test room was illuminated with a soft light. Animals were placed in the central platform facing the

closed arm and assessed during 5 minutes. Tests were videotaped and a trained observer registered time spent in the open arms, ratio of entries in the open arms to total arm entries, latency to enter the open arms and total entries in the 4 arms as an index of locomotion. An entry into an arm was recorded when the animal crossed the line that connected that arm with the central platform with all four legs.

2.3.5. Anxiety in the dark and light (DL). The DL test is based on the conflict between the inherent tendency of mice to explore a novel environment against their natural avoidance of a brightly lighted open field. The DL apparatus consisted of a polypropylene chamber divided in two compartments by a partition containing a small opening. One chamber was open and illuminated while the other was closed and dark. The behavioral test room was illuminated with a soft light. Each subject was placed in the dark chamber. Tests were videotaped and the latency of the first entry into the lit chamber, total time spent in the lit chamber and total number of crosses between chambers, were recorded manually over 5 minutes. The same animal was first evaluated in the EPM for 5 minutes and then immediately placed in the chamber for evaluation in the DL during 5 more minutes.

2.4. Plasma corticosterone determination.

Mice received drug injections 60 minutes before being sacrificed by decapitation under anesthesia. This time was chosen based on previous studies showing significant increments in corticosterone levels between 30 and 100 minutes after caffeine administration [8] and [29]. Blood samples were collected in heparinized (15 units/ml of blood) Eppendorf tubes and centrifuged at 4000 rpm for 10 minutes. Supernatant was taken and stored at -20°C until corticosterone determination. Plasma corticosterone levels were measured spectophotometrically using a commercially available enzymatic immunoassay kit (Rodents Corticosterone Enzyme Immunoassay System, OCTEIA Corticosterone; Immunodiagnostic Systems LTD, Boldon, England). The ng/ml of blood corticosterone concentration was determined using a logarithmic adjustment of the standard curve.

2.5. c-Fos visualization and quantification.

Mice were anesthesized and perfused 90 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 × for 5 minutes) and transferred into the primary antibody, anti-c-Fos (Calbiochem, Germany) for 24 h incubation. Following the primary antibody treatment, the sections were rinsed in PBS and incubated in the secondary antibody, anti-rabbit HRP conjugate, envision plus (DAKO, Denmark) for 1.5 h. The immunohistochemical reaction was developed using diaminobenzidine (DAB) as the chromagen (DAKO). Processed sections were then mounted to gelatin-coated slides, 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). Placements for the photographs were counterbalanced between right and left hemispheres for all the animals and structures. Images of the regions of interest were magnified at 20X and captured digitally using Stereo Investigator software. Cells that were positively labeled for c-Fos 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.

2.6. Statistics

Experiments used a between-groups design. Normally distributed and homogeneous data were evaluated by a parametric two-way analysis of variance (ANOVA) followed by non-orthogonal planned comparisons using the overall error term ([36] Keppel, 1991). Non-parametric data were analyzed with a Kruskal-Wallis one-way analysis by ranks. The Mann-Whitney U test was used to compare between two groups for the analysis of non-parametric data. A probability level of 0.05 or smaller was used to indicate statistical significance. Effect size calculations (R^2 values) were performed to assess the magnitude of the effect for every drug in every parameter tested [36]. With this type of effect size calculation, the magnitude of the treatment effect is independent of the number of animals, and is expressed as the proportion of total variance accounted for by treatment variance (for example $R^2 = 0.3$ reflects 30% of the variance explained) across experiments and measures (see Table. 2). Results are graphically depicted as means ±SEM. Statistics were conducted using STATISTICA 7 software.

3. Results

3.1. Effect of caffeine and theophylline on locomotor activity in the OF.

The two-way ANOVA (Treatment x Dose) showed a significant effect of the treatment factor [$F(1,67)= 6.33$, $p<0.05$], a significant effect of dose [$F(3,67)= 9.54$, $p<0.01$], and a significant treatment x dose interaction [$F(3,67)= 6.36$, $p<0.05$]. Planned comparison analysis revealed a significant stimulant effect of 50.0 mg/kg theophylline ($p<0.01$) and a depressant effect on locomotion of the highest dose of caffeine used (100.0 mg/kg) ($p<0.05$), both compared with their respective vehicle treatment. Caffeine and theophylline were significantly different from each other in their effects at the higher doses, 50.0 and 100.0 mg/kg ($p<0.01$), as measured by planned comparisons.

3.2. Effect of caffeine and theophylline on locomotor activity in the RW.

The two-way ANOVA (Treatment x Dose) showed a significant effect of the treatment factor [$F(1,64)= 3.75$, $p<0.05$], a significant effect of dose [$F(3,64)= 16.05$, $p<0.01$] and a significant treatment x dose interaction [$F(3,64)= 6.36$, $p<0.01$]. Planned comparisons analysis revealed that caffeine significantly decreased locomotion in the RW at all doses used (25.0, 50.0 and 100.0 mg/kg) compared with vehicle ($p<0.01$), while theophylline only showed this suppressant effect at the two highest doses (50.0 and 100.0 mg/kg) compared with vehicle treatment ($p<0.01$) (see Fig. 1B). Thus, caffeine seems to be more potent than theophylline at suppressing running in the RW.

3.3. Effect of caffeine and theophylline on rotarod performance.

The two-way ANOVA (Treatment x Dose) revealed that there was not an overall effect of treatment on rotarod performance [$F(1,46)= 0.09$, n.s]. However, a significant effect of dose [$F(3,46)= 29.81$, $p<0.01$], and a significant effect of treatment x dose interaction [$F(3,46)= 5.47$, $p<0.05$] were observed. Planned comparisons revealed that caffeine at doses of 50.0 and 100.0 mg/kg decreased time spent on the rotating rod ($p<0.01$) and theophylline only showed this effect at the highest dose (100 mg/kg) ($p<0.01$). Caffeine and theophylline were significantly different from each other at the dose of 50.0 mg/kg; caffeine induced a greater suppressant effect than theophylline at this dose ($p<0.01$) (see Fig. 1C). These results indicate that caffeine is more potent than theophylline at suppressing motor performance in the rotarod.

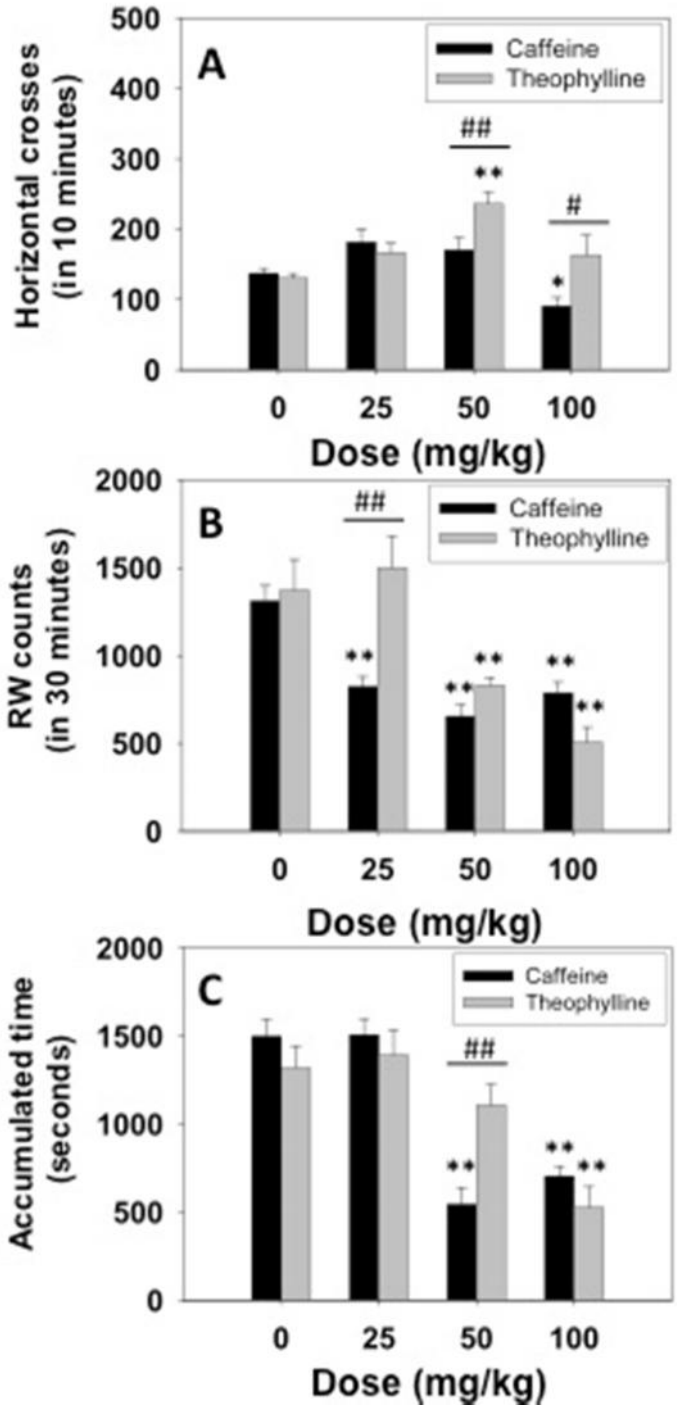

Fig. 1. Effect of caffeine and theophylline on A) horizontal locomotion in the OF (N=9-10 per group), B) running in the RW (N= 7-8 per group), and C) performance in the rotarod (N=7-8 per group). Data are expressed as mean (±SEM) number of counts during 10 minutes. *p<0.01**p<0.05 significantly different from vehicle. ##p<0.01 #p<0.05 significant differences between treatments at the same dose.

3.4. Effect of caffeine and theophylline on plasma corticosterone levels.

Figure 2 shows data on plasma corticosterone levels. A two-way ANOVA (Treatment x Dose) revealed a significant effect of treatment [$F(1,43)= 19.25$, $p<0.01$], a significant

effect of dose [F(3,43)= 37.65, p<0.01], and a significant treatment x dose interaction [F(3, 43)= 3.53, p<0.05]. Planned comparisons revealed that caffeine at all doses (25.0, 50.0 and 100.0 mg/kg) significantly increased plasma corticosterone levels (p<0.01). This effect was also observed after theophylline administration at all doses employed (25.0 mg/kg, p<0.05; 50.0 and 100.0 mg/kg p<0.01). Caffeine and theophylline were significantly different from each other at the two lower doses (25.0 and 50.0 mg/kg). Caffeine was more potent than theophylline at inducing corticosterone.

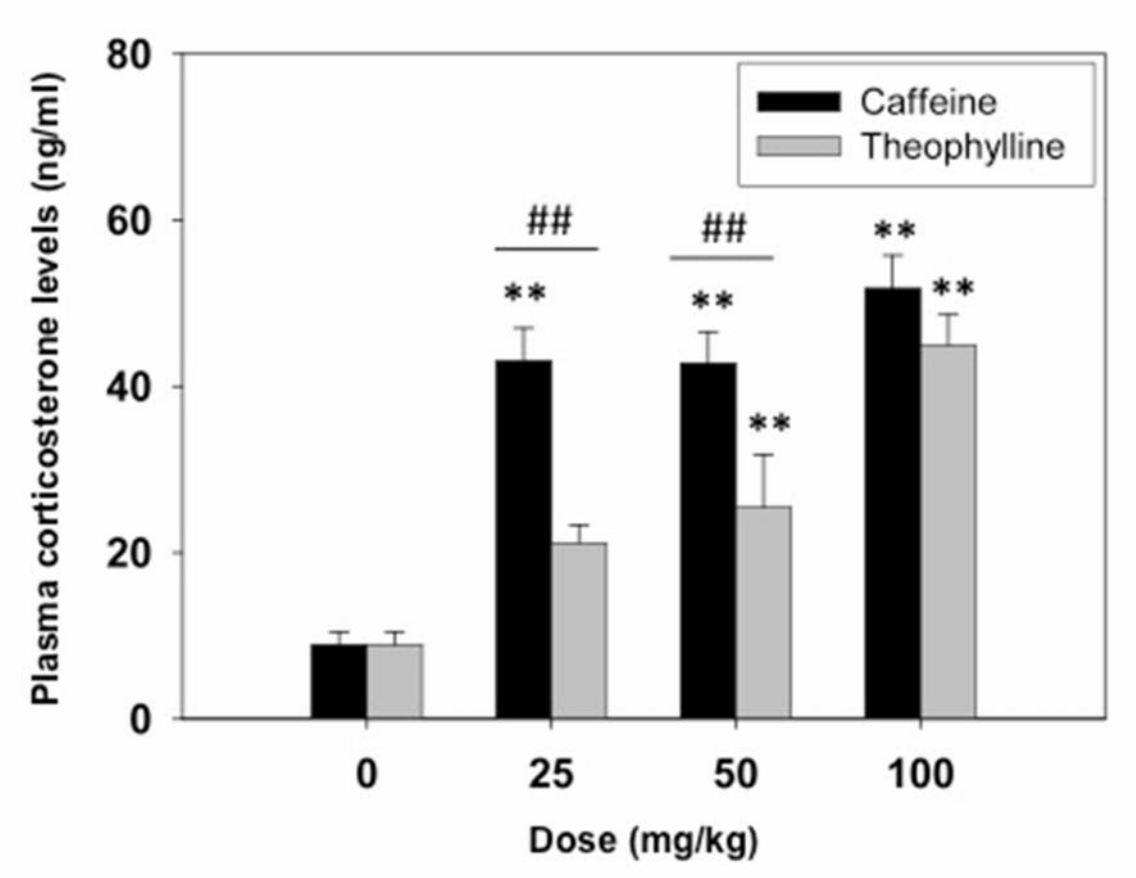

Fig. 2. Effect of caffeine and theophylline on plasma corticosterone levels (N=6-7 per group). Data are expressed as mean (± SEM) nanograms of corticosterone per mililiter of plasma. **p<0.01 significantly different from vehicle. ##p<0.01 significant differences between treatments at the same dose.

3.5. Effect of caffeine and theophylline in the EPM and in the DL.

For the anxiety experiments, only the two smallest doses of caffeine and theophylline (25.0 and 50.0 mg/kg) were used in order to minimize the impact of ataxia and incoordination on the anxiety measurements. Animals were evaluated first in the EPM and immediately after were evaluated in the DL. For the EPM results, a non-parametric

Kruskal-Wallis test of the median values for the dependent variable latency to enter into one of the open arms showed a significant effect [H(5 df)= 20.2, p<0.01]. The Mann-Whitney U test showed that caffeine at both doses (25.0 and 50.0 mg/kg) increased latency to enter into the open arm compared to the vehicle group [U=22.0, and U=27.0, p<0.01, respectively]. This effect was only observed with the highest dose of theophylline (50.0 mg/kg; U=27.0, p<0.01), indicating an anxiogenic-like effect of caffeine that was more potent than that of theophylline in this parameter (Fig. 3A). The non-parametric Kruskal-Wallis test for the dependent variable time in open arms showed a significant effect [H (5 df)=27.7, p<0.01]. Caffeine produced an anxiogenic effect as measured by the reduction in time spent in open arms. Thus, Mann-Whitney U test showed significant differences between both doses of caffeine (25.0 and 50.0 mg/kg) and vehicle [U=16.0, and U=18.0, p<0.01, respectively]. However, only the highest dose of theophylline (50.0 mg/kg) showed this significant decrement in time spent in open arms in relation to its control group [U=21.0, p<0.01]. Comparisons between caffeine and theophylline at the same dose revealed significant differences [U=47.5, p<0.05] between both drugs at dose of 25.0 mg/kg. However, this difference disappeared with the highest dose explored (50.0 mg/kg) in which both drugs reduced the time spent in open arms (Fig. 3B), suggesting a more potent anxiogenic effect of caffeine than theophylline. For the variable ratio of entries into open arms, the non-parametic Kruskal-Wallis test of the median values showed a significant effect [H(5 df)= 25.1, p<0.01]. Mann-Whitney U test revealed that both doses of caffeine (25.0 and 50.0 mg/kg) significantly reduced the ratio of entries into the open arms [U=16.0, p<0.01; U=25.0, p<0.05, respectively] compared to vehicle. However, consistently with previous results, this effect was only observed with the highest dose of theophylline (50.0 mg/kg) [U=24.0, p<0.01]. Comparisons between caffeine and theophylline at the same dose revealed significant differences [U=47.5, p<0.05] between both drugs at dose of 25.0 mg/kg. However, this difference disappeared with the highest dose explored (50.0 mg/kg) in which both drugs reduced the ratio of entries to open arm (Fig. 3C). Finally, for the total arm entries a parametric test was performed. The two-way factorial ANOVA (Treatment x Dose) showed a significant effect of the treatment [F(1,66)=3.77, p<0.05] and a significant effect of dose factor [F(2,66)=4.80, p<0.05] as well as a significant effect for the interaction [F(2,66)=4.02, p<0.05]. Planned comparisons showed that caffeine reduced total arm entries at the highest dose (50.0 mg/kg) compared to its vehicle (p<0.01) and compared to theophylline at the same dose

($p<0.05$). Consistently with the data obtained in the motor studies, this result indicates a suppressor effect of caffeine on locomotor activity at the high dose (Fig. 3D).

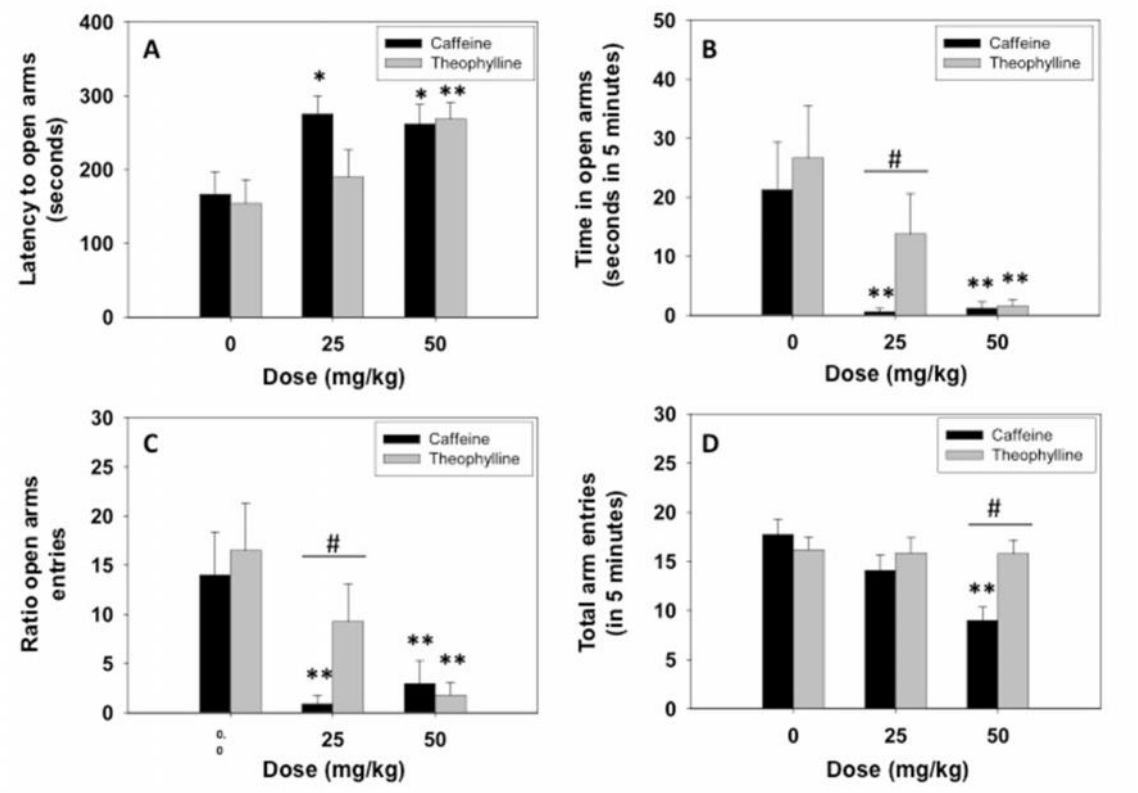

Fig. 3. Effect of caffeine and theophylline on the EPM (N=11-13 per group). Data are expressed as mean (±SEM) of **A)** latency (seconds) to enter an open arm, **B)** time (sec) spent in the open arms, **C)** ratio of open arm entries, and **D)** total arm entries during 5 minutes. ##$p<0.01$ significant differences between treatments at the same dose. **$p<0.01$ significant differences in ranks between treatments in A, or **$p<0.01$ significantly different from vehicle in B, C and D.

The effects of caffeine and theophylline in the DL are shown in figure 4 A-C. Although the results showed the same pattern as in the EPM, the non-parametic Kruskal-Wallis test of the median values showed that none of the variables explored (latency to lit compartment, time in lit compartment, and total number of crosses) were statistically affected by drug treatment.

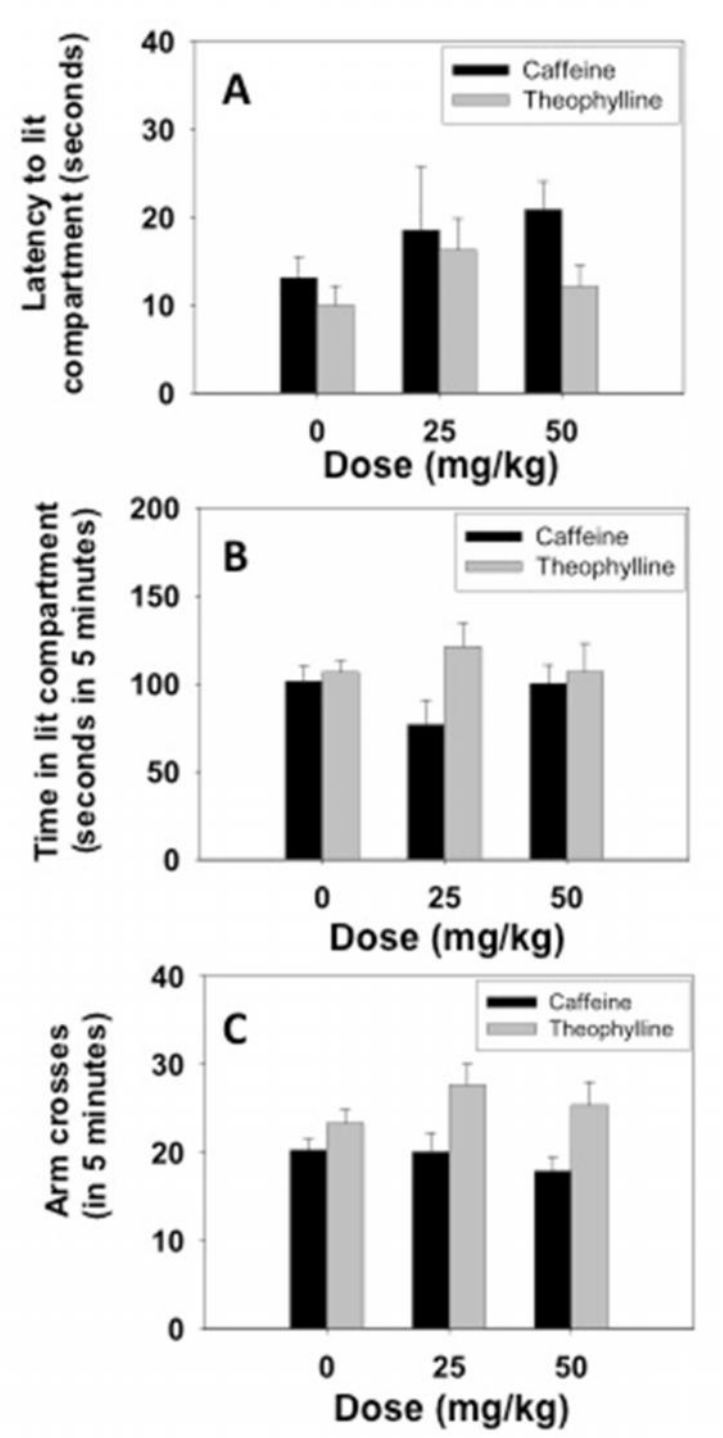

Fig. 4. Effect of caffeine and theophylline on the behavior of mice in the DL. Data are expressed as mean (±SEM) of A) latency (seconds) to lit compartment, B) time (seconds) in lit compartment, and C) crosses during 5 minutes.

3.6. Effect of caffeine and theoplhylline on c-Fos immunoreactivity in different brain areas.

The c-Fos immunoreactivity levels were analyzed separately for every brain area. The one-way ANOVA (Treatment x Dose) revealed an effect of treatment on c-Fos immunoreactivity in the anterior cingulate (ACC) [$F(2,16)= 16.36$, $p<0.01$). The post hoc test showed that only theophylline significantly increased c-Fos expression in ACC compared to vehicle ($p<0.01$) and compared to caffeine ($p<0.01$). A significant effect of treatment was also observed in the dorsomedial striatum (DMS) ($F(2,16)= 3.46$, $p<0.05$). Theophylline induced higher levels of c-Fos expression than caffeine in this striatal region ($p<0.05$). No statistically significant effects were observed in the other areas explored (dorsolateral (DLS), and ventrolateral striatum (VLS), accumbens (Acb) shell and core (see Table 1). These results suggest that theophylline seems to be more efficacious than caffeine at inducing c-Fos in the ACC and DMS regions.

	Vehicle	**Caffeine**	**Theophylline**
ACC	22.8 ± 13.7	34.2 ± 14.0	132.8 ± 18.8**##
Acb Core	10.4 ± 3.0	8.5 ± 3.8	9.1 ± 1.9
Acb Shell	5.9 ± 1.0	9.5 ± 1.8	9.6 ± 3.0
DMS	12.8 ± 3.3	5.5 ± 1.5	20.3 ± 5.7#
DLS	6.8 ± 1.7	6.9 ± 2.7	14.8 ± 7.2
VLS	3.3 ± 1.3	4.0 ± 1.1	3.5 ± 1.2

Table 1. Effect of caffeine and theophylline administration on c-Fos immunoreactivity in several brain areas (N=5-6 per group). Mean (±SEM) number of c-Fos positive cells per mm^2. **p<0.01 significantly different from vehicle, ##p<0.01 #p<0.05 significantly different from caffeine in the same structure.

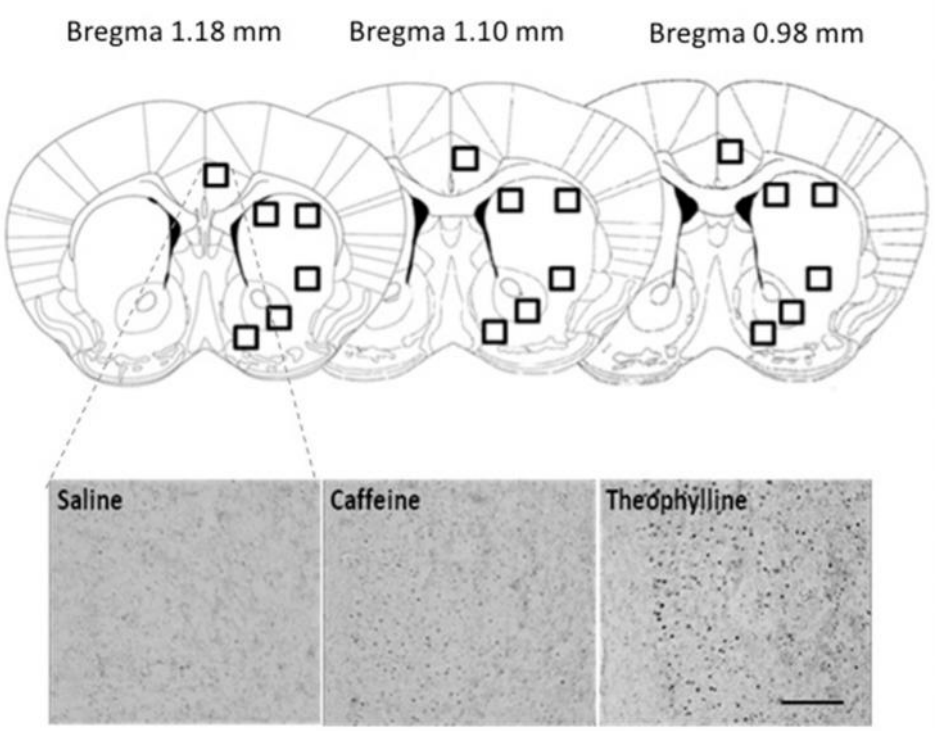

Fig. 5. Upper part: Diagram of coronal sections with bregma coordinates taken from Franklin and Paxinos 2007, showing location of the brain areas for c-Fos counting. Lower part: Photomicrographs of c-Fos staining in ACC from representative animals in the vehicle, caffeine 50 mg/kg, and theophylline 50 mg/kg groups. Low power images (20x), scale bar = 250 μm.

Dependent Variables	Caffeine	Theophylline
Open field	0.394	0.360
Running Wheel	0.375	0.499
Rotarod	0.823	0.545
Plasma corticosterone levels	0.886	0.689
Elevated plus-maze		
Time in open arms	0.298	0.190
Latency to open arms	0.225	0.172
Ratio of open arms entries	0.279	0.202
Total arm entries	0.589	0.001
Dark and Light		
Time in lit compartment	0.038	0.040
Latency to lit compartment	0.156	0.052
Crosses	0.074	0.040
C-Fos inmunochemistry		
ACC	0.034	0.704
AcbCore	0.014	0.232
AcbShell	0.235	0.127
DMS	0.249	0.109
DLS	0.010	0.136
VLS	0.109	0.001

Table 2. Effect size calculations (R^2 values). Marker of the magnitude of the effect for caffeine and theophylline in the behavioral, endocrine and biochemical parameters explored.

4. Discussion

The present work offers a comparative study, between two of the most well known and widely consumed methylxantines, caffeine and theophylline, at a range of doses that are

not usually explored (i.e., moderate to high doses), using animal models of motor performance and mood.

Evaluation of the motor stimulant actions of drugs is frequently conducted by analyzing the locomotor activity of animals in an OF [37,38]. This paradigm can offer an index of exploration induced by novelty when the animals are exposed to a chamber for the first time, and it has been demonstrated that psychostimulant drugs potentiate reaction to novelty in the OF, both in rodents as well as humans [39,40]. In our study the two lowest doses of caffeine showed a tendency to stimulate locomotion, but the effect was not statistically significant. Increases in locomotion with moderate doses of caffeine are clearly seen in studies that habituate mice to the OF, and thus reduce basal activity [6]. In the present study with non-habituated animals, caffeine produced a biphasic effect with a non-significant tendency to increase locomotion and clear suppression of locomotion at the highest dose, in consonance with previous studies using also high doses [6,24]. On the other hand, theophylline, under the same conditions, produced significant stimulant effects at 50 mg/kg, and no suppressant effects. Previous data show that theophylline at lower doses (10-20 mg/kg) than the ones used in the present study also increase motor activity in mice [41,32]. Thus, theophylline- and caffeine-treated groups at the two highest doses (50 and 100 mg/kg) were significantly different from each other, indicating that theophylline is less potent than caffeine at suppressing exploration. These results (see Fig. 1A) suggest that at this range of doses, theophylline seems to be more efficacious at stimulating locomotion while caffeine is either more efficacious or more potent at suppressing activity in a novel OF.

In research with rodents, one of the most common ways of studying voluntary and vigorous physical activities is wheel running. This paradigm induces high levels of locomotion [42,43], and is useful for the assessment of motor suppressant effects of drugs. In our experiment, caffeine suppressed wheel running at all doses while theophylline only showed this effect at the highest doses (50 and 100 mg/kg). These results indicate again that theophylline is less potent than caffeine at reducing spontaneous running behavior. Another relevant motor parameter is coordination and balance. In the present study, those aspects of locomotion were assessed using a rotating rod that forces the animal to move in order to avoid falling from the elevated rod. Thus, this paradigm has an aversive component and also is a way to evaluate forced locomotion. Once again, we observed a suppressant effect of caffeine at the two highest doses (50 and 100 mg/kg) while theophylline only showed this effect at the highest

dose. Thus, the effect size for caffeine in this parameter is greater than for theophylline (see table 2). While low to moderate doses of caffeine (3-30 mg/kg) have demonstrated not to produce motor incoordination in mice in the rotarod [22,44], the present results on moderate to high doses of caffeine are in agreement with a study that evaluated the stumbling frequency in the holeboard test as a measure of motor coordination after moderate to high doses of caffeine (30-120 mg/kg), and demonstrated a dose-dependent increase in stumbling frequency [25]. Our data on theophylline are also consistent with a previous study showing that intraventricular administration of theophylline (150 µg/5µl) in mice did not affect endurance on the rotarod [45].

In addition to these behavioral effects, the present range of doses produced an increase in corticosterone levels, indicating a strong endocrine stress response to administration of these methylxantines. More importantly, caffeine reached higher levels of corticosterone than theophylline at moderate doses (25 and 50 mg/kg), although these differences disappeared at the highest dose (100 mg/kg). These results suggest that both drugs are efficacious at inducing increments in corticosterone levels, but caffeine seems to be more potent as also shown by the effect size analysis (see table 2). Previous studies have showed dose-related increases in plasma corticosterone levels, associated with changes in ACTH [8,29], after acute administration of caffeine in rats at a broad range of doses [8,29,46,47]. However, there was a lack of information about theophylline on this parameter.

Caffeine also has been demonstrated to induce anxiety in some humans (for a review see [9]. In the present work, we confirmed that caffeine is an anxiogenic agent. In the EPM, moderate (25 mg/kg) and high doses (50 mg/kg) of caffeine increased the latency to enter into the open arm, and decreased the time spent and ratio of entries into the open arms, causing a significant preference for the protected sections of the maze, an index of its anxiogenic-like effects. These results are in accordance with previous experiments in rats and mice using similar range of doses (15-100 mg/kg) [30,31,48,49]. In contrast, theophylline only produced anxiogenic effects at the highest dose used (50 mg/kg). The total number of entries into the different compartments was only affected at the highest dose of caffeine, showing again a motor suppressant effect of caffeine but not of theophylline. This difference is also observed in the effect size calculations (see table 2). In our study, neither caffeine nor theophylline produced statistically significant effects in the DL paradigm. However, there was a tendency of caffeine to produce greater anxiogenic effects than theophylline. Conflicting results

have been reported in the DL test after caffeine administration [48,50]. While caffeine has previously demonstrated to have anxiogenic properties in the DL test at a range of doses similar to those used above (25-100 mg/kg) [48], another study did not observed this effect after caffeine administration (15-45 mg/kg) [50]. It is possible that in our results the lack of clear effects is due to the experimental procedure. Animals were evaluated in the DL immediately after being tested in the EPM, which could possibly have masked the anxiogenic effects of both drugs in this paradigm. However, this serial testing procedure has been previously used to assess anxiolytic and anxiogenic effects of other drugs, and both effects were constant in both paradigms [51,52]. It is possible then, that the anxiogenic reaction produced by methylxantines is not very strong, and habituates easily. In summary, the overall results suggest that caffeine is more potent than theophylline at inducing anxiety and at suppressing locomotion.

In spite of its anxiety inducing properties at medium to high doses, caffeine is orally self-administered in rodents at medium to low doses. CD1 mice, independently of their baseline anxiety levels, seem to consume caffeine at a low concentration that nevertheless yielded a dose of 35 mg/kg per day [53]. Consumption of caffeine, at least at lower doses (around 12 mg/kg per day) [55], seems to be regulated by A_{2A} rather than A_1 adenosine receptors ([54,55], since A_1 KO and WT counterparts seem not to differ in terms of caffeine consumption [54], but A_{2A} KO mice drink less caffeine than WT mice [54]. It has also been demonstrated that rats prefer to drink only very low concentrations of caffeine ([56]. Moreover, after pavlovian conditioning, rats develop preference vs. avoidance to caffeine associated flavor solutions in a dose dependent manner: flavors associated with low concentrations of caffeine produce preference and flavors associated with high ones produce avoidance [56]. It has also been demonstrated that high doses of caffeine such as the ones used in the present study (25-50 mg/kg) associated to a place generate avoidance in rats [57,58], while only lower doses induce place preference [57-59]. To our knowledge, there are no studies about the impact of theophylline on these parameters.

Finally, the pattern of c-Fos expression has been used in past research as an index of neuronal activation in different brain areas in which adenosine and dopamine receptors are abundant [60,61]. Thus, in the present study we included dorsal and ventral areas of the striatum as well as the two subregions of the Acb and an area of the frontal cortex; the ACC. All these structures seem to be important for behavioral activation, exertion of effort, habit formation, locomotion and voluntary movement in general [62,63], among

other processes. In this case, our results show a greater efficacy of theophylline than caffeine. Thus, c-Fos immunoreactivity after theophylline administration at a dose of 50 mg/kg is increased in ACC. The effect size for theophylline in ACC (R^2=0.704) was the biggest across all the structures, and was much larger than that of caffeine (R^2=0.034). Caffeine at this dose, did not induce c-Fos in any of the structures studied. Reports in the literature show that, at least in rats, caffeine at high doses (50-100 mg/kg) produces induction of *c-fos* expression or the amount of c-Fos protein in striatum and Acb [27,64,65], as well as in ACC [66]. Thus, it is possible that a higher dose of caffeine than the one used in the present study (e.g. 50 mg/kg) would induce c-Fos as well in mice. The present results showing an effect of theophylline on c-Fos is relevant due to the relative lack of studies on this methylxanthine. In mice, lower doses of theophylline (15 mg/kg) than the ones used in the present work did not induce c-Fos immunoreactivity in different areas of striatum or Acb [13,32]. However, a study in rats showed that a high dose of caffeine and of theophylline (100 mg/kg) did induce *c-fos* expression in the striatum [67]. Our results in mice indicate that the difference between the two drugs is evident at a lower dose. The mechanism of action for methylxanthines, specifically caffeine, on immediate early gene expression is not clear, but it has been related to dopamine regulation [65]. Previous studies in rats have demonstrated that dopamine D_1, and to a lesser extent D_2, receptor antagonists blocked potentiation of *c-fos* expression by a high dose of caffeine (100 mg/kg) in different areas of striatum [65]. In turn, the effect of caffeine on *c-fos* induced by dopamine D_1 and D_2 receptor blockade is probably regulated by the actions of caffeine on A_1 and A_{2A} receptors respectively [65,68,69]. In most mammals, A_{2A} receptors are highly concentrated in the striatum, with very low concentrations in prefrontal cortex [4]. However, A_1 receptors are highly concentrated in prefrontal cortex as well as striatum [4]. Considering the higher ratio of A_1 versus A_{2A} receptors in prefrontal cortex, it seems reasonable that the present results (induction of c-Fos only in ACC) are probably due to theophylline effects on A_1 receptors. However, previous studies in rats, using A_1 and A_{2A} receptor agonists alone or in combination, demonstrate that in order to produce changes in *c-fos* expression in cingulate cortex, is necessary to have combined stimulation of both receptors [70].

Taking all these results together, we have demonstrated that caffeine is more potent than theophylline at suppressing voluntary physical activity, motor exploration and coordination. In humans, one of the few experimental studies comparing both drugs

found that subjects receiving a single dose of caffeine (6 mg/kg) or of theophylline (4.5 mg/kg), showed a delayed exhaustion time in a cycling task compared to placebo, and there was not a significant difference between both drugs at these doses [71].

The average consumption of caffeine worldwide ranges from 100 mg to 400 mg per day [72]. However, with the introduction of energy drinks (which can contain up to 500 mg per unit) these levels are much higher in some consumers [2]. It has been reported that most of the beneficial effects of caffeine show a linear dose–response relationship up to about 300 mg, but at higher doses there is either a flattening of the curve, or impaired performane [73]. However, there are clear individual differences in response to some effects of caffeine, such as sleep disturbances or anxiogenic effects [74].

Thus, although caffeine is generally consumed with the purpose of potentiating an alert state, or improving endurance and performance, our results suggest that at high doses it can induce quite different actions. Our data do not support the idea that high doses of caffeine can be used to potentiate endurance and performance, specially, when physical activity is already performed at a high level (for a review see [75]).

The rank order in potency for the motor actions of methylxanthines has been established from more potent to less as paraxanthine, caffeine, theophylline and theobromine [67,76,77]. Behavioral and neurochemical reports confirmed this order [76-79]. However, there is a surprising lack of information about the less potent methylxantines. The present study offers a necessary comparison between caffeine and theophylline in a range of behaviors that can be affected after administration of these substances. Moreover, these results could help to better understand the potential effects of two nonselective adenosine receptor antagonists, caffeine and theophylline, that are being widely consumed in food, coffee and tea, or as “energy” drinks [71], and that are being proposed as possible therapeutic agents [80-84].

The separation between therapeutic efficacy and adverse side effects remains a challenge in the discovery and development of novel adenosine-based medicines, but this also is important for the naturally occurring ones. Although both methylxantines should have similar properties at low doses, caffeine is more potent than theophylline at inducing adverse effects at moderate and high doses; this would suggest that theophylline has a wider therapeutic window than caffeine. The present study can help to establish a range of doses that can induce undesirable side effects related to locomotion, anxiety and physiological stress responses in animal models. Future translational research should

assess the therapeutic window for each substance in disease animal models as well as human studies.

6. References

[1] Antoniou K, Papadopoulou-Daifoti Z, Hyphantis T, Papathanasiou G, Bekris E, Marselos M, Panlilio L, Müller CE, Goldberg SR, Ferré S. A detailed behavioral analysis of the acute motor effects of caffeine in the rat: involvement of adenosine A1 and A2A receptors. Psychopharmacology 2005;183:154-62.

[2] Reissig CJ, Strain EC, Griffiths RR. Caffeinated energy drinks-a growing problem. Drug Alcohol Depend 2009;99:1-10.

[3] Safranow K, Machoy Z. Methylated purines in urinary stones. Clinical Chem 2005;

51:1493-8.

[4] Fredholm BB, Ijzerman AP, Jacobson KA, Klotz KN, Linden J. International Union of Pharmacology. XXV. Nomenclature and classification of adenosine receptors. Pharmacol Rev 2001;53:527-52.

[5] Fredholm BB, Battig K, Holmen J, Nehlig A, Zvartau EE. Actions of caffeine in the brain with special reference to factors that contribute to ihallts widespread use. Pharmacol Rev 1999;51:83-133.

[6] El Yacoubi M, Ledent C, Ménard JF, Parmentier M, Costentin J, Vaugeois JM. The stimulant effects of caffeine on locomotor behaviour in mice are mediated through its blockade of adenosine A(2A) receptors. Br J Pharmacol 2000;129:1465-73.

[7] Correa M, Font L. Is there a major role of A2A adenosine receptor in anxiety? Front Biosci 2008;13:4058-70.

[8] Nicholson SA. Stimulatory effect of caffeine on plasmacorticosterone and pituitary-adrenocortical axis in the rat. Journal of Endocrinology 1989;122:535-43.

[9] Schiffmann S N, Fisone G, Moresco R, Cunha R, Ferre S. Adenosine A2A receptors and basal ganglia physiology. Prog. Neurobiol 2007; 83:277-92.

[10] Fredholm BB, Svenningsson P. Adenosine-dopamine interactions: development of a concept and some comments on therapeutic possibilities. Neurobiology 2003;61:55-9.

[11] Ferré S. An update on the mechanisms of the psychostimulant effects of caffeine. J Neurochem. 2008 ;105:1067-79.

[12] Collins LE, Galtieri DJ, Collins P, Jones SK, Port RG, Paul NE, Hockemeyer J, Müller CE, Salamone JD. Interactions between adenosine and dopamine receptor antagonists with different selectivity profiles: Effects on locomotor activity. Behavioural Brain Research 2010;211:148-55.

[13] Pardo M, Lopez-Cruz L, Valverde O, Ledent C, Baqi Y, Müller CE, Salamone JD, Correa M. Adenosine A2A receptor antagonism and genetic deletion attenuate the effects of dopamine D2 antagonism on effort-based decision making in mice. Neuropharmacology 2012;62:2068-77.

[14] Ferré S, Ciruela F, Quiroz C, Luján R, Popoli P, Cunha RA, Agnati LF, Fuxe K, Woods AS, Lluis C, Franco R. Adenosine receptor heteromers and their integrative role in striatal function.CientificWorldJournal 2007;7:74-85.

[15] Santerre JL, Nunes EJ, Kovner R, Leser CE, Randall PA, Collins-Praino LE, Lopez Cruz L, Correa M, Baqi Y, Müller CE, Salamone JD. The novel adenosine A(2A) antagonist prodrug MSX-4 is effective in animal models related to motivational and motor functions. Pharmacol Biochem Behav 2012;102:477-87.

[16] Morelli M, Frau L, Simola N. Alteration in the progression of dopamine neuron degeneration: may caffeine offer new perspective? Exp Neurol 2012;237:218-22.

[17] Schwarzschild MA. Caffeine in Parkinson disease: better for cruise control than snooze patrol?. Neurology 2012;79:616-8.

[18] Morelli M, Carta AR, Jenner P. Adenosine A2A receptors and Parkinson's disease. Handb Exp Pharmacol 2009;193:589-15.

[19] Ferré S, Popoli P, Giménez-Llort L, Rimondini R, Müller CE, Strömberg I, Ögren SO, Fuxe K. Adenosine/dopamine interaction: implications for the treatment of

Parkinson's disease. Parkinsonism Relat Disord 2001;7:235-41.

[20] Salamone JD, Collins-Praino LE, Pardo M, Podurgiel SJ, Baqi Y, Müller CE, Schwarzschild MA, Correa M. Conditional neural knockout of the adenosine A2A receptor and pharmacological A2A antagonism reduce pilocarpine-induced tremulous jaw movements: Studies with a mouse model of parkinsonian tremor. Eur Neuropsychopharmacol 23, 2013;972-7.

[21] Nunes EJ, Randall PA, Santerre JL, Given AB, Sager TN, Correa M, Salamone JD. Differential effects of selective adenosine antagonists on the effort-related impairments induced by dopamine D1 and D2 antagonism. Neuroscience 2010;170: 269-80.

[22] Connole L, Harkin A, Maginn M. Adenosine A1 receptor blockade mimics caffeine's attenuation of ethanol-induced motor incoordination. Basic Clin Pharmacol Toxicol 2004;95:299-04.

[23] Dar MS. The biphasic effects of centrally and peripherally administered caffeine on ethanol-induced motor incoordination in mice. J Pharm Pharmacol. 1988;40:482-7.

[24] Zhang Q, Yu YP, Ye YL, Zhang JT, Zhang WP, Wei WQ. Spatiotemporal properties of locomotor activity after administration of central nervous stimulants and sedatives in mice. Pharmacol biochem Behav 2011;97:577-85.

[25] Meyer L, Caston J. Repeated stress alters caffeine action on motor coordination in C57Bl6/J male mice. Brain Res 2005; 1039:171-6.

[26] Johansson B, Lindström K,Fredholm, BB. Differences in the regional and cellular localization of *c-los* mRNA induced by amphetamine, cocaine and caffeine in the rat. Neuroscience 1994;59:837-49.

[27] Bennett HJ, Semba K. Immunohistochemical localization of caffeine-induced c-Fos protein expression in the rat brain. J Comp Neurol 1998;401:89-98.

[28] Lin AS, Uhde TW, Slate SO, McCann UD. Effects of intravenous caffeine administered to healthy males during sleep. Depress Anxiety 1997;5:21-8.

[29] Patz MD, Day HE, Burow A, Campeau S. Modulation of the hypothalamo-pituitary adrenocortical axis by caffeine. Psychoneuroendocrinology. 2006;31:493-500.

[30] Bhattacharya SK, Satyan KS, Chakrabarti A. Anxiogenic action of caffeine: an experimental study in rats. Journal of Psychopharmacology 1997;11:219-24.

[31] Jain N, Kemp N, Adeyemo O, Buchanan P, Stone TW. Anxiolytic activity of adenosine receptor activation in mice. Br J Pharmacol 1995;116:2127-33.

[32] Pardo M, López-Cruz L, Valverde O, Ledent C, Baqi Y, Müller CE, Salamone JD, Correa M. Effect of subtype-selective adenosine receptor antagonists on basal or

haloperidol-regulated striatal function: studies of exploratory locomotion and c-Fos immunoreactivity in outbred and A(2A)R KO mice. Behav Brain Res 2013;247:217-26.

[33] Mally J, Stone TW. The effect of theophylline on parkinsonian symptoms. JPharm Pharmacol 1994;46:515-7.

[34] Mally J, Stone TW. Potential role of adenosine antagonist therapy in pathological tremor disorders. Pharmacol Ther 1996;72:243-50.

[35] Hirose M, Yokoyama H, Iinuma K. Theophylline impairs memory/learning in developing mice. Brain Dev 2004;26:448-52.

[36] Keppel G. Design and Analysis: a researchers handbook. Prentice-Hall: Englewood Cliffs, NJ;1991.

[37] Dunne F, O'Halloran A, Kelly JP. Development of a home cage locomotor tracking system capable of detecting the stimulant and sedative properties of drugs in rats. Prog Neuropsychopharmacol Biol Psychiatry 2007;31:1456-63.

[38] Himmel HM. Safety pharmacology assessment of central nervous system function in juvenile and adult rats: effects of pharmacological reference compounds.J Pharmacol Toxicol Methods 2008;58:129-46.

[39] Henry BL, Minassian A, van Rhenen M, Young JW, Geyer MA, Perry W; Translational Methamphetamine AIDS Research Center (TMARC) Group. Effect of methamphetamine dependence on inhibitory deficits in a novel human open-field paradigm. Psychopharmacology 2011; 215:697-707.

[40] Keck TM, Suchland KL, Jimenez CC, Grandy DK. Dopamine D4 receptor deficiency in mice alters behavioral responses to anxiogenic stimuli and the psychostimulant methylphenidate. Pharmacol Biochem Behav 2013;103:831-41.

[41] Malec D, Poleszak E. Involvement of adenosine receptors in dizocilpine-induced motor activity in mice. Pharmacol Rep 2006;58:101-6.

[42] Harri M, Lindblom J, Malinen H, Hyttinen M, Lapvetelainen T, Eskola S, Helminen HJ. Effect of access to a running wheel on behavior of C57BL/6J mice. Lab Anim Sci 1999;49:401-5.

[43] de Visser L, van den Bos R, Spruijt BM. Automated home cage observations as a tool to measure the effects of wheel running on cage floor locomotion. Behav Brain Res 2005;160:382-8.

[44] Batista LC, Prediger RD, Morato GS, Takahashi RN. Blockade of adenosine and dopamine receptors inhibits the development of rapid tolerance to ethanol in mice. Psychopharmacology 181, 2005, 714-721.

[45] Dar MS. Central adenosinergic system involvement in ethanol-induced motor incoordination in mice. J Pharmacol Exp Ther. 1990;255:1202-9.

[46] Spindel ER, Griffith I, Wurtman RJ . Neuroendocrine effects of caffeine. II Effects on tyrothropin and corticosterone secretion. J Pharmacol Exp Ther 1983;225:346-50.

[47] Kunin D, Gaskin S, Rogan F, Smith BR, Amit Z. Augmentation of corticosterone release by means of a caffeine-ethanol interaction in rats. Alcohol 2000;22:53-6.

[48] El Yacoubi M, Ledent C, Parmentier M, Costentin J, Vaugeois JM. The anxiogenic-like effect of caffeine in two experimental procedures measuring anxiety in the mouse is not shared by selective A(2A) adenosine receptor antagonists. Psychopharmacology (Berl) 2000;148:153-63.

[49] Baldwin HA, File SE. Caffeine-induced anxiogenesis: the role of adenosine, benzodiazepine and noradrenergic receptors. Pharmacol Biochem Behav 1989;32:181-6.

[50] Costall B, Jones BJ, Kelly ME, Naylor RJ, Tomkins DM. Exploration of mice in a black and white test box: validation as a model of anxiety. Pharmacol Biochem Behav 1989;32:777-85.

[51] Correa M, Manrique HM, Font L, Escrig MA, Aragon CM. Reduction in the anxiolytic effects of ethanol by centrally formed acetaldehyde: the role of catalase inhibitors and acetaldehyde-sequestering agents. Psychopharmacology 2008;200:455-64.

[52] Escrig MA, Pardo M, Aragon CM, Correa M. Anxiogenic and stress-inducing effects of peripherally administered acetaldehyde in mice: similarities with the disulfiram-ethanol reaction. Pharmacol Biochem Behav 2012;100:404-12.

[53] Vautrin S, Pelloux Y, Costentin J. Preference for caffeine appears earlier in non-anxious than in anxious mice. Neurosci Lett 2005;8:94-8.

[54] El Yacoubi M, Ledent C, Parmentier M, Costentin J, Vaugeois JM. Reducedappetite for caffeine in adenosine A(2A) receptor knockout mice. Eur J Pharmacol 2005;519:290-1.

[55] Rieg T, Schnermann J, Vallon V. Adenosine A_1 receptors determine effects of caffeine on total fluid intake but not caffeine appetite. Eur J Pharmacol. 2007;555:174-7.

[56] Fedorchak PM, Mesita J, Plater SA, Brougham K. Caffeine-reinforced conditioned flavor preferences in rats. Behav Neurosci 2002;116:334-46.

[57] Brockwell NT, Eikelboom R, Beninger RJ. Caffeine-induced place and taste conditioning: production of dose-dependent preference and aversion. Pharmacol Biochem Behav 1991;38:513-7.

[58] Patkina NA, Zvartau EE. Caffeine place conditioning in rats: comparison with cocaine and ethanol. Eur Neuropsychopharmacol 1998;8:287-91.

[59] Bedingfield JB, King DA, Holloway FA. Cocaine and caffeine: conditioned place preference, locomotor activity, and additivity. Pharmacol Biochem Behav 1998;61:291-6.

[60] Vontell R, Segovia KN, Betz AJ, Mingote S, Goldring K, Cartun RW, Salamone JD. Immunocytochemistry studies of basal ganglia adenosine A2A receptors in rat and human tissue. J Histotechnol 2010;33:41-7.

[61] Le Moine C, Svenningsson P, Fredholm BB, Bloch B. Dopamine-adenosine

interactions in the striatum and the globus pallidus: inhibition of striatopallidal neurons through either D2 or A2A receptors enhances D1 receptor-mediated effects on c-fos expression. J Neurosci 1997;17:8038-48.

[62] Salamone JD, Correa M. The mysterious motivational functions of mesolimbic dopamine. Neuron 2012;76:470-85.

[63] Voorn P, Vanderschuren LJ, Groenewegen HJ, Robbins TW, Pennartz CM. Putting a spin on the dorsal-ventral divide of the striatum. Trends Neurosci 2004;27:468-74.

[64] Svenningsson P, Ström A, Johansson B, Fredholm BB. Increased expression of c-jun, junB, AP-1, and preproenkephalin mRNA in rat striatum following a single injection of caffeine. J Neurosci 1995;15:3583-93.

[65] Dassesse D, Vanderwinden JM, Goldberg I, Vanderhaeghen JJ, Schiffmann SN. Caffeine-mediated induction of c-fos, zif-268 and arc expression through A1 receptors in the striatum: different interactions with the dopaminergic system. Eur J Neurosci 1999;11:3101-14.

[66] Singewald N, Salchner P, Sharp T. Induction of c-Fos expression in specific areas of the fear circuitry in rat forebrain by anxiogenic drugs. Biol Psychiatry 2003;53:275-83.

[67] Svenningsson P, Johansson B, Fredholm BB. Effect of different xanthines and phosphodiesterase inhibitors on c-fos expression in rat striatum. Acta Physiol Scand 1995;154:17-24.

[68] Svenningsson P, NergaÊrdh R, Fredholm BB. Regional differences in the ability of caffeine to affect haloperidol-induced striatal c-fos mRNA expression in the rat. Neuropharmacology 1998;37:331-7.

[69] Pollack AE, Fink JS. Adenosine antagonists potentiate D2 dopamine-dependent activation of Fos in the striatopallidal pathway. Neuroscience 1995;68:721-8.

[70] Karcz-Kubicha M, Quarta D, Hope BT, Antoniou K, Müller CE, Morales M, Schindler CW, Goldberg SR, Ferré S. Enabling role of adenosine A1 receptors in adenosine A2A receptor-mediated striatal expression of c-fos. Eur J Neurosci 2003;18:296-302.

[71] Greer F, Friars D, Graham TE. Comparison of caffeine and theophylline ingestion: exercise metabolism and endurance. J Appl Physiol, 2000;89:1837-44.

[72] Barone JJ, Roberts HR. Caffeine consumption. Food Chem Toxicol 1996;34:119-29.

[73] Smith A. Effects of caffeine on human behavior. Food Chem Toxicol. 2002;40:1243-55.

[74] Penolazzi B, Natale V, Leone L, Russo PM. Individual differences affecting caffeine intake. Analysis of consumption behaviours for different times of day and caffeine sources. Appetite. 2012;58:971-7.

[75] Ganio MS, Klau JF, Casa DJ, Armstrong LE, Maresh CM. Effect of caffeine on sport-specific endurance performance: a systematic review. J Strength Cond Res 2009;23:315-24.

[76] Logan L, Seale TW, Carney JM. Inherent differences in sensitivity to methylxanthines among inbred mice. Pharmacol Biochem Behav 1986;24:1281-6.

[77] Carney JM. Effect of caffeine, theophylline and theobromine on schedule controlled responding in rats. Br J Pharmacol 1984;75:451-4.

[78] Orrú M, Guitart X, Karcz-Kubicha M, Solinas M, Justinova Z, Barodia SK, Zanoveli J, Cortes A, Lluis C, Casado V, Moeller FG, Ferré S. Psychostimulant pharmacological profile of paraxanthine, the main metabolite of caffeine in humans. Neuropharmacology 2013;67:476-84.

[79] Randall PA, Nunes EJ, Janniere SL, Stopper CM, Andrew M. Farrar AM Sager TN, Baqi Y, Hockemeyer J, Müller CE, Salamone JD. Stimulant effects of adenosine antagonists on operant behavior: differential actions of selective A2A and A1 antagonist. Psychopharmacology 2011; 216:173-86.

[80] Wardas J, Konieczny J, Lorenc-Koci E. SCH 58261, an A2A adenosine receptor antagonist, counteracts parkinsonian-like muscle rigidity in rats. Synapse 2001;41:160-71.

[81] Morelli M, Pinna A. Interaction between dopamine and adenosine A2A receptors as a basis for the treatment of Parkinson's disease. Neurol Sci 2001;22:71-2.

[82] Correa M, Wisniecki A, Betz A, Dobson DR, O'Neill MF, O'Neill MJ, Salamone, JD. The adenosine A2A antagonist KF17837 reverses the locomotor suppression and tremulous jaw movements induced by haloperidol in rats: possible relevance to parkinsonism. Behav Brain Res 2004;148:47-4.

[83] Simola N, Fenu S, Baraldi PG, Tabrizi MA and Morelli M. Blockade of adenosine A2A receptors antagonizes parkinsonian tremor in the rat tacrine model by an action on specific striatal regions. Exp Neurol 2004;189:182-8.

[84] Salamone JD, Betz AJ, Ishiwari K, Felsted J, Madson L, Mirante B, Clark K, Font L, Korbey S, Sager TN, Hockemeyer J and Muller CE. Tremorolytic effects of adenosine A2A antagonists: implications for parkinsonism. Front Biosci 2008;13:3594-605.

CHAPTER 3:

Effect of caffeine and ethanol coadministration on social interaction and recognition in mice: role of adenosine receptors.

Abstract

Rationale. Caffeine and ethanol are frequently consumed in combination. Ethanol increases adenosine levels, but caffeine is a non-selective adenosine A_1/A_{2A} receptor antagonist. These receptors are highly expressed in striatum and olfactory tubercle, brain areas involved in exploration and social interaction in rodents. Ethanol modulates social interaction processes, but the role of adenosine in social behavior is still poorly understood.

Objectives. These studies were undertaken to study the impact of caffeine and ethanol and their combination on social behavior, and explore the involvement of A_1 and A_{2A} receptors on those actions.

Methods. Male CD1 mice were evaluated in a social interaction three-chamber paradigm, for preference of conspecific vs. object and for long-term recognition memory of familiar vs. novel conspecific.

Results. Caffeine (7.5-60.0mg/kg) decreased social preference in a dose dependent manner. Ethanol (0.25-1.5g/kg) showed a biphasic effect, blocking social preference at high doses. However, ethanol reversed some impairments induced by caffeine (15.0-30.0 mg/kg). CPT (A_1 antagonist) did not modify social preference on its own, or in combination with ethanol. MSX- (A_{2A} antagonist) increased social preference, but did not block totally ethanol-impairing effects on preference. Both, ethanol and caffeine, or their combination, produced amnesic effects. MSX-3 blocked the amnesic effects of low doses of ethanol but CPT did not.

Conclusions. Caffeine can reduce social interaction and preference possibly via A_1 receptor antagonism but not A_{2A}, since MSX3 potentiated rather than reduce social preference. Low doses of ethanol can counteract caffeine reduction in preference. Ethanol has a potent effect impairing recognition. However, adenosine antagonists do not seem to have a strong effect on the prevention of memory loss.

1. Introduction

Caffeine and alcohol are the most consumed psychoactive drugs worldwide. In recent times, it has become common to consume high doses of caffeine in combination with ethanol in order to reduce the intoxicating effects of the alcohol (Ferré and O'Brien 2011; López-Cruz et al. 2013; Correa et al. 2014). Caffeine and ethanol act on the adenosine system in distinct ways that can result in opposite physiological and behavioral effects. Caffeine is a non-selective adenosine antagonist that acts mainly on A_1 and A_{2A} receptors (Fredholm 1999), whereas ethanol has been demonstrated to increase the adenosinergic tone by inhibiting the endonucleotid transporter type-1, thus, blocking adenosine uptake (Nagy et al. 1990; Krauss et al. 1993), and also by increasing the synthesis of adenosine during ethanol metabolism (Carmichael et al. 1993).

Adenosine is a neuromodulator in the central nervous system (CNS) that plays an important role in the regulation of synaptic transmission and neuronal excitability (Sebastiao et al. 2009). Several subtypes of adenosine receptors are expressed in the brain, with A_1 and A_{2A} being the most abundant. A_{2A} receptors are expressed at high levels, and almost exclusively, in the striatum and olfactory bulbs and tubercle (Fredholm et al. 2001; Schiffmann et al. 1991), regions that are involved in the regulation of motivated (Hauber and Sommer, 2009; Salamone and Correa 2002; 2012), and social behaviors (Sano et al. 2008). However, A_1 receptors have a widespread distribution in the brain, with a somewhat higher concentration in hipoccampus (Schwarzschild et al. 2006; Fuxe et al. 2003).

Caffeine was shown to decrease social interaction at high doses in mice (60 mg/kg) (Hilakivi et al. 1989) and rats (20 and 40 mg/kg) (Baldwin and File, 1989; Baldwin et al. 1989), effects that have been suggested to be related to its anxiogenic actions (Baldwin et al. 1989; Hilakivi et al. 1989). However, other studies have shown that caffeine increases the number of social contacts after similar doses (20 mg/kg) in rats (Nadal et al. 1993). Ethanol consumption was shown to facilitate interactions with peers and to alleviate anxiety (Kirchner et al. 2006; Varlinskaya and Spear, 2002). In rodent models of social interaction, acute ethanol administration at low doses produces social facilitation (Nadal et al. 1993; Varlinskaya and Spear, 2009; Procópio-Souza et al. 2011), but dose-related decrements in social interaction after high doses also have been observed in mice (Hilakivi et al. 1989; Lister and Hilakivi, 1988). However, there are almost no reports of interactions between these two drugs in this important aspect of

motivated behavior (Hilakivi et al. 1989). In the only study so far, a high dose of caffeine (30 mg/kg) that did not modify the time spent engaged in social interaction by itself was able to reverse the impairment induced by a high dose of ethanol (2 g/kg) (Hilakivi et al. 1989). The same lack of information applies to the impact of caffeine-ethanol interactions in another important aspect of social behavior, long-term social recognition memory. In the only interaction study on this type of memory, a low dose of caffeine (5 mg/kg), blocked the retrograde memory impairments induced by a high dose of ethanol (3 g/kg) in a social odor recognition test in rats (Spinetta et al. 2008). The amnesic effect of ethanol is well known. Although ethanol at low doses was shown to act as a short-term social memory enhancer in mice (Manrique et al. 2005), high doses of ethanol can cause amnesia, or impaired retrieval of memory, after the drug wears off (Goodwin 1995; Hartzler and Fromme, 2003). This effect of ethanol could be explained by the fact that adenosine and adenosine receptor agonists impair short-term social recognition memory in rats (Prediger and Takahashi, 2005). On the other hand, selective A_1 and A_{2A} receptor antagonists can improve short-term social memory (Prediger and Takahashi, 2005).

The present work evaluated the effect of a broad range of doses of caffeine, in combination with ethanol, on social motivation as measured by preference towards a conspecific versus a neutral object. Our procedure minimized anxiety induced by aggression, avoiding whole-body contact. We also evaluated the impact of high doses of caffeine on plasma corticosterone levels (a measure of physiological stress) and assessed if a low dose of ethanol that improved caffeine-induced reductions in preference was able to modulate those hormonal levels. In a second phase of the behavioral test, long-term social recognition memory was studied 24 hours after the preference test took place. In addition, the role of A_1 and A_{2A} receptors on social motivation and memory were also evaluated using selective adenosine antagonists alone or in combination with ethanol.

2. Methods

2.1. Subjects

Adult male CD1 mice (30-45 g) were purchased from Janvier (France). Mice were housed in groups of three per cage, with standard laboratory rodent chow and tap water available *ad libitum*. They were maintained in the colony at 22 ± 1ºC with lights on from 8:00 to 20:00 hours. All experimental procedures complied with the European Community Council directive (86/609/ECC) for the use of laboratory animal subjects and with the "Guidelines for the Care and Use of Mammals in Neuroscience and Behavioral Research" (National Research Council 2003).

2.2. Drugs

Caffeine (Sigma-Aldrich, Spain) and MSX3 ((*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; synthesized at the laboratory of Dr. Christa E. Müller at the Pharmazeutisches Institut, Universität Bonn, Germany) were dissolved in 0.9% w/v saline. CPT (8-cyclopentyltheophylline; purchased from Sigma-Aldrich, Spain) was dissolved in distilled water (pH=8). All these drugs were administered IP intraperitoneally (IP) 30 minutes before testing. Ethanol (Panreac Quimica S.A., Spain) was diluted to 20% (v/v) in physiological saline (0.9 % w/v) and administered IP 10 minutes before testing. Saline solution was used as vehicle. These doses of ethanol were selected based on previous studies done in our laboratory with the same strain of mice (Correa et al. 2008).

2.3. Behavioral apparatus and testing procedures

Social preference and social recognition tests. The effects of adenosine antagonists on social preference were measured in a three-chambered social box (originally developed by Crawley 2004). The general procedure was adapted from Chévere-Torres and colleagues (2012). Every mouse had two consecutive habituation sessions in the chambers: in the first one, they freely explored the empty social arena during 15 minutes, and immediately there was a second exploration session, that lasted 30 minutes, in the presence of two wire cages, one in each of the side-compartments. After the 45 minute habituation period, different groups of animals received their corresponding treatment and were placed in an individual cage during 10 or 30 minutes (depending of drug). After this time, mice were placed in the center chamber of the social interaction

apparatus and test started. During the test session (10 minutes), the three-chambered arena contained a caged with a conspecific in one side, and in the other side there was a small wire cage with an object. The center compartment was empty (see **Figure 1** for a schematic on the procedure). The placement of the conspecific or the object was counterbalanced between animals. A trained experimenter who was unaware of the experimental conditions, registered manually time spent sniffing each target (conspecific versus object) as a measure of social preference. Vertical and horizontal locomotion were also registered. Twenty-four hours after the social preference test, mice were placed back in the central chamber and were subjected to a 10 minutes social recognition test (Moy et al. 2004). No drugs were administered before this second test. During the recognition test a novel mouse replaced the object, and the experimental mice were given the choice to interact with the familiar conspecific (same conspecific used in the social preference test the day before) versus a novel conspecific. Time sniffing each conspecific was registered.

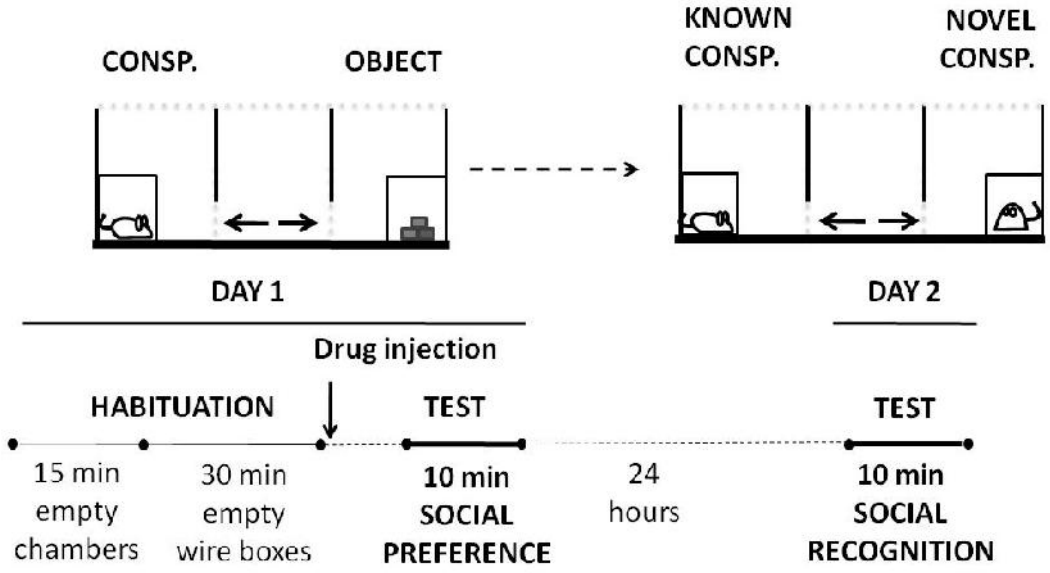

Figure 1. Schematic representation of social preference and social recognition tests settings and timeline.

Plasma corticosterone determination. Mice received caffeine (0.0, 15.0 or 30.0 mg/kg) plus vehicle or the dose of ethanol that in experiment 3 had been more effective at reversing the suppressant effects of caffeine on social preference (0.5 g/kg). Animals received injections 80 and 60 min respectively before being sacrificed by decapitation

under deep anesthesia. Animals were anesthetized with a 1.0 ml/kg IP injection of a cocktail solution containing 10.0 ml of 100 mg/ml ketamine plus 0.75 ml of 20.0 mg/ml xylazine (both from Phoenix Scientific, Inc., St. Joseph, MO, USA), 30 minutes before decapitation. Blood samples were collected in heparinized (15 units/ml of blood) Eppendorf tubes and centrifuged at 4000 rpm for 10 min. Supernatant was taken and stored at -20°C until corticosterone determination. Plasma corticosterone levels were measured spectrophotometrically using a commercially available enzymatic immunoassay kit (Rodents Corticosterone Enzyme Immunoassay System, OCTEIA Corticosterone; Immunodiagnostic Systems LTD, Boldon, England). Blood corticosterone concentration (ng/ml) was determined using a logarithmic adjustment of the standard curve.

2.4. Statistics

One-way ANOVA was used to analyze the effect of drug administration on the different dependent variables; time sniffing conspecific, object, familiar and novel conspecific, and vertical and horizontal locomotion. Two-way factorial ANOVA was used for the interaction studies. When the overall ANOVA was significant, non-orthogonal planned comparisons using the overall error term were used to compare each treatment with the control group (Keppel, 1991). For these comparisons, α level was kept at 0.05 because the number of comparisons was restricted to the number of treatments minus one. Student's t-test for dependent samples was used to analyse “preference” (e.g. conspecific vs. object, or familiar vs. novel conspecifics). A probability level of 0.05 or smaller was used to indicate statistical significance. Statistics were done using STATISTICA 7 software.

3. Results

Experiment 1: Effect of the non-selective adenosine A_1/A_{2A} antagonist caffeine on social preference and locomotion: impact on long-term social recognition memory.

Mice (N=44) were injected with saline or caffeine (7.5, 15.0, 30.0 or 60.0 mg/kg) 30 minutes before the social interaction test started. The following day (24 hours later) no drugs were administered and social recognition was evaluated as described before.

The one-way ANOVA revealed an overall effect of caffeine on time sniffing the conspecific ($F(4,39)=21.12$, $p<0.01$). Planned comparison analysis showed a significant

decrement on time spent sniffing the conspecific after caffeine administration at doses of 15.0, 30.0 and 60.0 mg/kg ($p<0.01$). The one-way ANOVA for the effect of caffeine on time sniffing the object ($F(3.39)=4.03$, $p<0.01$) was also significant, and the planned comparisons revealed that the same doses of caffeine (15.0, 30.0 and 60.0 mg/kg) decreased time sniffing the object compared to vehicle ($p<0.05$. $p<0.01$ and $p<0.01$, respectively). The Student's t-test for dependent samples was used to compare time spent sniffing the conspecific with time spent sniffing the object. The vehicle treated group spent more time exploring the conspecific than the object ($t=5.24$, $p<0.01$), and this pattern of behavior was also preserved after the administration of moderate doses of caffeine (7.5 and 15.0 mg/kg; $t=6.28$, $p<0.01$, $t=3.84$, $p<0.01$ respectively) but not after the highest doses of caffeine (30.0 and 60.0 mg/kg; $t=0.04$, $p=0.97$ and $t=2.15$, $p=0.06$ respectively) (**Fig 2. A**)

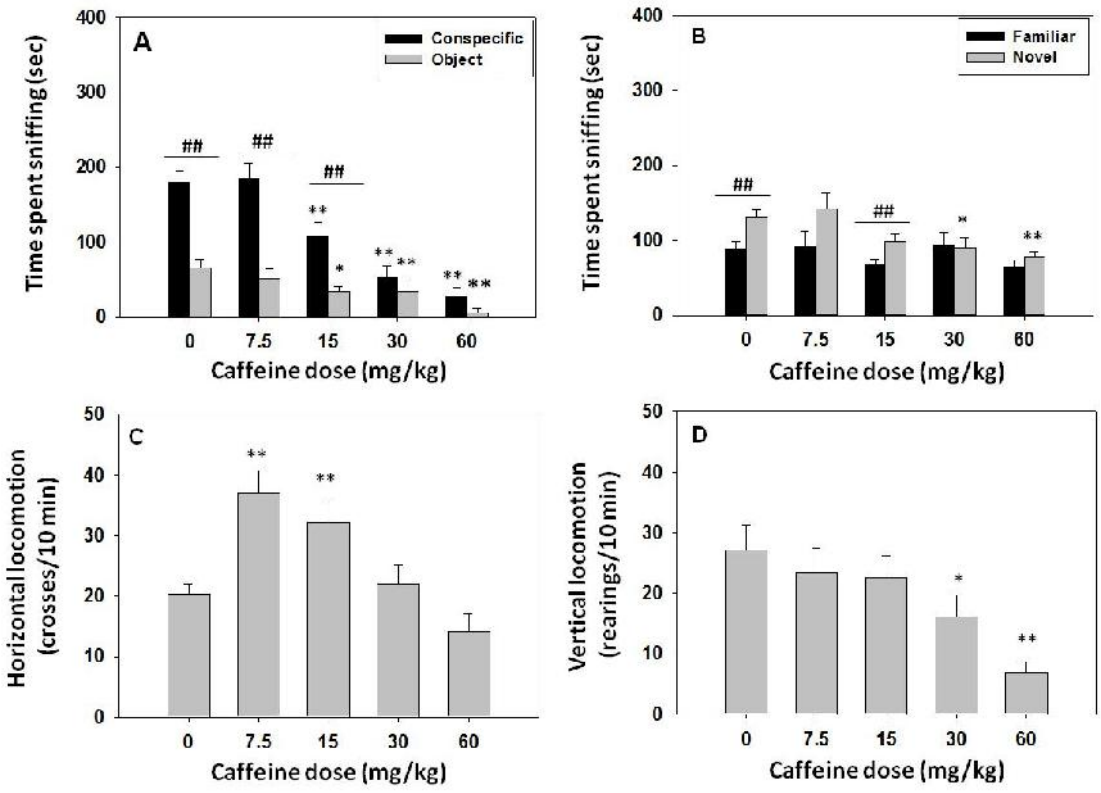

Figure 2. Effect of caffeine on social preference and recognition tests. Data are expressed as mean (±SEM) of time sniffing **A**) conspecific and object in the social preference test, **B**) familiar and novel cosnpecifics in the social recognition test, and **C**) horizontal and **D**) vertical locomotion during the social preference test. **p<0.01, *p<0.05 significant differences from vehicle for the same target. ##p<0.01 significant differences between time sniffing both targerts for the same dose of caffeine.

The one-way ANOVA revealed an overall effect of caffeine on horizontal locomotion ($F(4,39)=7.90$ $p<0.01$). Caffeine significantly increased horizontal locomotion at low to intermediate doses (7.5 and 15.0 mg/kg; $p<0.01$) compared to vehicle, but did not have a significant effect at higher doses. The one-way ANOVA for vertical locomotion ($F(4,39)=4.60$ $p<0.01$) was also significant, but for this dependent variable, planned comparisons revealed that the higher doses (30.0 and 60.0 mg/kg), significantly decreased vertical locomotion in comparison with the vehicle treated group ($p<0.05$ and $p<0.01$, respectively) (**Fig 2. C and D**). This increase in locomotion could be influencing the reduction in time dedicated to targeted exploration, more importantly, to conspecific exploration.

For the social recognition results, the one-way ANOVA revealed no significant effect of the previous treatment with caffeine on time spent sniffing the familiar conspecific ($F(4,39)=1.37$, n.s.). However, there was an overall effect of previous caffeine treatment on time sniffing the novel conspecific ($F(4,39)=3.83$, $p<0.01$). Planned comparisons revealed that the highest doses of caffeine (30.0 and 60.0 mg/kg) significantly decreased time spent sniffing the novel conspecific compared with vehicle ($p<0.05$ and $p<0.01$, respectively) **(Fig 2.B).** Student's t-test for dependent samples showed that the vehicle group spent more time sniffing the novel conspecific than sniffing the familiar one ($t=-3.40$, $p<0.01$) and this was also observed in the group that received 15.0 mg/kg of caffeine ($t=-3.31$, $p<0.01$), but not 7.5 mg/kg, 30.0 or 60.0 mg/kg ($t=1.58$, $p=0.17$; $t=0.16$, $p=0.87$; $t=-1.14$, $p=0.29$, respectively) **(Fig 2.B).**

Experiment 2: Effect of ethanol on social preference and locomotion: impact on long-term social recognition memory.

In this experiment mice (N=45) received saline or ethanol (0.25, 0.5, 1.0 or 1.5 g/kg) 10 minutes before been evaluated in the social preference test. The following day, the same animals were tested for social recognition memory.

Ethanol treatment, as shown by the one-way ANOVA, had a significant effect on time sniffing the conspecific ($F(4,40)=20.12$, $p<0.01$), and planned comparisons revealed that ethanol at the lowest dose (0.25 g/kg) increased direct conspecific exploration ($p<0.01$) in comparison with vehicle treatment, while higher doses decreased time with conspecific (1.0 and 1.5 g/kg, $p<0.05$ and $p<0.01$ respectively). The one-way ANOVA for time sniffing the object ($F(4,40)=4.45$, $p<0.01$) was also significant. However, only

the highest dose of ethanol (1.5 g/kg) significantly reduced ($p<0.01$) time spent sniffing the object compared to the vehicle treated group (**Fig 3.A**). When comparing time exploring both stimuli in the same animals, Student t-test for dependent samples showed that in the vehicle group there was a significant difference in time spent sniffing the conspecific versus the object ($t=-8.28$, $p<0.01$), a pattern that was repeated at all doses of ethanol (0.25 g/kg, $t=-5.49$, $p<0.01$; 0.5 g/kg, $t=-5.75$, $p<0.01$; 1.0 g/kg, $t=2.61$, $p<0.05$; 1.5 g/kg $t=-2.76$, $p<0.01$) (**Fig 3.A**). Thus, independently of the ethanol dose used, all groups explored more the conspecific than the object.

There were no significant effect of ethanol treatment on total crosses ($F(4,40)=0.59$, n.s.) (**Fig 3.C**) and on vertical locomotion ($F(4,40)=2.25$, n.s.) (**Fig 3.D**).

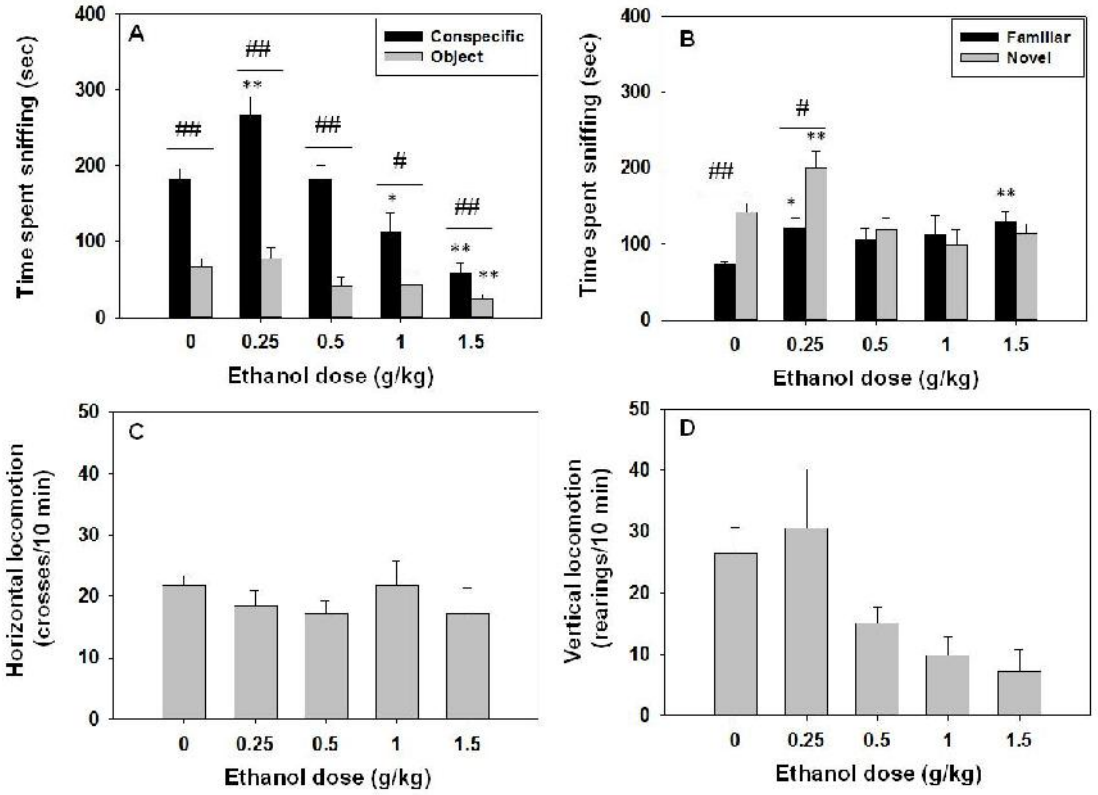

Figure 3. Effect of ethanol in social preference and recognition tests. Data are expressed as mean (±SEM) of time sniffing **A**) conspecific and object in the social preference test, **B**) familiar and novel cosnpecifics in the social recognition test, and **C**) horizontal and **D**) vertical locomotion during the social preference test. **p<0.01, *p<0.05 significant differences from vehicle for the same target. ##p<0.01 #p<0.05 significant differences between time sniffing both targerts for the same dose of ethanol.

One day after the social interaction test took place, social recognition was evaluated, and the results of the one-way ANOVA showed an overall effect of previous exposure

to ethanol on time sniffing the familiar conspecific ($F(4.40)=2.08$, $p<0.05$). Ethanol at doses of 0.25 and 1.5 g/kg increased time sniffing the familiar conspecific ($p<0.05$ and $p<0.01$ respectively) compared to the group previously treated with vehicle. A significant effect of ethanol administered the previous day was also observed on time sniffing the novel conspecific ($F(4,40)=5.78$, $p<0.01$). Only the lowest dose of ethanol (0.25 g/kg) increased time sniffing the novel conspecific in comparison with the vehicle group ($p<0.01$) **(Fig 3.B).** Student's t-test for dependent samples showed significant differences in the vehicle group between time spent sniffing familiar versus novel conspecific. These animals spent more time sniffing the novel than familiar conspecific ($t=5.32$, $p<0.01$), a pattern that was only observed on the group that had received the lower dose of ethanol (0.25 g/kg, $t=2.46$, $p<0.05$), suggesting that ethanol, even at doses that had no effect on social exploration the day before (0.5 g/kg), can impair social recognition 24 hours after been administered.

Experiment 3: Effect of caffeine-ethanol coadministration on social preference and locomotion: impact on long-term social recognition memory.

For experiment 3 mice (N=74) received and injection of saline or caffeine (15.0 or 30.0 mg/kg; 30 minutes before being tested) plus vehicle or a dose of ethanol (0.5 or 1.0 g/kg; 10 minutes before test), and were evaluated for social preference and locomotion. The following day, the same animals were tested in the social recognition test.

Factorial ANOVA (caffeine x ethanol) on time sniffing the conspecific showed overall effects of caffeine ($F(2,65)=13.33$, $p<0.01$), and ethanol ($F(2,65)=9.97$, $p<0.01$) and also a significant interaction ($F(4,65)=8.99$, $p<0.05$). Planned comparisons confirmed that when compared with the vehicle-vehicle group only the highest dose of ethanol used in the present study (1.0 g/kg) reduced conspecific exploration ($p<0.05$), and that the two doses of caffeine (15.0 and 30.0 mg/kg) selected for this experiment also reduced social exploration ($p<0.01$). In terms of the interactions, the group that received the lowest dose of caffeine (15.0 mg/kg) in combination with the lowest dose of ethanol (0.5 g/kg) was significantly different ($p<0.01$) from the group that had received that dose of caffeine but no ethanol, pointing to a reversal effect of ethanol on the caffeine-induced impairment. However, the effect of this dose of caffeine was not reversed when given in combination with the highest dose of ethanol (1.0 g/kg). As for the impairing effect on conspecific exploration observed in the group that had received the highest

dose of caffeine (30.0 mg/kg) plus vehicle, this effect was partially reversed by the two doses of ethanol ($p<0.05$ and $p<0.01$ respectively). (**Fig 4.A**).

The factorial ANOVA (Caffeine x Ethanol) for the dependent variable time sniffing the conspecific did not show a significant effect of caffeine ($F(2,65)=1.31$, n.s.), of ethanol ($F(2,65)=1.69$, n.s.) or the interaction ($F(4,65)=0.71$, n.s.), (**Fig 4.B**).

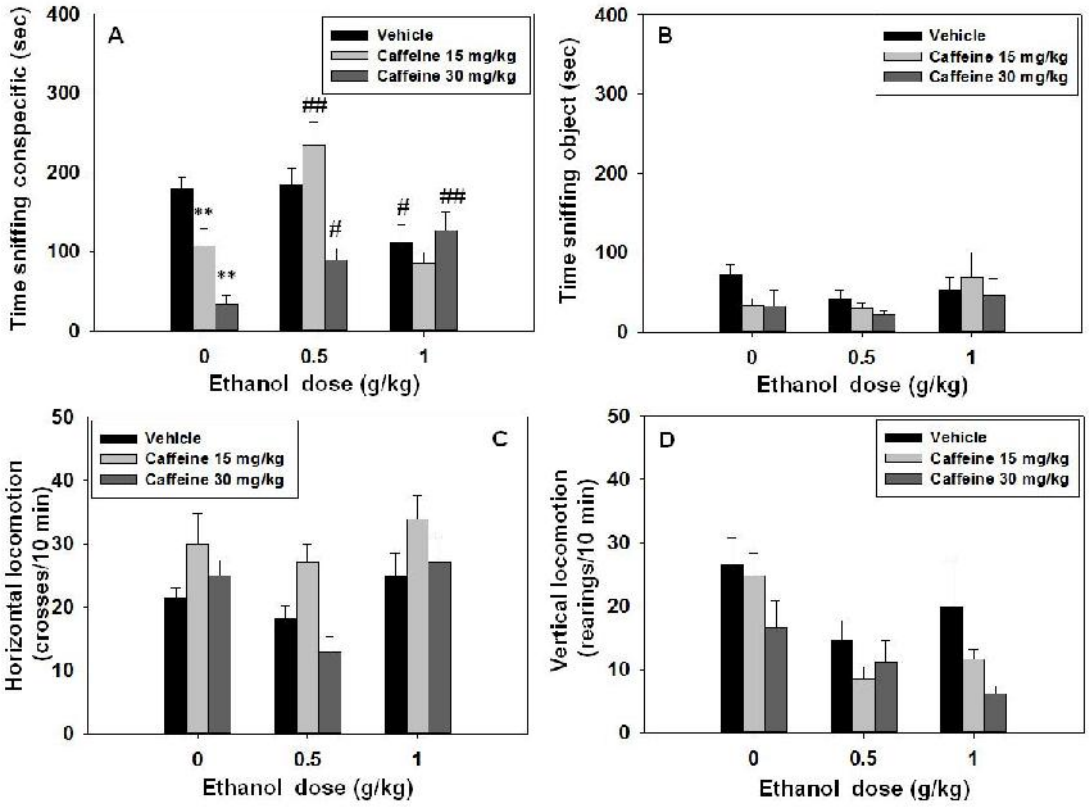

Figure 4. Effect of caffeine plus ethanol interaction in the social preference test. Data are expressed as mean (±SEM) of time sniffing **A**) conspecific, **B**) object, **C**) horizontal and **D**) vertical locomotion during the social preference test. **p<0.01, *p<0.05 significantly different from the vehicle group in the same dose of ethanol. ##p<0.01 #p<0.05 significantly different from the group that received the same dose of caffeine plus ethanol 0.0 g/kg.

Factorial ANOVA (Caffeine x Ethanol) for total crosses as a measure of horizontal locomotion revealed an overall effect of caffeine ($F(2,65)=7.22$, $p<0.01$), and ethanol ($F(2,65)=6.27$, $p<0.01$), but no significant interaction ($F(4,65)=0.77$, n.s.), (**Fig. 4.C)**. A separate factorial ANOVA for vertical locomotion showed the same pattern of results. It revealed an effect of caffeine ($F(2,65)=4.23$, $p<0.05$) and of ethanol ($F(2,65)=7.74$, $p<0.01$), but no significant caffeine-ethanol interaction ($F(4,65)=0.81$, n.s.), (**Fig. 4.D**).

The results for the impact of these pharmacological manipulations on social recognition memory evaluated the day after the drug injection, and the preference test, are shown in **Table 1.** The factorial ANOVA (Caffeine x Ethanol) showed an overall effect of caffeine ($F(2,65)=3.72$, $p<0.05$), and of ethanol ($F(2,65)=8.27$, $p<0.01$) on time sniffing the familiar conspecific. However, there was no significant caffeine x ethanol interaction ($F(4,65)=1.49$, n.s.). The factorial ANOVA for variable time sniffing a novel conspecific revealed a significant effect of caffeine ($F(2,65)=3.43$, $p<0.05$), but no significant effect of ethanol ($F(2,65)=2.37$, n.s.), and a significant interaction effect ($F(2,65)=0.91$, $p<0.01$). The Student's t-test for dependent samples comparing time spent sniffing familiar conspecific versus novel conspecific revealed that the group that had received vehicle-vehicle injections the day before spent significantly more time sniffing the novel conspecific than the familiar conspecific ($t=4.96$, $p<0.01$), and the same was true for the animals treated with caffeine (15.0 mg/kg) plus saline ($t=2.85$, $p<0.05$). However, caffeine 30 mg/kg plus saline impaired social recognition the day after, since there was no difference between the time spent exploring the two conspecifics ($t=0.15$, n.s.) as expected from the results in experiment 1. All doses of ethanol employed (0.5 and 1.0 g/kg) impaired social recognition memory as it had been observed in experiment 2 ($t=0.47$, n.s., $t=-0.43$, n.s., respectively). Moreover, combining caffeine (15.0 or 30.0 mg/kg) with ethanol (0.5 or 1.0 g/kg) did not improve the impairing effect produced by ethanol (caffeine 15 mg/kg plus ethanol 0.5 g/kg; $t=-0.82$, n.s.; caffeine 15 mg/kg plus ethanol 1.0 g/kg, $t=1.49$, n.s.; caffeine 30.0 mg/kg plus ethanol 0.5 g/kg, $t=1.69$, n.s.; caffeine 30.0 mg/kg plus ethanol 1.0 g/kg $t=0.66$, n.s.).

Time sniffing (sec)						
Etoh (g/kg)	**0.0**		**0.5**		**1**	
Caff (mg/kg)	**Familiar**	**Novel**	**Familiar**	**Novel**	**Familiar**	**Novel**
0.0	87.5±9.1	136.4±12.1##	111.4±14.5	124.1±16.6	115.9±21.7	106±19.1
15.0	71.2±7.1	100.1±13.2#	120.6±27.3	98.1±12.5	72.1±10.5	102.3±21.7
30.0	33.0±11.1	31.9±21.1	103.6±11.8	137.2±21.9	83.3±11.2	91.1±12.7

Table 1. Effect of caffeine-ethanol coadministration on social recognition memory. Data are expressed as mean ± SEM of time in seconds sniffing the novel and the familiar conspecifics. ##p<0.01, #p<0.05 significant differences between time in familiar vs time in novel conspecific for the same treatment group.

Experiment 4: Effects of caffeine-ethanol co-administration on plasma corticosterone levels.

Independent groups of mice (N=35) received one injection of saline or caffeine (15.0 or 30.0 mg/kg) plus a second injection of saline or ethanol (0.5 g/kg). Blood samples were extracted 80 minutes after caffeine administration (60 minutes after ethanol administration). Data are shown in **Table 2.** A two-way ANOVA (caffeine x ethanol) showed a significant effect of caffeine treatment on plasma corticosterone levels ($F(2,24)=21.59$, $p<0.01$). However, ethanol did not produce a significant effect ($F(1,24)=0.16$, n.s), and there was not a significant interaction ($F(2,24)=1.14$, n.s). Thus, ethanol did not modify the increase in corticosterone produced by caffeine, suggesting that the effects seen in experiment 3 were not the result of a reduction on stress levels.

	Plasma corticosterone levels (ng/ml)	
Caffeine / Etoh (g/kg) (mg/kg)	**0.0**	**0.5**
0.0	38.3 ± 11.6	36.8 ±17.8
15.0	76.4 ± 12.0	90.4 ± 9.0
30.0	128.3 ± 16.7	129.6 ± 14.5

Table 2. Effects of caffeine (0.0, 15.0 or 30.0 mg/kg) plus ethanol (0.0 or 0.5 g/kg) on corticosterone levels. Data are expressed as mean (±SEM) plasma corticosterone levels (ng/ml).

Experiment 5: Effect of the selective adenosine A_1 receptor antagonist CPT on social preference and locomotion. Impact on long-term social recognition memory.

Mice (N=37) were injected with vehicle or CPT at doses of 3.0, 6.0, or 9.0 mg/kg 30 minutes before being tested in the social preference task. The following day (24 hours later) the same animals were tested in the social recognition test.

The effect of CPT on time sniffing the conspecific was analyzed by a one-way ANOVA, but revealed no significant effect ($F(3,33)=2.13$, n.s.). However, the one-way

ANOVA on the effect of CPT on time sniffing the nonsocial target was significant ($F(3,33)=5.21$, $p<0.01$). Planned comparison revealed that CPT significantly decreased time spent exploring the object at all doses of CPT in comparison with the vehicle group ($p<0.01$) (**Fig. 5.A**). Student's t-test for dependent samples showed significant differences in time sniffing the conspecific vs. the object in all the groups. Animals spent more time sniffing the conspecific after saline ($t=5.37$, $p<0.05$), CPT 3.0 mg/kg ($t=11.25$, $p<0.01$), CPT 6.0 mg/kg ($t=6.38$, $p<0.01$), and CPT 9.0 mg/kg ($t=5.95$, $p<0.01$).

These doses of CPT did not affect horizontal ($F(3,33)=1.03$, n.s.) or vertical locomotion ($F(3,33)=1.42$, n.s.) as analyzed by one-way ANOVA's (**Fig. 5.C and 5.D**).

For the social recognition test, the one-way ANOVA's did not show a significant effect of CPT dose on time sniffing the familiar conspecific ($F(3,33)=0.14$, n.s.) or on time sniffing the novel conspecific ($F(3,33)=0.02$, n.s.). Student's t-test for dependent samples showed significant differences between time spent sniffing the novel versus the familiar conspecific in the vehicle group ($t=-3.82$, $p<0.01$), as expected when animals recognized the previously explored conspecific, and this effect was also observed in the animals that had received the highest dose of CPT 9.0 mg/kg the day before ($t=-3.25$, $p<0.05$), but not the lower doses (CPT 3.0 mg/kg, $t=-0.96$, n.s.; and CPT 6.0 mg/kg $t=-0.79$, n.s.) (**Fig 5.B**).

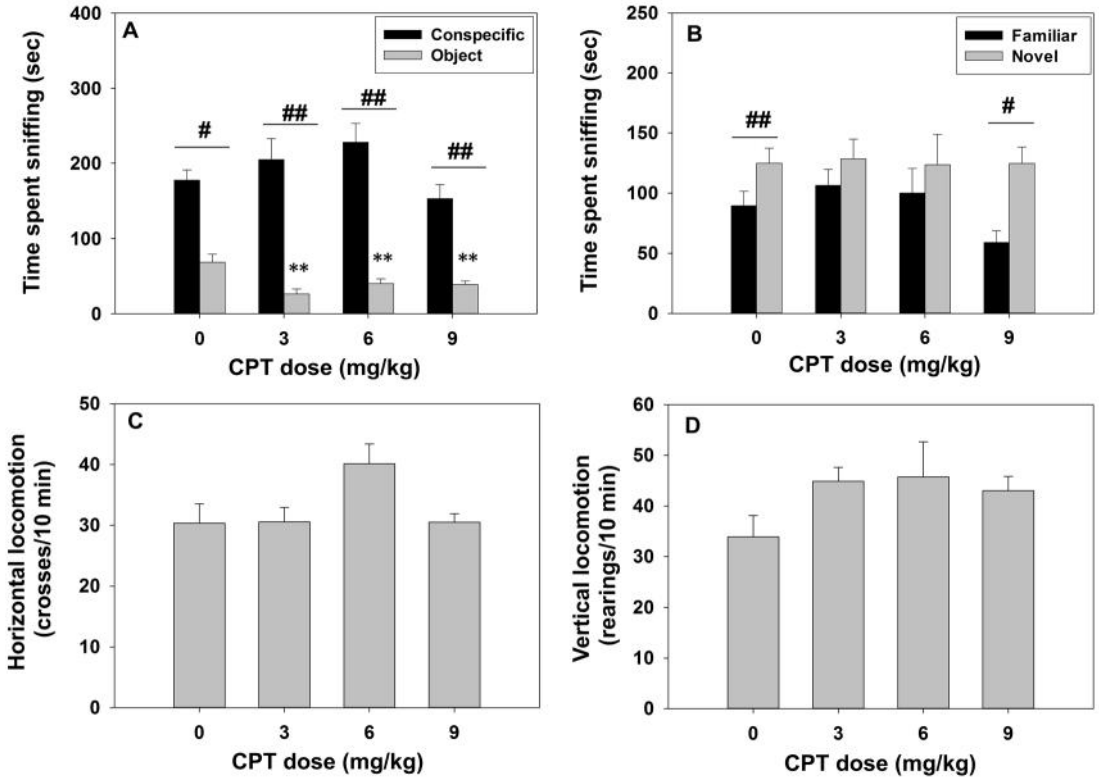

Figure 5. Effect of CPT in the social preference and recognition tests. **A**) conspecific and object in the social preference test, **B**) familiar and novel cosnpecifics in the social recognition test, and **C**) horizontal and **D**) vertical locomotion during the social preference test. **p<0.01 significant differences from vehicle for the same target. ##p<0.01 #p<0.05 significant differences between time sniffing both targerts for the same dose of CPT.

Experiment 6: Effect of CPT–ethanol co-administration on social preference and locomotion: impact on long-term social recognition memory.

Mice (N=60) received and injection of vehicle or CPT 6.0 mg/kg 20 minutes before the test and a second injection of vehicle or ethanol (0.5 or 1.0 g/kg) 10 minutes before the social preference test started. The following day, the same animals were tested in the social recognition test with no drug been administered.

A factorial ANOVA (CPT x Ethanol) showed an overall effect of ethanol ($F(2,41)=5.33$, n.s.), but no significant effect of CPT ($F(1,41)=0.32$, n.s) or CPT-ethanol interaction ($F(2,41)=1.60$, n.s.) on time sniffing the conspecific. (). The factorial ANOVA for time sniffing the object did not reveal a significant effect of CPT ($F(1,41)=0.43$, n.s.), of ethanol ($F(2,41)=1.46$, ns), or of the interaction ($F(2,41)=2.21$, n.s.) either (**Fig 6.A**).

For the social recognition test the factorial ANOVA (CPT x Ethanol) did not show a significant effect of CPT ($F(1,41)=1.06$, n.s.), of ethanol ($F(2,41)=0.97$, n.s.), or of the

interaction (F(2,41)=0.05, n.s.) on time sniffing the familiar conspecific (**Fig 6.B**) The factorial ANOVA for the variable time sniffing the novel conspecific, did not show an overall effect of CPT (F(1,41)=0.38, n.s), ethanol (F(2,41)=1.78, n.s.) or CPT-ethanol interaction (F(2,41)=1.11, n.s.)

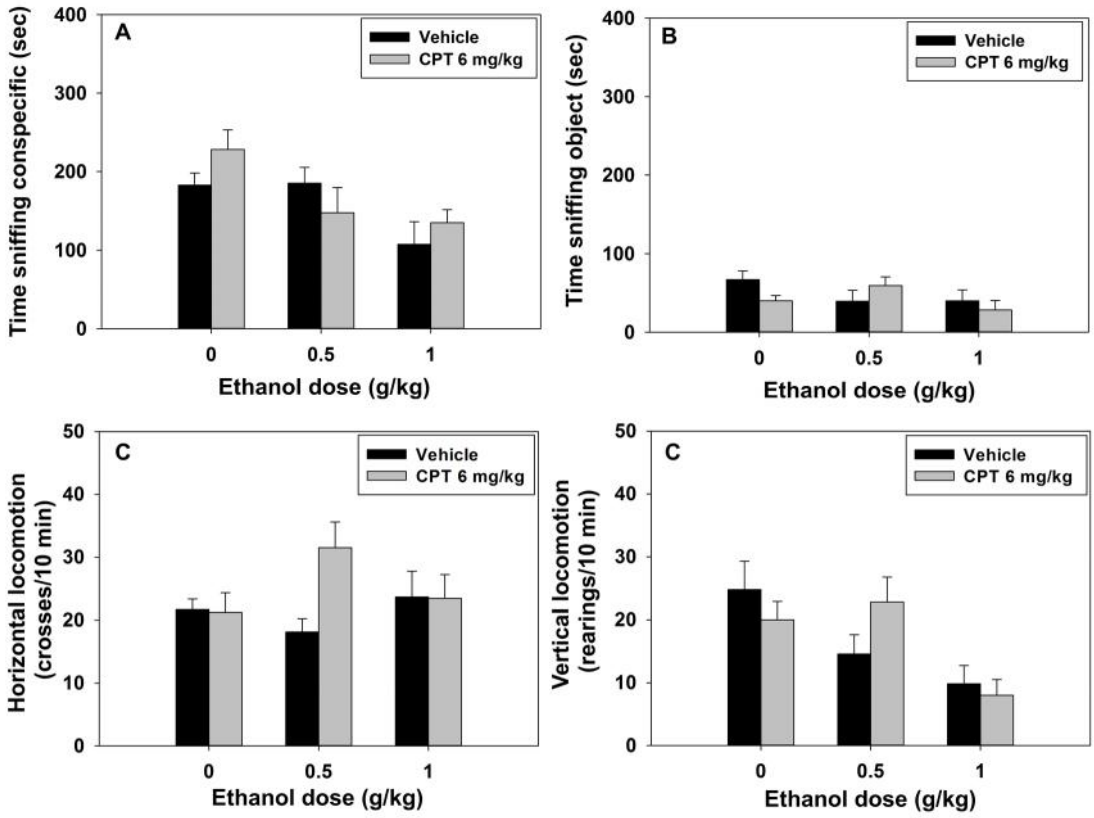

Figure 6. Effect of CPT plus ethanol interaction on the social preference test. Data are expressed as mean (±SEM) of time sniffing **A**) conspecific, **B**) object, **C**) horizontal and **D**) vertical locomotion during the social preference test.

Student's t test for dependent samples showed significant differences between time sniffing the novel vs. familiar conspecific in control group (t=4.7, p<0.01). Ethanol as well as happened in the experiment 2 impaired social recognition at all doses employed (0.5 g/kg, t=0.47, n.s. and 1 g/kg, t=0.14, n.s). CPT (6 mg/kg) as occurred in experiment 6 also impaired social memory since t-student test for dependent samples did not showed differences between time sniffing novel vs. familiar conspecific in this treatment group (t=0.79, n.s). None of the doses of ethanol co-administered with CPT (6

mg/kg) reverted such effect (0.5 g/kg; t=0.99, p=0.35 and 1.0 g/kg; t=0.14, p=0.89) (**Table 3**).

Time sniffing (sec)						
Etoh (g/kg)	**0.0**		**0.5**		**1.0**	
CPT (mg/kg)	**Familiar**	**Novel**	**Familiar**	**Novel**	**Familiar**	**Novel**
0.0	74.1±4.5	139.4±12..4##	122.6±12.4	124.1±16.6	99.0±22.0	102.5±12.4
6.0	100.1±20.5	123.5±25.3	111.0±14.6	172.3±27.8	119.6±23.3	105.0±27.8

Table 3. Effects of CPT-ethanol combination on social recognition memory. Data are expressed as mean ±SEM of time in seconds sniffing novel and familiar conspecifics. ##p<0.01 significant differences between time in familiar vs time in novel conspecific for the same dose of CPT and ethanol.

Experiment 7: Effect of the selective adenosine A_{2A} receptor antagonist MSX-3 on social preference and locomotion. Impact on long-term social recognition memory.

Different groups of mice (N=36) received an acute administration of vehicle or MSX-3 at dose of 1.5, 3.0, 6.0 mg/kg, 30 minutes before the social interaction test. The same animals were tested 24 hours later in the social recognition test.

The one-way ANOVA revealed an overall effect of MSX-3 on time spent sniffing the conspecific ($F(3,32)=4.58$, $p<0.01$), and planned comparison showed that all doses increased significantly time sniffing the social target (MSX-3 1.5 mg/kg, $p<0.05$; MSX3 3.0 mg/kg and MSX-3 6.0 mg/kg, $p<0.01$) compared with the vehicle treated group. The one-way ANOVA for the dependent variable time spent exploring the object was also significant ($F(3,32)=3.63$, $p<0.05$). MSX-3 significantly decreased the time exploring the object at all doses employed (MSX-3 1.5 mg/kg, $p<0.05$; MSX3 3.0 mg/kg and MSX-3 6.0 mg/kg, $p<0.01$) when compared with the vehicle group. Student t-test for dependent samples demonstrated that there were significant differences in time spent sniffing the conspecific versus the object in the vehicle group ($t=12.96$, $p<0.01$), but also in all the MSX-3 treated groups (MSX-3 1.5 mg/kg, $t=7.96$, $p<0.01$; MSX-3 3.0 mg/kg, $t=10.33$ $p<0.01$, and MSX-3 6.0 mg/kg, $t=6.87$ $p<0.01$) (**Fig 7.A**).

The impact of MSX-3 on locomotion is shown in **Fig 7.C. and 7.D**. The ANOVA for the effect of MSX-3 on horizontal locomotion was significant ($F(3,32)=3.66$, $p<0.05$), and planned comparisons showed a significant effect of all doses of MSX-3 on total crosses between compartments as a measure of horizontal locomotion (MSX-3 1.5 mg/kg and MSX-3 3.0 mg/kg, $p<0.05$; and MSX-3 6.0 mg/kg, $p<0.01$). However, the one-way ANOVA for vertical locomotion was not significant ($F(3,32)=1.83$, n.s.).

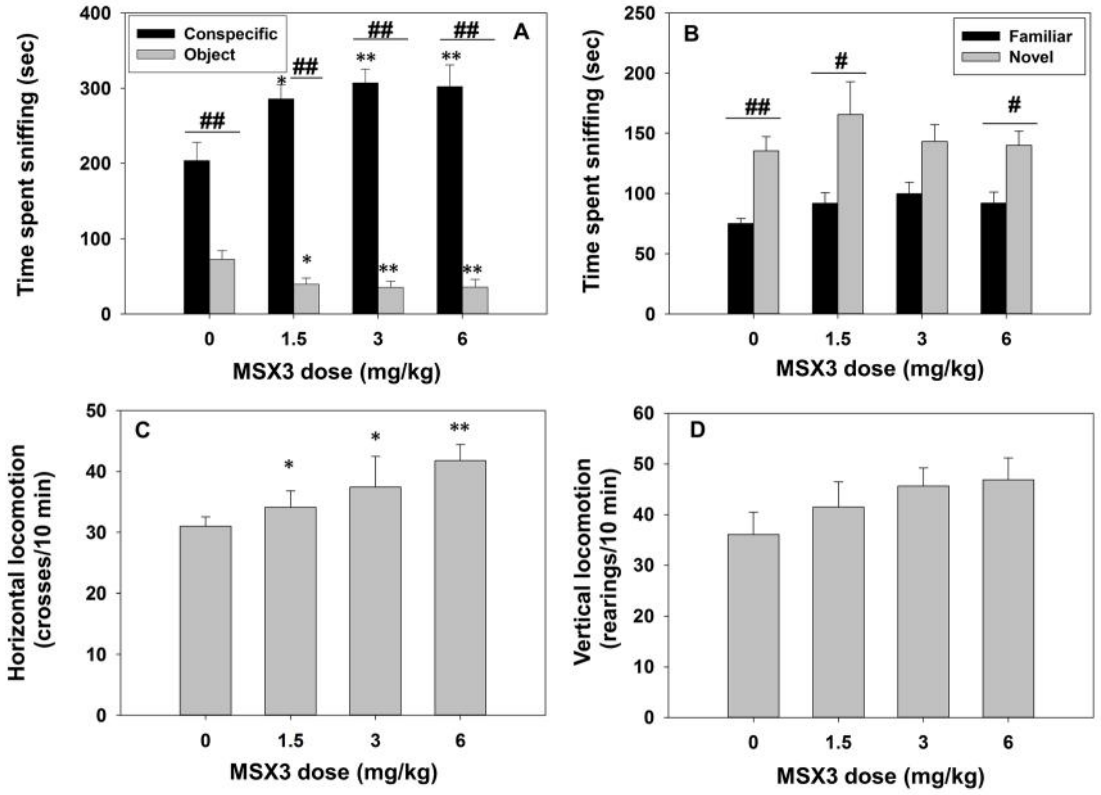

Figure 7. Effect of MSX3 in social preference and recognition tests. Data are expressed as mean (±SEM) of time sniffing **A**) conspecific and object in the social preference test, **B**) familiar and novel cosnpecifics in the social recognition test, and **C**) horizontal and **D**) vertical locomotion during the social preference test. **p<0.01, *p<0.05 significant differences from vehicle for the same target. ##p<0.01 #p<0.05 significant differences between time sniffing both targerts for the same dose of MSX3.

For the social recognition test, the one-way ANOVA's revealed no significant effect of MSX-3 on time spent sniffing the familiar conspecific ($F(3,32)=1.83$, n.s.), and also no significant effect of this drug on novel conspecific exploration ($F(3,32)=0.61$, n.s.) (**Fig 7.B**). Student's t-test for dependent samples showed significant differences between time spent sniffing novel versus familiar conspecific in the vehicle group (t=-4.71,

$p<0.01$), as expected, and this pattern was also observed in the MSX-3 1.5 mg/kg, ($t=-2.64$, $p<0.05$) and the MSX-3 6.0 mg/kg groups ($t=-2.42$, $p<0.05$). The intermediate dose of MSX-3 3.0 mg/kg almost reach significant levels ($t=-2.13$, $p=0.06$). Thus MSX-3 administered the day before did not affect social recognition memory.

Experiment 8: Effect of MSX3-ethanol co-administration on social preference and locomotion. Impact on long-term social recognition memory.

Mice (N=50) received a dose of vehicle or of the lowest dose of MSX-3 (1.5 mg/kg) that was effective in experiment 7. MSX-3 was administered 20 minutes before test, and 10 minutes before the social preference test a second injection of vehicle or ethanol (0.5 or 1.0 g/kg) was administered. The following day, the same animals were tested for social long-term memory.

A factorial ANOVA (MSX-3 x ethanol) revealed an overall effect of MSX-3 ($F(1,43)=40.65$, $p<0.01$), and ethanol ($F(2,43)=3.36$, $p<0.05$) on time sniffing the conspecific. However, there was not a significant interaction effect with this variable ($F(2,43)=0.34$, n.s.) (**Fig 8.A**). The factorial ANOVA for time sniffing the object did not reveal a significant effect of MSX-3 ($F(1,43)=1.45$, n.s.), or ethanol ($F(2,43)=0.49$, $p=0.61$), and no significant interaction ($F(2,43)=2.23$, n.s.) either (**Fig 8.B**).

Total crosses between compartment as a measure of horizontal locomotion were overall affected by MSX-3 ($F(1,43)=21.18$, $p<0.01$), but not by ethanol ($F(2,43)=2.42$, n.s.), and there was not a significant interaction either ($F(2,43)=0.30$, n.s.). The one-way ANOVA for vertical locomotion revealed a significant effect of ethanol ($F(2,43)=3.99$, $p<0.05$), but no effect of MSX3 ($F(1,43)=2.27$, n.s.), and no significant interaction ($F(2,43)=0.11$, n.s.).

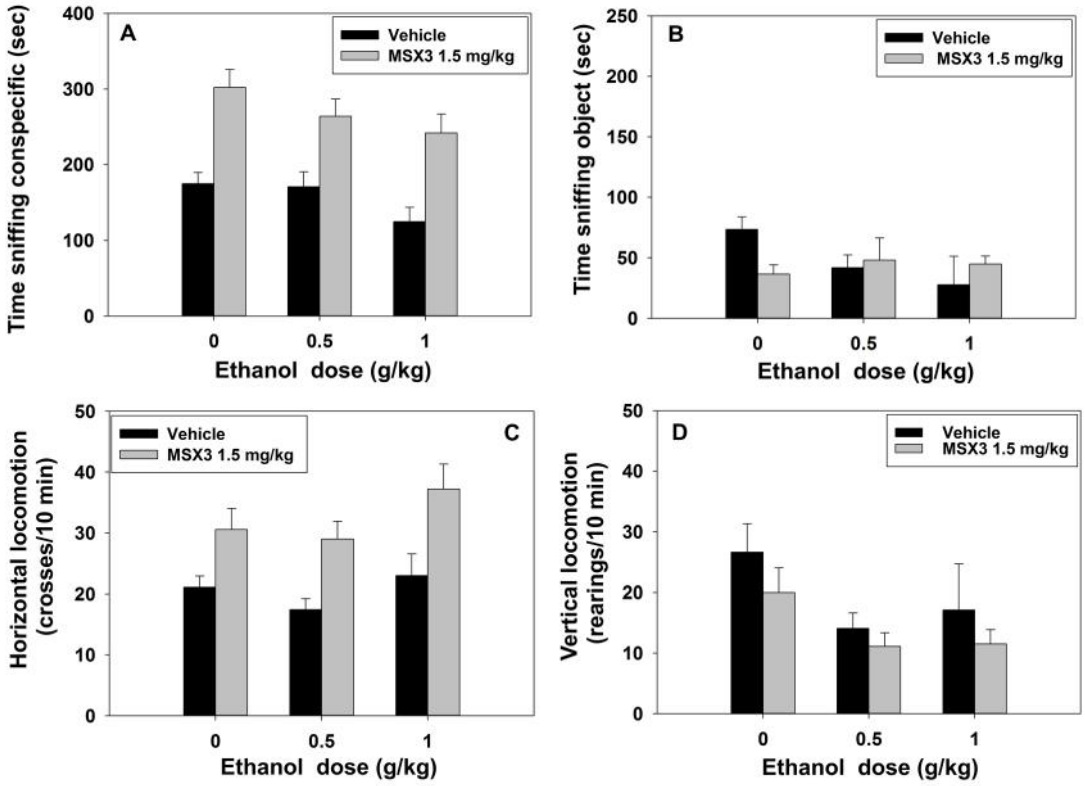

Figure 8. Effect of MSX3 plus ethanol interaction in the social preference test. Data are expressed as mean (±SEM) of time sniffing **A**) conspecific, **B**) object, **C**) horizontal and **D**) vertical locomotion during the social preference test.

As for the impact of these drugs on recognition of the conspecific presented during the preference test, the factorial ANOVA (MSX-3 x Ethanol) for time sniffing the familiar conspecific showed a significant effect of ethanol ($F(2,43)=6.97$, $p<0.01$), but did not show an effect of MSX-3 ($F(1,43)=0.02$, n.s.), and no MSX-3 x ethanol interaction on this variable ($F(2,43)=2.14$, n.s.) (**Table 4**). Another factorial ANOVA for the variable time sniffing the novel conspecific, did not reveal an effect of MSX-3 ($F(1,43)=0.14$, n.s.), it did not show a significant effect of ethanol although it was close to significance ($F(2,43)=2.73$, $p=0.08$), and the interaction was not significant ($F(2,43)=0.43$, n.s.). When comparing the behavior of every group of animals in the exploration of the known and novel conspecific, the control group that had been treated with vehicle-vehicle the day before spent significantly more time sniffing the novel conspecific vs. the familiar conspecific as expected if the animal recognizes the known conspecific ($t=4.71$, $p<0.01$). This result was also observed in animals treated with MSX-3 1.5 mg/kg plus vehicle ($t=2.64$, $p<0.05$), and with MSX-3 1.5 mg/kg plus the lowest dose of

ethanol 0.5 g/kg (t=2.52, p<0.05). However, the group treated with MSX-3 1.5 mg/kg plus the highest dose of ethanol 1.0 g/kg showed memory impairment since the time spent exploring both conspecifics was not different (t=-0.93, n.s.). There were no differences between time sniffing the novel conspecific vs. novel conspecific either in mice that had received vehicle plus ethanol 0.5 g/kg (t=0.62, n.s.) or vehicle plus ethanol 1.0 g/kg (t=-0.21, n.s.). Thus, it seems that MSX-3 had a preventive effect only when the dose of ethanol was low.

	Time sniffing (sec)					
Etoh (g/kg)	**0.0**		**0.5**		**1.0**	
MSX3 (mg/kg)	**Familiar**	**Novel**	**Familiar**	**Novel**	**Familiar**	**Novel**
0.0	75.1±4.9	138.3±14.0##	105.6±11.9	118.7±13.3	120.0±24.6	114.6±19.8
1.5	84.1±10.9	160.1±24.8#	68.7±7.8	109.3±12.3#	142.6±20.0	117.8±12.2

Table 4. Effects of MSX3-ethanol combination on social recognition memory. Data are expressed as mean ±SEM of time in seconds sniffing novel or familiar conspecifics. ##p<0.01, #<p0.05 significant differences between time in familiar vs time in novel conspecific for the same dose of MSX3 and ethanol.

4. Discussion

In the present study, we characterize the impact of two of the most commonly consumed drugs of abuse, caffeine and alcohol, on motivation for social contact as manifested by social preference or avoidance, and also on consolidation of social memories. We evaluated the possibility of a common mechanism of action for both drugs via the adenosine system. Thus, we hypothesized that low to intermediate doses of alcohol could lead to an increase in adenosine levels that would counteract the effect of caffeine, which acts as a non-selective A_1 and A_{2A} antagonist. For that purpose, the effects of selective A_1 and A_{2A} receptor antagonists were also assessed alone or in combination with ethanol.

Our results show that the suppressing effects of high doses of caffeine on social approach and preference can be counteracted by low doses of ethanol, but this reversal effect reaches a ceiling when ethanol starts to mildly impair social approach and

preference on its own. Ethanol at the low dose did not improve social approach by reducing the physiological stress response induced by caffeine, which increased plasma corticosterone levels at these doses. However, since there is not a clear direct relationship between endocrine measures of stress and behavioral anxiety measures (Marquez et al. 2006), we cannot rule out anxiety as the cause of these changes in social preference and approach. In fact, social interaction has been mostly used to evaluate anxiety in rodents, because it was found that anxiolytics increase time spent in active social interaction while anxiogenic drugs decrease social contact independently of any change in activity (File and Hyde 1979; Guy and Gardner, 1985). Thus, the reduction in social preference observed after caffeine administration could be explained by an increase in anxiety, since doses ranging from 25 to 100 mg/kg have been demonstrated to have a potent anxiogenic effect in this strain of mice as seen in the elevated plus maze (López-Cruz et al. 2013). It is also possible that anxiolysis induced by ethanol could be playing a role in potentiating social interaction as suggested by previous researchers (Nadal et al. 1993; Hilakivi et al, 1993). However, it cannot be the only explanation for this effect since doses of ethanol that induced anxiolysis in this strain of mice (0.5 and 1.0 g/kg) in an elevated plus maze (Correa et al. 2008) were not able to reverse social preferences to normal levels. Moreover, in the present study we used a procedure developed to minimize anxiety in the experimental mouse by eliminating the possibility of physical aggression since the target mouse was enclosed in a wire cage (Crawley, 2004; Moy et al. 2007). Thus, in this paradigm it is possible to assess preference or avoidance for social interaction based on free choice. Furthermore, none of the pharmacological manipulations used in the present series of studies produced a significant avoidance for the compartment where the conspecific was located (data not shown). The effects of caffeine and ethanol alone or in combination on social behavior do not seem to be mediated by their effects on locomotion either, because the range of doses used do not clearly impair locomotion, and an increase in locomotion induced by the lowest doses of caffeine (7.5 and 15.0 mg/kg) seem to be unrelated to social exploration.

Although a strength of the present study was the use of a broad range of doses for all drugs, including the studies of drug interaction (most of the previous studies have used a single dose approach), it is not clear that the effect of high doses of caffeine were mediated by its actions on adenosine A_1 and A_{2A} receptors, since neither of the selective

adenosine receptors reduced social interaction at the doses tested. Because in the present paradigm the experimental mouse has to explore a broad area that separates the two targets (conspecific and object), we selected doses of caffeine and selective adenosine antagonists based on results from previous work showing no impairing effects on ambulation and rearing in an open field (López-Cruz et al. 2013; Pardo et al. 2013), in order to avoid the possibility of mediating variables related to motor function. Thus, the A_1 antagonist CPT did not produce a significant change in social approach and preference, although mice spent more time in the conspecific compartment at the low doses (data not shown), and there was no interaction with ethanol on these parameters. It is possible, however, that higher doses of CPT could mimic the effects of caffeine on social preference. On the other hand, the A_{2A} receptor antagonist MSX-3 did have a significant effect, increasing preference for the social target and reducing it for the object. It is also worth noting that although general exploration (crossings between the 3 compartments) increased, MSX-3 did not disturb focused social exploration. Moreover, there was no significant interaction between MSX-3 and ethanol on any of these parameters; the improving effect of MSX-3 on preference was maintained at the same level independently of the dose of ethanol (0.5 or 1.0 g/kg) that the animals received. Consistently, high levels of social interaction have been observed in A_{2A} receptor KO mice, and these animals were not affected by a dose of ethanol (1.0 g/kg) that impaired social interaction (López-Cruz et al. submitted). Interestingly, A_{2A}KO mice showed an anxiogenic profile, which again argues against a straight relationship between anxiety and social interaction (López-Cruz et al. submitted).

A decrease in exploring a familiar conspecific when a new one is also present has been interpreted as an index of social recognition (Thor and Holloway, 1982; Moy et al. 2004; Crawley, 2004), which some authors consider to be also an index of preference for novelty seeking (Costa et al. 2014). Whatever the interpretation, it is required that the animal consolidates a memory for the familiar conspecific. Adenosine seems to modulate short-term social memory in rats by acting on both A_1 and A_{2A} receptors, with adenosine receptor agonists and antagonists respectively disrupting and enhancing social recognition memory (Prediger and Takahashi, 2005). Thus, the selective A_1 agonist CCPA and the A_{2A} agonist DPMA disrupted juvenile recognition in adult rats (Prediger and Takahashi, 2005). This impairment of short-term social memory induced by adenosine agonists was reversed by caffeine, the A_1 antagonist DPCPX, and the A_{2A}

antagonist ZM24138 (Prediger and Takahashi, 2005). Moreover, acute administration of caffeine or selective A_{2A} antagonists reversed the disruption of social recognition memory in ageing rats (Prediger et al. 2005a) and also in spontaneously hypertensive rats (Prediger et al. 2005b) in which some alterations in the adenosinergic neurotransmission have been reported (Matias et al. 1992; Cunha et al. 1995; Lopes et al. 1999; Davies et al. 1987). However, all these studies evaluated short-term social memory and not long-term social memory.

If the recognition test is carried 24 hours after the first presentation it can be considered as a test of long-term memory processes. The development and consolidation of long-term potentiation seems to be also modulated by adenosine receptor-dependent mechanisms in the hippocampus (Tanaka et al. 1990; de Mendonca and Ribeiro 1994; Hauber and Bareiss, 2001). Data from the present study indicates that caffeine at high doses impaired recognition on the following day, especially at those doses (30.0 and 60.0 mg/kg) that had reduced relative preference for social interaction the day before. Thus, mice explored familiar and novel conspecifics equally, which could be explained by the fact that animals had explored the conspecific much less time the day before than animals under control conditions. It is possible that the ability of caffeine to improve memory at low doses could be seen under different experimental conditions. In fact, theophylline has been demonstrated to facilitate long-term spatial reference memory in retention sessions, but not in working memory, both of which are tasks that are highly dependent on hippocampus (Hauber and Bareiss, 2001). Thus, when the nature of the task involves optimal performance during basal conditions is very difficult to improve performance.

It is well know that ethanol can produce amnesic effects and impair retrieval of memories after the drug wears off (Goodwin 1995; Hartzler and Fromme 2003; Gulick and Gould, 2007; 2009). Ethanol-induced memory impairments can be produced by disruption of attention, and also by affecting neural mechanisms involved in memory consolidation such as the adenosinergic system (Tanaka et al. 1990; Gulick and Gould, 2007; 2009). In experiment 2, ethanol, even at doses that did not impair social interaction (0.5 g/kg), impaired social recognition 24 hours later. In spite of been a situation of low performance, caffeine (15.0 or 30.0 mg/kg) co-administration was not able to block the amnesic effects of ethanol. A previous study in rats explored the effect of caffeine-ethanol interaction on long-term memory using social odors (Spinetta et al.

2008). In that study ethanol was administered immediately after exposure to the social odor, and a recognition test was performed 24 hours later (Spinetta et al. 2008). Caffeine, at a low dose that did not have an effect on its own (5 mg/kg), was able to prevent the disruptive effects of ethanol (1.0 g/kg) on memory consolidation (Spinetta et al. 2008). It is possible that in our study lower doses of caffeine could have improved ethanol-induced deficits. The behavioral effects induced by methylxantines at low doses are likely to be mediated by an nonselective adenosine A1/A2A receptor blockade, while higher doses might involve additional mechanisms such as inhibition of phosphodiesterases (Nehlig et al. 1992; Hauber and Bareiss, 2001).

As for the role of selective adenosine receptor antagonists, it appears that although CPT did not affect social interaction, it mildly impaired long-term social recognition at low doses, an effect that was not observed at high doses. CPT was not able to reverse the ethanol-induced impairment of recognition memory. In contrast, the selective A_{2A} antagonist MSX-3, which increased preference for the conspecific when administered alone, did not impair social recognition, and was able to block the amnesic effect of the lower dose of ethanol (0.5 g/kg). Thus, in our studies a selective A_{2A} antagonist was able to improve social memory under conditions of suboptimal performance (ethanol amnesic effects), but not under optimal performance (i.e., non-treated animals). This improvement in memory might be due to actions on processes involved in learning, such as attention and wakefulness, but may also be related to direct actions on learning and memory.

Although it is clear that normal social interaction is required for normal retrieval of social memories, the data from the present studies indicate a relative independence between social preference and social long-term memory processes. The results available at the present moment also suggest that A_1 receptors do not seem to regulate social motivation and social recognition, since blocking their tonic activity has very little effect. A_1 receptor antagonists appear to play only a modest role in the regulation of dopamine-dependent aspects of motivated behaviors (Nunes et al. 2013; Salamone and Correa 2012). Moreover, because selective A_1 and A_{2A} antagonists did not mimic the effects of caffeine, it is possible that blockade of both receptors is necessary for producing a caffeine-like action. Alternately, it is possible that at high doses caffeine may not be acting solely as an adenosine antagonist. Thus, although an increase in adenosine levels could be mediating ethanol effects, the usefulness of highly caffeinated

drinks in counteracting ethanol-induced impairments on these normal social processes is questionable.

5. References

- Baldwin HA, Johnston AL, File SE (1989) Antagonistic effects of caffeine and yohimbine in animal tests of anxiety. Eur J Pharmacol 159(2):211-215.

- Baldwin HA, File SE (1989) Caffeine-induced anxiogenesis: the role of adenosine, benzodiazepine and noradrenergic receptors. Pharmacol Biochem Behav 32(1):181-186.

- Carmichael FJ, Israel Y, Crawford M, Minhas K, Saldivia V, Sandrin S, Campisi P, Orrego H. (1991) Central nervous system effects of acetate: Contribution to the central effects of ethanol. Journal of Pharmacology and Experimental Therapeutics 259:403-408.

- Chévere-Torres I, Maki JM, Santini E, Klann E (2012) Impaired social interactions and motor learning skills in tuberous sclerosis complex model micenovelty seeking behavior during decision making. Behav Neurosci 128(5):556-566. doi.org/10.1037/a0037128

- Correa M, San Miguel N, López-Cruz N, Bayarri P, Monferrer L, Salamone JD (2014). Caffeine Modulation of Alcohol Intake: Impact on its psychomotor effects and withdrawal. Pp: 89-111. In: Caffeine: Consumption, Side Effects and Impact on Performance and Mood. Tolley, A.S. (editor). NOVA Science Publishers, Inc.

- Costa VD, Tran VL, Turchi J, Averbeck BB (2014). Dopamine modulates novelty seeking behavior during decision making. Behav Neurosci ;128(5):556-66. doi: 10.1037/a0037128.

- Crawley JN (2004) Designing mouse behavioral tasks relevant to autistic-like behaviors. Ment Retard Dev Disabil Res Rev 10:248–258.

- Cunha RA (2001) Adenosine as a neuromodulator and as a homeostatic regulator in the nervous system: different role, different sources and different receptors. Neurochem Int 38:107-125.

- Davies LP, Hambley JW, Johnston GAR (1987). Reduced adenosine deaminase activity in the CNS of spontaneously hypertensive rats. Neurochem Int 10:533-536. doi:10.1016/0197-0186(87)90081-7

- de Mendonca A, Ribeiro JA (1994). Endogenous adenosine modulates long-term potentiation in the hippocampus. Neuroscience 62(2):385-390.

- File SE, Hyde JR (1978). Can social interaction be used to measure anxiety? British Journal of Pharmacology 62(1):9-24.

- Fredholm BB, Battig K, Holmen J, Nehlig A, Zvartau E (1999). Actions of caffeine in the brain with special reference to factors that contribute to its widespread use. Pharmacol. Rev.51:83-130. Doi: 0031-6997/99/5101-0083$03.00/0.

- Fredholm BB, IJzerman AP, Jacobson KA, Klotz KN, Linden J. (2001) International Union of Pharmacology. XXV. Nomenclature and classification of adenosine receptors. Pharmacol Rev 53:527-552.

- Fuxe K, Agnati LF, Jacobsen K, Hillion J, Canals M, Torvinen M. et al. (2003). Receptor heteromerization in adenosine A2A receptor signaling: relevance for striatal function and Parkinson's disease. Neurology 61,S19-23. doi: 10.1212/01.WNL.0000095206.44418.5C

- Goodwin DW (1995) Alcohol amnesia. Addiction 90:315-317. doi: 10.1080/09652149542292

- Gulick D, Gould TJ (2007) Acute ethanol has biphasic effects on short- and long-term memory in both foreground and background contextual fear conditioning in C57BL/6 mice. Alcohol Clin Exp Res 31(9):1528-1537. doi: 10.1111/j.1530-0277.2007.00458.x

- Gulick D, Gould TJ (2009) Effects of ethanol and caffeine on behavior in C57BL/6 mice in the plus-maze discriminative avoidance task. Behav Neurosci 123(6):1271-1278.

- Guy AP, Gardner CR. (1985) Pharmacological characterisation of a modified social interaction model of anxiety in the rat. Neuropsychobiology 13(4):194-200.

- Hartzler B, Fromme K (2003) Fragmentary and en bloc blackouts: similarity and distinction among episodes of alcohol-induced memory loss. J Stud Alcohol 64:547-550. doi: http://dx.doi.org/10.15288/jsa.2003.64.547

- Hauber W, Bareiss A (2001) Facilitative effects of an adenosine A1/A2 receptor blockade on spatial memory performance of rats: selective enhancement of reference memory retention during the light period. Behav Brain Res 118:43-52. doi:10.1016/S0166-4328(00)00307-7

- Hauber W., Sommer S (2009) Prefrontostriatal circuitry regulates effort-related decision making. Cereb. Cortex. 19(10):2240-2247.

- Hilakivi LA, Durcan MJ, Lister RG (1989) Effects of caffeine on social behavior, exploration and locomotor activity: interactions with ethanol. Life Sci. 44(8):543-553.

- Keppel G. Design and analysis: A researcher's handbook. Englewood Cliffs, New

Jersey: Prentice-Hall; 1991.

- Kirchner TR, Sayette MA, Cohn JF, Moreland RL, Levine JM (2006) Effects of alcohol on group formation among male social drinkers. J Stud Alcohol 67:785-793. doi: http://dx.doi.org/10.15288/jsa.2006.67.785

- Krauss SW, Ghirnikar RB, Diamond I, Gordon AS (1993) Inhibition of adenosine uptake by ethanol is specific for one class of nucleoside transporters. Mol Pharmacol 44(5):1021-1026.

- Lister RG, Hilakivi LA (1988) The effects of novelty, isolation, light and ethanol on the social behavior of mice. Psychopharmacology (Berl) 96(2):181-187. doi: 10.1007/BF00177558

- Lopes LV, Cunha RA, Ribeiro JA (1999) Increase in the number, G protein coupling, and efficiency of facilitatory adenosine A2A receptors in the limbic cortex, but not striatum, of aged rats. J Neurochem 73:1733-1738.

- López-Cruz L, Pardo M, Salamone JD, Correa M (2014) Differences between the nonselective adenosine receptor antagonists caffeine and theophylline in motor and mood effects: studies using medium to high doses in animal models. Behav Brain Res 1270:213-222. doi: 10.1016/j.bbr.2014.05.020.

- López-Cruz L, Salamone JD, Correa M (2013) The Impact of Caffeine on the behavioral Effects of Ethanol Related to Abuse and Addiction: A Review of Animal Studies. J Caffeine Res 3(1):9-21. doi: 10.1089/jcr.2013.0003

- López-Cruz, L, Carbó-Gas M, Pardo, M, Bayarri, P et al. 2015. Motivation for social interaction and impact of anxiety levels in A2A receptor KO mice: involvement of anterior cingulate and amygdala. Genes Brain and Behavior. Submitted.

- Manrique HM, Miquel M, Aragon CM (2005) Brain catalase mediates potentiation of social recognition memory produced by ethanol in mice. Drug Alcohol Depend 79(3): 343-350. doi:10.1016/j.drugalcdep.2005.02.007

- Matias A, Zimmer FJ, Keil R (1992) Decreased affinity of central A1 adenosine receptors in spontaneously hypertensive rats. Naunyn- Schmied Arch Pharmacol 315:59.

- Moy SS, Nadler JJ, Perez A, Barbaro RP, Johns JM, Magnuson TR, Piven J, Crawley JN (2004) Sociability and preference for social novelty in five inbred strains: an approach to assess autistic-like behavior in mice. Genes Brain Behav 3:287-302. doi: 10.1111/j.1601-1848.2004.00076.x

- Nadal RA, Pallares MA, Ferré NS (1993) The effects of caffeine in the social interaction test and on exploration in rats: comparison with ethanol and clorazepate. Behav Pharmacol 4:501-508. doi.org/10.1097/00008877-199310000-00005

- Nagy LE, Diamond I, Casso DJ, Franklin C, Gordon AS (1990) Ethanol increases extracellular adenosine by inhibiting adenosine uptake via the nucleoside transporter. J Biol Chem 265(4):1946-1951.

- Nehlig A, Daval J-L, Debry G (1992) Caffeine and the central nervous system: mechanisms of action, biochemical, metabolic and psychostimulant effects. Brain Res Rev 17:139-179. doi:10.1016/0165-0173(92)90012-B

- Prediger RD, Batista LC, Takahashi RN (2005a) Caffeine reverses age-related deficits in olfactory discrimination and social recognition memory in rats. Involvement of adenosine A1 and A2A receptors. Neurobiol Aging 26(6):957-964. Doi: http://dx.doi.org/10.1016/j.neurobiolaging.2004.08.012

- Prediger RD, Fernandes D, Takahashi RN (2005b). Blockade of adenosine A2A receptors reverses short-term social memory impairments in spontaneously hypertensive rats. Behav Brain 159(2):197-205.

- Prediger RD, Takahashi RN (2005) Modulation of short-term social memory in rats by adenosine A1 and A(2A) receptors. Neurosci Lett 376(3):160-165. doi:10.1016/j.neulet.2004.11.049

- Procópio-Souza R, Fukushiro DF, Trombin TF, Wuo-Silva R, Zanlorenci LH, Lima AJ, Ribeiro LT, Corrêa JM, Marinho EA, Kameda SR, Andersen ML, Tufik S, Frussa-Filho R (2011) Effects of group exposure on single injection-induced behavioral sensitization to drugs of abuse in mice. Drug Alcohol Depend 118(2-3):349-359. doi: 10.1016/j.drugalcdep.2011.04.017.

- Salamone JD, Correa M (2002) Motivational views of reinforcement: implications for understanding the behavioral functions of nucleus accumbens dopamine. Behav Brain Res 137(1-2):3-25.

- Salamone JD, Correa M (2012) The mysterious motivational functions of mesolimbic dopamine. Neuron 76: 470–485. doi: http://dx.doi.org/10.1016/j.neuron.2012.10.021

- Sano H, Nagai Y, Miyakawa T, Shigemoto R,Yokoi M (2008) Increased social interaction in mice deficient of the striatal medium spiny neuron-specific phosphodiesterase 10A2. J Neurochem 105:546-556. Doi: 10.1111/j.1471-4159.2007.05152.x

- Schiffmann SN, Libert F, Vassart G (1991). Distribution of A2A receptor mRNA in the human brain. Neurosciences Letters 130(2):177-181. doi:10.1016/0304-3940(91)90391-6

- Schwarzschild MA, Agnati L, Fuxe L, Chen J, Morelli M (2006) Targeting adenosine A2A receptors in Parkinson's disease. Trends Neurosci 29(11):647-654. doi:10.1016/j.tins.2006.09.004

- Sebastião AM, Ribeiro JA (2009) Adenosine receptors and the central nervous system. Handb Exp Pharmacol 193:471-534. doi: 10.1007/978-3-540-89615-9_16.

- Spinetta MJ, Woodlee MT, Feinberg LM, Stroud C, Schallert K, Cormack LK, Schallert T (2008) Alcohol-induced retrograde memory impairment in rats: prevention by caffeine. Psychopharmacology (Berl) 201(3):361-371. doi:10.1007/s00213-008-1294-1295.

- Tanaka Y, Sakurai M, Goto M, Hayashi S (1990). Effect of xanthine derivatives on hippocampal long-term potentiation. Brain Res 522:63-68.

- Thor D, Holloway W (1982) Social memory of the male laboratory rat. J Comp Physiol Psych 96:1000-1006.

- Varlinskaya EI, Spear LP (2002) Acute effects of ethanol on social behavior of adolescent and adult rats: role of familiarity of the test situation. Alcohol Clin Exp Res 26:1502-1511. doi: 10.1111/j.1530-0277.2002.tb02449.x

- Varlinskaya EI1, Spear LP. Ethanol-induced social facilitation in adolescent rats: role of endogenous activity at mu opioid receptors. Alcohol Clin Exp Res. 2009 Jun;33(6):991-1000. doi: 10.1111/j.1530-0277.2009.00920.x.

CHAPTER 4:

Motivation for social interaction and impact of anxiety levels in A_{2A} receptor KO mice: involvement of anterior cingulate and amygdala.

Abstract

Social interaction paradigms evaluate the natural preference of animals for exploring other conspecifics and the ability to differentiate between familiar versus novel ones. Anxiety is one of the factors that can induce avoidance of social interaction. It has been demonstrated that blockade of adenosine A_{2A} receptors can potentiate motivation for natural reinforcers, but can also induce anxiety. However, the role of adenosine receptors in motivation for social interaction has not been widely explored. In the present study, A_{2A} knockout (A_{2A}KO) and wild-type (WT) mice were assessed for social and anxiety-related behaviors. c-Fos immunoreactivity was evaluated as a measure of neuronal activation in brain areas involved in motivational and emotional processes. Although A_{2A}KO mice showed an anxiogenic profile, they displayed higher levels of sociability than WT mice. WT mice displayed a typical pattern of social recognition 24 hours later, but not A_{2A}KO mice, which explored equally both conspecifics. There were no differences between strains in aggressiveness or social odor preferences. c-Fos immunoreactivity in A_{2A}KO mice was higher in anterior cingulate and amygdala compared to WT mice. An anxiolytic dose of ethanol eliminated differences between strains in social preference, and impaired conspecific recognition in WT mice. In conclusion, A_{2A}KO mice tend to engage more in social exploration and are less sensitive to social novelty. In these animals, there seems to be a dissociation between baseline and ethanol-related anxiety and motivation for social interaction. Thus, A_{2A} receptors appear to be potential targets for the improvement of pathologies related to social function.

Key words: social preference, social recognition, adenosine, anxiety, aggressive behavior, c-Fos, marble-burying test.

Introduction

Adenosine is a central nervous system (CNS) neuromodulator that in the brain acts mainly via the activation of high affinity A_1 and A_{2A} G-protein coupled receptors (Fredholm et al., 2001). While A_1 receptors are widely distributed in the brain, A_{2A} receptors are predominantly localized and highly concentrated in the basal ganglia. Nucleus accumbens and caudate/putamen have a high concentration of adenosine A_{2A} receptors (Ferré et al., 2004; Jarvis and Williams, 1989; Vontell et al., 2010), and considerable evidence indicates that those adenosine receptors interact with dopamine receptors in the regulation of the activational component of motivated behaviors such as actively seeking natural reinforcers (i.e.: food or sucrose) (Salamone and Correa, 2009). Adenosine A_{2A} receptors are also highly concentrated in the olfactory tubercle (Vontell et al., 2010), and to a much lesser extent, in amygdala (Fredholm et al., 2001; Schiffmann et al., 2007), both important regions for the regulation of social behaviors in rodents (Sano et al., 2008).

Although adenosine has been demonstrated to modulate processes involved in social interaction such as exploration (Florio et al., 1997), arousal (Dunwiddie & Worth, 1982), anxiety (Correa & Font, 2008), and memory (Zarrindast & Shafaghi, 1994), the role of adenosine and adenosine A_{2A} receptors in seeking social interaction has not been widely explored. In some studies, caffeine, a non- selective adenosine receptor antagonist (A_1/A_{2A}) has been shown to decrease social interaction in rodents at high doses, which has been interpreted as an axiogenic effect (Daldwin et al., 1989; Hilakivi et al., 1989). However, lower doses seem to potentiate social contact (Nadal et al., 1993). Anxiolytic drugs such as ethanol (Correa et al., 2008), which increases the brain adenosinergic tone (Nagy et al., 1990), has also been demonstrated to increase social interaction at low doses (Nadal et al., 1993) but decrease it at high doses (Hilakivi et al., 1989).

On the other hand, caffeine and selective adenosine antagonists for A_1 and A_{2A} receptors improve short-term social memory in rats (Prediger & Takahashi, 2005), while ethanol impairs social recognition in mice (Manrique et al., 2005). Thus, it is not clear how adenosine regulates social motivation or social memory, and if adenosine modulation of anxiety can reduce social exploration in rodents.

In the present study, we focused on the impact of A_{2A} receptor deletion (A_{2A} receptor knockout, A_{2A}KO) on motivation for social exploration, as well as social memory, in mice. These A_{2A}KO mice have been shown to be more aggressive, and more anxious, and they display lower levels of locomotion than their wild type (WT) counterparts (Ledent et al., 1997; Berrendero et al., 2003). However, their patterns of social behavior have not been previously explored. Thus, the present work evaluates the performance of A_{2A}KO mice on tests of social preference, recognition, and anxiety. The impact of an anxiolytic dose of ethanol on social behaviors was also explored in these animals. Because perseverative behavior, aggressiveness or odor detection problems could regulate social behavior patterns in rodents (Liebenauer & Slotnick et al., 1996; Bortolato et al., 2011; Doty, 1986), these processes were also evaluated. Finally, expression of the immediate early-gene product c-Fos as a measure of neuronal activation was evaluated in different A_{2A} receptor containing regions, as well as areas that are important for the regulation of motivation and emotion.

Materials and methods

Subjects

Male mice lacking the A_{2A} adenosine receptor and WT littermates (N=9 and 10 respectively) were generated from a CD1 background by C. Ledent at Universite Libre de Bruxelles (Belgium), as previously reported (Ledent et al., 1997). All animals weighted 30-40 g at the beginning of the study and were housed in groups of 3 or 4 animals per cage with water and food available *ad libitum*. The colony was kept at temperature of 22 $\pm$ 2 ºC with lights on from 8:00-20:00h. 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).

Drugs

Ethanol (Panreac Quimica S.A., Spain) was diluted to 20% (v/v) in physiological saline (0.9 % w/v) and administered intraperitoneally (IP) 10 minutes before testing. Saline solution was used as vehicle. The dose of ethanol used (1.0 g/kg) was based in previous studies with the same strain (Correa et al., 2008).

Behavioral apparatus and testing procedures

The behavioral test room was illuminated with a soft light, and external noise was attenuated. All tests were videotaped and dependent variables were later registered by a trained observer unaware of the experimental condition.

Anxiety in the dark and light box (DL)

The DL test is based on the conflict between the inherent tendencies of mice to explore a novel environment vs. their natural avoidance of a brightly lit open field. The DL apparatus consisted of a polypropylene chamber divided in two compartments by a partition containing a small opening. One compartment was open and illuminated while the other was enclosed and dark. Initially each subject was placed in the dark compartment. Latency to enter the lit compartment, latency to go back into the dark compartment, total time spent in the lit compartment and total number of crosses between chambers were recorded manually over 5 min.

Anxiety in the elevated plus maze (EPM)

The EPM consists of two open and two enclosed arms arranged in a plus configuration. This anxiety paradigm measures the avoidance that rodents show to elevated open spaces. Animals were placed in the central platform facing a closed arm and assessed during 5 minutes. Time spent in the open arms, ratio of entries into the open arms to total arm entries, and latency to enter the open arms as measures of anxiety were evaluated. Total number of entries in the four arms was recorded as an index of locomotion. An entry into an arm was recorded when the animal crossed the line that connected that arm with the central platform with all four legs.

Marble-burying task

Burying behavior in rodents involves the displacement of bedding material in an effort to cover an object as a defensive mechanism against potentially threatening objects (Pinel & Treit 1978), and it can be extended in time as a sign of perseverative or repetitive behavior (Thomas et al., 2009). Mice were placed individually in clean cages containing fresh bedding (5-6 cm deep) on top of which were placed 25 black marbles arranged in five evenly spaced rows of five marbles each. Testing lasted 5 minutes. Number of non-buried marbles at the end of this period was recorded.

Olfactory preference test

Although contact is important, olfaction is a key component of social interaction in mice (Bluthe & Dantzer, 1993). Animals were compared in their preference between a non-social odor and a social odor. The social odor was obtained by rubbing a cotton ball off the body of an unknown mouse. The non-social odor consisted of a drop of a floral essence in a cotton ball. This experiment took place in the three-chamber box used for the social preference and novelty experiments. Testing lasted 10 minutes and time spent sniffing each target (social vs. non-social odor) was registered by a trained observer unaware of the experimental condition.

Social preference and social recognition tests.

Sociability was measured in a three-chambered social box (Crawley, 2004), and the general procedure was adapted from Chévere-Torres and colleagues (2012). Mice received two habituation sessions in the social arena in two consecutive sessions. In the first session, they freely explored the empty social arena during 15 minutes, and then a second exploration session (30 minutes) was allowed to be in the presence of two wire cages, one in each of the chamber sides. After these two habituations (45 minutes total), the social preference test lasted 10 minutes and started by placing the animal in the empty middle compartment. Mice were allowed to explore the three-chambered arena, which in one chamber contained a caged with a conspecific, and in the opposite side chamber a cage with an object (**Fig 4A**). The placement of the conspecific or object was counterbalanced between animals. Time spent sniffing each target (conspecific vs. object) and time spent in each compartment were evaluated as measures of social preference. Vertical and horizontal locomotion in all compartments were also registered as indices of motor behavior. The following day (24 hours after social preference test) mice were placed in the central chamber and were evaluated during 10 minutes in a "social novelty test". During this test a novel caged mouse replaced the object. Thus, mice were given the choice to interact with a familiar conspecific (same conspecific used in the social preference test) versus a novel conspecific (**Fig 4B**). The same parameters were registered. The index of social recognition is based upon comparing the time spent investigating the novel mouse vs. the more familiar one (Moy et al., 2004).

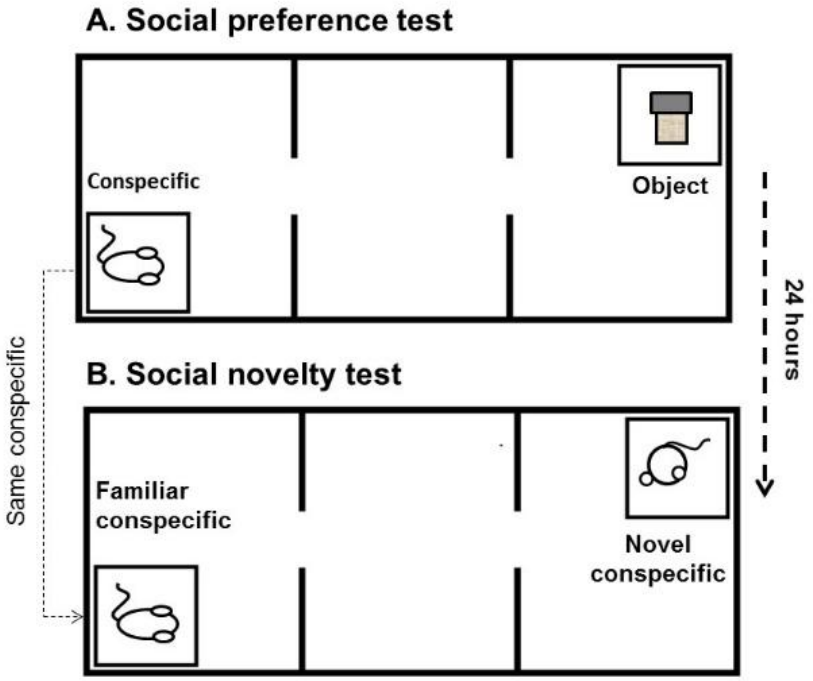

Fig 4. Schematic illustration of social preference (A) and social novelty tests (B).

Aggressive behavior

Since our social interaction paradigm avoids body contact, aggressive behavior was evaluated by registering tail rattle frequency during the social recognition test (10 minutes). Tail rattling was defined as rapid vibrations of the tail, which has been classified as reflecting threat behavior during aggressive encounters (Krsiak, 1979).

c-Fos visualization and quantification

Mice were anesthetized and perfused after the social preference and novelty tests. 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 cryostat (Microm HM 560, 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) and transferred into the primary antibody, anti-c-Fos (Calbiochem, Germany) for 24 h incubation. Following the primary antibody treatment, the sections were rinsed in PBS (3 × for 5 minutes) and incubated in the secondary antibody, anti-rabbit HRP conjugate, envision plus (DAKO,

Denmark) for 1.5 h. The immunohistochemical reaction was developed using diaminobenzidine (DAB) as chromogen (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 that were positively labeled for c-Fos were quantified with ImageJ software (v. 1.42, National Institutes of Health sponsored image analysis program) in three or four sections per animal, and the average value per mm^2 was used for statistical analysis.

Statistical analysis

Normally distributed data with homogeneity of variance were evaluated by Student's t-test for independent samples, and Mann-Whitney U test was used to analyse non-parametric data. Student's t-test for dependent samples was used to analyse “preference” (e.g. conspecific vs. object). A probability level of 0.05 or smaller was used to indicate statistical significance. Statistics were done using STATISTICA 7 software.

Results

Experiment 1: Anxiety in the DL test.

Statistical analysis revealed that KO mice spent significantly less time in the lit compartment ($t=-2.56$, $p<0.05$), and had a higher latency to enter into the lit compartment ($U=27,0$, $p<0.05$) compared to their WT counterparts (**Figs. 1A and B**). Latency to go back to the dark compartment did not reach statistical significance ($U=50.0$, n.s.) (**Fig. 1C**). There were no differences between strains in the total number of crosses between compartments ($t=-0.84$, n.s.) (**Fig. 1D**).

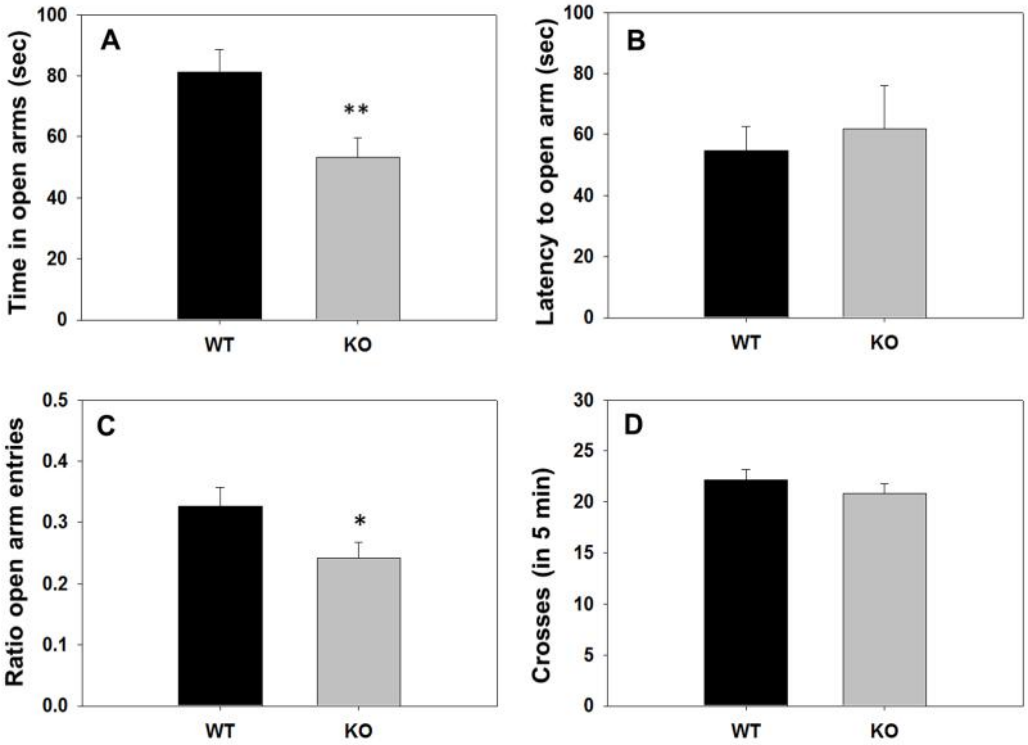

Fig 1. WT and KO mice performance in the elevated plus-maze. Data are expressed as mean (±SEM) of time spent in the open arms (**A**), latency to enter in one of the open arms for the first time (**B**), ratio of open arm entries versus total entries (**C**) and total crosses between arms (**D**). **p<0.01, *p<0.05 significant differences between strains.

Experiment 2: Anxiety in the EPM test.

Also in this test, KO mice displayed an anxiogenic profile in comparison with their WT counterparts. They spent significantly less time in the open arms ($t=-2.84$, $p<0.05$), and had a lower ratio of entries into the open arms compared to WT mice ($t=-2.15$, $p<0.05$) (**Fig. 2A** and **C**). No differences in latency to enter into an open arm for the first time ($U=49.0$, n.s.) (**Fig. 2B**) or in total number of crosses (**Fig. 2D**) were observed between the strains in this paradigm ($t=0.98$, n.s.). Thus, in both paradigms, locomotion does not seem to be the source of differences in anxiety.

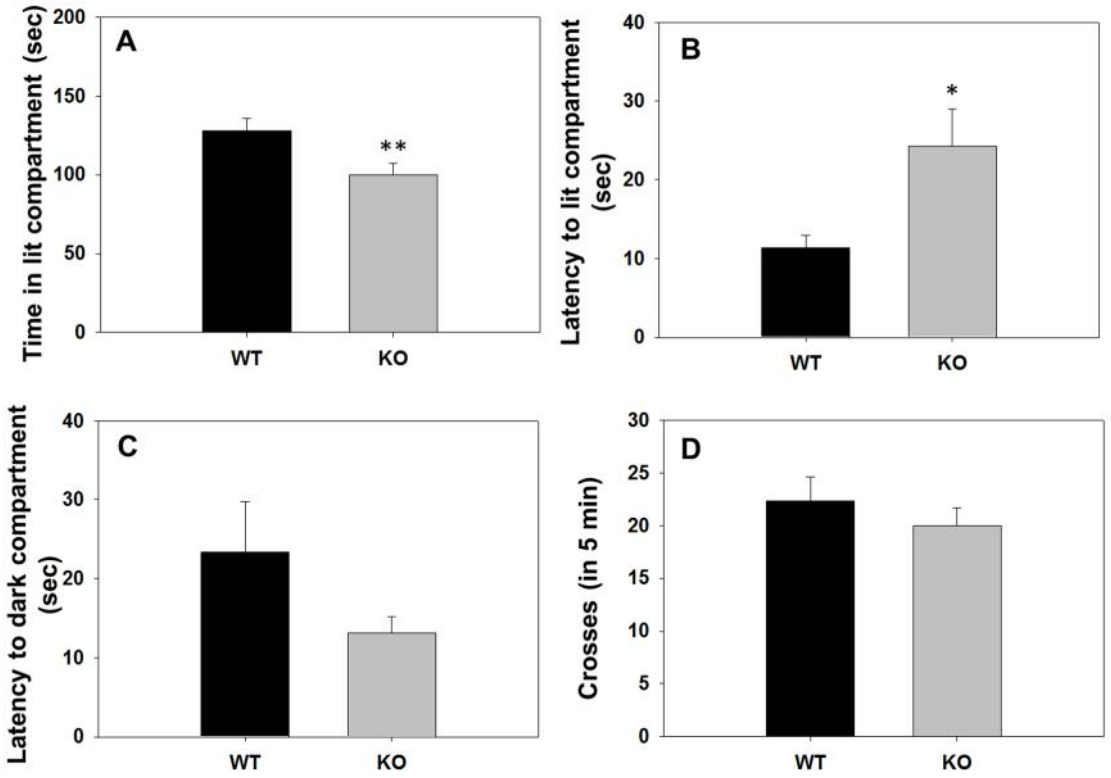

Fig 2.WT and KO mice performance in the dark and light box. Data are expressed as mean (±SEM) of time in lit compartment (**A**), latency to enter the lit compartment (**B**), latency back to the dark compartment (**C**) and number of crosses between compartments. **p<0.01, *p<0.05 significant differences between strains.

Experiment 3: Anxiety-perseverative behavior in the marble burying test.

The Mann-Whitney U test showed no differences between WT and KO mice in number of non-buried marbles in 5 minutes (U=116.5, n.s.), although there was a non-significant tendency of the KO mice to bury more marbles than WT in the same period of time **(Fig. 3A).**

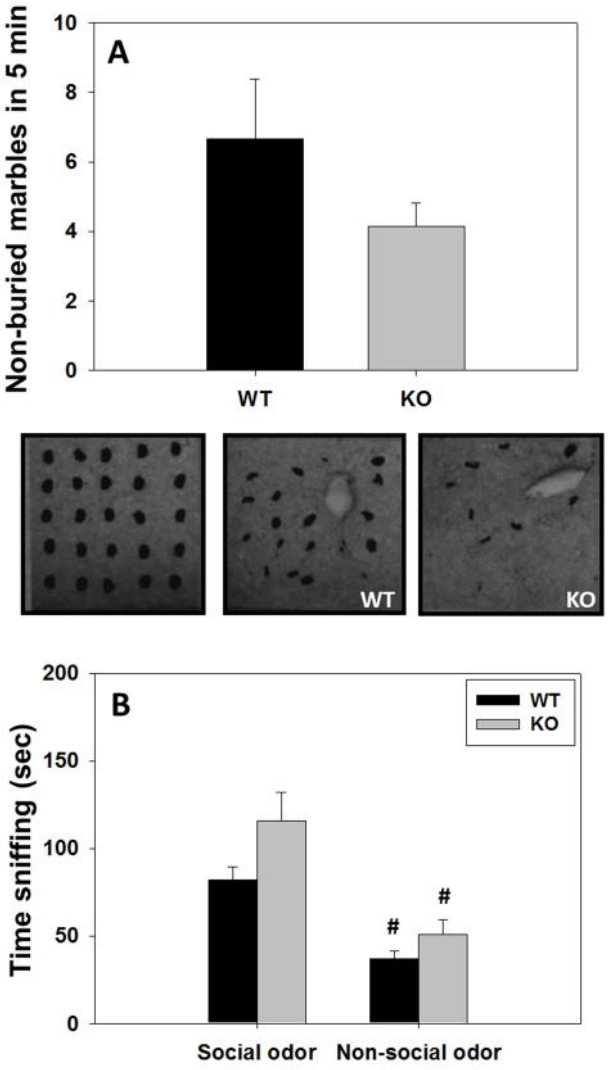

Fig 3. WT and KO mice performance in the marble burying test, and the odor preference test. Data are expressed as mean (±SEM) of number of non-buried marbles (**A**), and time sniffing the cottons with the social and non-social odors (**B**). #p<0.05 significant differences in time spent exploring the different odors in the same strain.

Experiment 4: Odor preference tests.

The Student's t-test showed that both strains of mice, KO and WT, spent significantly more time exploring social odors than non-social odors (t=-8,44, p<0.01, t=-5.83, p<0.01, respectively). Although there was a tendency for the KO mice to spend more time sniffing social odors than the WT mice, this effect did not reach statistical significance (t=-1.78, n.s.) (**Fig. 3B**).

Experiment 5: Social preference test.

In the social preference test, when comparing preference between stimuli for each strain, the Student's t-test for dependent samples showed that both types of mice spent

significantly more time sniffing a conspecific than an object; KO (t=7.36, p<0.01) and WT (t=3.23, p<0.05). They also remained in the compartment with the conspecific longer than in the object compartment; KO (t=3.80, p<0.01) and WT (t=2.44, p<0.05). Thus both WT and KO mice showed a clear social vs. non-social preference. In addition, Student's t-test for independent samples comparing both strains for each stimuli indicated that KO mice spent more time sniffing the conspecific compared to their WT counterparts (t= -2.32, p<0.05), and they also spent more time in the compartment with the conspecific than their WT counterparts (t=-2.03, p<0.05). There were no significant differences between strains in relation to object exploration (t=0.26, n.s). However, a Student's t-test for independent samples showed that KO mice spent less total time in non-social compartments (object plus middle compartments) than WT mice (t=-3.01, p<0.05). These results suggest that KO mice allocate more time into social exploration than WT mice. There was not a significant effect of the strain on horizontal (t=1.18, p= n.s) and vertical locomotion (t=0.82, p= n.s). For all these data see **Fig. 5A to 5D**.

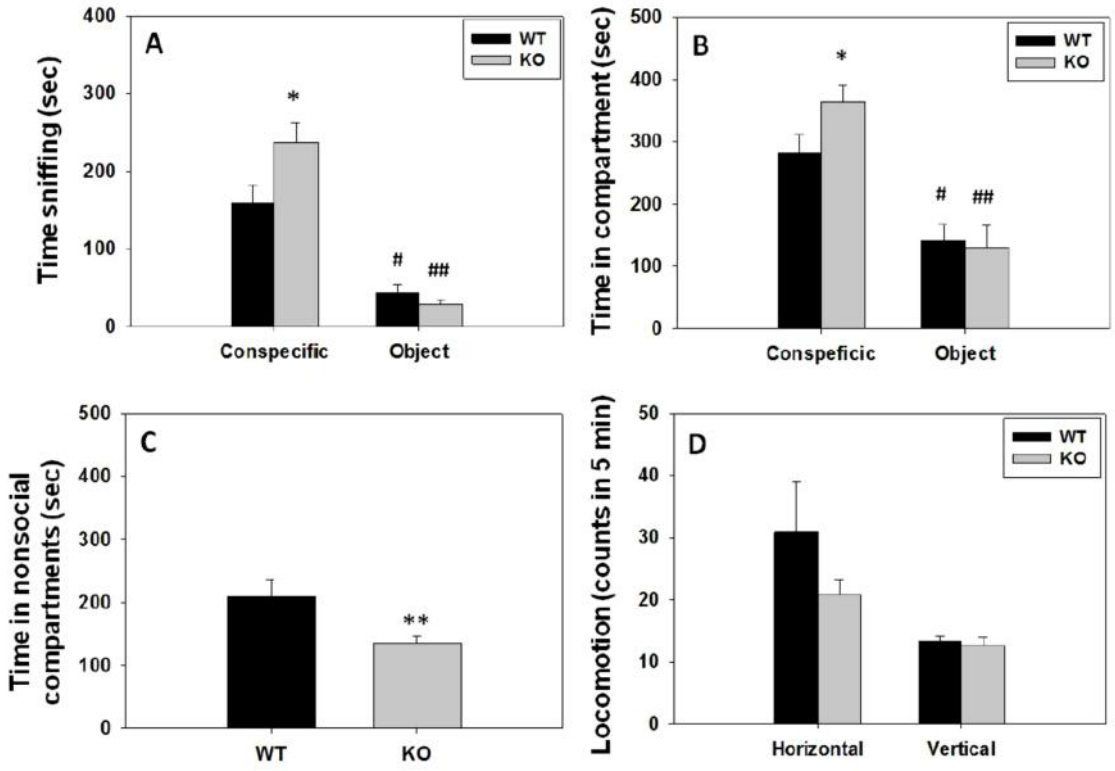

Fig 5. WT and KO mice performance in the social preference test. Data are expressed as mean (±SEM) of time sniffing the conspecific or the object (**A**), time spent in the compartments were the conspecific or the object are located (**B**), total time spent in non-social compartments (middle plus object) (**C**), and total

horizontal and vertical locomotion (**D**). *p<0.05, **p<0.01 significant differences between strains. #p<0.01, ##p<0.05 significant differences in the same strain.

Experiment 6: Social recognition test, and aggressive behavior.

In the social recognition test, WT mice spent more time sniffing a novel conspecific than a familiar one (t=-2.81, p<0.05), and the tendency was the same in the variable time in compartments (novel versus familiar conspecific), though it did not reach statistical significance (t=-1.84, n.s.). Thus, control mice displayed a normal recognition pattern. However, in KO mice, there were no differences either in time sniffing familiar versus novel conspecific (t=0.42, n.s.), or in time spent in those compartments (t=0.96, n.s.) (**Fig. 6A and B**). When comparing between strains, the Student's t-test showed that KO mice spent more time sniffing the familiar conspecific (t=-2.33, p<0.05), and remained more time in the familiar conspecific compartment (t=-2.61, p<0.05) in comparison with WT mice, showing again a higher level of social exploration. The Mann-Whitney U test did not show differences between WT and KO mice in threat behavior evaluated as the number of total tail-rattling behaviors during the social recognition test; neither during interaction with familiar conspecifics, nor with novel mice (U=26.00, n.s., U=20.5, n.s.) (**Fig. 6C**). No differences in total crosses and rearing were observed between strains (t=2.08, n.s.; t=1.78, n.s., respectively) (Fig.6D).

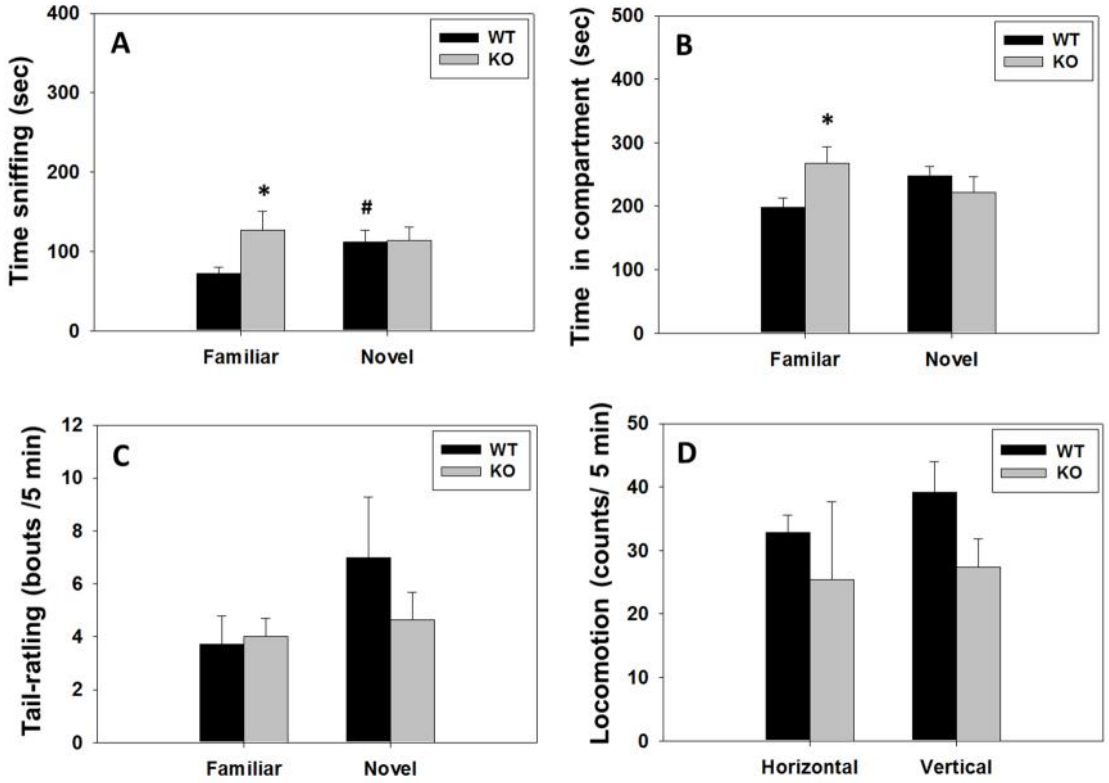

Fig 6. WT and KO mice performance in the social novelty test. Data are expressed as mean (±SEM) of time sniffing the familiar or the novel conspecifics (**A**), time spent in compartments (**B**), number or tail ratling bouts during conspecific exploration (**C**), and total horizontal and vertical locomotion (**D**). *p<0.05, significant differences between strains. #p<0.05 significant differences in the same strain.

Experiment 7: c-Fos immunoreactivity in different brain areas.

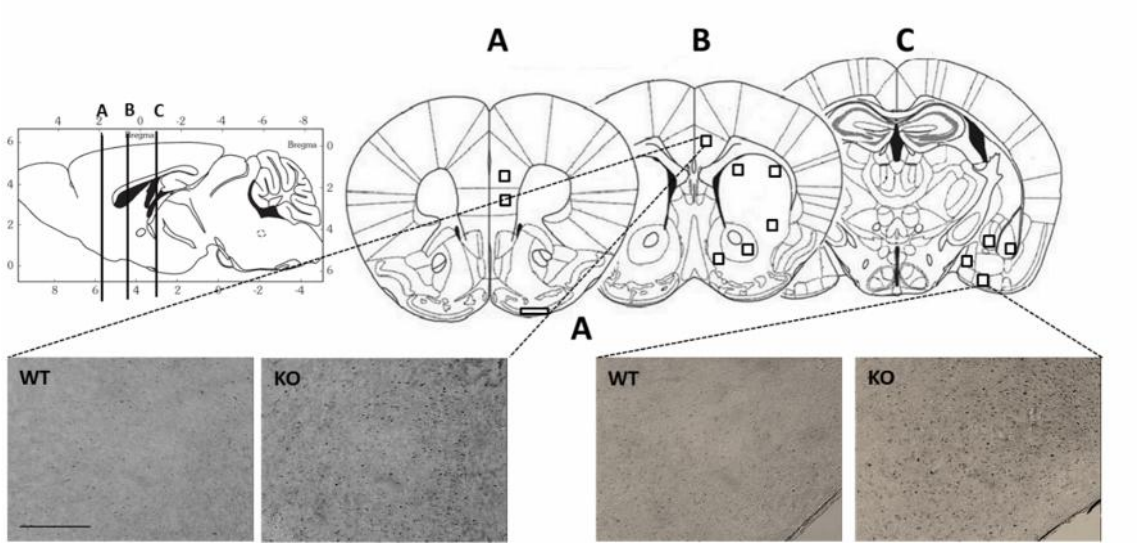

Fig 7. Left part: Sagital plane of the mouse brain with bregma coordinates: 1.94 mm (**A**), 1.18 mm (**B**) and -1.34 mm (**C**). Right part: Diagram of coronal sections with bregma coordinates (**A**, **B** and **C**)

showing location of the brain areas for c-Fos counting taken from Franklin and Paxinos, 2007. Lower part: Photomicrographs of c-Fos immunoreactivity staining in ACg and ACo from representative WT and KO animals. Images at 20x, scale bar = 250 μm.

Differences in c-Fos immunoreactivity between WT and KO mice were assessed in brain areas rich in A_{2A} receptors, some of which are traditionally implicated in social exploration. These data are shown in **Table 1 and Fig. 7.** When analyzing data for c-Fos immunoreactivity in brain regions such as all prefrontal cortex, all striatum and all amygdala, there were no significant differences between strains either in cortical or in striatal regions (t=-1.66, n.s., t=-1.17, n.s. respectively). However, in amygdala KO mice showed higher c-Fos immunoreactivity than their WT counterparts (t=-2.47, $p<0.05$).

Separate analysis for every specific brain area showed significant differences in c-Fos staining between WT and KO mice in ACg. KO mice showed significantly more c-Fos immunoreactivity than WT in this region (t=-2.24, $p<0.05$). However no differences between strains were observed in the other cortical regions explored, PrL and IL (t=-0.88, n.s; t=-0.72, n.s, respectively). There were no differences in c-Fos immunoreactivity in regions of dorsal striatum; DMS and DLS (t=0.44, n.s. and t=0.75 n.s., respectively), or ventral striatum; AcbC and AcbSh (t=0.95, n.s. and t=1.00, n.s., respectively). Although KO mice showed almost double c-Fos staining in the OT, this difference did not reach statistical significance (t=-1.64, n.s). The same pattern was observed in specific amygdala regions, in which no differences were seen between WT and KO in c-Fos expression in individual nuclei; BLA, CeA, MeA, or ACo (t=-0.54, n.s; t=-0.44, n.s; t=-1.50, n.s; t=1.49, n.s, respectively).

	Strain	
Brain area	**WT**	**KO**
Prefrontal Cortex		
ACg	61.5 ± 14.9	100.0 ± 8.6*
PrL	21.7 ± 3.2	32.4 ± 11.8
IL	23.2 ± 5.3	33.3 ± 13.0
Striatum		
DMS	53.8 ± 13.7	38.6 ± 12.8
DLS	23.7 ± 5.9	21.3 ± 4.6
AcbSh	21.7 ± 4.3	21.7 ± 6.8
AcbC	27.4 ± 5.7	27.9 ± 7.1
Olfactory system		
OT	7.6 ± 2.8	14.5± 3.2
Amygdala *		
BLA	9.1 ±1.8	18.24 ± 5.2
CeA	18.6 ± 6.9	23.52 ± 8.7
MeA	34.5 ± 12.3	58.80 ± 9.4
ACo	26.9 ± 4.9	54.5 ± 17.8

TABLE 1. c-Fos immunoreactivity in several brain areas of WT and KO mice (N=5-6 per group). Mean (±SEM) number of c-Fos positive cells per mm^2. *p<0.05 significant differences between strains. ACg anterior cingulated cortex; PrL, prelimbic cortex; IL, infralimbic cortex; DMS, dorsomedial striatum; DLS, dorsolateral striatum; AcbSh, nucleus accumbens shell; AcbC, nucleus accumbens core; OT, Olfactory tubercle; BLA, basolateral nucleus of amygdala; CeA, central nucleus of amygdala; MeA, medial nucleus of amygdala; ACo, anterior cortical nucleus of the amygdala.

Experiment 8: Effect of an anxiolytic dose of ethanol on social preference.

After receiving an anxiolytic dose of ethanol (1.0 g/kg) both strains behave similarly in the social preference test **(Fig. 8A and B)**. A Student's t-test for dependent samples showed that both strains spent significantly more time sniffing a conspecific than an object; WT ($t=6.84$, $p<0.01$) and KO ($t=4.01$, $p<0.01$). In addition, WT mice remained in the compartment with the conspecific longer than they did in the object compartment

(t=3.51, p<0.01). This tendency was also observed in KO mice, however it did not reach statistical significance (t=1.72, n.s.). When comparing both strains, the Student's t-test for independent samples showed no differences between WT and KO mice after receiving ethanol, neither in time sniffing the conspecific (t=0.35, n.s.), nor in time exploring the object (t=-1.45, n.s.). Both strains also were not different in time spent in compartments (conspecific and object, t=0.41 n.s., t=-1.34, n.s., respectively). Time in non-social compartments (middle plus object compartment, **Fig. 8C**) was not different between both strains after ethanol administration (t=0.01, n.s.). Thus, both strains showed a clear but equal social preference after ethanol administration. Horizontal locomotion after receiving ethanol was significantly lower in KO than in WT (t=2.60, p<0.05), but no differences were observed in vertical locomotion (t=1.49, n.s) **(Fig. 8D).**

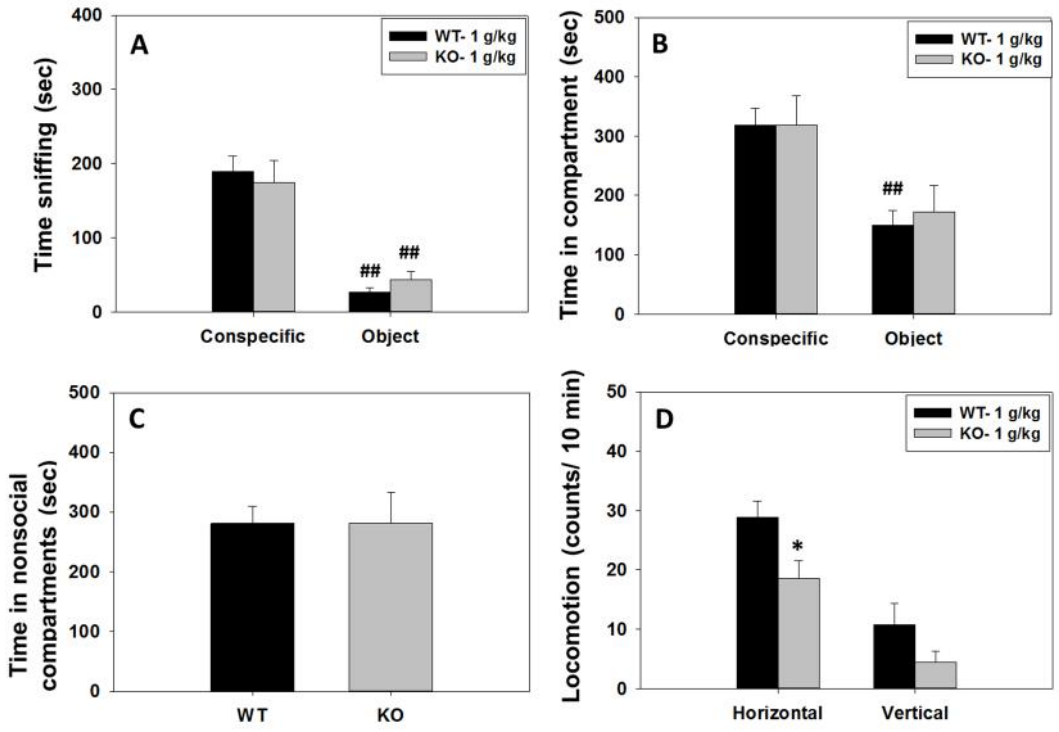

Fig 8. WT and KO mice performance in the social preference test after receiving 1.0 g/kg of ethanol. Data are expressed as mean (±SEM) of time sniffing the conspecific or the object (**A**), time spent in the compartments were the conspecific or the object are located (**B**), total time spent in non-social compartments (middle plus object compartments) (**C**), and total horizontal and vertical locomotion (**D**).

Experiment 9: Effect of an anxiolytic dose of ethanol administered before the preference test on social recognition the following day.

Ethanol produced recognition memory impairments in both strains of mice, since both WT and KO mice spent the same amount of time (or even more) with the familiar conspecific compared to the novel one. Thus, Student's t-test for dependent samples revealed that WT mice spent significantly more time sniffing the familiar conspecific than the novel conspecific ($t=2.91$, $p<0.05$, **Fig. 9A**), and there was no differences in time in compartments (familiar vs. novel) ($t=0.82$, lllln.s., **Fig. 9B)**. KO mice spent similar time sniffing novel and familiar conspecifics ($t=1.11$, n.s.), and they spent more time in the familiar conspecific compartment than in the novel conspecific compartment ($t=2.74$, $p<0.05$), which suggests a lack of recognition. When comparing between strains, KO mice spent significantly more time sniffing the novel conspecific than did the WT mice ($t=-2.91$, $p<0.01$), although there were no differences in time spent in the novel conspecific compartment ($t=0.15$, n.s.). There were no differences between strains in time sniffing the familiar conspecific ($t=-0.90$, n.s.), however, KO mice spent significantly more time than their WT counterparts ($t=-2.43$, $p<0.05$) in that compartment. Thus, KO mice had a tendency to spend more time with both conspecifics compared to WT animals. Finally, a dose of ethanol administered 24 hrs before seemed to have an impact on vertical locomotion ($t=2.02$, $p<0.05$) in KO mice, however horizontal locomotion was not affected ($t=1.74$, n.s).

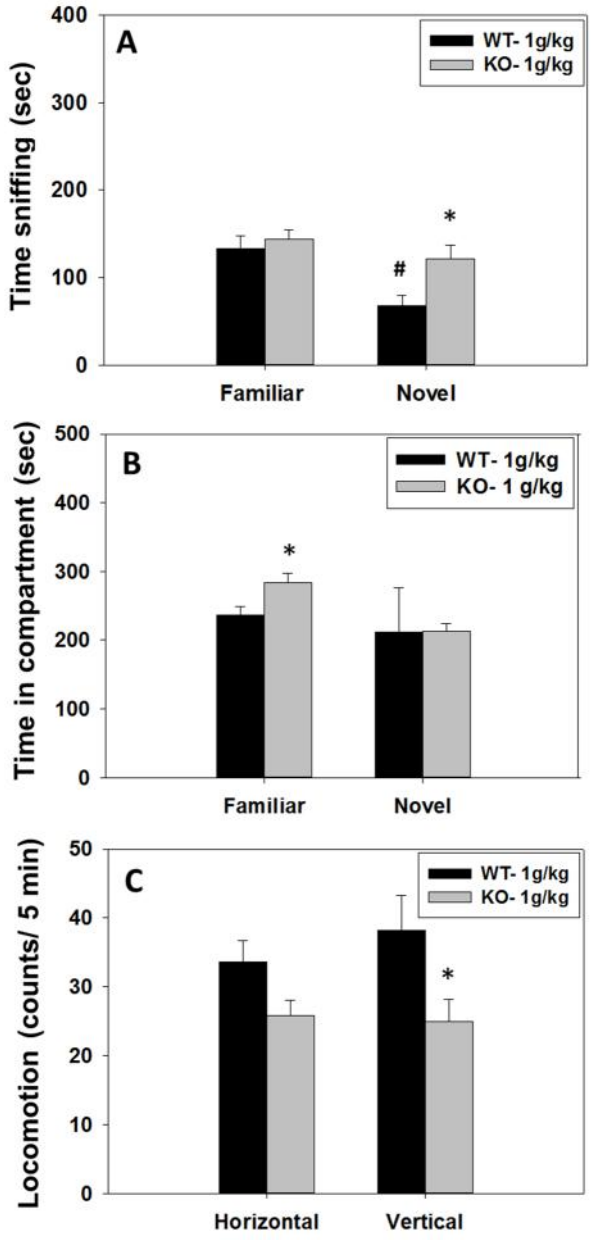

Fig 9. WT and KO mice performance in the social novelty test after receiving 1.0 g/kg of ethanol. Data are expressed as mean (±SEM) of time sniffing the familiar or the novel conspecifics (**A**), time spent in compartments (**B**), and total horizontal and vertical locomotion (**C**). *p<0.05 significant differences between strains. #p<0.05 significant differences in the same strain.

Discussion

The present study evaluated anxiety and social behavior patterns in A_{2A}KO mice. The results demonstrate that the lack of A_{2A} receptors induces an anxiogenic pattern of behavior that does not seem to impair social behaviors. Thus A_{2A}KO mice, in comparison with WT mice, were more anxious in the DL box, delaying the first entrance into the lit compartment, and spending less time there. The same pattern of results was observed in the EPM, in which A_{2A}KO mice spent less time in the open arms and had a lower ratio of entries to the open arms compared with total entries. Our results in the anxiety paradigms are in agreement with previous studies (Ledent et al. 1997; Berrendero et al. 2003). In spite of been more anxious, A_{2A}KO mice are more

sociable than WT mice; they spent more time exploring or in proximity to conspecifics. Traditionally, social interaction paradigms in rodents have been used as models of anxiety (File & Hyde, 1978; File & Seth, 2003). Thus, increases in social interaction after drug administrations have been interpreted as reflecting anxiolytic actions, whereas a specific decrease in social interaction has been considered as being due to an anxiogenic effect (File & Seth, 2003). However, this correspondence between anxiety levels and social interaction is not always clear (Egashira et al., 2007), as it has been shown by the present results.

Social preference and social novelty tests in the three-chamber social paradigm provide information about central aspects of social behavior, such as social motivation or sociability, as well as novelty seeking or recognition of conspecifics. Sociability in this case is defined as propensity to spend time with another conspecific compared to time spent alone (Moy et al., 2004) or with an object in our case. Preference for social novelty is defined as propensity to spend time with a novel conspecific and can be interpreted in terms of novelty seeking (Costa et al., 2014), or also of recognition and memory of the already know animal (Moy et al., 2004; Crawley, 2004). In our study, A_{2A}KO mice showed poor recognition of a familiar mouse, allocating equal amounts of time exploring both conspecifics. This lack of social recognition could not be explained by deficits in spatial memory, since A_{2A}KO mice have previously been shown to have better results in spatial memory tasks such as the Y test in comparison with WT mice (Wang et al. 2006). Although greater exploration of a novel versus familiar conspecific is a normal pattern of social recognition in rodents (Moy et al., 2004), it is possible that since these mice seem more sociable they do not show distinctive preferences when the two stimuli are conspecifics.

Lower horizontal and vertical activity has been previously reported for A_{2A}KO mice in an open field (Ledent et al. 1997; Chen et al. 2007; Berrendero et al. 2003; Pardo et al. 2013). However, in the present study, no significant differences in baseline locomotion were observed between A_{2A}KO mice and their WT counterparts in any of the paradigms. Thus, differences between WT and A_{2A}KO mice in this social task do not seem to be influenced by differences in motor parameters leading to less exploration of the chambers.

A number of factors could be influencing this potentiated sociability observed in the A_{2A}KO mice. More aggressive tendencies, avoidance of non-social odors, or repetitive and perseverative behaviors when checking other conspecifics, all could underlie an apparent increase in sociability. For that reason we explored those behaviors. In the marble test, A_{2A}KO mice did not differ from WT controls, although the KO animals did show a tendency to bury marbles in 5 minutes. Pharmacological studies established this paradigm as a model of anxiety-related behavior (Broekkamp et al., 1986), although it also has been suggested as test for preservative or repetitive behavior (Thomas et al., 2009). Is possible that our conditions generated high levels of anxiety in both strains in this paradigm, making difficult to observe clear differences in burying behavior. Odor cues are known to be important for rodents in different contexts (Schellinck & Brown, 1998) and many behavioral tasks designed for mice depend on these cues. Olfactory deficits could interfere with performance in our social tests and produce false positive results. Thus, accurate assessment of olfaction is critical for proper interpretation of mice behaviors within the social domain (Yang and Crawley, 2009). In addition, A_{2A} receptors are highly concentrated in the olfactory tubercle and olfactory bulbs (Kaelin-Lang et al., 1999; Vontell et al., 2010). In experiment 4 of the present study, when a social odor (rubbed in a cotton ball) was presented concurrently to a non-social odor (floral odor in a cotton ball) in the three-chambered box, all animals spent more time exploring the social odor, and no differences in non-social odor exploration were observed between WT and A_{2A}KO mice. There was a tendency for A_{2A}KO to spend more time than WT sniffing social cues, which could suggest a stronger sociability in KO mice, though this effect was not significant. Previous studies have demonstrated that A_{2A}KO mice displayed an increased number of attacks and tail rattles, as well as a decreased latency to attack the intruder in the resident-intruder test of aggression (Ledent et al., 1997). However, no differences between WT and A_{2A}KO mice were observed in our experiment when evaluating the number of tail rattles as a measure of basal levels of threat behavior towards the cage enclosed conspecific, possibly because our setting did not potentiate aggressive behaviors.

Analyses of the c-Fos immunoreactivity data indicated that A_{2A}KO mice showed greater neuronal activation in brain regions that are important for the regulation of social behavior. Amygdala is a region in which adenosine A_1 and A_{2A} receptors have been identified (Brass et al., 1986; Svenningsson et al., 1997, 1999; Rosin et al., 1998), and

this region, especially the medial nucleus, has been implicated in processing social information in humans (Critchley et al., 2000) and in rodents (Young et al., 2002). In our study, there was greater c-Fos immunoreactivity in A_{2A}KO mice compared with WT mice in the amygdala. OT is an area rich in A_{2A} receptors that also is very important for social behavior in rodents (Wesson et al., 2011), which receives inputs from the accessory and main olfactory systems (Ubeda-Bañona et al., 2007; Martinez-Marcos, 2009). Although there was a tendency for the number of c-Fos positive cells in the OT to be higher in A_{2A}KO compared to WT animals, there was not a significant difference. However, A_{2A}KO mice did show significantly higher levels of c-Fos immunoreactivity in ACg compared to WT animals. ACg is an important prefrontal area that is involved in the regulation of aspects of motivation (Schweimer et al., 2005). Allocating more time into social exploration (as the KO mice did) indicates increased preference, which is an index of the directional component of motivation. ACg has also been implicated in enhancing stimulus discrimination (Schweimer et al., 2005).

Previous studies using WT and A_{2A}KO mice with the same CD1 background as the ones used in the present study have shown how A_{2A}KO mice have increased sensitivity to the anxiolytic effects of low doses of ethanol (Houchi et al. 2008). Thus, we tried to assess if a low dose of ethanol that has been shown to be anxiolytic in CD1 mice in previous studies (Correa et al., 2008) can regulate social interaction in A_{2A}KO mice. Although ethanol did not change social exploration in WT mice, it did eliminate differences between WT and A_{2A}KO animals. Since ethanol increases adenosine levels (see López-Cruz et al., 2013), it is possible that an ethanol-induced increase in adenosine tone can counteract the effects of reduced A_{2A} receptor transmission in KO animals, possibly by acting on A_1 receptors. An increase in adenosine after receiving ethanol can also explain a reduction in locomotion in A_{2A}KO mice seen in the social test. Thus, our studies with A_{2A}KO mice do not support the idea of a simple and direct relationship between anxiety and social interaction in these mice, since ethanol reduced social exploration in A_{2A}KO mice but it induced anxiolysis in these animals (Houchi et al. 2008). In addition, ethanol disrupted social recognition the following day in both strains, with a bigger impact on WT animals that spent less time with the novel animals than the familiar ones. A_{2A}KO mice spent equal time with both conspecifics, suggesting that they had a higher preference for social contact overall.

Thus, the present results suggest that the A_{2A} receptors are potential targets for the regulation of social function, an aspect of behavior that seems to be affected in a variety of neuropsychiatric disorders (Landau et al., 1991; Mueser et al., 1991). Future studies should investigate pharmacological antagonists with different selectivity profiles for A_1 and A_{2A} receptors in order to elucidate the role of the adenosine system in socially motivated behaviors. Moreover, since A_{2A} receptors are localized in regions that also are rich in neuropeptides such as oxytocin and vasopressin, which are important for the establishment of social attachment (Tobin et al., 2010; Ferguson et al., 2011), A_{2A} receptors could be also important for modulating the actions of those neuropeptides.

References

Baldwin, H.A., Johnston, A.L., File, S.E.(1989) Antagonistic effects of caffeine and yohimbine in animal tests of anxiety. *Eur J Pharmacol* **159(2**), 211-215.

Berrendero, F., Castane, A., Ledent, C., Parmentier, M., Maldonado, R. & Valverde, O. (2003) Increase of morphine withdrawal in mice lacking A2a receptors and no changes in CB1/A2a double knockout mice. *Eur J Neurosci* **17**, 315–324.

Bluthe, R.M. & Dantzer R. (1993). Role of the vomeronasal system in vasopressinergic modulation of social recognition in rats. Brain research. 604, 1-2.

Bortolato, M., Chen, K., Godar, S. C., Chen, G., Wu, W., Rebrin, I., et al. (2011). Social Deficits and Perseverative Behaviors, but not Overt Aggression, in MAO-A Hypomorphic Mice. *Neuropsychopharmacology* **36(13),** 2674-2688.

Brass, K.M., Newby, A.C., Wilson, V.S., & Snyder, S.H. (1986). Adenosine-containing neurons in the brain localized by immunocytochemistry. Journal of Neuroscience. **6**,1952-1961.

Broekkamp, C.L., Rijk, H.W., Joly-Gelouin, D., & Lloyd, K.L. (1986) Major tranquillizers can be distinguished from minor tranquillizers on the basis of effects on marble burying and swim-induced grooming in mice. Eur J Pharmacol. **126(3)**,223-229.

Chen, J.F., Sonsalla, P.K., Pedata, F., Melani, A., Domenici, M.R., Popoli, P., Geiger, J., Lopes, L.V. & de Mendonca, A. (2007) Adenosine A2A receptors and brain injury: broad spectrum of neuroprotection, multifaceted actions and "fine tuning" modulation. *Prog Neurobiol* **83**, 310–331.

Chévere-Torres, I., Maki, J.M., Santini, E. & Klann, E. (2012) Impaired social interactions and motor learning skills in tuberous sclerosis complex model mice expressing adominant/negative form of tuberin. *Neurobiol Dis* **45**, 156–164.

Correa, M. & Font, L. (2008) Is there a major role of A2A adenosine receptor in anxiety? *Front Biosci* **13**, 4058–4070.

Correa, M., Manrique, H.M., Font, L., Escrig, M.A., Aragon, C.M.G. (2008) Reduction in the anxiolytic effects of ethanol by centrally formed acetaldehyde: the role of catalase inhibitors and acetaldehyde-sequestering agents. *Psychopharmacology* **200 (4)**,455-464.

Costa, V.D., Tran, V.L., Turchi, J. & Averbeck, B.B.(2014). Dopamine modulates novelty seeking behavior during decision making. *Behav Neurosci*.**128(5**),556-66.

Crawley, J.N. (2004) Designing mouse behavioral tasks relevant to autistic-like behaviors. *Ment Retard Dev Disabil Res Rev* **10**, 248–258.

Critchley, H.D., Daly, E.M., Bullmore, E.T., Williams, S.C.R., Amelsvoort, T., Robertson, D.M, Rowe, A., Phillips, M., McAlonan, G., Howlin, P. & Murphy, D.G. (2000) The functional neuroanatomy of social behavior: Changes in cerebral blood flow when people with autistic disorder process facial expressions. *Brain* **123**, 2203–2212.

Doty, R.L. (1986). Odor-guided behavior in mammals. *Experientia* 42, 257–271.

Dunwiddie, T.V. & Worth, T. (1982) Sedative and anticonvulsant effects of adenosine analogs in mouse and rat. *J Pharmacol Exp Ther* **220**, 70–76.

Egashira, N., Tanoue, A., Matsuda, T., et al. (2007). Impaired social interaction and reduced anxiety-related behavior in vasopressin V1a receptor knockout mice. *Behav Brain Res* ***178*** **(1)**,123-127.

Ferguson JN, Aldag JM, Insel TR, & Young LJ. (2001) Oxytocin in the medial amygdala is essential for social recognition in the mouse. J *Neurosci* **15**;21:8278-85.

Ferré, S., Ciruela, F., Canals, M., et al. (2004) Adenosine A2A-dopamine D2 receptor-receptor heteromers. Targets for neuro-psychiatric disorders. *Parkinsonism Relat Disord*. **10,**265-71.

File, S. E., & Hyde, J. R. (1978). Can social interaction be used to measure anxiety? *British Journal of Pharmacology* ***62*****(1)**, 19-24.

File, S.E. & Seth, P. (2003) A review of 25 years of the social interaction test. *Eur J Pharmacol* **463**, 35–53. Review.

Florio, C., Rosati, A.M., Traversa, U. & Vertua, R. (1997) Inhibitory and excitatory effects of adenosine antagonists on spontaneous locomotor activity in mice. *Life Sci* **60**, 1477–1486.

Franklin, K. B. J &., Paxinos G. (2007). The Mouse Brain in Stereotaxic Coordinates, 3rd Edn. Elsevier Academic Press. San Diego.

Fredholm, B.B., Ijzerman, A.P., Jacobson, K.A., Klotz, K.N. & Linden, J. (2001) International Union of Pharmacology. XXV. Nomenclature and classification of adenosine receptors. *Pharmacol Rev* **53**, 527–552.

Hilakivi, L.A., Durcan, M.J. & Lister, R.G. (1989) Effects of caffeine on social behavior, exploration and locomotor activity: interactions with ethanol. *Life Sci* **44**, 543–553.

Houchi, H., Warnault, V., Barbier, E., Dubois, C., Pierrefiche, O., Ledent, C., Daoust, M., & Naassila, M. (2008) Involvement of A2A receptors in anxiolytic, locomotor and motivational properties of ethanol in mice. *Genes Brain Behav* **7(8)**,887-898.

Jarvis, M.F. & Williams, M.(1989). Direct autoradiographic localization of adenosine A_2 receptors in the rat brain using the A_2-selective agonist, [3H]CGS 21680. *Eur J Pharmacol*.**168(2),**243-246.

Kaelin-Lang, A., Lauterburg, T. & Burgunder JM.(1999). Expression of adenosine A2a receptors gene in the olfactory bulb and spinal cord of rat and mouse. Neurosci Lett. **261(3)**, 189-191.

Krsiak, M. (1979) Effects of drugs on behaviour of aggressive mice. *Br J Pharmacol* **65**, 525–533.

Landau, S. & Moore, L.A. (1991). Social skill deficits in children with attention-deficit hyperactivity disorder. *School Psychology Review* **20(2),** 235-251.

Ledent, C., Vaugeois, J.M., Schiffmann, S.N., Pedrazzini, T., El Yacoubi, M., Vanderhaeghen, J.J., Costentin, J., Heath, J.K., Vassart, G. & Parmentier, M. (1997) Aggressiveness, hypoalgesia and high blood pressure in mice lacking the adenosine A2a receptor. *Nature* **388,** 674–678.

Liebenauer, L.L. & Slotnick BM.(1996) Social organization and aggression in a group of olfactory bulbectomized male mice. *Physiol Behav* **60(2),**403-409.

López-Cruz L., Salamone, J.D., Correa, M.(2013) The Impact of Caffeine on the behavioral Effects of Ethanol Related to Abuse and Addiction: A Review of Animal Studies. *J Caffeine Res* 3(1),9-21.

Manrique, H.M., Miquel, M.,& Aragon, C.M. (2005) Brain catalase mediates potentiation of social recognition memory produced by ethanol in mice. *Drug Alcohol Depend* **79(3)**, 343-350.

Moy, S.S., Nadler, J.J., Perez, A., Barbaro, R.P., Johns, J.M., Magnuson, T.R., Piven, J. & Crawley, J.N. (2004) Sociability and preference for social novelty in five inbred strains: an approach to assess autistic-like behavior in mice. *Genes Brain Behav* **3**, 287–302.

Mueser, K.T., Bellack, A.S., Douglas, M.S. & Morrison, R.L. (1991) Prevalence and stability of social skill deficits in schizophrenia. *Schizophr Res* **5**, 167–176.

Nadal, R.A., Pallares, M.A., & Ferré, N.S. (1993) The effects of caffeine in the social interaction test and on exploration in rats: comparison with ethanol and clorazepate. *Behav Pharmacol* **4**, 501-508.

Nagy, L.E., Diamond, I., Casso, D.J., Franklin, C. & Gordon, A.S. (1990) Ethanol increases extracellular adenosine by inhibiting adenosine uptake via the nucleoside transporter. *J Biol Chem* **265**, 1946–1951.

Pardo, M., Lopez-Cruz, L., Valverde, O., Ledent, C, Baqi. Y, Müller, C.E., Salamone, J.D., & Correa, M. (2013). Effect of subtype-selective adenosine receptor antagonists on basal or haloperidol-regulated striatal function: studies of exploratory locomotion and c-Fos immunoreactivity in outbred and A2AR KO mice. *Behav Brain Res* **247**,217-226.

Pinel, J.P.J. & Treit, D. (1978) Burying as a defensive response in rats. *J Comp Physiol Psychol* **92**, 708–712.

Prediger, R.D. & Takahashi, R.N. (2005) Modulation of short-term social memory in rats by adenosine A1 and A(2A) receptors. *Neurosci Lett* **376**, 160–165.

Rosin, D.L., Robeva, A., Woodard, R.L., Guyenet, P.G., & Linden, J. (1998) Immunohistochemical localization of adenosine A_{2A} receptors in the rat central nervous system. Journal of Comparative Neurology. **401**,163–186.

Salamone, J.D. & Correa, M.(2009). Dopamine/adenosine interactions involved in effort-related aspects of food motivation. *Appetite*. **53**, 422-525.

Sano, H., Nagai, Y., Miyakawa, T., Shigemoto, R. & Yokoi, M. (2008) Increased social interaction in mice deficient of the striatal medium spiny neuron-specific phosphodiesterase 10A2. *J Neurochem* **105**, 546–556.

Schellinck, H.M. & Brown, R. (1998) Searching for the source of urinary odors of individuality in rodents. In Johnston, R.E., Müller-Schwarze, D. & Sorensen, P. (eds),

Advances in Chemical Signals in Vertebrates. Kluwer Academic/Plenum Press, New York, pp. 267–280.

Schiffmann, S. N., Fisone, G., Moresco, R., Cunha, R. & Ferré, S. (2007) Adenosine A_{2A} receptors and basal ganglia physiology. *Prog Neurobiol* **83**, 277–292.

Schweimer, J., Saft, S. & Hauber, W. (2005). Involvement of catecholamine neurotransmission in the rat anterior cingulate in effort-related decision making. *Behav Neurosci* **119**, 1687–1692.

Svenningsson, P., Hall, H., Sedvall, G., & Fredholm, B.B. (1997). Distribution of adenosine receptors in the postmortem human brain: an extended autoradiographic study. Synapse. **27**,322–335.

Svenningsson, P., Le Moine, C., Fisone, G. & Fredholm, B.B. (1999) Distribution, biochemistry and function of striatal adenosine A2A receptors. *Prog Neurobiol* **59**, 355–396.

Thomas, A., Burant, A., Bui, N., Graham, D., Yuva-Paylor, L.A. & Paylor, R. (2009) Marble burying reflects a repetitive and perseverative behavior more than novelty-induced anxiety. *Psychopharmacology (Berl)* **204**, 361–373.

Tobin, V. A., Hashimoto, H., Wacker, D. W., Takayanagi, Y., Langnaese, K., & Caquineau, C., Ludwig, M. (2010). An intrinsic vasopressin system in the olfactory bulb is involved in social recognition. *Nature* ***464*****(7287**), 413–417.

Ubeda-Bañona I., Novejarque A., Mohedano-Moriano A., Pro-Sistiaga P., de la Rosa-Prieto C., Insausti R., Martinez-Garcia F., Lanuza E. & Martinez-Marcos A. (2007). Projections from the posterolateral olfactory amygdala to the ventral striatum: neural basis for reinforcing properties of chemical stimuli. *BMC Neurosci*. **8**, 103.10.1186/1471-2202-8-103

Vontell, R., Segovia, K.N., Betz, A.J., Mingote, S., Goldring K., Cartun, W.R. & Salamone, J.D. (2010). Immunocytochemistry Studies of Basal Ganglia Adenosine A_{2A} Receptors in Rat and Human Tissue. *Journal of Histotechnology*. 1(33), 41-47.

Wang, J.H., Ma, Y.Y. & van den Buuse, M. (2006) Improved spatial recognition memory in mice lacking adenosine A2A receptors. *Exp Neurol* **199**, 438–445.

Wesson, D. W., & Wilson, D. A. (2011). Sniffing out the contributions of the olfactory tubercle to the sense of smell: hedonics, sensory integration, and more? *Neuroscience and Biobehavioral Reviews*, ***35*****(3),** 655–668.

Yang, M. & Crawley, J.N. (2009) Simple behavioral assessment of mouse olfaction. *Curr Protoc Neurosci* Chapter 8:Unit 8.24.

Young, L.J. (2002) The neurobiology of social recognition, approach, and avoidance. *Biol Psychiatry* **51**, 18–26.

Zarrindast, M.R. & Shafaghi, B. (1994) Effects of adenosine receptor agonists and antagonists on acquisition of passive avoidance learning. *Eur J Pharmacol* **256**, 233–239.

CHAPTER 5:

Does caffeine have a therapeutic role in depression?: relevance for the treatment of motivational symptoms.

Abstract

Major depressive disorder is one of the most common and debilitating psychiatric conditions. Some of its motivational symptoms, such as anergia (lack of self-reported energy) and fatigue, are resistant to traditional treatments with serotonin uptake inhibitors, so new pharmacological targets are being investigated. Limited epidemiological data indicate that caffeine consumption has an impact in some aspects of depressive symptomatology. In animal studies, drugs that act on adenosine receptors are being assessed for their effects on the modulation of behavioral functions related to depression. Caffeine is a non-selective adenosine antagonist that binds to both A_1 and A_{2A} receptors, and has been shown to modulate behavior in classical animal models of depression. This review focuses on the effects of caffeine and selective adenosine antagonists on different aspects of depression in humans, as well as in animal models. The effects of caffeine on motivational symptoms of depression such as anergia and psychomotor slowing receive particular attention. In that regard, the ability of caffeine to reverse the anergia induced by dopamine antagonism or depletion is of special interest. In conclusion, it appears that caffeine and adenosine antagonists could have potential as therapeutic agents for the treatment of motivational dysfunction in depression.

KEYWORDS: caffeine, depression, adenosine, anergia, fatigue, amotivation, anxiety.

Major Depression Disorder: symptomatology and current treatment

Major depression disorder (MDD) is one of the most debilitating psychiatric disorders in the world (World Health Organization, 2004), and the most commonly diagnosed (American Psychiatric Association, 2013). The Diagnostic and Statistical Manual (DSM) in its last edition (DMS-V) defines this disorder as a set of symptoms that include depressed mood, decreased interest or pleasure in almost all activities nearly every day, appetite changes (changes in body weight), sleep disturbances, feelings of worthlessness or guilt, diminished ability to concentrate or indecisiveness, psychomotor agitation or retardation and fatigue or loss of energy (American Psychiatric Association, 2013).

Symptoms such psychomotor retardation, fatigue and loss of energy are related to the activational component of motivation. Motivated behavior is directed towards or away from particular stimuli, but it also is characterized by a high degree of activity, effort, vigor, and persistence (Salamone and Correa, 2002). People with depression commonly show profound activational impairments, such as lassitude, listlessness, fatigue and anergia (low self-reported energy) that affect their motivation (Tylee et al., 2002; Stahl, 2002). In fact, among depressed people, energy loss and fatigue are the second most commonly reported symptoms, only behind depressed mood itself (Tylee et al., 1999), and depressed patients with anergia are more common than patients with anxiety related symptoms (Tylee et al., 2002). Furthermore, in a factor analytic study of depressed patients, “lack of energy” was a factor that correlated highly with problems such as energy/fatigability, inability to work, and psychomotor retardation, loading most strongly onto a second order general depression factor (Gullion and Rush 1998). Moreover, many people with major depression have fundamental deficits in reward seeking, exertion of effort, and effort-related decision making that do not simply depend upon any problems that they may have with experiencing pleasure (Treadway et al., 2012). Lack of energy is the symptom most highly correlated with impaired social function in depressed patients, and is closely related to various work-related problems such as days in bed, days of lost work, and low work productivity (Swindle, 2001). In addition, this cluster of symptoms can be highly resistant to treatment (Stahl, 2002), and they are the best predictors of lack of remission after antidepressant drug treatment (Stahl, 2002; Gorwood et al., 2014).

Treatments for the motivational and activational symptoms in depression.

The severity of effort-related motivational symptoms in depression is related to problems with social function, employment absence, and treatment outcomes (Tylee et al. 1999; Stahl 2002). Patients with high scores in psychomotor retardation also have longer duration of illness, an earlier age of onset, and more depressive episodes (Calugi et al., 2011; Gorwood et al., 2014). These symptoms are a predictor of delayed response to treatment with either interpersonal psychotherapy or selective serotonin (5-HT) reuptake inhibitor pharmacotherapy (Frank et al., 2011), often remaining as residual symptoms even in patients in remission (Stahl, 2002; Fava et al. 2014; Gorwood et al., 2014).

Most of the present treatment strategies for MDD focus on drugs that block the inactivation (i.e., inhibitors of enzymatic breakdown or uptake) of the monoamine neurotransmitters 5-HT and norepinephrine (NE). The classical antidepressants include monoamine oxidase inhibitors (MAOIs), which affect one of the major catabolic enzymes for monoamines (Quitkin et al., 1979), and drugs that inhibit uptake of one or more monoamines (Feighner, 1999; Richelson et al., 1982; Yildiz et al., 2002). Although 5-HT and NE reuptake inhibitors have become the most frequently prescribed medications for MDD, they fail to induce symptom remission in 40%-60% of all patients (Rush and Trivedi, 1995; Fava et al., 2014), and it is widely accepted that at least 20% of all depressed patients do not respond adequately to most antidepressant drugs (Crown et al., 2002). Many common antidepressants, including 5-HT transport inhibitors such as fluoxetine, are relatively ineffective at treating anergia and fatigue, and in fact, can induce or exacerbate these symptoms (Padala et al. 2012; Stenman and Lilja 2013; Fava et al. 2014).

Interestingly, some clinical studies suggest that drugs that inhibit dopamine (DA) transport, such as the catecholamine uptake inhibitor bupropion, are relatively more effective than 5-HT uptake inhibitors for treating effort-related motivational symptoms (Rampello et al. 1991; Stahl 2002; Demyttenaere et al. 2005; Pae et al. 2007). Furthermore, a recent paper (Bell et al. 2013) reports that individual differences in behavioral traits can differentiate between depressed patients that are more responsive to bupropion (i.e., motivated, achievement-oriented, active, exercise-oriented people) vs. fluoxetine (people with mood problems, irritability, and rumination). Stimulant drugs that are not considered to be antidepressants in the classical sense, such as

methylphenidate and modafinil, have been shown to increase energy and motivation in depressed patients (Zisook et al., 2006). Thus, clinical studies, together with preclinical investigations (e.g. Salamone et al. 2006, 2007; Salamone and Correa, 2012; Argyropoulos and Nutt, 2013; Yohn et al., 2015ab), have led to the suggestion that DA systems and related circuits are particularly involved in effort-related motivational symptoms.

In addition to DA, another possible therapeutic target for the anergia component of depression is the adenosinergic system. In the present review, we focus on studies that assessed the effect of caffeine and selective adenosine antagonists on different aspects of depression in humans, as well as in animal models of depression, with special emphasis on motivational/psychomotor symptoms.

Caffeine consumption, depression and related mood symptoms.

Caffeine is a naturally occuring methylxanthine that acts mainly as a non-selective A_1 and A_{2A} adenosine receptor antagonist (Fredholm et al., 1999). This methylxantine is found in common beverages including coffee, tea, soft drinks, and products containing cocoa, as well as a variety of medications and dietary sources (Barone and Roberts 1996; Andrews et al., 2007). Thus, caffeine ranks as one of the most commonly consumed dietary ingredients throughout the world (Heckman et al., 2010). Daily intake of caffeine among consumers in US is about 280 mg, and higher intakes are estimated in some European countries (Gilbert, 1984; Barone and Roberts, 1996). Caffeine is typically consumed in order to increase alertness, arousal, activation and self-reported energy (Malinauskas et al., 2007; Smith et al., 2002). Its consumption has been related to changes in cognitive performance and mood in the normal population (Smith et al., 2002), as well as in people with fatigue (Childs and de Wit, 2001).

There are very few studies on the relation between caffeine consumption and depression-related symptoms, and in many cases, its use is related to self-medication patterns. Some of these studies focus on the role of caffeine as a drug that prevents depression, while others discuss caffeine as a possible treatment for existing depression. Thus, in a longitudinal study in women free from depressive symptoms at baseline, high levels of caffeine consumption (>550 mg/day) were negatively correlated with the appearance of depressive symptoms (Lucas et al., 2011). In fact, the relative risk for depression was higher for those women with lower caffeine consumption (<100

mg/day; Lucas et al., 2011). However, in women with multiple sclerosis, high doses of caffeine (>400 mg/day) increased the prevalence of MDD (Patten et al., 2000). Moreover, in non-clinical samples, although caffeine consumption at moderate doses was related to decreases in suicide risk (Kawachi et al., 1996; Tanskanen et al., 2004), excessive consumption (750 mg/day) was correlated with a higher risk of suicide (Tanskanen et al., 2004; Kawachi et al., 1996). Thus, from the present studies, it seems that intermediate levels of caffeine consumption (300-550 mg/day) may produce beneficial effects in non-clinical populations, but not in people with some neurological pathologies. Higher doses appear to have negative effects, even in non-clinical populations.

Multiple reports have lent support to the idea that depressed people could use caffeine as self-medication. It has been reported that psychiatric patients show a relatively high degree of caffeine consumption compared to the normal population (Greden et al, 1978; Scott et al, 1989; Rihs et al., 1996, Leibenluft et al., 1993). This appears to be particularly true in patients that have experienced depressive symptoms (Leibenlugt et al., 1993). Different profiles of patients (i.e. with alcohol dependence, seasonal affective disorder and people with MDD) have been shown to have higher levels of caffeine consumption after experiencing depressive symptoms (as shown by the Halmilton Rating Scale for depression; Leibenluft et al., 1993; Halmilton et al., 1967). Among youth with depression, there is higher caffeine consumption that in the general population (Whalen et al., 2008). Moreover, the degree of caffeine consumption seems to be a predictor of improvement of somatic symptoms and hostility in depressed patients medicated with fluoxetine (Worthington et al., 1998), suggesting that caffeine could be an effective co-treatment for some of the symptoms of depression.

Impact of caffeine on energy/fatigability and behavioral activation in humans.

A wide range of studies have demonstrated that caffeine can increase alertness and subjectively reported energy, and also can reduce fatigue (Lieberman et al., 2001; Yu et al., 1991; Johnson et al., 1990; Smith et al., 1992; 1997). Caffeine has also been demonstrated to increase feelings of efficiency, self-confidence, motivation to work (Fredholm et al., 1999), to increase the desire to socialize (Griffiths et al., 1990; Silverman et al., 1994; Griffiths and Mumford, 1995), and to improve psychomotor performance (Rees et al., 1999). The behavioral effects of caffeine can be

influenced by baseline arousal levels and also by the nature of the task requirements. It has been argued that the effects of caffeine on fatigue should be most clearly evident in situations of low baseline arousal or high fatigue, or in tasks placing high demands on controlled processing (Bachrach, 1966; Liberman et al., 1986; Weiss and Laties, 1962). In fact, beneficial effects of caffeine have been observed in people in low states of alertness, such as after benzodiazepines administration (Johnson et al., 1990), sleep loss (Bonnet et al., 1995), when the person is suffering from a common cold (Smith et al., 1997), or when the experiment is done in the early morning (Smith, 1992). In addition, a broad range of studies have reported effects of caffeine withdrawal on different markers of motivation using descriptors such as fatigue, decreased energy or vigor, lethargy, amotivation for work, etc. (for a review see Juliano and Griffiths, 2004). For example, in controlled studies, after 10 days of high levels of caffeine consumption (1,250 mg/day), withdrawal results in increased subjective ratings of headache, sleepiness, laziness and fatigue, as well as decreased alertness, activation and vigor (evaluated with the Profile of Mood State, POMS) (Griffiths et al., 1986). Abstinence from intermediate doses in daily coffee and cola consumers (average of 579 mg/day) increased ratings of drowsy/sleepy, fatigue/tired, lazy/sluggish/slow-moving, and decreased ratings of active/energetic/excited, and motivated to work, as well as performance in psychomotor tasks (Liguori and Hughes, 1997). Even at low doses (100 mg/day, in a controlled study), caffeine withdrawal increased ratings of lethargy, fatigue, tiredness, and sluggishness, and decreased ratings of energy, motivation and urge to work (Griffiths et al., 1990). Silverman et al. (1992) reported that caffeine withdrawal in non-depressed moderate caffeine users increased fatigue and decreased self-reported vigor, and also increased the number of people with abnormally high scores on the Beck Depression Inventory.

Effect of caffeine and adenosine antagonists on classic animal models of depression.

Preclinical studies have attempted to elucidate the effect of caffeine and selective adenosine antagonists on classic animal models of depression (El Yacoubi 2001). Two of the classic tests for the assessment of antidepressant properties of different substances in rodents are the forced swim test (FST) and the tail suspension test (TST). In the FST, animals are placed in an inescapable cylinder filled with water, and after an

extended period of swimming, eventually become immobile (Porsolt et al., 1977; Petit de Mouliere et al., 2005). The TST is based on the observation that a mouse suspended by the tail shows alternate periods of agitation and immobility (Sterú et al., 1985). Classical antidepressants reduce immobility time in these paradigms, which have become the classical models for evaluating the antidepressant effects of drugs or showing depressive symptoms induced by behavioral manipulations (Armario and Nadal 2013).

There are a number of stress-based models used to study behavioral processes related to depression. Learned helplessness has been considered as one of the factors leading to the development of depression in vulnerable individuals that suffer stressful life events (Abelaira et al., 2013). Learned helplessness can be produced in animal models in which the depressive-like state is induced either by chronic uncontrollable and unpredictable stressors (CUS), typically electrical foot-shock, and subsequently fails to escapable shock (Overmier and Seligman, 1967). In addition to deficits in escape and avoidance, animals that develop learned helplessness show decreases in weight gain, increased immobility in the FST or TST, and reduced locomotion, all symptoms associated to some degree with depression (Maier and Seligman, 1976). More recently, the chronic mild stress (CMS) model was developed. CMS is induced by irregular exposure to a combination of different types of stressors over a period of weeks (Katz et al., 1981; Willner, 2005). These conditions reduce sucrose consumption in rodents (Willner, 2005). After the administration of substances with antidepressant properties, animals exposed to CUS or CMS consume normal levels of sucrose (Willner, 2005), and display escape-directed behaviors, reducing time of immobility (Porsolt et al., 1977; Steru et al., 1985).

All these tests and manipulations have been used to study the potential therapeutic properties of caffeine and selective adenosine antagonists or genetic deletion of adenosine receptors in rodents. In one of the seminal papers, Porsolt and colleagues (1978) demonstrated that an acute dose of caffeine reduced immobility time in the FST in Sprague-Dawley rats. In later studies, this effect has been confirmed using other strains of rats and mice, after acute or repeated administration of a broad range of doses and using diverse animal tests (see table 1). Consistent with the effects of caffeine, selective adenosine A_{2A} receptor antagonists have also been effective in these tests. Thus, SCH 58261 and istradefinille (KW6002), reduced total immobility time in both

the TST and the FST in mice (El Yacoubi, 2001). SCH 58261 also reduced immobility time in a selectively bred 'helpless' CD1 mice strain in the TST (El Yacoubi, 2001). Moreover, A_{2A} receptor knockout (A_{2A}KO) mice showed reductions in immobility time compared to wild type (WT) animals in both tests (El Yacoubi, 2001).

Using the learned helplessness model for inducing depressive symptoms, it has been demonstrated that acute doses as well as chronic administration of caffeine can reduce the impact of CUS. Thus, caffeine prevented as well as reversed CUS-induced behavioral and physiological signs of depression such as decreased weight gain, increased corticosterone levels, escape behavior impairments in a shuttle box, increased immobility time in the FST and TST, increased anxiety, and decreased sucrose consumption, locomotion and spatial reference memory (see table 1). In agreement with these findings, mice that received the selective A_{2A} receptor antagonist istradefylline, as well as constitutive A_{2A}KO mice, were protected from the CUS-induced behavioral impairments in the FST, TST, and memory tests (Kaster et al., 2015), suggesting a key role for A_{2A} receptors in acute and chronic stress-induced depressive effects.

Based on these results some researchers have focused on adenosine receptor antagonists, including caffeine, as tools to reverse behavioral impairments induced by pharmacological manipulations of the adenosine system (Pechlivanova et al., 2012; Minor et al., 2008; 1994; Woodson et al., 1998; Kulkarni and Mehta, 1985; Hunter et al., 2003). Thus, a high dose of adenosine (100 mg/kg, intraperitoneally; IP), or its analog 1-chloroadenosine (2.0 mg/kg, IP) induce immobility in the FST in mice, and caffeine and theophylline (8.0 mg/kg, IP), reversed this effect (Kulkarni and Metha, 1985). Theophylline is a psychoactive methylxanthine found in tea and other substances, and is also a metabolite of caffeine that acts as a non-selective adenosine antagonist for A_1/A_{2A} receptors (Gu et al., 1992). In contrast, Kaster and colleagues (2004) used low doses of adenosine administered via two different routes of administration (IP: 1-10 mg/kg, and intracerebroventricular, ICV: 0.1-10 ug/site), and observed antidepressant-like effects in the FST and TST. Moreover, the A_1 adenosine receptor agonist, adenosine N6-cyclohexyladenosine (CHA) (0.05-1.0 mg/kg, IP), and the A_{2A} agonist N6-[2-(3,5-dimethoxyphenyl)- 2-(methylphenyl)ethyl]adenosine (DPMA) (1.0-5.0 mg/kg, IP) also decreased the immobility time in this test (Kaster et al., 2004). In addition, pretreatment with non-effective doses of caffeine (3.0 mg/kg, IP), the A_1 antagonist DPCPX, and the A_{2A} antagonist ZM241385, inhibited the

antidepressant effect induced by a low dose of adenosine in the FST (Kaster et al., 2004). These authors also observed that a higher dose of adenosine (50.0 mg/kg, IP) did not have antidepressant effects (Kaster et al., 2004). As a whole, these studies suggest that adenosine might elicit antidepressant actions only at low doses (1.0-10.0 mg/kg, IP), having no effect at intermediate doses (50.0 mg/kg) and depressant-like effects at higher doses (100 mg/kg) (Kulkarni and Metha, 1985).

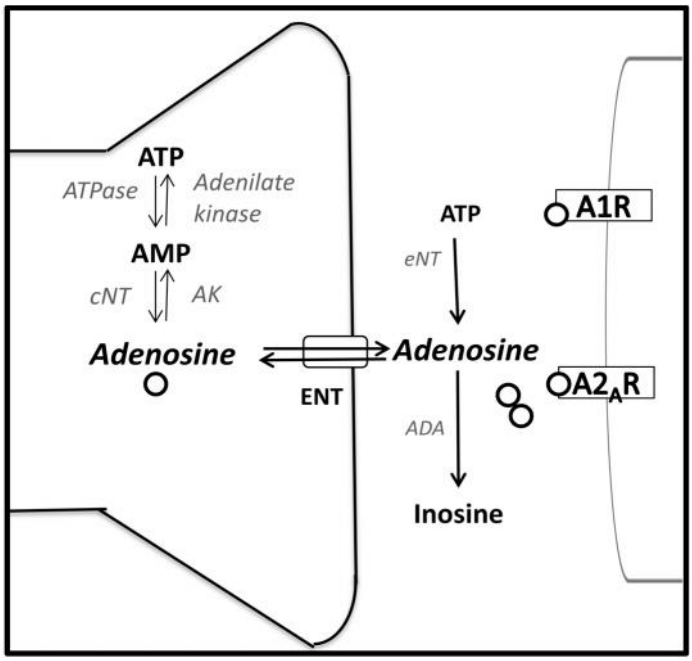

Figure 1. Adenosine synthesis and metabolism. Abbreviations: ADA, adenosine deaminase; AK, adenosine kinase; A1R and A2AR, adenosine A_1 and A_{2A} receptors, cNT: cytosolic endo-nucleotidase; ENT, equilibrative nucleoside transporter; eNT, exo-nucleotidase. (Adapted from Ruby, 1999).

Consistent with the results from studies using high doses of IP adenosine, increases in central adenosine transmission have been also associated with escape deficits in the inescapable shock paradigm (Minor et al., 1994a; Woodson et al., 1998; Kurlarni and Mehta, 1985; Minor and Hanff, 2015). Thus, it has been demonstrated that ICV administration of NBTI (S-(4-nitrobenzyl)-6-theoinosine), an equilibrative nucleoside transport (ENT) blocker that increases extracellular adenosine levels by blocking its reuptake, impaired escape latency in rats (Jacobson et al., 1992; Noji et al., 2004; Minor et al., 2008). Moreover, ICV administration of erytrho-9(2-hydroxy/3/nonyl adenine (ENHA), a selective adenosine deaminase (ADA) inhibitor which blocks adenosine

metabolism, mimicked the effect of inescapable shock (Woodson et al., 1998). Low doses of caffeine reversed the escape deficits induced by EHNA (Woodson et al., 1998). The reversal effects of caffeine appear to be specific to actions on adenosine receptors, and not as a general psychomotor stimulant effect, since amphetamine exacerbated the behavioral impairments induced by inescapable shocks. Moreover, the amphetamine-induced impairment was reversed by caffeine and theophylline (Minor et al., 1994b), and also by the A_{2A} antagonist CSC, but not by the selective A_1 adenosine antagonist DPCPX (Minor et al., 2008). Injections of glutamate into prefrontal cortex have been shown to impair escape performance (Petty et al., 1985), and later work reported that caffeine can reverse these glutamate-induced escape deficits (Hunter et al, 2003). This pattern of results is consistent with studies showing that increases in glutamate are counterbalanced by an increase in adenosine production and release (Deckert and Gleiter, 1994).

Caffeine has also been used to enhance the effect of monoaminergic antidepressants (especially 5HT and NE uptake inhibitors) that are been used in clinical practice and also have been shown to reduce immobility in classical animal tests of depression. Thus, caffeine administered at doses that do not have an effect on their own can potentiate the effects of desipramine, imipramine, duloxetine, fluoxetine and paroxetine on FST performance (Robles-Molina et al., 2012; Kale et al., 2014; Szopa et al., 2016). In addition, a low dose of caffeine can also enhance the behavioral and neurochemical effects of bupropion, which blocks catecholamine uptake (Kale et al., 2014).

Impact of caffeine on behavioral activation and effort-related processes: preclinical studies

In the animal literature, as with the human data, there are studies showing how caffeine and selective adenosine antagonists affect the willingness to work depending on the demands of the task. Caffeine and theophylline produced rate-dependent effects on lever pressing to obtain palatable food in rats (Randall et al., 2011). Caffeine (5.0-20.0 mg/kg, IP) and theophylline (10.0-40.0 mg/kg, IP) increased responding on the schedule that generated low baseline rates of responding (a fixed interval 240 seconds (FI-240 sec) schedule). In contrast, caffeine and theophylline decreased responding on a fixed ratio 20 (FR20) schedule that typically generates high rates of responding (Randall et al., 2011) (see table 2). The A_{2A} antagonists MSX-3 and istradefylline increased FI-240 sec

lever pressing but did not suppress FR20 lever pressing in the dose range tested. In fact, there was a tendency for istradefylline to increase FR20 responding at a moderate dose. A_1 antagonists failed to increase lever-pressing rate, and actually DPCPX decreased FR20 responding at higher doses. These results suggest that the work potentiating effects of methylxantines are mediated by their actions on adenosine A_{2A} receptors, while their A_1 receptor antagonist action could be mediating the suppressant effects. Progressive ratio (PR) schedules, which require gradually increasing work output, have been also employed to explore the effect of caffeine on motivation to work for sucrose or food reinforcement in rats and monkeys (Sheppard et al., 2012; Retzbach et al., 2014; Buffalo et al., 1993). Acutely and chronically moderate doses of caffeine elevated PR lever pressing for sucrose (Sheppard et al., 2012; Retzbach et al., 2014). Caffeine had no effect on inactive lever presses suggesting that this increase was not due to an increase in general motor activity (Retzbach et al., 2014). However, in rhesus monkeys intravenous (IV) caffeine decreased percent of task completed, and breakpoint in a PR for palatable food (Buffalo et al., 1993), possibly because this dose directly administered in the blood stream resulted in higher levels in the brain. Thus, it seems that low-to-moderate doses of caffeine increase behavioral output in tasks that evaluate willingness to work for a reinforcer, while high doses decrease responding.

Caffeine modulation of DA-related postsynaptic signaling.

As described above, caffeine is a non-selective adenosine receptor antagonist. In the brain, adenosine acts upon both A_1, and A_{2A} G-protein-coupled receptors (Fredholm et al., 2011; Jackobson and Gao, 2006). The distribution of adenosine receptors within the brain (Fredholm et al., 2011) allows a wide range of effects, including modulation of other neurotransmitter systems (Cuhna-Reis et al., 2007). Thus, adenosine A_{2A} receptors are highly expressed in DA rich areas such as neostriatum and accumbens (Acb) (Shiffmann et al., 1991; deMet et al., 2002). In fact, it has been demonstrated that in these areas, there is a functional interaction between DA D_2 and adenosine A_{2A} receptors (see Figure 2), which are co-localized on enkephalin-containing medium spiny neurons, form hetromeric complexes, and converge onto the same signal transduction pathways in an antagonistic manner (Ferré, 2008; Ferré et al., 1997, 2008; Fink et al., 1992; Fuxe et al., 2003). Similarly, A_1 and D_1 receptors antagonistically interact in substance P-containing medium spiny neurons (Ferré et al., 1997; 2008).

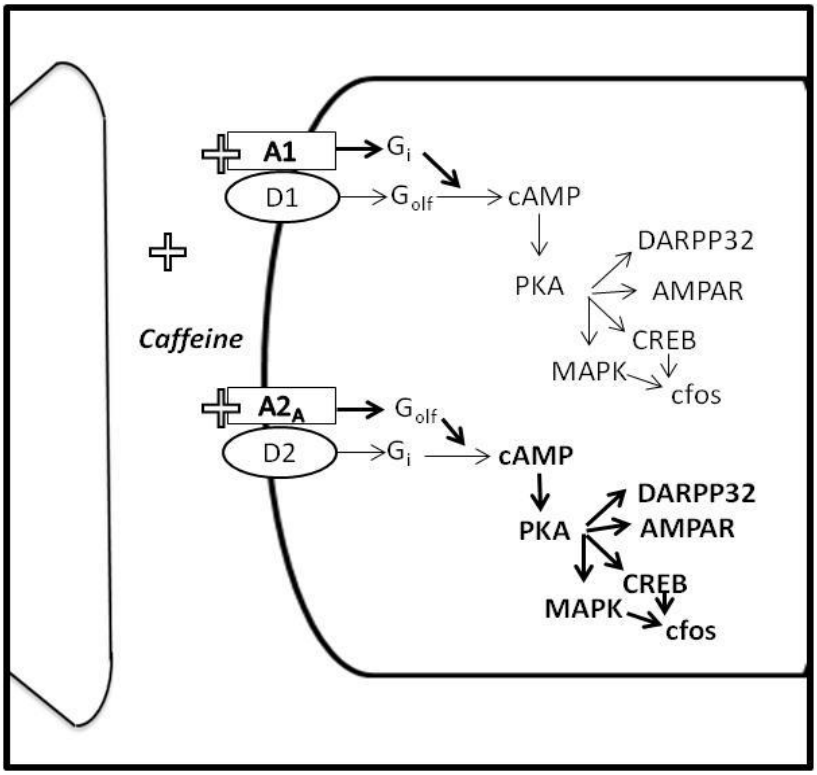

Figure 2. Impact of caffeine on the functional interaction between adenosine and DA receptors. A_1R and $A_{2A}R$: adenosine A_1 and A_{2A} receptors, D_1: DA type 1 receptor, D_2: DA type 2 receptor (adapted from Ferré 2008).

The behavioral significance of this interaction has frequently been studied in the context of neostriatal motor functions and pathologies (Correa et al., 2004; Ferré et al., 1997; Collins et al., 2010; Pinna et al., 2007; Simola et al., 2004, 2006; Wardas et al., 2001). Thus, selective A_{2A} receptor antagonists are been tested in clinical trials for pathologies involving DAergic dyfunctions such as Parkinson disease (LeWitt et al., 2008), and positive results indicate that they can be used as adjuvant therapies (Hung and Schwarzschild, 2014). In fact, istradefylline is currently approved in Japan for use in treating Parkinson's disease. Caffeine's actions on A_1 and A_{2A} adenosine receptors (Ferré, 2008), has promoted its study as an alternative preventive or therapeutic tool for Parkinsonian symptoms (Prediger, 2010). Moreover, within the last few years, the motivational significance of DA-adenosine receptor interactions has become apparent with regard to processes such as behavioral activation and effort-related decision-making, which could have significance for the treatment of depression and other pathologies (Salamone et al., 2006; 2009; 2010). The next two sections will review the

literature related to DA-adenosine interactions in pathological symptoms related to effort-based decision-making.

Effort-related decision-making in depression: Clinical significance and animal models.

Activational aspects of motivation (i.e., vigor, persistence, work output) are highly adaptive because they enable organisms to overcome obstacles or work-related response costs that separate them from significant stimuli (Salamone and Correa, 2002, 2012; van den Bos et al., 2006). An important feature of adaptive behavior, in the face of work-related challenges, is effort-related decision making. Frequently, organisms must make cost/benefit analyses in which they weigh the value of a stimulus relative to the cost of obtaining it, and such decisions involve effort-relateed costs (Salamone and Correa, 2002, 2012; Salamone et al., 2007). These processes are important for both normal and pathological aspects of motivation. For example, people with MDD show a reduced likelihood of selecting high effort activities in human tasks of effort-related decision making (Treadway et al. 2012; Yang et al., 2014).

Extensive animal research has demonstrated that Acb DA is a key mediator of effort-based decision-making processes (for a review see Salamone and Correa, 2012). In preclinical studies, 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. Interference with DA transmission produces a shift in effort-related choice behavior, biasing animals towards instrumental behaviors that involve less effort or lower activity. One such procedure is a T-maze task that provides an effort-related challenge by having a vertical barrier in the arm with the higher reward density (HD) vs. an arm that contains a lower density of reward (LD) and has no barrier (Salamone et al. 1994; Cousins et al. 1996; Mott et al., 2009; Pardo et al., 2012). With this procedure, rodents choose to climb the barrier to get more reward in 90% of the trials, once they have been trained (Cousins et al. 1996; Pardo et al., 2012). In operant tasks animals are given a choice between lever pressing for the more preferred reward (in FR5, FR7, or 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 the concurrent FR5/free reward choice task, untreated rats typically spend most time pressing the lever for the preferred reward and eat little of the freely available food or fluids (Salamone et al., 1991, 2002;

Pardo et al., 2015). In contrast, rats tested on the PR/chow free feeding choice task show more individual variability, and tend to disengage more readily from the PR lever pressing component because of the increasing work requirement (Randall et al., 2012, 2014). Research with these concurrent choice tasks has shown that interference with DA transmission via DA depletions or DA receptor antagonism typically biases rodents towards the low effort-low reward option (Salamone et al., 1991; 2009; Worden et al., 2009; Pardo et al., 2012; Randall et al., 2012, 2014; Yohn et al., 2015a,b,2016a,b).

Using these effort-related choice procedures it has been demonstrated that the catecholamine depleting agent and vesicular transport inhibitor (VMAT-2) tetrabenazine (TBZ) can shift effort-based decision making across multiple behavioral tasks (Nunes et al., 2013; Randall et al., 2014; Yohn et al., 2015a,b, 2016a,b; Pardo et al., 2015). TBZ has been shown to deplete monoamines, with its greatest impact being upon striatal DA (Pettibone et al., 1984; Tanra et al., 1995; Nunes et al., 2013). TBZ is used as a therapeutic drug to treat Huntington's disease patients, but it also induces side effects that include symptoms of depression, including fatigue, in humans (Frank 2010; Guay 2010). Because of its neurochemical and behavioral effects, TBZ is a useful tool for animal models of depression, and TBZ has previously been employed in studies that use the FST and TST rodent models of depression (Preskhorn et al. 1984; Kent et al. 1986; Wang et al. 2010). Recent studies have demonstrated that the effort-related effects of TBZ are attenuated by the catecholamine uptake blocker bupropion, which is a commonly used antidepressant, and also by the selective DA uptake blocker GBR12909 (Nunes et al., 2013; Randall et al., 2014; Yohn et al., 2016a). In contrast, other classical drugs for the treatment of depression, such as the 5-HT uptake inhibitors fluoxetine and citalopram and the NE uptake inhibitor desipramine, failed to reverse the effects of TBZ, and higher doses even led to further behavioral impairments (Yohn et al., 2016a,b).

In addition to DA, adenosine also is involved in effort related decision-making processes (Farrar et al., 2007, 2010; Hauber and Sommer, 2009; Nunes et al., 2010; Salamone et al., 2007, 2009). Microinjections of the adenosine A_{2A} agonist CGS 21680 into the Acb produced effects on instrumental behavior and effort-related choice that resembled those produced by Acb DA antagonism or depletion (Font et al., 2008; Mingote et al., 2008). In addition, considerable evidence indicates that DA D_2 and adenosine A_{2A} receptors interact to regulate effort-related functions (Salamone and Correa, 2009, 2012). Thus, adenosine A_{2A} antagonists such as MSX-3, MSX-4, and

istradefylline were able to reverse the shift in effort-based choice that was induced by administration of the D_2 antagonists haloperidol and eticlopride (Farrar et al., 2007, 2010; Salamone et al., 2009; Mott et al., 2009; Worden et al., 2009; Nunes et al., 2010; Pardo et al., 2012; Santerre et al., 2012). Moreover, A_{2A} KO mice were resistant to the effects of haloperidol on performance of the T-maze barrier task (Pardo et al., 2012). In contrast, adenosine A_1 antagonists DPCPX and CPT were ineffective at reversing the effort-related effects of either the D_1 antagonist ecopipam or the D_2 antagonist eticlopride (Salamone et al., 2009; Nunes et al, 2010; Pardo et al., 2012, 2015).

The effects of caffeine and theophylline on effort-related choice behavior after the administration of D_2 antagonists have also been reported in rats tested on the concurrent FR5/chow feeding choice task. Caffeine (5.0, 10.0 and 20.0 mg/kg, IP) partially attenuated the effects of haloperidol, increasing the lever pressing and decreasing chow intake in haloperidol-treated rats (Salamone et al., 2009). Similarly, theophylline (10.0 and 15.0 mg/kg) reversed the effects induced by D_2 antagonism in mice tested on the T-maze barrier task (Pardo et al., 2012). Furthermore, using the T-maze task, our laboratory recently conducted an experiment in CD1 male mice assessing the impact of caffeine (0.0, 2.5, 5.0 or 10.0 mg/kg, IP 30 min before test) on arm selection before and after DA depletion via TBZ (0 or 4.0 mg/kg, IP 120 min before test). Although a single dose of caffeine (10.0 mg/kg) significantly reduced latency to get access to the food in the first 10 trials in the phase in which there was no barrier (t-test for dependent samples; ($t=2.2$, $p<0.05$), it did not increase HD arm selection or food consumption (see figure 3).

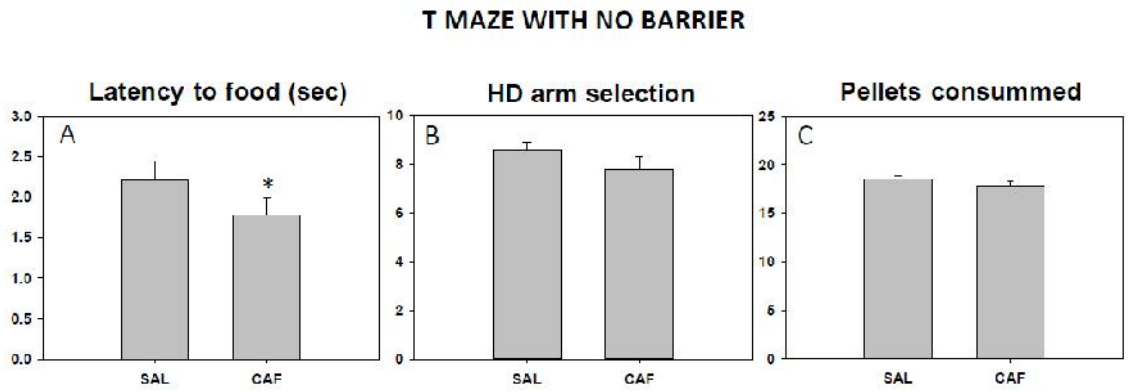

Figure 3. Impact of caffeine (10.0 mg/kg, IP 30 minutes before test) on performance in a T maze in which the high density (HD) and the low density (LD) arms had no barrier. Data for the 10 first trials. A) Average latency to reach the food (seconds) *$p<0.05$ different from saline. B) Number of HD arm selection. C) Number of total pellets consumed.

Caffeine was effective at reversing the reduction in HD arm selection (repeated measures ANOVA for HD arm selection; $F(3,28)=8.32$, $p<0.01$) and the concurrent increase in LD arm selection ($F(3,28)=7.53$, $p<0.01$) when there was a 14 cm barrier in the HD arm and animals had received a dose of TBZ that shifted behavior towards the low effort option. Thus, a dose of 5.0 mg/kg of caffeine reversed the impairing effect of the DA depleting agent TBZ on selection of the high effort choice (see figure 4).

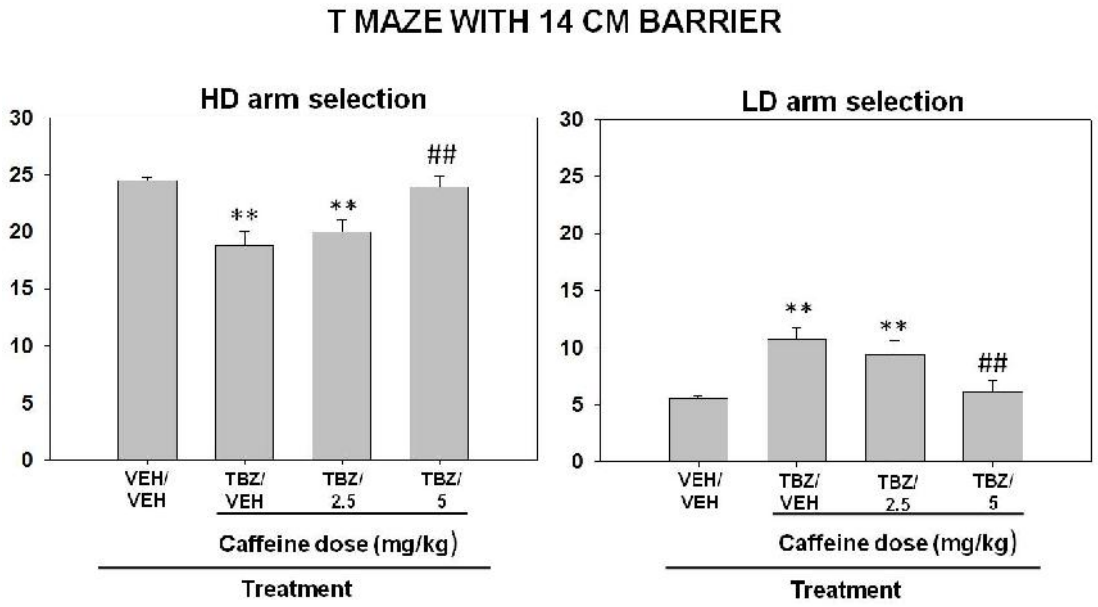

Figure 4. Impact of TBZ (4 mg/kg, IP administered 120 minutes before test) on HD arm (A) and LD arm (B) selection and reversal with different doses of caffeine in a T maze with a 14 cm barrier in the HD arm. Mean ± SEM of number of trials in which animals choose HD or LD arm (**p<0.01 different from VEH/VEH. ##p<0.01 different from TBZ/VEH.)

Furthermore, several recent papers have reported that the adenosine A_{2A} antagonist MSX-3 can reverse the effort-related effects of TBZ across multiple tasks (Nunes et al., 2013; Randall et al., 2014; Yohn et al., 2015a). Taken together with the results of studies showing that A_{2A} but not A_1 receptor antagonists can reverse the effort-related effects of D_2 antagonism, these results suggest that the ability of caffeine to reverse the effects of DA antagonism and depletion may depend largely upon blockade of A_{2A} receptors.

Mental fatigue associated with high attentional demands can also be overcome by the use of psychostimulants such as amphetamine or caffeine (Peeling and Dawson, 2007;

Silber et al., 2006). In cost/benefit decision-making tasks involving 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). Under basal conditions, animals chose high effort/high reward trials more than low-effort/low reward trials. However, there are substantial individual differences in baseline performance. Amphetamine increases the selection of high effort/high reward trials in animals that usually do not choose this option under baseline conditions, but it decreases the selection of the high cognitive demand trials in animals that usually choose them. A high dose of caffeine (20.0 mg/kg) decreased choice of high effort/high reward trials in animals that usually choose them as did amphetamine, but caffeine did not increase the selection in the ones that usually did not choose them (Cocker et al., 2012). Thus, it appears that cognitive arousal and attention are components of this task that are not improved by caffeine, which, on the other hand, seems to benefit selection of responses that require high levels of physical effort.

Translational studies in humans have employed tasks that evaluate effort-based decision making processes in normal humans as well as psychiatric patients. The effort expenditure for rewards task (EEfRT; Treadway et al., 2009), is based on the operant lever pressing and T-maze choice tasks described above (Salamone et al., 1991, 1994, 2002). In the human version of this task, subjects choose on each trial between a high cost/high reward option (HC/HR) and low cost/low reward option (LC/LR) to obtain different monetary rewards. The HC/HR trials required 100 button presses with the non-dominant little finger within 21 seconds, and subjects were eligible to win higher amounts that varied per trial between $1.24-4.30. In contrast, the LC/LR option only required 30 button presses with the dominant index finger during 7 seconds, and subjects could win $1.00 for each successfully completed trial. The rewards were not guaranteed if they completed the task, thus some trials were “win” trials while others were “no win” trials. Participants were provided with probability cues during the choice session, leading to three levels of probability of a win trial: high (88%), medium (50%), and low (12%). Based on these percentages, participants could choose between the HC/HR trial and the LC/LR trial. Patients with MDD were significantly less likely to make HC/HR choices relative to controls, and this result was not related with depression-related differences in psychomotor speed (Treadway et al., 2012). The probability of reward was an important factor, as the impairment in MDD patients was

greater when reward probability, and thus baseline selection of the high effort option, was highest (Treadway et al., 2012). The effects of caffeine on this task in depressed patients has not been explored, but it was assessed in nonpathological human subjects (Wardle et al., 2014). A single dose of caffeine (200 mg) significantly increased the speed of responses compared to placebo, but did not have an effect on percentage of HC/HR choices (Wardle et al., 2014). In fact, caffeine decreased effortful choices in high cardiovascular responders (subjects with high arterial pressure in response to caffeine) (Wardle et al., 2014). These results are different from previous human studies reporting that a major psychomotor stimulant, amphetamine, was able to increase HC/HR choice (Wardle et al., 2011), and also with studies showing that, during exercise, caffeine decreases the perception of effort in humans (Doherty and Smith, 2005) and improves performance particularly during endurance testing (Doherty and Smith, 2004).

Conclusions and further directions

Although many available treatments for MDD provide relief for some individuals with depressed mood, no single therapeutic option provides a full and permanent recovery for all the symptoms of MDD in the majority of patients (McClintock et al., 2011). Clinicians have come to emphasize the importance of effort-related motivational symptoms in depression (Tylee et al., 1999; Stahl 2002; Demyttenaere et al,. 2005; Salamone et al., 2006), because even among patients in remission, anergia and psychomotor retardation are pervasive symptoms (Gorwood et al., 2014). Thus, novel pharmacological targets are being investigated in clinical and preclinical studies. There are promising results shown in human epidemiological studies, as well as research with animal models, characterizing the potential effect that caffeine and selective adenosine receptor antagonists could have on these symptoms. It is worth noting that the epidemiological studies have reported mixed outcomes in humans depending on the amount of caffeine consumed. Thus, whereas some studies reveal a relation between caffeine consumption and decreased risk for developing depression (Lucas et al., 2011), and different reports demonstrate the use of caffeine as a self-medication among depressed patients (Leibenlugt et al., 2003), other studies show a relation between high levels of caffeine consumption and increased risk of suicide (Tanskanen et al., 2004). Thus, it seems clear that more controlled studies are needed to explore the effect of caffeine on the wide variety of symptoms observed in patients with MDD.

Systematic studies on the effects of caffeine in animal models of depression and anergia have shown the efficacy of this methylxanthine at improving parameters related to initiation and maintenance of behavior in order to escape an aversive situation, but also in order to pursue valued reinforcers and achieve goals (Pechilivanova et al., 2012; Hunter et al., 2003; Minor et al., 2008; Woodson et al., 1998; Kulkarni and Metha et al., 1985; Salamone et al., 2006; Randall et al., 2011). As with the human data, these actions are dependent on the dose, since high doses not only fail to improve depression-like symptoms, but can in fact promote anxiety (Correa and Font 2008; López-Cruz et al., 2014). Both in humans and in animal studies, the therapeutic actions of caffeine also seem to be dependent on the basal state; for example it seems to be effective when subjects are under a state or fatigue, tiredness or sleepiness (Bonnet et al., 1995; Johnson et al., 1990; Smith 1992), or when the DAergic system is compromised (Salamone et al., 2009; present data), and such effects are less evident when humans and rodents are assessed under "normal" conditions. Other methylxantines such as theophylline, and several A_{2A} selective antagonists, have also been shown to reverse motivational impairments induced by DA antagonism or depletion in animal models of anergia (Salamone et al., 2009; Farrar et al., 2009; Mott et al., 2009; Pardo et al., 2012). Adenosine A_{2A} receptors appear to be involved in these processes, probably through their interaction with DA D_2 receptors in the Acb, a striatal region that is highly involved in the activational component of motivation (for a review see Salamone and Correa, 2012).

Consistently, it has been demonstrated in human studies that the rank order of clinical effectiveness in depressed patients with psychomotor retardation paralleled the specificity of antidepressants as DA-mimetic agents (Rampello et al., 1991). Antidepressants such as bupropion have been demonstrated to have therapeutic effects on motivational symptoms in humans (Pae et al., 2005) and to stimulate effort-related behavioral output in animals (Randall et al., 2014). In animal studies, caffeine has been shown to improve the effects of antidepressants such as bupropion, duloxetine and desipramine (Kale et al., 2014; Robles-Molina et al., 2012; Szopa et al., 2016). These studies have helped to identify caffeine as a potential enhancer of antidepressant pharmacotherapy (for a review, Kale et al., 2010). This suggestion is consistent with the clinical trials for antiparkinsonian effects showing that A_{2A} antagonists can be a useful adjuvant in the treatment of motor symptoms (Hung and Schwarzschild, 2014). However, determination of the predominant symptomatology in individual patients may

be an important key to therapeutic success. In patients affected by anxious depression, preferential inhibition of 5-HT reuptake may be a more effective selective inhibition of DA reuptake (Rampello et al., 1995), and caffeine in those types of depressed patients may actually worsened the anxiety symptoms.

Table 1. Effect of caffeine in classical animal tests of depression.

Caffeine dose	Animal model	Species/strain	Behavioral effects	Reference
15-20 mg/kg, IP. Acute	FST	Sprague-Dawley rats	Decreased immobility	Porsolt et al., 1978 Kitada et al., 1981
1 mg/kg, IP. Repeated 3 times	FST	Wistar Rats	Decreased immobility	Enríquez-Castillo, et al., 2008
10 mg/kg, 5 mg/kg IP. Acute	FST, TST	Swiss mice	Decreased immobility. Potentiated antidepressant effect of bupropion and duloxetine	Kale et al., 2014
10-50 mg/kg, 5 mg/kg IP. Acute	FST	Albino Swiss mice	Decreased immobility. Potentiated antidepressant effect of imipramine, desipramine, fluoxetine, paroxetine, escitalopram and reboxetine.	Spoza et al., 2016
3.1-30 mg/kg, 0.31-1 mg/kg, IP. Acute	FST	Balb-c mice	Decreased immobility. Potentiated antidepressant effect of desipramine	Robles-Molina et al., 2012
3 mg/kg, IP. Acute	FST, TST	Swiss mice	Reversed: immobility induced by adenosine	Kaster et al., 2004
8 mg/kg, IP. Acute	FST	Wistar mice	No effect on its own. Reversed: immobility induced by adenosine and 2-chloroadenosine.	Kulkarni and Mehta et al., 1985
7 mg/kg, IP. Acute	FST	Sprague Dawley Rats	Reversed: immobility induced by reserpine	Minor et al., 2015
8 mg/kg, Oral. 4 weeks	CUS induction	Wistar Rats	Reversed: immobility in FST, weight loss, hypolocomotion, anxiety, decreased sucrose consumption, and core temperature	Pechlivanova et al., 2012
7 mg/kg, IP. Acute	CUS induction	Sprague Dawley Rats	No effect on its own Reversed: escape deficits induced by glutamate injection in prefrontal cortex.	Hunter et al, 2003
7 mg/kg, IP. Acute	CUS induction	Sprague Dawley Rats	Reversed: escape deficits induced by ENT blocker	Minor et al., 2008.
10 mg/kg, IP. Acute	CUS induction	Sprague Dawley Rats	Reversed: escape deficits induced by ICV administration of EHNA	Woodson et al., 1998

1 g /L , Orally. 6 weeks (before and after CUS)	CUS induction	C57BL/6 mice	Prevented: immobility in FST and TST, weight loss, increased levels of corticosterone, reduction in sucrose preference, and decreased spatial reference memory	Kaster et al., 2015

Table 2. Effect of caffeine in behavioral activation and effort-based decision making tests.

Caffeine dose	Animals model	Species/ strain	Behavioral effects	Reference
5-20 mg/kg, 10-40 mg/kg, IP. Acute	FI-240 FR20	Sprague Dawley Rats	Increased lever pressing Decreased lever pressing	Randall et al., 2011
6.25-25.0 mg/kg, IP Acute.	FR2 PR	Sprague Dawley Rats	Increased lever pressing for visual stimuly and sucrose	Sheppard et al., 2012
5 mg/kg, IP. Chronic 10 days	FR4	Sprague Dawley Rats	Increased lever pressing for sucrose.	Retzbach et al., 2014
10 mg/kg, IV. Acute	PR	Rhesus Monkey	Decreased breakpoint for palatable food.	Buffalo et al., 1993
5-20 mg/k, IP. Acute	FR5/Free chow concurrent choice	Sprague Dawley Rats	Increase lever pressings and decrease free chow intake in haloperidol-treated rats	Salamone et al., 2009
20 mg/kg, IP. Acute	Cognitive effort task (rCET)	Long Evans Rats	Decreased choice of high effort/high reward trials in “workers”. No increase in “slackers”.	Cocker et al., 2012

Bibliography

- Abelaira, H.M., Réus, G.Z., Quevedo, J. (2013). Animal models as tools to study the pathophysiology of depression. Rev Bras Psiquiatr. 35 (Suppl2):S112-120. doi: 10.1590/1516-4446-2013-1098.

- American Psychiatric Association (2013). Diagnostic and statistical manual of mental disorders (5th ed.). Washington, DC.

- Andrews, K.W., Schweitzer, A., Zhao, C., Holden, J.M., Roseland, J.M., Brandt, M., et al. (2003). The caffeine contents of dietary supplements commonly purchased in the US: analysis of 53 products with caffeine-containing ingredients. Anal Bioanal Chem 389,231-239. doi:10.1007/s00216-007-1437-2.

- Argyropoulos, S.V., Nutt, D.J. (2013). Anhedonia revisited: is there a role for dopamine-targeting drugs for depression? J Psychopharmacol 27,869-877. doi: 10.1177/0269881113494104.

- Armario, A., Nadal, R. (2013). Individual differences and the characterization of animal models of psychopathology: a strong challenge and a good opportunity. Front Pharmacol. 4:137. doi: 10.3389/fphar.2013.00137.

- Bachrach, H. (1966). Note on the psychological effects of caffeine. Psychol Rep. 18(1),86.

- Barone, J.J., Roberts, H.R. (1996). Caffeine consumption. Food Chem Toxicol. 4(1),119-129.

- Bell, D.A., Shipman, M., Cleves, M., Siegelmand, J. (2013). Which Drug For Which Patient? Is There a Fluoxetine Responding Versus a Bupropion Responding Personality Profile? Clin Pract Epidemiol Ment Health. 9,142–147. doi: 10.2174/1745017901309010142

- Bonnet, M.H., Gomez, S., Wirth, O., Arand, D.L. (1995). The use of caffeine versus prophylactic naps in sustained performance. Sleep. 18(2),97-104.

- Buffalo, E.A., Gillam, M.P., Allen, R.R., Paule, M.G. (1993). Acute effects of caffeine on several operant behaviors in rhesus monkeys. Pharmacol Biochem Behav. 46(3),733-737.

- Calugi, S., Cassano, G.B., Litta, A., Rucci, P., Benvenuti, A., Miniati, M., Lattanzi, L., Mantua, V., Lombardi, V., Fagiolini, A., Frank, E. (2011). Does psychomotor retardation define a clinically relevant phenotype of unipolar depression? J Affect Disord. 129(1-3),296-300. doi: 10.1016/j.jad.2010.08.004.

- Childs, E., de Wit, H. (2008). Enhanced mood and psychomotor performance by a caffeine-containing energy capsule in fatigued individuals. Exp. Clin. Psychopharmacol. 16(1),13-21. doi: 10.1037/1064-1297.16.1.13.

- Cocker, P.J., Hosking, J.G., Benoit, J., Winstanley, C.A. (2012). Sensitivity to cognitive effort mediates psychostimulant effects on a novel rodent cost/benefit decision-making task. Neuropsychopharmacology. 37,1825-1837. doi: 10.1038/npp.2012.30.

- Collins, L.E., Galtieri, D.J., Collins, P., Jones, S.K., Port, R.G., Paul, N.E., Hockemeyer, J., Müller, C.E., Salamone, J.D. (2010) Interactions between adenosine and dopamine receptor antagonists with different selectivity profiles: Effects on locomotor activity. Behav Brain Res. 25;211(2):148-155.

- Correa, M., Font, L., (2008). Is there a major role for adenosine A_{2A} receptors in anxiety? Front Biosci. 1:4058-4070. http://dx.doi.org/10.2741/

- Correa, M., Wisniecki, A., Betz, A., Dobson, D.R, O'Neil,l M.F., O'Neill, M.J., et al. (2004). The adenosine A2A antagonist KF17837 reverses the locomotor suppression and tremulous jaw movements induced by haloperidol in rats: possible relevance to parkinsonism. Behavioural Brain Research. 148,47-54. doi:10.1016/S0166-4328(03)00178-5.

- Cousins, M.S., Atherton, A., Turner, L., Salamone, J.D. (1996). Nucleus accumbens dopamine depletions alter relative response allocation in a T-maze cost/benefit task. Behavioural Brain Research. 74:189-197.

- Crown, W.H., Finkelstein, S., Berndt, E.R., Ling, D., Poret, A.M., Rush, A.J., et al. (2002). The impact of treatment-resistant depression on health care utilization and costs. J. Clin. Psychiatry. 63,963-971. doi: 10.4088/JCP.v63n1102.

- Cunha-Reis, D., Fontinha, B.M., Ribeiro, J.A., Sebastião, A.M. (2007). Tonic adenosine A1 and A2A receptor activation is required for the excitatory action of VIP on synaptic transmission in the CA1 area of the hippocampus. Neuropharmacology. 52,313-320. doi:10.1016/j.neuropharm.2006.08.003.

- Deckert, J., Gleiter, C.H. (1994). Adenosine-an endogenous neuroprotective metabolite and neuromodulator. J. Neural. Transm. Suppl. 43,23-31.

- DeMet, E.M., Chicz-DeMet, A. (2002). Localization of adenosine A2A-receptors in rat brain with [3H]ZM-241385. Naunyn. Schmiedebergs Arch. Pharmacol. 366,478-481. doi: 10.1007/s00210-002-0613-3.

- Demyttenaere, K., De Fruyt, J., Stahl, S.M. (2005). The many faces of fatigue in major depressive disorder. International Journal of Neuropsychopharmacology, 8, 93-105.
- Demyttenaere, K., Jaspers, L. (2008). Bupropion and SSRI-induced side effects. J Psychopharmacol. 22(7),792-804
- Doherty, M., Smith, P.M. (2005). Effects of caffeine ingestion on rating of perceived exertion during and after exercise: a meta-analysis. Scand. J. Med. Sci. Sports.15,69-78. Doi: 10.1111/j.1600-0838.2005.00445.x.
- El Yacoubi, M., Ledent, C., Parmentier, M., Bertorelli, R., Ongini, E., Costentin, J., et al. (2001). Adenosine A2A receptor antagonists are potential antidepressants: evidence based on pharmacology and A2A receptor knockout mice. Br. J. Pharmacol. 134,68-77. doi: 10.1038/sj.bjp.0704240
- Enríquez-Castillo, A., Alamilla, J., Barral, J., Gourbière, S., Flores-Serrano, A.G., Góngora-Alfaro, et al. (2008). Differential effects of caffeine on the antidepressant-like effect of amitriptyline in female rat subpopulations with low and high immobility in the forced swimming test. Physiol Behav 94,501-509. doi:10.1016/j.physbeh.2008.03.004.
- Farrar, A.M., Pereira, M., Velasco, F., Hockemeyer, J., Muller, C.E., Salamone, J.D. (2007). Adenosine A2A receptor antagonism reverses the effects of dopamine receptor antagonism on instrumental output and effort-related choice in the rat: implications for studies of psychomotor slowing. Psychopharmacology. 191,579-586. doi: 10.1007/s00213-006-0554-5.
- Farrar, A.M., Segovia, K.N., Randall, P.A., Nunes E.J., Collins, L.E., Stopper, C.M. et al. (2010). Nucleus accumbens and effort-related functions: behavioral and neural markers of the interactions between adenosine A2A and dopamine D2 receptors. Neuroscience. 166(4),1056-1067. doi:10.1016/j.neuroscience.2009.12.056
- Fava, M., Ball, S., Nelson, J.C., Sparks, J., Konechnik, T., Classi, P., Dube, S., et al. (2014). Clinical relevance of fatigue as a residual symptom in major depressive disorder. Depress. Anxiety. 31,250-257. doi: 10.1002/da.22199.
- Feighner, J.P. (1999). Mechanism of action of antidepressant medications. J Clin Psychiatry. 60 Suppl 4:4-11; discussion 12-3.
- Ferré, S. (2008). An update on the mechanisms of the psychostimulant effects of caffeine. J. Neurochem. 105,1067-1079. doi: 10.1111/j.1471-4159.2007.05196.x.
- Ferré, S., Fredholm, B.B., Morelli, M., Popoli, P., Fuxe, K. (1997). Adenosine-dopamine receptor-receptor interactions as an integrative mechanism in the basal

ganglia. Trends. Neurosci. 20,482-487. doi: http://dx.doi.org/10.1016/S0166-2236(97)01096-5.

- Ferré, S., Ciruela, F., Borycz, J., Solinas, M., Quarta, D., Antoniou, K., Quiroz, C., Justinova, Z., Lluis, C., Franco, R., Goldberg, S.R. (2008). Adenosine A1–A2A receptor heteromers: new targets for caffeine in the brain. Frontiers in Bioscience. 13,2391-2399. doi:10.2741/2852.

- Fink, J.S., Weaver, D.R., Rivkees, S.A., Peterfreund, R.A., Pollack, A.E., Adler, E.M., et al. (1992). Molecular cloning of the rat A2 adenosine receptor: selective co-expression with D2 dopamine receptors in rat striatum. Mol Brain Res. 14,186–195. doi:10.1016/0169-328X(92)90173-9

- Font, L., Mingote, S., Farrar, A.M., Pereira, M., Worden, L., Stopper, C., et al. (2008). Intra-accumbens injections of the adenosine A2A agonist CGS 21680 affect effort-related choice behavior in rats. Psychopharmacology (Berl). 199(4),515-526. doi: 10.1007/s00213-008-1174-z.

- Frank, E., Cassano, G. B., Rucci, P., Thompson, W. K., Kraemer, H. C., Fagiolini, A., et al. (2011). Predictors and Moderators of Time to Remission of Major Depression with Interpersonal Psychotherapy and SSRI Pharmacotherapy. Psychological Medicine, 41(1), 151–162. http://doi.org/10.1017/S0033291710000553.

- Frank S. (2010). Tetrabenazine: the first approved drug for the treatment of chorea in US patients with Huntington disease. Neuropsychiatr Dis Treat. 6:657-665. doi: 10.2147/NDT.S6430.

- Fredholm, B.B., Battig, K., Holmen, J., Nehlig, A., Zvartau, E. (1999). Actions of caffeine in the brain with special reference to factors that contribute to its widespread use. Pharmacol. Rev.51,83-130. Doi: 0031-6997/99/5101-0083$03.00/0.

- Fredholm, B.B., IJzerman, A.P., Jacobson, K.A., Linden, J., Müller, C.E. (2011). International Union of Basic and Clinical Pharmacology. LXXXI. Nomenclature and Classification of adenosine Receptors—An Update. Pharmacological Reviews. 63(1):1-34. doi: 10.1124/pr.110.003285.

- Fuxe, K., Agnati, L.F, Jacobsen, K., Hillion, J., Canals, M., Torvinen, M. et al. (2003). Receptor heteromerization in adenosine A2A receptor signaling: relevance for striatal function and Parkinson's disease. Neurology. 61,S19-23. doi: 10.1212/01.WNL.0000095206.44418.5C

- Gilbert, R.M. (1984). Caffeine consumption. In: Spiller GA (ed) The methylxanthine beverages and foods: chemistry, consumption, and health effects. Alan R. Liss, New York 185-213.

- Gorwood P., Richard-Devantoy, S., Baylé, F., Cléry-Melun, M.L. (2014). Psychomotor retardation is a scar of past depressive episodes, revealed by simple cognitive tests. European Neuropsychopharmacology. 24,1630-1640. doi: 10.1016/j.euroneuro.2014.07.013.

- Greden, J.F., Fontaine, P., Lubetsky, M., Chamberlin, K. (1978). Anxiety and depression associated with caffeinism among psychiatric inpatients. American. Journal. of Psychiatry, 135,963-966.

- Griffiths, R.R., Mumford, G.K. (1995). Caffeine – a drug of abuse? In: Bloom FE, Kupfer DJ (eds) Psychopharmacology: the fourth generation of progress. Raven Press, New York, pp 1699-1713.

- Griffiths, R.R., Bigelow, G.E., Liebson, I.A. (1986). Human coffee drinking: reinforcing and physical dependence producing effects of caffeine. J Pharmacol Exp Ther. 239(2),416-25

- Griffiths, R.R., Evans, S.M., Heishman, S.J., Preston, K.L., Sannerud, C.A., Wolf, B., et al. (1990). Low-dose caffeine discrimination in humans. J Pharmacol Exp Ther. 252,970-978.

- Gu, L., Gonzalez, F.J., Kalow, W., Tang, B.K. (1992). Biotransformation of caffeine, paraxanthine, theobromine and theophylline by cDNA-expressed human CYP1A2 and CYP2E1. Pharmacogenetics. 2(2),73-77. doi: 10.1097/00008571-199204000-00004

- Guay, D.R. (2010).Tetrabenazine, a monoamine-depleting drug used in the treatment of hyperkinectic movement disorders. Am J Geriatr Pharmacother. 8,331-373. doi: 10.1016/j.amjopharm.2010.08.006.

- Gullion, C.M., Rush, A.J. (1998). Toward a generalizable model of symptoms in major depressive disorder. Biol Psychiatry. 44,959-972. doi:10.1016/S0006-3223(98)00235-2.

- Hamilton, M. (1960). A rating scale for depression. J Neurol Neurosurg Psychiatry 23:56-62.

- Hauber, W., Sommer, S. (2009). Prefrontostriatal circuitry regulates effort-related decision making. Cereb. Cortex [Epub ahead of print].10.1093/cercor/bhn241

- Heckman, M.A., Weil, J., Gonzalez de Mejia, E. (2010). Caffeine (1, 3, 7-trimethylxanthine) in foods: a comprehensive review on consumption, functionality,

safety, and regulatory matters. J. Food. Sci. 75,77-87. doi: 10.1111/j.1750-3841.2010.01561.x.

- Hung, A.Y., Schwarzschild, M.A. (2014). Treatment of Parkinson's disease: what's in the non-dopaminergic pipeline?. Neurotherapeutics.11,34-46. doi: 10.1007/s13311-013-0239-9.

- Hunter, A.M., Balleine, B.W., Minor, T.R. (2003). Helplessness and escape performance: glutamate-adenosine interactions in the frontal cortex. Behav. Neurosci. 117,123-135. doi: http://dx.doi.org/10.1037/0735-7044.117.1.123.

- Jacobson, K.A., Gao, Z.G. (2006). Adenosine receptors as therapeutic targets. Nat Rev Drug Discov. 5(3247-64.

- Jacobson, K.A., van Galen, P.J., Williams, M. (1992). Adenosine receptors: pharmacology, structure-activity relationships, and therapeutic potential. J Med Chem 35,407-422. doi**:** 10.1021/jm00081a001

- Johnson, L.C., Spinweber, C.L., Gomez, S.A. (1990). Benzodiazepines and caffeine: effect on daytime sleepiness, performance, and mood. Psychopharmacology (Berl). 101,160-167. Doi: 10.1007/BF02244120.

- Juliano, L.M., Griffiths, R.R. (2004). A critical review of caffeine withdrawal: empirical validation of symptoms and signs, incidence, severity, and associated features. Psychopharmacology.176,1-29. doi. 10.1007/s00213-004-2000-x

- Kale, P.P., Addepalli, V. (2014). Augmentation of antidepressant effects of duloxetine and bupropion by caffeine in mice. Pharmacol. Biochem. Behav. 124,238-244. doi:10.1016/j.jecm.2010.09.001

- Kaster, M.P., Rosa, A.O., Rosso, M.M., Goulart, E.C., Santos, A.R., Rodrigues, A.L. (2004). Adenosine administration produces an antidepressant-like effect in mice: evidence for the involvement of A1 and A2A receptors. Neurosci Lett. 355,21-24. doi:10.1016/j.neulet.2003.10.040.

- Kaster, M.P., Machado, N.J., Silva, H.B., Nunes, A., Ardais, A.P., Santana, M., et al., (2015). Caffeine acts through neuronal adenosine A2A receptors to prevent mood and memory dysfunction triggered by chronic stress. Proc Natl Acad Sci U S A. 112(25),7833-7838. doi: 10.1073/pnas.1423088112.

- Katz, R.J., Roth, K.A., Carroll, B.J. (1981). Acute and chronic stress effects on open field activity in the rat: implications for a model of depression. Neurosci Biobehav Rev. 5(2),247-251.

- Kawachi, I., Willett, W.C., Colditz, G.A., Stampfer, M.J., Speizer, F.E. (1996). A prospective study of coffee drinking and suicide in women. Arch. Intern. Med. 156, 521-525. doi:10.1001/archinte.1996.00440050067008.

- Kent, T.A., Preskorn, S.H., Glotzbach, R.K., Irwin, G.H. (1986). Amitriptyline normalizes tetrabenazine-induced changes in cerebral microcirculation. Biol Psychiatry. 21:483-491. doi: 10.1016/0006-3223(86)90190-3

- Kitada, Y., Miyauchi, T., Satoh, A., Satoh, S. (1981). Effects of antidepressants in the rat forced swimming test. Eur J Pharmacol. 72(2-3),145-152.

- Kulkarni, S.K, Metha, A.K. (1985). Purine nucleoside-mediated immobility in mice: Reversal by antidepressants. Psychopharmacology, 85,460-463. doi: 10.1007/BF00429665.

- Leibenluft, E., Fiero, P.L., Bartko, J.J., Moul, D.E., Rosenthal. N.E. (1993). Depressive symptoms and the self-reported use of alcohol, caffeine, and carbohydrates in normalvolunteers and four groups of psychiatric outpatients. Am J Psychiatry. 150(2),294-301. doi: 10.3109/10884609409021713.

- LeWitt, P.A., Guttman, M., Tetrud, J.W., Tuite, P.J., Mori, A., Chaikin, P., Sussman, N.M. (2008). 6002-US-005 Study Group. Adenosine A2A receptor antagonist istradefylline (KW-6002) reduces "off" time in Parkinson's disease: a double-blind, randomized, multicenter clinical trial (6002-US-005). Ann Neurol. 63(3),295-302.

- Lieberman, H.R., Wurtman, R.J., Emde, G.G., Coviella, I.L. (1987). The effects of caffeine and aspirin on mood and performance. J Clin Psychopharmacol. 7,315-320. Doi:271-0749/87/0705-0315$02.00/0

- Liguori, A., Hughes, J.R (1997). Caffeine self-administration in humans: 2. A within-subjects comparison of coffee and cola vehicles. Exp Clin Psychopharmacol. 5(3),295-303.

. - López-Cruz, L., Pardo, M., Salamone, J.D., Correa, M. (2014). Differences between the nonselective adenosine receptor antagonists caffeine and theophylline in motor and mood effects: studies using medium to high doses in animal models. Behav Brain Res. 270,213-22. doi: 10.1016/j.bbr.2014.05.020.

- Lucas, P.B., Pickar, D., Kelsoe, J., Rapaport, M., Pato, C., Hommer, D. (2011). Effects of the acute administration of caffeine in patients with schizophrenia. Biol Psychiatry.28,35-40. doi:0006-3223/90/$00.00.

- Maier, S.F., Seligman, M.E.P. (1976) Learned helplessness: theory and evidence. J Exp Psychol. 105,3-46.

- Malinauskas, B.M., Aeby, V.G., Overton, R.F., Carpenter-Aeby, T., Barber-Heidal, K. (2007). A survey of energy drink consumption patterns among college students. Nutr J. 31, 6-35. doi:10.1186/1475-2891-6-35.

- McClintock, S.M., Husain, M.M., Wisniewski, S.R., Nierenberg, A.A., Stewart, J.W., Trivedi, M.H., Cook, I., Morris, D,, Warden, D., Rush, A.J. (2011). Residual symptoms in depressed outpatients who respond by 50% but do not remit to antidepressant medication. J Clin Psychopharmacol. 31(2):180-6. doi: 10.1097/JCP.0b013e31820ebd2c.

- Mingote, S., Font, L., Farrar, A.M., Vontell, R., Stopper, C.M., Port R.G., et al. (2008). Nucleus accumbens adenosine A2A receptors regulate exertion of effort by acting on the ventral striatopallidal pathway. J Neuroscience. 28,9037-9046. doi: 10.1523/JNEUROSCI.1525-08.2008.

- Minor, T.R., Hanff, T.C. (2015). Adenosine signaling in reserpine-induced depression in rats. Behavioural Brain Research. 286,184-91. doi: 10.1016/j.bbr.2015.02.032

- Minor, T.R., Winslow, J.L., Chang, W.C. (1994a). Stress and adenosine: II. Adenosine analogs mimic the effect of inescapable shock on shuttle-escape performance in rats. Behav Neurosci. 108,265-276. doi: http://dx.doi.org/10.1037/0735-7044.108.2.265.

- Minor, T. R., Chang, W. C., Winslow, J. L. (1994b). Stress and adenosine: I. Effect of methylxanthine and amphetamine stimulants on learned helplessness in rats. Behav Neurosci. 108, 254–264.

- Minor, T.R., Rowe, M., Cullen, P.K., Furst, S. (2008). Enhancing brain adenosine signaling with the nucleoside transport blocker NBTI (S-(4-nitrobenzyl)-6-theoinosine) mimics the effects of inescapable shock on later shuttle-escape performance in rats. Behav Neurosci. 122(6),1236-47. doi: 10.1037/a0013143.

- Mott, A.M., Nunes, E.J., Collins, L.E., Port, R.G., Sink, K.S., Hockemeyer, J., et al. (2009). The adenosine A2A antagonist MSX-3 reverses the effects of the dopamine antagonist haloperidol on effort-related decision making in a T-maze cost/benefit procedure. Psychopharmacology (Berl). 204(1),103-112. doi: 10.1007/s00213-008-1441-z.

- Noji, T., Karasawa, A., Ksuka, H. (2004). Adenosine uptake inhibitors. European Journal of Pharmacology, 495,1-16.

- Nunes, E.J., Randall, P.A., Santerre, J.L., Given, A.B., Sager, T.N., Correa, M., et al. (2010). Differential effects of selective adenosine antagonists on the effort-related

impairments induced by dopamine D1 and D2 antagonism. Neuroscience. 170, 268-280. doi: 10.1016/j.neuroscience.2010.05.068.

- Nunes, E.J., Randall, P.A., Hart, E.E., Freeland, C., Yohn, S.E., Baqi, Y., et al., (2013). Effort-related motivational effects of the VMAT-2 inhibitor tetrabenazine: implications for animal models of the motivational symptoms of depression. J Neurosci 33,19120-19130.

- Overmier, J.B., Seligman, M.E. (1967). Effects of inescapable shock upon subsequent escape and avoidance responding. J Comp Physiol Psychol. 63(1),28-33.

- Padala, P.R., Padala, K.P., Monga, V., Ramirez, D.A., Sullivan, D.H. (2012). Reversal of SSRI-associated apathy syndrome by discontinuation of therapy. Ann. Pharmacother. 46(3),e8. doi: 10.1345/aph.1Q656.

- Pae, C.U., Lim, H.K., Han, C., Patkar, A.A., Steffens, D.C., Masand, P.S. (2007). Fatigue as a core symptom in major depressive disorder: overview and the role of bupropion. Expert Rev Neurother. 7(10),1251-1263. doi:10.1586/14737175.7.10.1251.

- Pardo, M., Lopez-Cruz, L., Valverde, O., Ledent, C., Baqi, Y., Müller, C.E., Salamone, J.D., Correa, M. (2012). Adenosine A2A receptor antagonism and genetic deletion attenuate the effects of dopamine D2 antagonism on effort-based decision making in mice. Neuropharmacology. 62, 2068-2077. doi: 10.1016/j.neuropharm.2011.12.033.

- Pardo, M., López-Cruz, L., San Miguel, N., Salamone, J.D., Correa, M. (2015). Selection of sucrose concentration depends on the effort required to obtain it: studies using tetrabenazine, D1, D2, and D3 receptor antagonists. Psychopharmacology (Berl). 232(13):2377-91. doi: 10.1007/s00213-015-3872-7.

- Patten, S.B., Metz, L.M., Reimer, M.A. (2000). Biopsychosocial correlates of lifetime major depression in a multiple sclerosis population. Mult. Scler. 6,115-120. doi: 10.1177/135245850000600210.

- Pechlivanova, D.M., Tchekalarova, J.D., Alova, L.H., Petkov, V.V., Nikolov, R.P., and Yakimova, K.S. (2012). Effect of long-term caffeine administration on depressive-like behavior in rats exposed to chronic unpredictable stress. Behav. Pharmacol. 23,339-347. doi: 10.1097/FBP.0b013e3283564dd9.

- Peeling, P., Dawson, B. (2007). Influence of caffeine ingestion on perceived mood states, concentration, and arousal levels during a 75-min university lecture. Adv Physiol Educ. 31,332-335. Doi: 10.1152/advan.00003.2007.

- Petit-Demouliere, B., Chenu, F., Bourin, M. (2005). Forced swimming test in mice: a review of antidepressant activity. Psychopharmacology (Berl). 177,245-255. doi:10.1007/s00213-004-2048-7.

- Pettibone, D.J., Totaro, J.A., Pflueger, A.B. (1984) Tetrabenazine-induced depletion of brain monoamines: characterization and interaction with selected antidepressants. Eur J Pharmacol 102,425-430.

- Petty, F., McChesney, C., Kramer, G. (1985). Intracortical glutamate injection produces helpless-like behavior in the rat. Pharmacol. Biochem. Behav. 22,531-533. doi:10.1016/0091-3057(85)90270-9.

- Pinna, A., Pontis, S., Borshini, F., Morelli, M. (2007). Adenosine A2A receptor antagonists improve deficits in initiation of movement and sensory motor integration in the unilateral 6-hydroxydopamine rat model of Parkinson's disease. Synapse 61(8),606-614. doi: 10.1007/s40263-014-0161-7.

- Porsolt, R.D., Bertin, A., Jalfre, M. (1977a). Behavioral despair in mice: a primary screening test for antidepressants. Arch. Int. Pharmacodyn. Ther. 229,327-336.

- Porsolt, R.D., Le Pichon, M., Jalfre, M. (1977b). Depression: a new animal model sensitive to antidepressant treatments. Nature. 266,730-732. doi:10.1038/266730a0

- Porsolt, R.D., Anton, G., Blavet, N., Jalfre, M. (1978). Behavioral despair in rats: a new model sensitive to antidepressant treatments. Eur J Pharmacol. 47:379-381.

- Prediger, R.D. (2010). Effects of caffeine in Parkinson's disease: from neuroprotection to the management of motor and non-motor symptoms. J Alzheimers Dis. 20 Suppl 1,205-220. doi: 10.3233/JAD-2010-091459.

- Preskorn, S.H., Kent, T.A., Glotzbach, R.K., Irwin, G.H., Solnick, J.V. (1984). Cerebromicrocirculatory defects in animal model of depression. Psychopharmacology (Berl). 84(2),196-199.

- Quitkin, F., Rifkin, A., Klein, D.F. (1979). Monoamine oxidase inhibitors. A review of antidepressant effectiveness. Arch Gen Psychiatry. 36(7):749-760. doi:10.1001/archpsyc.1979.01780070027003.

- Rampello, L., Nicoletti, G., Raffaele, R. (1991). Dopaminergic hypothesis for retarded depression: a symptom profile for predicting therapeutical responses. Acta. Psychiatr. Scand. 84,552-554. doi: 10.1111/j.1600-0447.1991.tb03193.x.

- Rampello, L., Nicoletti, G., Raffaele, R., Drago, F. (1995). Comparative effects of amitriptyline and amineptine in patients affected by anxious depression. Neuropsychobiology. 31(3),130-141. doi:10.1159/000119183.

- Randall, P.A, Nunes, E.J, Janniere, S.L, Stopper, C.M, Farrar, A.M, Sager, T.N, et al. (2011). Stimulant effects of adenosine antagonists on operant behavior: differential actions of selective A2A and A1 antagonists. Psychopharmacology 216,173-186.

- Randall, P., Pardo M., Nunes, E., López Cruz, L., Vemuri, V.K., Makriyannis, A., et al., (2012). PLoS ONE, 7, p. e47934

- Randall, P.A., Lee, C.A., Podurgiel, S.J., Hart, E., Yohn, S.E., Jones, M., Rowland, M. et al. (2014). Bupropion increases selection of high effort activity in rats tested on progressive ratio/chow feeding choice procedure: Implications for treatment of effort-related motivational symptoms. The International Journal of Neuropsychopharmacology. doi: 10.1093/ijnp/pyu017.

- Rees, K., et al (1999). The influences of age and caffeine on psychomotor and cognitive function. Psychopharmacology (Berl).145,181-188.

- Retzbach, E.P., Dholakia, P.H., Duncan-Vaidya, E.A. (2014). The effect of daily caffeine exposure on lever-pressing for sucrose and c-Fos expression in the nucleus accumbens in the rat. Physiol Behav. 135,1-6. doi: 10.1016/j.physbeh.2014.05.038.

- Richelson, E. (1982). Pharmacology of antidepressants in use in the United States. J Clin Psychiatry.43,4-13.

- Rihs, M., Muller, C., Baumann, P. (1996). Caffeine consumption in hospitalized psychiatric patients. Eur Arch Psychiatry Clin. Neurosci. 246,83-92. doi: 10.1007/BF02274898.

- Robles-Molina, E., Millán, D., Hong, E., Villafaña, S. (2012). Increased antidepressant-like effect of desipramine combined with central stimulants (caffeine and amphetamine) in mice. Cent. Eur. J. Biol. 7(3), 391-396. doi: 10.2478/s11535-012-0028-1.

- Rush, A.J., Trivedi, M.H. (1995). Treating depression to remission. Psychiatric. Annals. 25,704-709. doi: 10.1002/gps.2443.

- Salamone, J.D., Correa, M. (2002). Motivational views of reinforcement: implications for understanding the behavioral functions of nucleus accumbens dopamine. Behav. Brain. Res.137,3-25. doi:10.1016/S0166-4328(02)00282-6.

- Salamone, J.D., Correa, M. (2009). Dopamine/adenosine interactions involved in effort-related aspects of food motivation. Appetite. 53,422-525. doi:10.1016/j.appet.2009.07.018.

- Salamone, J.D., Correa, M. (2012). The mysterious motivational functions of mesolimbic dopamine. Neuron 76(3),470-85.

- Salamone, J.D., Steinpreis, R.E., McCullough, L.D., Smith, P., Grebel, D., Mahan, K. (1991). Haloperidol and nucleus accumbens (DA) depletion suppress lever pressing for food but increase free food consumption in a novel food choice procedure. Psychopharmacology. 104, 515-521. doi: 10.1007/BF02245659.

- Salamone, J.D., Cousins, M.S., Bucher, S. (1994). Anhedonia or anergia? Effects of haloperidol and nucleus accumbens (DA) depletion on instrumental response selection in a T-maze cost/benefit procedure. Behav Brain Res. 65,221-229. doi:10.1016/0166-4328(94)90108-2.

- Salamone, J.D., Arizzi, M.N., Sandoval, M.D., Cervone, K.M., Aberman, J.E. (2002). (DA) antagonists alter response allocation but do not suppress appetite for food in rats: contrast between the effects of SKF 83566, raclopride, and fenfluramine on a concurrent choice task. Psychopharmacology. 160,371-380. doi: 10.1007/s00213-001-0994-x.

- Salamone, J.D., Correa, M., Mingote, S.M., Weber, S.M., Farrar, A.M. (2006). Nucleus accumbens dopamine and the forebrain circuitry involved in behavioral activation and effort-related decision making: Implications for understanding anergia and psychomotor slowing in depression. Current. Psychiatry. Reviews. 22,267-280. doi: http://dx.doi.org/10.2174/157340006776875914.

- Salamone, J.D., Correa, M., Farrar, A., Mingote, S.M. (2007). Effort-related functions of nucleus accumbens dopamine and associated forebrain circuits. Psychopharmacology. 191,461-482. doi:10.1007/s00213-006-0668-9.

- Salamone, J.D., Farrar, A.M., Font, L., Patel, V., Schlar, D.E., Nunes, E.J., et al. (2009). Differential actions of adenosine A1 and A2A antagonists on the effort-related effects of dopamine D2 antagonism. Behav Brain Res. 201,216-222. doi: 10.1016/j.bbr.2009.02.021.

- Salamone, J.D., Correa, M., Farrar, A.M., Nunes, E.J., Collins, L.E. (2010). Role of dopamine–adenosine interactions in the brain circuitry regulating effort-related decision making: insights into pathological aspects of motivation. 3,377-392. http://dx.doi.org/10.2217/fnl.10.19.

- Santerre, J.L., Nunes, E.J., Kovner, R., Leser, C.E., Randall, P.A., Collins-Praino, L.E. et al. (2012). The novel adenosine A(2A)antagonist prodrug MSX-4 is effective in animal models related to motivational and motor functions. Pharmacol Biochem Behav. 102,477-487. doi:10.1016/j.pbb.2012.06.009.

- Schiffmann, S.N., Jacobs, O.P., Vanderhaegen, J.J. (1991). Striatal restricted adenosine A2 receptor (RDC8) is expressed by enkephalin but not substance P neurons:

an in situ hybridization study. J Neurochem 57,1062-1067. doi:10.1111/j.1471-4159.1991.tb08257.x

- Scott, N.R., Chakraborty, J., Marks, V. (1989). Caffeine consumption in the United Kingdom: a retrospective study. Food Sciences and Nutrition. 42F,183-191.

- Sheppard, B.A., Gross, S.C., Pavelka, S.A, Hall, M.J., Palmatier, M.I. (2012). Caffeine increases the motivation to obtain non-drug reinforcers in rats. Drug Alcohol Depend. 124(3),216-222. doi: 10.1016/j.drugalcdep.2012.01.008.

- Silber, B.Y., Croft, R.J., Papafotiou, K., Stough, C. (2006). The acute effects of D-amphetamine and methamphetamine on attention and psychomotor performance. Psychopharmacol (Berl). 187,154-169. doi: 10.1007/s00213-006-0410-7.

- Silverman, K., Evans, S.M., Strain, E.C., Griffiths, R.R. (1992). Withdrawal syndrome after the double-blind cessation of caffeine consumption. N. Engl. J. Med. 327(16),1109-1114.

- Silverman, K., Mumford, G.K., Griffiths, R.R. (1994). Enhancing caffeine reinforcement by behavioral requirements following drug ingestion. Psychopharmacology 114,424-432.

- Simola, N., Fenu, S., Baraldi, P.G., Tabrizi, M.A., Morelli, M. (2004). Blockade of adenosine A2A receptors antagonizes parkinsonian tremor in the rat tacrine model by an action on specific striatal regions. Exp Neurol 189,182-188. doi:10.1016/j.expneurol.2004.05.027.

- Simola, N., Fenu, S., Baraldi, P.G., Tabrizi, M.A., Morelli, M. (2006). Dopamine and adenosine receptor interaction as basis for the treatment of Parkinson's disease. J Neurol Sci. 248,48-52. doi:10.1016/j.jns.2006.05.038

- Smith, A. (2002). Effects of caffeine on human behavior. Food. Chem. Toxicol. 40,1243-1255. doi:10.1016/S0278-6915(02)00096-0.

- Smith, A., Kendrick, A.M., Maben, A.L. (1992). Effects of breakfast and caffeine on performance and mood in the late morning and after lunch. Neuropsychobiology. 26,198-204. doi:10.1159/000118920.

- Smith, A., Thomas, M., Perry, K., Whitney, H. (1997). Caffeine and the common cold. J Psychopharmacol. 11,319-324. doi: 10.1177/026988119701100406.

- Szopa, A., Poleszak, E., Wyska, E.,Serefko, A., Wośko, S., Wlaź, A., et al., (2016). Caffeine enhances the antidepressant-like activity of common antidepressant drugs in the forced swim test in mice. (2016). Naunyn Schmiedebergs Arch Pharmacol. 389,211–221.

doi: 10.1007/s00210-015-1189-z.

- Stahl, S.M. (2002). The psychopharmacology of energy and fatigue. J. Clin. Psychiatry. 263,7-8. doi: 10.4088/JCP.v63n0102.

- Stenman, E., Lilja, A. (2013). Increased monoaminergic neurotransmission improves compliance with physical activity recommendations in depressed patients with fatigue. Med Hypotheses. 80(1),47-49. doi: 10.1016/j.mehy.2012.10.007.

- Steru, L., Chermat, R., Thierry, B., Simon, P. (1985). The tail suspension test: a new method for screening antidepressants in mice, Psychopharmacology. 85,367-370. doi: 10.1007/BF00428203.

- Swindle, R. (2001). Energy and improved workplace productivity in depression. In: Investing in Health: The Social and Economic Benefits of Health Care Innovation. Elsevier Science. 14,23-341.

- Szopa, A., Poleszak, E., Wyska, E., Serefko, A., Wośko, S., Wlaź, A., Pieróg, M., Wróbel, A., Wlaź, P. (2016). Caffeine enhances the antidepressant-like activity of common antidepressant drugs in the forced swim test in mice. Naunyn Schmiedebergs Arch Pharmacol. 389(2):211-221. doi: 10.1007/s00210-015-1189-z

- Tanra, A.J., Kagaya, A., Okamoto, Y., Muraoka, M., Motohashi, N., Yamawaki, S. (1995). TJS-010, a new prescription of oriental medicine, antagonizes tetrabenazine-induced suppression of spontaneous locomotor activity in rats. Prog Neuropsychopharmacol Biol Psychiatry 19(5),963-971.

- Tanskanen, A., Tuomilehto, J., Viinamaki, H., Vartiainen, E., Lehtonen, J., and Puska, P. (2000). Heavy coffee drinking and the risk of suicide. Eur. J. Epidemiol. 16, 789-799.doi: 10.1023/A:1007614714579.

- Treadway, M.T., Buckholtz, J.W., Schwartzman, A.N., Lambert, W.E., and Zald, D.H. (2009). Worth the 'EEfRT'? The effort expenditure for rewards task as an objective measure of motivation and anhedonia. PLoS One. 4(8):e6598. doi:10.1371/journal.pone.0006598.

- Treadway, M.T., Bossaller, N.A., Shelton, R.C., and Zald D.H. (2012). Effort-based decision-making in major depressive disorder: a translational model of motivational anhedonia. J. Abnorm. Psychol. 121,553-558. doi: 10.1037/a0028813.

- Tylee, A., Gastpar, M., Lepine, J.P., and Mendlewicz, J. (1999). DEPRES II (Depression Research in European Society II): a patient survey of the symptoms, disability and current management of depression in the community. Int Clin Psychopharmacol. 14,139-151.doi: 10.1097/00004850-199905030-00001.

- van den Bos, R., van der Harst, J., Jonkman, S., Schilders, M., Spruijt, B. (2006). Rats assess costs and benefits according to an internal standard. Behav Brain Res 171,350-354.

- Wang, H., Chen, X., Li, Y., Tang, T.S., Bezprozvanny, I. (2010). Tetrabenazine is neuroprotective in Huntington's disease mice. Mol Neurodegener. 5,18. doi: 10.1186/1750-1326-5-18.

- Wardas, J., Konieczny, J., Lorenc-Koci, E. (2001). SCH 58261, an A(2A) adenosine receptor antagonist, counteracts parkinsonian-like muscle rigidity in rats. Synapse. 41:160-171. doi:10.1016/S0006-8993(03)02759-8

- Wardle, M.C., Treadway, M.T., Mayo, L.M., Zald, D.H., de Wit, H. (2011). Amping up effort: effects of d-amphetamine on human effort-based decision-making. J Neurosci. 31(46),16597-16602. doi: 10.1523/JNEUROSCI.4387-11.2011.

- Wardle, M.C., Treadway, M.T., de Wit, H. (2012). Caffeine increases psychomotor performance on the effort expenditure for rewards task. Pharmacol Biochem Behav. 102,526-531. doi: 10.1016/j.pbb.2012.06.016.

- Weiss, B., Laties, V. G. (1962). Enhancement of human performance by caffeine and the amphetamines. Pharmacol Rev., 14,1-36.

- Whalen, D.J., Silk, J.S., Semel, M., Frobes, E.E., Ryan, N.D., Axelson, D.A., et al. (2008). Caffeine consumption, sleep, and affect in the natural environments of depressed youth and healthy controls. J Pediatr Psychol 33,358-367. doi: 10.1093/jpepsy/jsmo86.

- Willner P. (2005). Chronic mild stress (CMS) revisited: consistency and behavioural-neurobiological concordance in the effects of CMS. Neuropsychobiology. 52(2),90-110.doi: 10.1159/000087097.

- Woodson, J.C., Minor, T.R., and Job, R.F. (1998). Inhibition of adenosine deaminase by erythro-9-(2-hydroxy-3-nonyl)adenine (EHNA) mimics the effect of inescapable shock on escape learning in rats. Behav Neurosci. 112,399-409. http://dx.doi.org/10.1037/0735-7044.112.2.399.

- Worden, L.T., Shahriari, M., Farrar, A.M., Sink K.S, Hockemeyer, J., Müller, C., et al. (2009).The adenosine A2A antagonist MSX-3 reverses the effort-related effects of dopamine blockade: differential interaction with D1 and D2 family antagonists. Psychopharmacology. 203,489-499. doi: 10.1007/s00213-008-1396-0.

- World Health Organization. The Global Burden of Disease: 2004 Update. Available at www.who.int/healthinfo/global_burden_disease/2004_report_update/en/index.html.

- Worthington, J., Fava, M., Agustin, C., Alpert, J., Nierenberg, A.A., Pava, J.A., Rosenbaum, J.F. (1996). Consumption of alcohol, nicotine, and caffeine among depressed outpatients. Relationship with response to treatment. Psychosomatics. 37,518-522.

- Yang, X. H., Huang, J., Zhu, C. Y., Wang, Y. F., Cheung, E. F., Chan, R. C., et al. (2014). Motivational deficits in effort-based decision making in individuals with subsyndromal depression, first-episode and remitted depression patients. Psychiatry Res. 220,874–882. doi: 10.1016/j.psychres.2014. 08.056.

- Yıldız, A., Gönül, A.S., Tamam, L. (2002). Mechanism of Actions of Antidepressants: Beyond the Receptors. BCP 12,194-200.

- Yohn, S.E., Thompson, C., Randall, P.A., Lee, C.A., Müller, C.E., Baqi, Y., Correa, M., Salamone, J.D. (2015a). The VMAT-2 inhibitor tetrabenazine alters effort-related decision making as measured by the T-maze barrier choice task: reversal with the adenosine A2A antagonist MSX-3 and the catecholamine uptake blocker bupropion. Psychopharmacology 232,1313-1323. doi: 10.1007/s00213-014-3766-0.

- Yohn, S.E., Santerre, J.L., Nunes, E.J., Kozak, R., Podurgiel, S.J., Correa, M., Salamone, J.D. (2015b). The role of dopamine D1 receptor transmission in effort-related choice behavior: Effects of D1 agonists. Pharmacol Biochem Behav. 135,217-226. doi:10.1016/j.pbb.2015.05.003.

- Yohn, S.E., Collins, S.L., Contreras-Mora, H.M., Errante, E.L., Rowland, M.A., Correa, M., et al., (2016a). Salamone JD. Not All Antidepressants Are Created Equal: Differential Effects of Monoamine Uptake Inhibitors on Effort-Related Choice Behavior. Neuropsychopharmacology. 41(3),686-94. doi: 10.1038/npp.2015.188.

- Yohn, S.E., Lopez-Cruz, L., Hutson, P.H., Correa, M., Salamone, J.D. (2016b). Effects of lisdexamfetamine and s-citalopram, alone and in combination, on effort-related choice behavior in the rat. Psychopharmacology (Berl). 233(6),949-60.doi: 10.1007/s00213-015-4176-7.

- Yu, G., Maskray, V., Jackson, S.H., Swift, C.G., Tiplady, B. (1991). A comparison of the central nervous system effects of caffeine and theophylline in elderly subjects. Br J Clin Pharmacol. 32,341-345.

- Zisook, S., Rush, A.J., Haight, B.R., Clines, D.C., Rockett, C.B. (2006). Use of bupropion in combination with serotonin reuptake inhibitors. Biol Psychiatry. 59(3),203-210. doi:10.1016/j.physbeh.2008.03.004.

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CHAPTER 6:

Caffeine reverses the shift in preference from high to low effort reinforcing activities induced by dopamine depletion: relation to DARPP32 phosphorylation patterns.

Abstract

The mesolimbic dopamine (DA) system plays a critical role in behavioral activation and effort-based decision-making. DA depletion produces anergia (shifts to low effort options) in effort-based tasks. Caffeine, the most consumed stimulant in the world, acts as an adenosine A_1/A_{2A} receptor antagonist, and DA D_1 and D_2 receptors are co-localized with adenosine A_1 and A_{2A} receptors. In the present work, we evaluated the effect of caffeine on anergia induced by the VMAT-2 inhibitor tetrabenazine (TBZ), which produces DA depletions. Anergia was evaluated in a three-chamber T-maze task in which animals can chose between running on a wheel (RW) vs. sedentary activities such as consuming sucrose or sniffing a neutral odor. Independent groups of animals were evaluated for voluntary motor activity in the RW and sucrose consumption. DA tissue levels after TBZ were evaluated with HPLC. TBZ-caffeine interactions were evaluated on DARPP-32 phosphorylation patterns as an intracellular marker of DA receptor activity in striatum. In the T-maze, control mice spent more time running and much less consuming sucrose, and also did very little sniffing. TBZ (4.0 mg/kg) reduced DA tissue levels and also shifted preferences, reducing selection of the reinforcer that involved vigorous activity (RW), but increasing consumption of a reinforcer that required little effort (sucrose), at doses that had no effect on independent measures of appetite or locomotion in the RW. This suggests that DA depletion produced anergia, but did not affect the primary motivating effects of sucrose. Caffeine at doses that had no effect on their own reversed TBZ effects on the T-maze and the RW. Caffeine also suppressed TBZ-induced pDARPP-32(Thr34) expression, suggesting a role for D_2-A_{2A} interaction.

Key words: Decision-making, motivation, behavioral activation, dopamine, adenosine, sucrose, running wheel

Running title: Caffeine reversal of dopamine depletion-induced anergia

1. Introduction

Motivated behavior is directed towards or away from particular stimuli, but it also is characterized by a high degree of activity, effort, vigor, and persistence (Salamone and Correa, 2002; 2012). These activational aspects of motivation are highly adaptive, because they enable organisms to overcome the work-related obstacles that separate them from significant stimuli (Salamone and Correa, 2002, 2012). Activation-related dysfunctions, such as anergia, and fatigue, are an important and debilitating set of symptoms seen in major depression, Parkinson disease (PD), schizophrenia and other pathologies (Caligiuri and Ellwanger, 2000; Salamone and Correa, 2012; Friedman et al. 2007; Tellez et al. 2005**;** Tylee et al. 1999; Demyttenaere et al. 2005). Thus, it has been demonstrated that early Parkinsonian patients have subjective reports of lack of energy (Friedman et al. 2007; Nomoto et al. 2014), and reduced selection of high-effort activities (Elbers et al. 2009), and people with depression show a decrease -n selection of high effort/high reward options when compared with healthy controls (Treadway et al. 2012).

Several lines of evidence have identified dopamine (DA), particularly in nucleus accumbens (Nacb), as a critical component of the brain circuitry regulating behavioral activation and effort-related processes (Salamone and Correa, 2002, 2012; Mai et al. 2012). Interference with DA transmission can affect allocation of effort on tasks that assess effort-based choice behavior, biasing individuals towards lower effort alternatives (Salamone et al. 2007; Floresco et al. 2008; Hauber and Sommer, 2009). In these tasks animals have the option of vigorously working (lever pressing or climbing a barrier) to get access to preferred reinforcers versus approaching and consuming a less preferred food or sucrose solution that requires less effort to obtain (Salamone et al. 1991, 1994, 2002; Randall et al. 2012; Pardo et al. 2012, 2015; Mott et al. 2009; Yohn et al., 2015). The catecholamine depleting agent and vesicular transport inhibitor (VMAT-2) tetrabenazine (TBZ) used to treat Huntington's disease patients, has demonstrated to deplete monoamines, with its greatest impact being upon striatal DA (Pettibone et al. 1984; Tanra et al. 1995; Nunes et al. 2013). TBZ's main side effects include fatigue, Parkinsonism, and depression (Frank, 2009, 2010; Guay, 2010). TBZ also has been demonstrated to induce shifts in behavior towards low effort/low reward options in effort-based decision-making tasks in rodents (Pardo et al. 2015; Yohn et al. 2015; Nunes et al. 2013).

In addition to DA, the neuromodulator adenosine appears to be involved in the regulation of the activational component of motivated behaviors (Salamone and Correa 2009; Pereira et al. 2011). DA and adenosine receptors are co-localized (D_2-A_{2A} and D_1-A_1), and they converge onto the same signal transduction pathways, having opposite effects on the adenylyl cyclase-related signal transduction cascade (Ferré et al. 2004). Striatal areas such as neostriatum and Nacb are very rich in these types of adenosine receptors (Demet et al. 2002; Ferré et al. 2008, 2004). Thus, adenosine antagonists are been proposed as therapeutic agents to counteract symptoms induced by DA dysfunctions (Jenner 2014). In human patients with PD, istradefylline (a A_{2A} antagonist) has been demonstrated to reduce feelings of fatigue, depression and listlessness (Nomoto et al. 2014). Thus far, istradefylline is the only adenosine A2A antagonist that is approved for clinical use, and it is available in Japan. Caffeine is a natural methylxantine that acts mainly as a non-selective adenosine A_1 and A_{2A} receptor antagonist (Fredholm et al. 1999). In humans, caffeine has been shown to increase subjectively reported energy, and motivation to work, and to reduce fatigue and improve psychomotor performance (Lieberman et al. 2001; Yu et al. 1991; Johnson et al., 1990; Smith et al. 1992; 1997; Fredholm et al. 1999; Rees et al. 1999). It has been argued that the most powerful therapeutic effects of caffeine would be expected in situations of high fatigue (Liberman et al. 1987; Weiss and Laties, 1962).

The present studies investigated the impact of TBZ, caffeine and their combination on a novel T-maze task developed to assess preferences between active versus sedentary sources of reinforcement (adapted from Correa et al. 2016). This T-maze task does not involve work in order to get access to a reinforcer, as previous tasks developed in our laboratory (Pardo et al. 2012; Yohn et al. 2015), but instead offers the choice to freely engage in wheel running, or to consume palatable pellets containing 50% sucrose or, as a third alternative, to sniff into a hole with a neutral non-social odor. In addition, we evaluated the impact of TBZ and caffeine even at higher doses in independent groups of animals that were not in a choice situation and only had access to pellets or to a RW. Striatal levels of DA after TBZ administration were evaluated, and markers of D_1 or D_2 receptor activity (phosphorylated forms of DARPP-32; pDARPP-32(Thr34) and (Thr75) were quantified by western blot after these manipulations.

2. Methods

2.1. Subjects.

CD1 adult male mice (N=119) purchased from Janvier, France S.A. were 15-17 weeks old (30-45 g) at the beginning of the study. Mice were housed in groups of three or four per cage, with standard laboratory rodent chow and tap water available *ad libitum*. The colony was kept at a temperature of 22±2 ºC with lights on from 08:00 to 20:00 h. All animals were under a protocol approved by the Institutional Animal Care and Use Committee of Universitat Jaume I, and all experimental procedures complied with European Community Council directive (86/609/ECC). All efforts were made to minimize animal suffering, and to reduce the number of animals used.

2.2. Drugs.

Tetrabenazine (TBZ) [(*R,R*)-3-Isobutyl-9,10-dimethoxy-1,3,4,6,7,11b-hexahydro-pyrido[2,1-*a*]isoquinolin-2-one] (CIMYT Quimica SL, Spain), was dissolved in a 20% dimethylsulfoxide (DMSO) solution mixed with saline and pH adjusted with 1 N HCl to bring the final solution to pH 5.5. DMSO (20%v/v) was used as its control. TBZ was administered 120 min before testing. Caffeine (Sigma-Aldrich, Spain) was dissolved in 0.9% w/v saline. Saline solution was used as its vehicle control. Caffeine was administrated 30 min before test. All solutions were administered intraperitoneally (IP).

2.3. Behavioral and biochemical procedures

Three-choice running wheel T-maze task. The T-maze apparatus consisted of a central corridor with two opposed arms. Each arm provided a different type of stimuli (for details, see Fig 1). In one of them sweet pellets (TestDietTM, 50% sucrose, 45 mg each) were available, in another arm there was a RW, and in the third arm there was a hole with a cotton ball soked with a neutral non-social odor. Training as well as test sessions lasted 15 minutes. Mice were trained 5 days a week. Training phase 1: to avoid neophobia to the sweet tasting pellets, animals were enclosed in that arm with the food during 5 sessions. Training phase 2: during 2 more weeks animals were exposed, one 15 min session a day to the T-maze with free access to the three stimuli. Test phase: This phase lasted during 4 more weeks. For each week there were 4 baseline sessions plus a testing session in which animals either received drug injections. Sessions were videotaped and a trained observer unaware of the drug condition register manually

accumulated time spent in the RW, consuming the sucrose pellets, or sniffing the hole, and crosses into the arms or time spent in the arms of the T-maze. These measures were taken based on previous studies (Correa et al., 2016). Time was selected as the main dependent measure because it allowed us to evaluate the three conditions with the same units. Time allocation is a useful measure of preference, relative reinforcement value, and response choice (Baum and Rachlin, 1969). Testing sessions started two hours after light period onset. The behavioral test room was illuminated with a soft light, and external noise was attenuated.

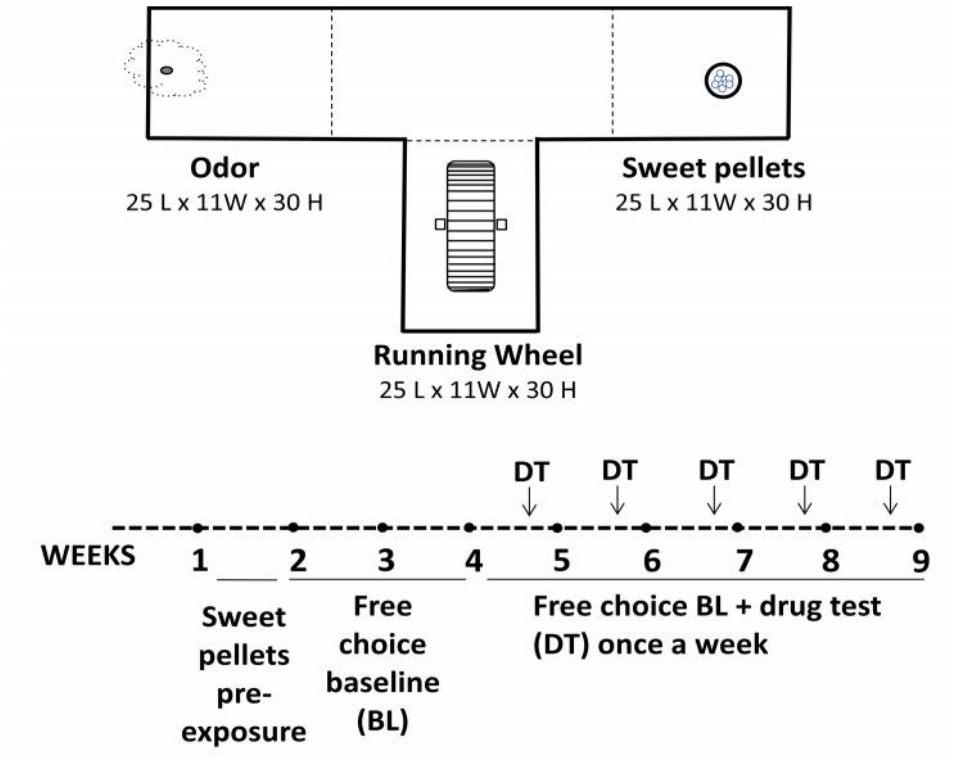


Figure 1. Schematic representation of the 3-choice RW T-maze task settings and experiments timeline.

Sweet pellets intake. Mice were individually placed in testing chambers (32 x 15 x 13 cm) identical to their home cages, during a 30 min session per day, 5 days/week. The testing chambers contained a glass plate with 30 pellets (45 mg each, with a 50% sucrose composition). At the end of the session, mice were immediately removed from the chamber, returned to their respective home cages, and number of pellets consumed was determined. Each animal was exposed to this procedure for 4 weeks before testing started.

Running Wheel (RW) locomotion. 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. Locomotor activity was registered by an electrical counter connected to the wheel. A completed turn of the wheel was registered as 4 counts. Animals placed in the cage had free access to the wheel. Animals were trained during 3 weeks to achieve a stable baseline of locomotion (30 min sessions per day).

High Performance Liquid Chromatography (HPLC) for DA level determination. Brain samples were extracted after TBZ administration. Mice were anesthetized with carbon dioxide for 30 s and decapitated. Brains were quickly removed and frozen on a Leitz Wetzlar microtome. Coronal sections 750 µm thick were cut through the striatum. A 16-gauge stainless-steel tube was used to dissect bilateral cylindrical samples from the ventral striatum. These tissue samples were then placed in 200 µl of 0.1 N perchloric acid, and then homogenized, centrifuged, and frozen. The supernatant was subsequently analyzed for DA content using HPLC with electrochemical detection (ESA Coulochem II system). The electrochemical parameters were as follows: channel 1= − 100 mV, channel 2 = +200 mV, and guard cell = +350 mV. Each liter of mobile phase contained 27.6 g sodium phosphate monobasic, 8.0% of methanol 750 µl of 0.1M EDTA, and 2875 µl of 0.4M sodium octyl sulfate dissolved in deionized ultrapure H_2O with a final pH of 4.5. The flow rate was 1.0 ml/min.

Western blotting. 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 (30µg) of striatum 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% Bovine Serum Albumin (BSA) in TBS-Tween 0.1% for one hour and later incubated with polyclonal rabbit anti- DARPP32 (1:1000, Cell Signalling), DARPP32-Thr75 (1:500, Cell Signalling and DARPP32-Thr34 (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 film signals were scanned and levels of the band density were blind processed and quantified by densitometry with ImageJ software. Every sample was replicated at least twice to ensure the reproducibility of the method.

2.4. Experiments

Behavioral experiments used a within-groups design, in which each mouse received all treatments once per week over consecutive weeks. No dose sequence was repeated across different animals in any of the experiments.

Experiment 1. Impact of TBZ, caffeine and their combination on preference between concurrently available reinforcers in the T-maze

Experiment 1.1. Effect of TBZ on T-maze preferences. Mice (N=9) received vehicle or TBZ (1.0, 2.0 and 4.0 mg/kg) 120 minutes before the test.

Experiment 1.2. Effect of caffeine on T-maze preferences. A different group of mice (N=9) was injected with caffeine (1.25, 2.5 and 5.0 mg/kg) or saline 30 minutes before test started.

Experiment 1.3. Reversal of TBZ induced effects in the T-maze by different doses of caffeine. After being trained as described above, mice (N=8) received two injections: DMSO plus saline, TBZ (4.0 mg/kg) plus saline, and TBZ (4.0 mg/kg) plus caffeine (1.25, 2.5 and 5.0 mg/kg). TBZ was injected 120 min before test started, while caffeine was injected 30 min before test.

Experiment 2. Impact of TBZ, caffeine and their combination on independent tests of sucrose consumption or locomotion in the RW.

Experiment 2.1. Effect of caffeine and TBZ on sucrose consumption. Mice (N=8) were exposed to sucrose pellets daily during 6 weeks (30 minutes session). When the animals reached a stable level of intake, they were injected with TBZ at doses of 2.0, 4.0 and 8.0 mg/kg or DMSO 120 minutes before test. Another group of mice (N=9) was injected with caffeine at doses of 1.25, 2.5, 5.0 and 10.0 or saline 30 minutes before the intake test.

Experiment 2.2. Effect of caffeine and TBZ on locomotion in the RW. After RW training, mice (N=9) received TBZ (1.0, 2.0, and 4.0 mg/kg) or DMSO 120 minutes

before the locomotion test. A second group of mice (N=10) received injections of caffeine (2.5, 5.0 and 10.0 mg/kg) or saline 30 minutes before testing. A third group of mice (N=9) received a combination of treatments: DMSO plus saline or TBZ (8.0 mg/kg) plus saline or TBZ (8.0 mg/kg) plus caffeine (2.5, 5.0 and 10.0 mg/kg). Caffeine or saline vehicle was injected 30 min before the RW test while TBZ or DMSO vehicle were administered 120 min before the test began.

Experiment 3. Effect of TBZ on DA tissue levels in striatum. Mice (N=8 per condition) were injected with DMSO or TBZ (4 or 8 mg/kg). The striatum was extracted 120 min after drug administration. Samples were processed and analyzed with HPLC in order to quantify DA tissue levels.

Experiment 4. Effect of TBZ and caffeine co-administration on DARPP-32, pDARPP-32(Thr75) and pDARPP-32(Thr34) levels in striatum. Mice (N=6-8 per condition) were injected with DMSO plus saline or TBZ (8 mg/kg) plus saline or with TBZ (8 mg/kg) plus caffeine (10.0 mg/kg) before brain extraction. Striatum samples were analyzed by western blotting for DARPP-32, pDARPP-32(Thr75) and pDARPP-32(Thr34).

2.5. Statistical Analyses.

All the behavioral experiments followed a within-subjects design, and were analyzed with repeated measures analysis of variance (ANOVA). Behavioral data on the interaction between TBZ and caffeine were analyzed using a two way-factorial ANOVA. When the overall ANOVA was significant, non-orthogonal planned comparisons using the overall error term were used to compare each treatment with the vehicle control group (Keppel, 1991). For these comparisons, α level was kept at 0.05 because the number of comparisons was restricted to the number of treatments minus one. Biochemical studies were analyzed with one-way ANOVA. All data were expressed as mean ± SEM, and significance was set at $p<0.05$. STATISTICA 7 software was used for statistical analyses of the data.

3. Results

Experiment 1. Impact of TBZ, caffeine and their combination on preference between concurrently available reinforcing activities in the T-maze

Experiment 1.1. Effect of TBZ on T-maze preference. Repeated measures ANOVA revealed an overall effect of TBZ dose on time spent running in the RW ($F(3,24)=6.83$, $p<0.01$), and time spent eating ($F(3,24)=2.94$, $p<0.05$), but no significant effect on time sniffing the neutral odor ($F(3,24)=1.43$, n.s.) **(Fig 2A-C).** Planned comparisons showed a significant decrement in time running in the RW after TBZ injection at doses of 2.0 and 4.0 mg/kg compared with the vehicle group ($p<0.01$). There was also a significant increase in the time eating after the highest dose of TBZ (4.0 mg/kg) compared with the vehicle condition ($p<0.01$) **(Fig 2A and B).** The repeated measures ANOVA did not yield a significant effect of TBZ on total crosses as a measure of locomotion ($F(3,24)=0.52$, n.s.) **(Fig 2D)**. Thus none of these doses of TBZ produced an impairment on locomotion.

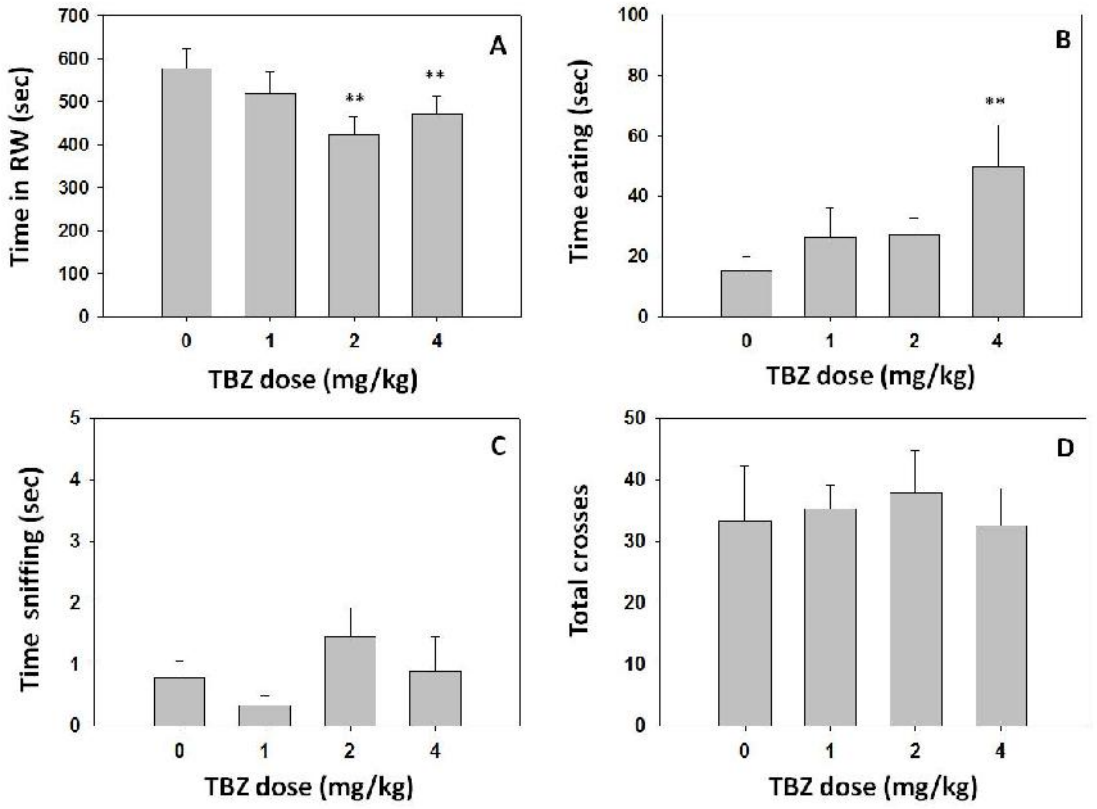


Figure 2. Effect of TBZ in the 3-choice RW T-maze task. Data are expressed as mean (±SEM) of time (seconds) spent interacting with each stimuli (**A**, **B** and **C**) or number of crosses between

the compartments where the stimuli were located (**D**), during a 15 minutes session. **A**) Time with RW, **B**) time eating **C**) time sniffing the non-social odor, and **D**) number of crosses between the 3 compartments. **p<0.01 significantly different from vehicle.

Experiment 1.2. Effect of caffeine on T-maze preference. Repeated measures ANOVA for caffeine treatment showed no significant effects in any of the 3 variables: time running in the RW ($F(3,24)=0.18$, n.s.), time spent eating ($F(3,24)=0.92$, n.s.) and time sniffing the neutral odor ($F(3,24)=0.81$, n.s.) **(Fig 3A-C)**. There was not an effect of caffeine on total crosses ($F(3,24)=0.10$, n.s.) either **(Fig 3D)**.

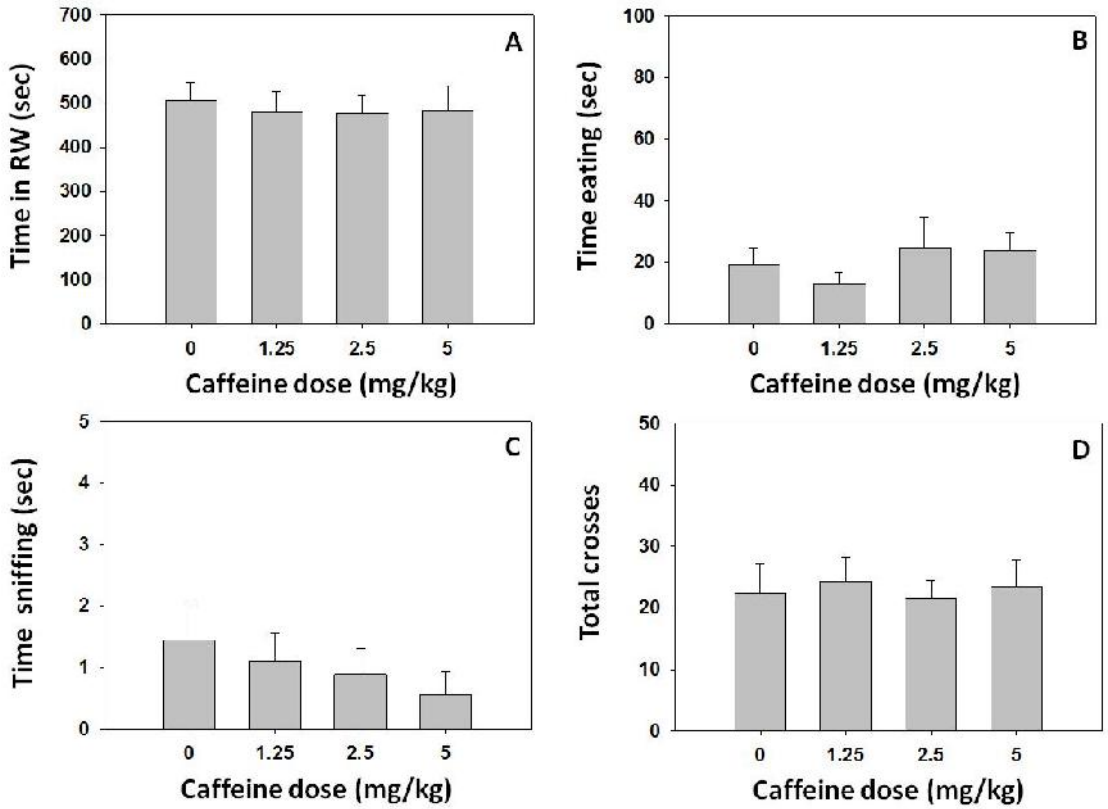


Figure 3. Effect of caffeine in the 3-choice RW T-maze task. Data are expressed as mean (±SEM) of time (seconds) spent interacting with each stimuli (**A**, **B** and **C**) or number of crosses between the compartments where the stimuli were located (**D**), during a 15 minutes session. **A**) Time with RW, **B**) time eating **C**) time sniffing the non-social odor, and **D**) number of crosses between the 3 compartments.

Experiment 1.3. Reversal of TBZ-induced effects in the T-maze by caffeine. Repeated measures ANOVA showed an overall effect of treatment on time running in the RW ($F(4,28)=4.57$, $p<0.01$) and on time eating ($F4,28)=3,63$, $p<0.05$), but not on time sniffing ($F(4,28)=0.71$, n.s.) **(Fig 4A-C)**. Planned comparisons revealed a significant decrease in time running in the RW after administration of TBZ/VEH compared with control condition (VEH/VEH) ($p<0.01$), and this decrement was reversed by the two highest doses of caffeine that were co-administered with TBZ (2.5 mg/kg, $p<0.05$, and 5.0 mg/kg, different from TBZ/VEH, $p<0.01$) **(Fig 4A).** Planned comparisons revealed that TBZ/VEH administration increased the time spent eating compared with control group (VEH/VEH) ($p<0.05$). This increase was reversed by the co-administration of caffeine at all doses (1.25, 5.0 mg/kg $p<0.01$ and 2.5 mg/kg, $p<0.05$) **(Fig 4B)**. Repeated measures ANOVA did not reveal an effect of treatment on total crosses between compartments ($F(4,28)=0.51$, n.s.) **(Fig 4D).**

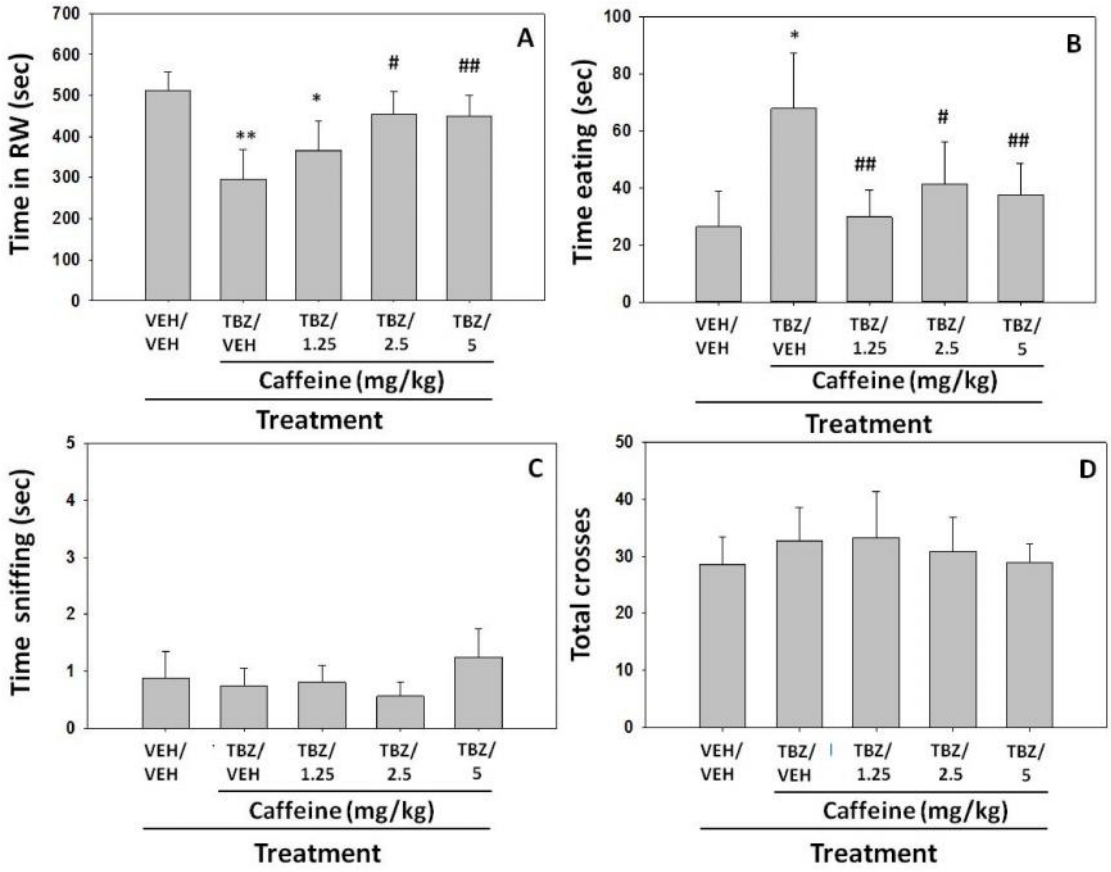


Figure 4. Effect of TBZ plus caffeine in the 3-choice RW T-maze task. Data are expressed as mean (±SEM) of time (seconds) spent interacting with each stimuli (**A**, **B** and **C**) or number of crosses between the compartments where the stimuli were located (**D**), during a 15 minutes

session. **A**) Time with RW, **B**) time eating **C**) time sniffing the non-social odor, and **D**) number of crosses between the 3 compartments. **p<0.01, *p<0.05 significantly different from VEH-VEH. ##p<0.01, #p<0.05 significantly different from TBZ-VEH.

Experiment 2. Impact of TBZ, caffeine and their combination on independent tests of sucrose consumption and locomotion in the RW.

Experiment 2.1. Effect of TBZ and caffeine on sucrose consumption. Repeated measures ANOVAs did not show significant overall effects of treatment with TBZ (0, 1.0, 2.0 and 4.0 mg/kg) or caffeine (0.0, 1.25, 2.5 and 5.0 mg/kg) on total intake of sweet pellets. Thus the ANOVA for TBZ ($F(3,24)=2.38$, n.s.), and for caffeine ($F(3,21)=1.33$, n.s.) did not yield significant effects, indicating that they had no effect on pellet consumption when there was no alternative reinforcer (see **Fig 5A and B**).

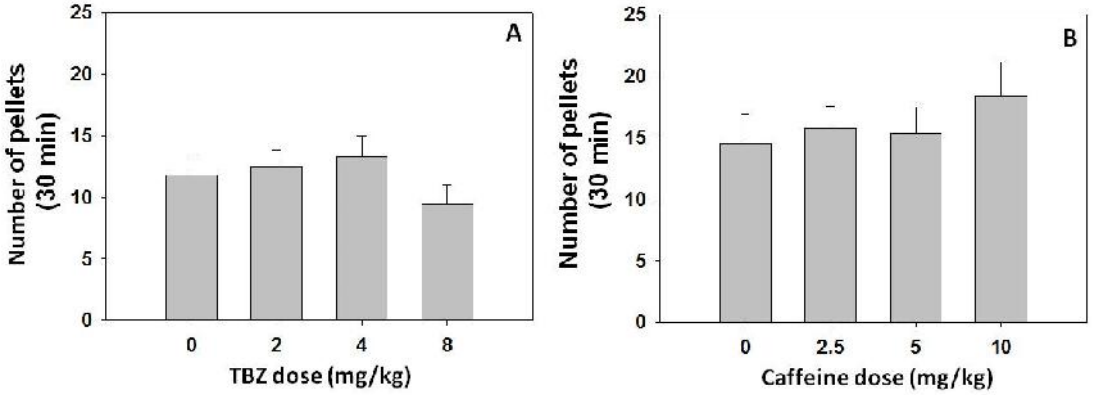


Figure 5. Effect of TBZ (**A**) and caffeine (**B**) on sucrose pellets consumption. Data are expressed as mean (±SEM) of number of pellets consummed during 30 minutes.

Experiment 2.2. Effect of TBZ and caffeine on locomotion in the RW. Repeated measures ANOVA indicated a significant effect of TBZ treatment ($F(3,24)=7.44$, $p<0.01$) on locomotion (**Fig 6A**). Planned comparisons revealed that TBZ at the highest dose used in the present experiment (8.0 mg/kg) significantly decreased locomotion compared with the vehicle group ($p<0.01$). However, the repeated measures ANOVA did not reveal a significant effect of caffeine treatment ($F(3,27)=1.44$; n.s) on locomotion in the RW, even at higher doses (**Fig 6B**). In the third experiment the highest dose of TBZ that had suppressed locomotion (8.o mg/kg) was used to study the

potential of caffeine to reverse TBZ-induced locomotor suppression. Repeated measures ANOVA across conditions (VEH/VEH, TBZ/VEH, TBZ-Caffeine 2.5, 5.0 or 10.0 mg/kg) yielded a significant effect on RW locomotion ($F(4,32)=3.44$, $p<0.01$). Planned comparisons indicated that TBZ (8.0 mg/kg) suppressed locomotion compared to control group (VEH/VEH) ($p<0.01$). All doses of caffeine (2.5, 5.0 and 10.0 mg/kg) reversed the locomotor suppression induced by 8.0 mg/kg TBZ. Thus, TBZ/VEH was significantly different from all the TBZ plus caffeine conditions (2.5, 5.0 mg/kg, $p<0.05$, and 10.0 mg/kg, $p<0.01$) **(Fig 6C**).

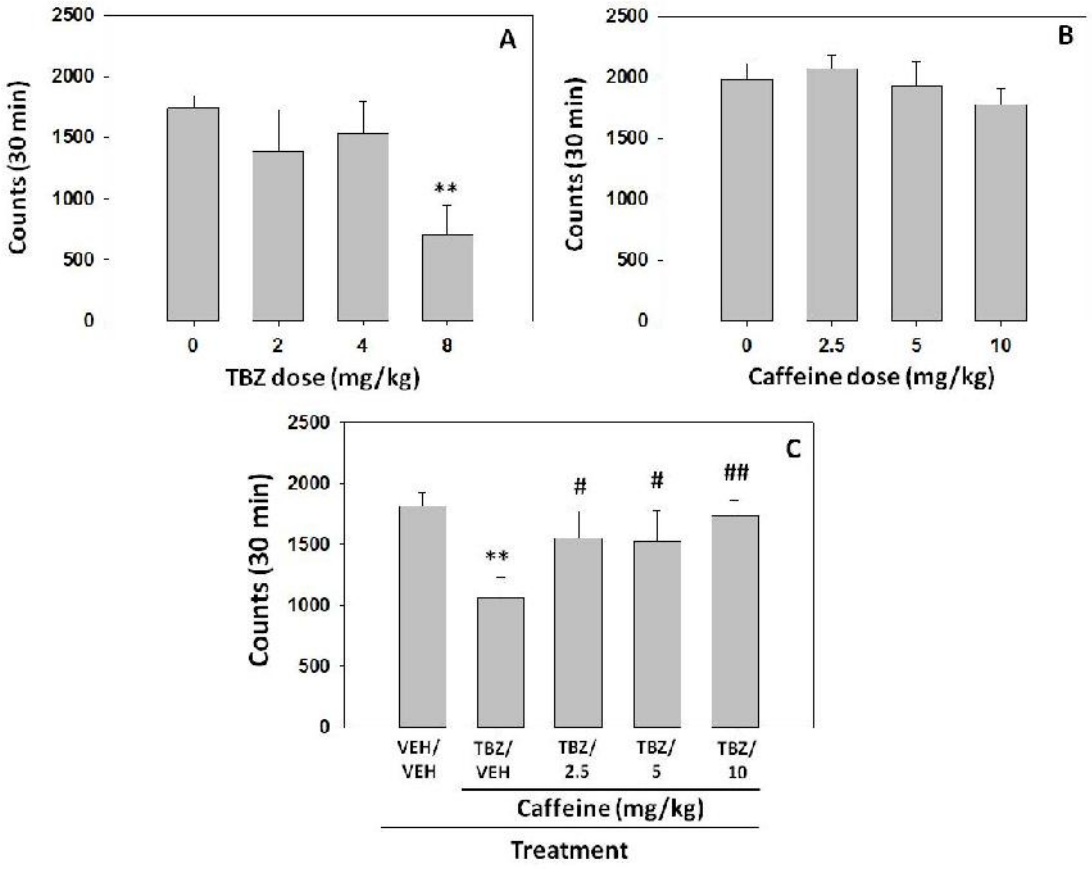


Figure 6. Effect of TBZ (**A**), caffeine (**B**) and their combination (**C**) on locomotion in the RW. The dose of TBZ used in **C** was 8 mg/kg. Data are expressed as mean (±SEM) of counts in the RW during 30 minutes. **p<0.01 significantly different from vehicle. ##p<0.01, #p<0.05 significantly different from TBZ-VEH.

Experiment 3. Effect of TBZ on DA tissue levels in striatum. The one way between-groups ANOVA revealed an overall significant effect of treatment on DA tissue levels

in the striatum ($F(2,28)=5.16$, $p<0.05$). Planned comparisons revealed a significant reduction in DA tissue levels after administration of TBZ at doses of 4.0 and 8.0 mg/kg compared with control group ($p<0.05$ and $p<0.01$, respectively) **(Fig 7).**

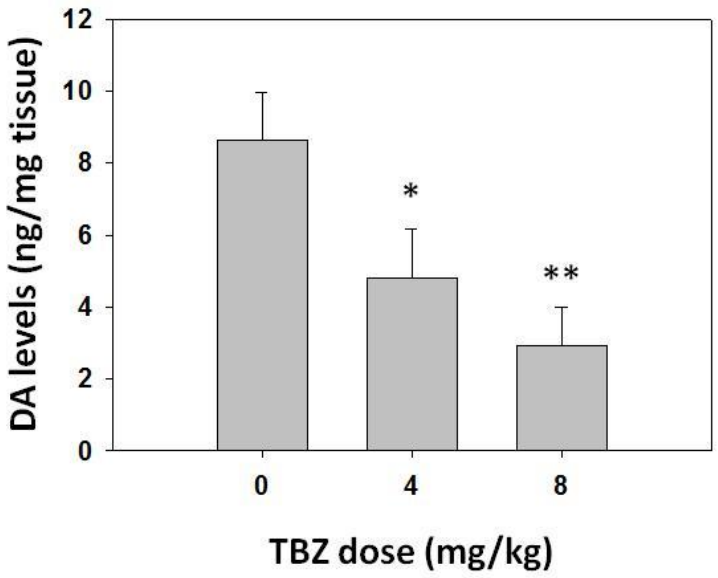


Figure 7. Effect of TBZ (4 or 8 mg/kg) on DA levels in striatum. Data are expressed as mean (±SEM) of ng per mg of DA in tissue. **p<0.01, *p<0.05 significantly different from vehicle.

Experiment 4. Effect of TBZ and caffeine coadministration on DARPP-32, pDARPP-32(Thr75) and pDARPP-32(Thr34) levels in striatum. One way ANOVA showed an overall effect of treatment on DARPP-32 levels ($F(2,19)=3.70$, $p<0.05$). The *post hoc* analysis showed a significant increase of DARPP-32 in the TBZ plus saline treated group compared with control group ($p<0.05$) and with the TBZ plus caffeine group ($p<0.05$) **(Fig 8A)**. A one way ANOVA for pDAPPP-32(Thr75) levels did not show an overall effect of treatment on this marker ($F(2,12)=1.86$, $p=1.19$) although a non significant increment was observed after TBZ treatment **(Fig 8B).** Finally, the one way ANOVA revealed a significant effect of treatment on pDARPP-32(Tr34) ($F(2,10)=43.9$, $p<0.01$). The post hoc analysis showed a significant increase of this marker after TBZ treatment ($p<0.05$) compared to control, and compared to the group that also received caffeine ($p<0.05$), indicating a reversal of TBZ effects by caffeine **(Fig 8C).**

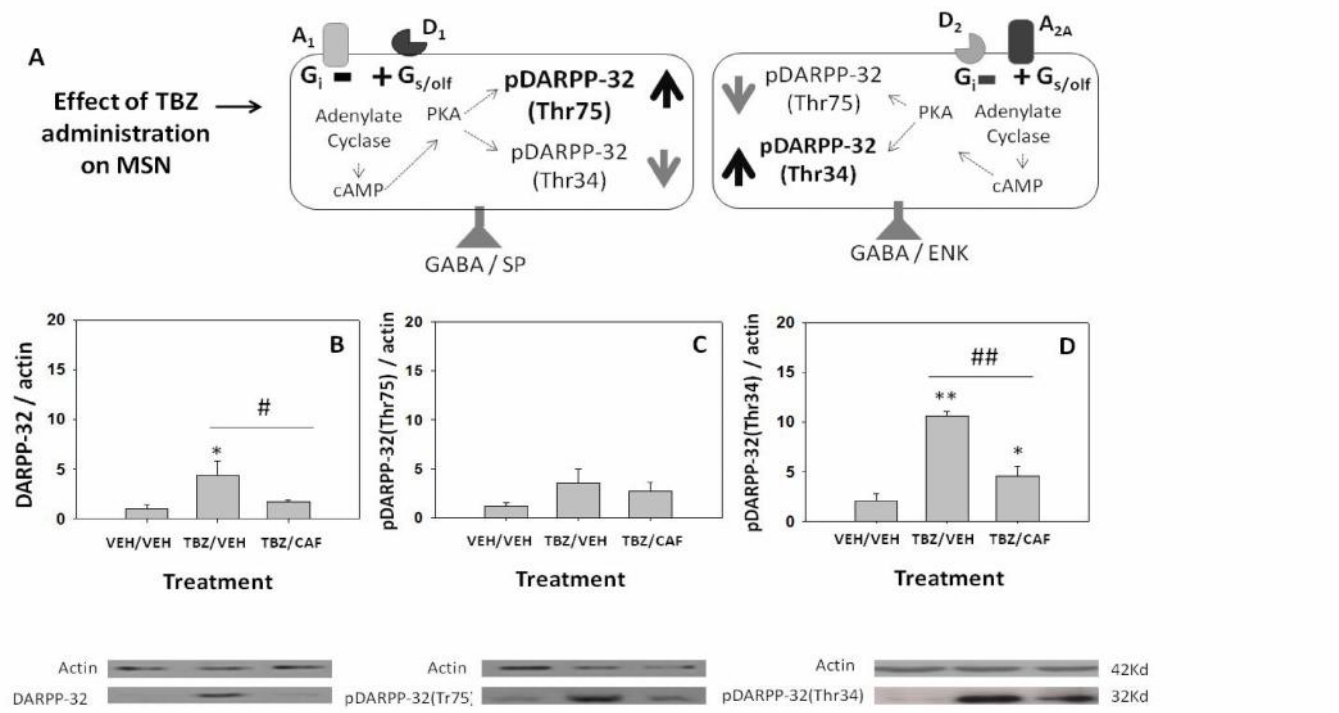


Figure 8. (**A**) Diagram showing effect of DA depletion on DARPP-32 phosphorylation patterns. Effect of TBZ-caffeine interaction on DA-related markers of signal transduction. The doses used were: TBZ 8.0 mg/kg and caffeine 10 mg/kg. Data are expressed as mean (±SEM) of density units of DARPP-32 (**B**), pDARPP-32(Thr75) (**C**) and pDARPP-32(Thr34) (**D**). **p<0.01, *p<0.05 significant differences from VEH-VEH. ##p<0.01, #p<0.05 significant differences from TBZ-VEH group.

4. Discussion

The present study assessed the impact of the VMAT-2 inhibitor TBZ on the choice between voluntary engagement in vigorous and highly preferred physical activity vs. other sources of reinforcement that could be obtained with little effort, such as sucrose consumption. TBZ was administered at doses that were shown to reduce tissue levels of DA in ventral striatum. In the first experiment, mice were evaluated in a T-maze in which they could freely distribute their time between running on a RW or consuming or exploring other reinforcers that require minimal behavioral activation (sucrose pellets or non-social odor). Under basal conditions, mice spent most of the time running (60%), and much less time eating (2%) or sniffing the neutral odor (0.1%). Consistent with this finding, previous studies have demonstrated that running has a high motivational value, since animals work to unlock a wheel (Collier et al. 1990; Iversen, 1993; Belke and

Heyman, 1994), to turn on a motorized wheel (Kavanau, 1967) or to gain access to areas containing a wheel (Sherwin, 1996; Sherwin and Nicol, 1996). In addition, wheel running, as well as the after effect or running, can be used as the motivational stimulus for the establishment of conditioned place preference (CPP) (Lett et al. 2000; Torst and Hauber, 2014).

After TBZ administration choice behavior was altered; time spent running was reduced, but time consuming sucrose was actually increased, which demonstrates a shift to a low effort option. No changes were observed in time sniffing the neutral odor. This set of results is consistent with previous studies in a similar two-options T-maze, in which the D_2 antagonist haloperidol shifted relative preference from RW to sucrose pellets in mice (Correa et al., 2016). Haloperidol has previously been demonstrated to shift behavior in mice towards low effort alternatives in a T-maze barrier task in which animals have to climb a barrier in order to get a higher quantity of food in every trial (Pardo et al. 2012). TBZ was previously shown to reduce selection of high effort/high reward options in rats, using effort-based decision-making paradigms such as the T-maze barrier task (Yohn et al. 2012), operant tasks with concurrent lever pressing for preferr5ed highly palatable food versus free feeding standard chow (Salamone et al. 2012; Nunes et al. 2013), or lever pressing for high concentrations of sucrose versus free access to low sucrose concentrations (Pardo et al. 2015). Furthermore, TBZ also has been shown to have these effects when injected into Nacb core, reducing lever pressing for the palatable food and increasing chow intake (Nunes et al. 2013). However, free consumption of foods or sweet solutions, preferences between different types or amounts of foods, or facial expressions that reflect hedonic reactivity after sucrose intake, were not affected in rats after TBZ administration (Nunes et al. 2013; Pardo et al. 2015; Yohn et al. 2015), demonstrating that DA depletion does not simply affect primary food motivation. Similarly, in the present results from experiment 3, even higher doses of TBZ than the ones used in the T-maze did not change sucrose consumption when animals had no alternative option present during the testing session. Thus, as previously demonstrated, DA depletion with TBZ does not affect primary motivation for food or sucrose when little work is involved (Nunes et al. 2013; Pardo et al. 2015). Furthermore, although the after effect of running can contribute to the establishment of emotional Pavlovian memories, as seen by the development of CPP, this after effect is not DA-dependent (Trost and Hauber, 2014), and it does not seem to

be playing a role in the present results, since mice did not change the amount of time that they spent in the RW compartment (data not shown) even after the administration of doses of TBZ that reduced time spent running on the wheel. It is also important to emphasize that the present results are not merely due to motor incapacity, because the higher dose of TBZ used in the T-maze (4.0 mg/kg) did not impair voluntary locomotion in a RW when there was no other reinforcer available (experiment 2.2).

The non-selective adenosine antagonist caffeine when administered alone did not change the relative preference of mice in this T-maze test, even when given at high doses (up to 10.0 mg/kg). However, caffeine (2.5 and 5.0 mg/kg) was able to reverse the change in relative preference induced by TBZ (4.0 mg/kg), shifting preferences by increasing time in the RW and decreasing time spent eating sucrose in TBZ-treated animals. Furthermore, caffeine (1.25-10 mg/kg) reversed the suppression of RW locomotion induced by a high dose of TBZ (8.0 mg/kg) that also significantly reduced DA tissue levels. Consistent with the present results, previous studies showed a similar interaction between DA antagonists or depletions and adenosine receptor antagonism or deletion (Farrar et al. 2007; Mott et al. 2009; Worden et al. 2009; Correa et al. 2016; Yohn et al. 2013; 2015). Thus, A_{2A} KO mice were resistant to the effects of the D_2 antagonist haloperidol in the two-option T-maze paradigm (Correa et al., 2016). A_{2A} KO mice did not shift time allocation from RW towards sucrose after haloperidol administration, as did the wild type mice (Correa et al. 2016). These KO mice were also resistant to the effect of haloperidol in a simple RW (Pardo et al., 2013), and in the T-maze barrier choice task (Pardo et al., 2012). Theophylline, which is another methylxantine, was able to palliate the anergia-like effect induced by haloperidol in the T-maze barrier task in mice (Pardo et al., 2012). In rats, the selective adenosine A_{2A} antagonist MSX-3 was shown to reverse the effects of the D_2 antagonist haloperidol and TBZ in several different types of effort-based decision making paradigms (Farrar et al., 2007; Nunes et al., 2013; Yohn et al., 2015; Pereira et al., 2011).

TBZ significantly increased postsynaptic intracellular DA markers, including DARPP-32 and one of its phosphorylated forms, pDARPP-32(Thr34). These TBZ-induced increases in markers of DA-related signal transduction were significantly reversed by caffeine. In contrast, the induction of pDARPP-32(Thr75) expression by TBZ did not reach significance. These results suggest that there is a substantial action of TBZ on neurons containing D_2 receptors (Nunes et al. 2013). DA D_2 and adenosine A_{2A}

receptors are co-localized on enkephalin-containing medium spiny neurons (MSNs), while D_1 and A_1 receptors are co-localized on substance P-containing MSNs (Nunes et al. 2013; Ferré, 2008; Ferré et al. 2004). D_2 and A_{2A} receptors are capable of forming heteromers, and also converge onto the same signal transduction mechanisms, having opposite effects on intracellular signaling cascades (Fuxe et al. 2003; Ferré, 2008). Reductions in D_2 receptor transmission have been shown to increase expression of pDARPP-32(Thr34) (Svenningsson et al. 2004; Bateup et al. 2008; Yger and Girault, 2011; Bonito-Oliva et al. 2011; Santerre et al. 2012; Nunes et al., 2013; see Fig 8). Previous studies in rats using immunohistochemical techniques have demonstrated that TBZ increased both phosphorylated forms of DARPP-32 (-Thr75 and -Thr34) in Nacb shell and core, but in different populations of neurons (Nunes et al., 2013). Adenosine antagonists acting on A_1 or A_{2A} receptors generally produce opposite effects to TBZ on these markers. Thus, it has been demonstrated that the selective A_{2A} receptor antagonist MSX3 reduced the increase of pDARPP-32(Thr34) induced by TBZ in enkephalin-positive neurons that also contain D_2 receptors (Nunes et al., 2013). However, this A_{2A} antagonist did not reverse the induction of pDARPP-32(Thr75) in D_1 containing neurons, which probably reflects the fact that D_1 receptors are not extensively co-localized with A_{2A} receptors (Nunes et al., 2013). In the present results, caffeine was able to reverse the induction of pDARPP-32(Thr34) expression produced by TBZ, pointing to a predominant effect of both drugs on D_2-A_{2A} receptors situated in striatal enkephalin-containing MSN.

In summary, the present results are consistent with the hypothesis that DA is involved in effort-related processes, and support the concept that adenosine receptors interact with DA in modulation these functions (Salamone et al., 2009; Santerre et al., 2012; Pardo et al., 2012; Nunes et al., 2013; Randall et al., 2014; Yohn et al., 2015). This study illustrates the ability of the 3-choice T-maze task with active vs. passive reinforcing activities to demonstrate the involvement of DA in the activational component of motivation, which is consistent with previous studies showing that DA antagonism was able to specifically shift preferences away from effortful sources of reinforcement (Correa et al., 2016). The 3-choice T-maze task offers the chance to study preferences between qualitatively different reinforcers in addition to food, which is in contrast to previous tasks that involved choices between different quantities of food (Pardo et al., 2012; Yohn et al., 2014) or foods with different palatability (Salamone et al., 2006;

Farrar et al., 2010; Pardo et al., 2015). The present results indicate that DA depletion with TBZ reduces the relative intrinsic reinforcing characteristics of wheel running in an empirical sense, in a manner similar to DA antagonism (Correa et al., 2016). The intrinsic reinforcing value of voluntary physical activities such as lever pressing, barrier climbing, or wheel running is of critical importance for understanding several aspects of motivation and decision-making (Salamone et al., 1997, 2016; Salamone and Correa 2002, 2012; Hosking et al. 2014).

The present work has potential clinical relevance, because DA has been implicated in aspects of depression such as anergia, psychomotor slowing, decreased energy levels and fatigue (Stahl, 2002; Salamone et al., 2006; Treadway and Zald, 2011). In addition, a lack of physical activity can contribute to the development of depression (Lambert 2006). Moreover, 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). Caffeine has been demonstrated to improve motor symptoms in PD patients and in animal models (Postuma et al., 2012; Qi and Li 2014), and it can enhance the antidepressant-like activity of common antidepressant drugs in traditional tests of depression such as the forced swim test (Szopa et al., 2016). The present results suggest that studies with caffeine and more selective adenosine antagonists may offer useful clues for developing novel treatments for this set of symptoms.

5. References

- Bateup, H.S., Svenningsson, P., Kuroiwa, M., Gong, S., Nishi, A., Heintz, N., Greengard, P., 2008. Cell type-specific regulation of DARPP-32 phosphorylation by psychohostimulant and anti-psychotic drugs. Nat. Neurosci. 11,932-939.

- Belke, T.W., Oldford, A.C., Forgie, M.Y., Beye, J.A., 2005. Responding for sucrose and wheel-running reinforcement: effect of D-amphetamine. Behav. Pharmacol. 16, 219-225.

- Bonito-Oliva, A., Feyder, M., Fisone, G., 2011. Deciphering the Actions of Antiparkinsonian and Antipsychotic Drugs on cAMP/DARPP-32 Signaling. Front. Neuroanat. 5,38.

- Cagniard, B., Balsam, P.D., Brunner, D., Zhuang, X., 2006. Mice with chronically elevated dopamine exhibit enhanced motivation, but not learning, for a food reward.Neuropsychopharmacology. 31(7),1362-1370.

- Collier, G.H., Johnson, D.F., Naveira, J., CyBulski, K.A., 1989. Ambient temperature and food costs: effects on behavior patterns in rats. Am J Physiol 257,1328-1334.

- Correa, M., Pardo, M., Bayarri, P., López-Cruz L., San Miguel, N., Valverde, O., Ledent, C., Salamone, J.D., 2016. Choosing voluntary exercise over sucrose consumption depends upon dopamine transmission: effects of haloperidol in wild type and adenosine A2AKO mice. Psychopharmacology (Berl). 233(3),393-404.

- DeMet, E.M., Chicz-DeMet, A., 2002. Localization of adenosine A2A-receptors in rat brain with [3H] ZM-241385. Naunyn Schmiedebergs Arch Pharmacol 366,478-481.

- Demyttenaere, K., De Fruyt, J., Stahl, S.M., 2005. The many faces of fatigue in major depressive disorder. Int. J. Neuropsychopharmacol. 8,93-105.

- Elbers, R., van Wegen, E.E., Rochester, L., Hetherington, V., Nieuwboer, A., Willems, A.M., Jones, D., Kwakkel, G., 2009. Is impact of fatigue an independent factor associated with physical activity in patients with idiopathic Parkinson's disease? Mov. Disord. 24(10),1512-1518.

- Farrar, A.M., Segovia, K.N., Randall, P.A., Nunes, E.J., Collins, L.E., Stopper, C.M., Port, R.G., Hockemeyer, J., Müller, C.E., Correa, M., Salamone, J.D, 2010. Nucleus accumbens and effort-related functions: behavioral and neural markers of the interactions between adenosine A2A and dopamine D2 receptors. Neuroscience. 166(4),1056-1067.

- Fava, M., Ball, S., Nelson, J.C., Sparks, J., Konechnik, T., Classi, P., Dube, S., 2014. Thase ME. Clinical relevance of fatigue as a residual symptom in major depressive disorder.Depress. Anxiety. 31(3),250-257.

- Ferré, S., 1997. Adenosine—dopamine interactions in the ventral striatum.
Implications for the treatment of schizophrenia. Psychopharmacology. 133,107–120.

- Ferré, S., Ciruela, F., Canals, M., Marcellino, D., Burgueno, J., Casado, V., Hillion, J., Torvinen, M., Fanelli, F., Benedetti, Pd. Pd., Goldberg, S.R., Bouvier, M., Fuxe, K., Agnati, L.F., Lluis, C., Franco. R,, Woods, A,. 2004. Adenosine A2A—dopamine D2 receptor—receptor heteromers. Targets for neuro-psychiatric disorders. Parkinsonism Relat. Disord. 10,265-271.

- Ferré, S., Quiroz, C., Woods, A.S., Cunha, R., Popoli, P., Ciruela, F., Lluis, C., Franco. R., Azdad, K., Schiffmann, S.N., 2008. An update on adenosine A2A-dopamine D2 receptor interactions: implications for the function of G protein-coupled receptors. Curr. Pharm. Des. 14,1468-1474

- Floresco, S.B., Tse, M.T., Ghods-Sharifi, S., 2008. Dopaminergic and glutamatergic regulation of effort- and delay-based decision making. Neuropsychopharmacology 33,1966 -1979.

- Frank, S., 2009. Tetrabenazine as anti-chorea therapy in Huntington Disease:an open-label continuation study. Huntington Study Group/TETRA-HD Investigators. BMC Neurol 9,62.

- Frank, S., 2010. Tetrabenazine: the first approved drug for the treatment of chorea in US patients with Huntington's disease. Neuropsychiatr. Dis. Treat. 5,657-665.

- Fredholm, B.B., Bättig, K., Holmén, J., Nehlig, A., Zvartau, E.E., 1999. Actions of caffeine in the brain with special reference to factors that contribute to its widespread use. Pharmacol. Rev. 51,83-133.

- Friedman, J.H., Brown, R.G., Comella C, Garber, C.E., Krupp, L.B., Lou J., Marsh, L., Taylor, C.C., 2007. Fatigue in Parkinson's disease: a review. Mov. Disord. 22,297-308.

- Fuxe, K., Agnati, L.F, Jacobsen, K., Hillion, J., Canals, M., Torvinen, M Tinner-Staines, B., Staines, W., Rosin, D., Terasmaa, A., Popoli, P., Leo, G., Vergoni, V., Lluis, C., Ciruela,F., Franco ,R., - Ferré, S.,2003. Receptor heteromerization in adenosine A2A receptor signaling: relevance for striatal function and Parkinson's disease. Neurology. 61,19-23.

- Guay, D.R., 2010. Tetrabenazine, a monoamine-depleting drug used in the treatment of hyperkinectic movement disorders. Am. J. Geriatr. Pharmacother. 8,331-373.

- Hauber, W., Sommer, S., 2009. Prefrontostriatal circuitry regulates effort-related decision making. Cereb. Cortex. 19(10), 2240-2247.

- Hosking, J.G., Cocker, P.J., Winstanley, C.A., 2014. Dissociable Contributions of Anterior Cingulate Cortex and Basolateral Amygdala on a Rodent Cost/Benefit Decision-Making Task of Cognitive Effort. Neuropsychopharmacology. 39(7),1558-1567.

- Iversen, I.H., 1993. Techniques for establishing schedules with wheel running as reinforcement in rats. J Exp Anal Behav. 60(1),219-238.

- Johnson, L.C., Spinweber, C.L., and Gomez, S.A., 1990. Benzodiazepines and caffeine: effect on daytime sleepiness, performance, and mood. Psychopharmacology (Berl) 101,160-167.

- Kavanau, J.L.,1967. Behavior of captive white-footed mice. Science 155,1623-1639.

- Lambert, K.G., 2006. Rising rates of depression in today's society: consideration for the roles of effort-based rewards and enhanced resilience in day-to-day functioning. Neurosci Biobehav Rev 30, 319-324.

- Lett, B.T., Grant, V.L., Koh, M.T., Flynn, G., 2002. Prior experience with wheel running produces cross-tolerance to the rewarding effect of morphine. Pharmacol Biochem Behav 72(1–2),101-105.

- Lieberman, H.R., 2001. The effects of ginseng, ephedrine and caffeine on cognitive performance, mood and energy. Nutr. Rev- 59,91-102.

- Mai, B., Sommer, S., Hauber, W., 2012. Motivational states influence effort based decision making in rats: the role of dopamine in the nucleus accumbens. Cogn Affect Behav. Neurosci. 12,74-84.

- Mott, A.M., Nunes, E.J., Collins, L.E., Port, R.G., Sink, K.S., Hockemeyer, J., Müller, C.E., Salamone, J.D., 2009. The adenosine A2A antagonist MSX-3 reverses the effects of the dopamine antagonist haloperidol on effort-related decision making in a T-maze cost/benefit procedure. Psychopharmacology (Berl). 204,103-112.

- Nomoto, M., Nagai, M., Nishikawa, N. 2014. Clinical nonmotor aspect of A2A antagonist in PD treatment. Int. Rev. Neurobiol. 119,191-194.

- Nunes, E.J., Randall, P.A., Hart, E.E., Freeland, C., Yohn, S.E., Baqi, Y., Müller, C.E., López-Cruz, L., Correa, M., Salamone, J.D., 2013. Effort-related motivational

effects of the VMAT-2 inhibitor tetrabenazine: implications for animal models of the motivational symptoms of depression. J. Neurosci. 33(49),19120-19130.

- Pardo, M., López-Cruz, L., San Miguel, N., Salamone, J.D., Correa, M., 2015. Selection of sucrose concentration depends on the effort required to obtain it: studies using tetrabenazine, D1, D2, and D3 receptor antagonists. Psychopharmacology (Berl). 232(13),2377-2391.

- Pardo, M., Lopez-Cruz, L., Valverde, O., Ledent, C., Baqi, Y,. Müller, C.E., Salamone, J.D., and Correa, M., 2012. Adenosine A2A receptor antagonism and genetic deletion attenuate the effects of dopamine D2 antagonism on effort-based decision making in mice. Neuropharmacology. 62, 2068-2077.

- Pardo, M., Lopez-Cruz, L., Valverde, O., Ledent, C., Baqi, Y., Müller, C.E., Salamone, J.D.,Correa, M., 2012. Adenosine A2A receptor antagonism and genetic deletion attenuate the effects of dopamine D2 antagonism on effort-based decision making in mice.Neuropharmacology. 62(5-6),2068-2077.

- Pardo, M., López-Cruz, L., Valverde, O., Ledent, C., Baqi, Y., Müller, C.E., Salamone J.D., Correa, M. (2013). Effect of subtype-selective adenosine receptor antagonists on basal or haloperidol-regulated striatal function: studies of exploratory locomotion and c-Fos immunoreactivity in outbred and A(2A)R KO mice. Behav. Brain. Res. 247,217-226.

- Pereira, M., Farrar, A.M., Hockemeyer, J., Müller, C.E., Salamone, J.D., Morrell, J.I., 2011. Effect of the adenosine A2A receptor antagonist MSX-3 on motivational disruptions of maternal behavior induced by dopamine antagonism in the early postpartum rat. Psychopharmacology. 213,69-79.

- Pettibone, D.J., Totaro, J.A., Pflueger, A.B., 1984. Tetrabenazine-induced deple- tion of brain monoamines: characterization and interaction with selected antidepressants. Eur J. Pharmacol. 102,425-430.

- Postuma, R.B., Lang, A.E., Munhoz, R.P., Charland, K., Pelletier, A., Moscovich, M., Filla, L., Zanatta, D., Rios, R.S., Altman, R., Chuang, R., Shah, B., 2012. Caffeine for treatment of Parkinson disease: a randomized controlled trial. Neurology. 79(7),651-658.

- Qi, H., Li, S.X., 2014. Dose-response meta-analysis on coffee, tea and caffeine consumption with risk of parkinson's disease. Geriatr. Gerontol. Int. 14 (2),430-439.

- Randall, P.A., Pardo, M., Nunes, E.J., Lopez-Cruz L., Vemuri, V.K., Makriyannis, A., Baqi, Y., Müller, C.E., Correa, M., Salamone, J.D., 2012. Dopaminergic modulation of

effort-related choice behavior as assessed by a progressive ratio chow task: pharmacological studies and role of individual differences. PLoS One. 7,e47934.

- Rees, K., Allen, D., Lader, M., 1999. The influences of age and caffeine on psychomotor and cognitive function. Psychopharmacology (Berl) 145,181-188.

- Salamone, J.D., Arizzi, M., Sandoval. M.D., Cervone, K.M., Aberman, J.E., 2002. Dopamine antagonists alter response allocation but do not suppress appetite for food in rats: contrast between the effects of SKF 83566, raclopride and fenfluramine on a concurrent choice task. Psychopharmacology 160, 371-380.

- Salamone, J.D., Correa, M., 2009. Dopamine/adenosine interactions involved in effort-related aspects of food motivation. Appetite. 53,422-525.

- Salamone, J.D., Correa, M., 2002. Motivational views of reinforcement: implications for understanding the behavioral functions of nucleus accumbens dopamine. Behav. Brain. Res. 137(1–2),3-25.

- Salamone, J.D., Correa, M., 2009. Dopamine/adenosine interactions involved in effort-related aspects of food motivation. Appetite 53(3),422-425.

- Salamone, J.D., Correa, M., 2012. The mysterious motivational functions of mesolimbic dopamine. Neuron. 76, 470–485

- Salamone, J.D., Correa, M., Farrar, A., Mingote, S.M., 2007. Effort-related functions of nucleus accumbens and associated forebrain circuits. Psychopharmacology (Berl) 191,461-482.

- Salamone, J.D., Correa, M., Farrar, A., Mingote, S.M., 2007. Effort-related functions of nucleus accumbens dopamine and associated forebrain circuits. Psychopharmacology (Berl) 191(3),461-482.

- Salamone, J.D., Correa, M., Mingote, S.M., Weber, S.M., Farrar, A.M., 2006. Nucleus accumbens and the forebrain circuitry involved in behavioral activation and effort-related decision making: implications for understanding anergia and psychomotor slowing in depression. Curr Psychiatr Rev 2,267-280.

- Salamone, J.D., Correa, M., Yohn, S., Lopez-Cruz, L., San Miguel, N., Alatorre, L., 2016. The pharmacology of effort-related choice behavior: Dopamine, depression, and individual differences. Behav- Processes. Pii, S0376-6357(16)30029-8

- Salamone, J.D., Cousin, M.S., Synder, B.J., 1997. Behavioral functions of nucleus accumbens dopamine: empirical and conceptual problems with the anhedonia hypothesis. Neurosci. Biobehav. Rev. 21, 341–359.

- Salamone, J.D., Cousins, M.S., Bucher, S., 1994. Anhedonia or anergia? Effects of haloperidol and nucleus accumbens (DA) depletion on instrumental response selection in a T-maze cost/benefit procedure. Behav. Brain. Res. 65,221-229.

- Salamone, J.D., Farrar, A.M., Font, L., Patel, V., Schlar, D.E., Nunes, E.J., Collins, L.E., Sager, T.N., 2009. Differential actions of adenosine A1 and A2A antagonists on the effort-related effects of D2 antagonism. Behav. Brain. Res. 201,216 -222.

- Salamone, J.D., Steinpreis, R.E., McCullough, L.D., Smith, P., Grebel, D., Mahan, K., 1991. Haloperidol and nucleus accumbens (DA) depletion suppress lever pressing for food but increase free food consumption in a novel food choice procedure. Psychopharmacology. 104,515-521.

- Salamone, J.D., Nunes, E.J., Randall, P.A., Huizenga, M., Nowak, V., Pardo, M., Correa, M., 2012. Development of rodent models of the motivational symptoms of depression: Comparison of the effects of the monoamine depleting agents reserpine and tetrabenazine on effort-related decision making. Program No. 923.05. 2012 Neuroscience Meeting Planner, New Orleans, LA: Society for Neuroscience, Online.

- Santerre, J.L., Nunes, E.J., Kovner, R., Leser, C.E., Randall, P.A., Collins-Praino, L.E., Lopez-Cruz L., Correa, M., Baqi, Y., Müller, C.E., Salamone, J.D., 2012. Behavioral studies with the novel adenosine A2A antagonist MSX-4: reversal of the effects of D2 antagonism. Pharmacol. Biochem. Behav. 102,477-487.

- Sherwin, C.M., 1996. The use and perceived importance of three resources which provide caged laboratory mice the opportunity for extended locomotion. Appl. Anim. Behav. Sci. 48,203-214.

- Sherwin, C.M., Nicol, C.J., 1996. Reorganization of behaviour in laboratory mice, Mus musculus, with varying cost of access to resources. Anim Behav 51:1087-1093.

- Smith, A., Kendrick, A.M., Maben, A.L., 1992. Effects of breakfast and caffeine on performance and mood in the late morning and after lunch. Neuropsychobiology. 26,198-204.

- Smith, A., Thomas, M., Perry, K., and Whitney, H., 1997. Caffeine and the common cold. J. Psychopharmacol. 11,319-324.

- Stahl, S.M., 2002. The psychopharmacology of energy and fatigue. J. Clin. Psychiatry. 63(1):7-8.

- Svenningsson, P., Nishi, A., Fisone, G., Girault, J.A., Nairn, A.C., Greengard, P., 2004. DARPP-32: an integrator of neurotransmission. Annu. Rev. Pharmacol. Toxicol. 44,269-296.

- Szopa, A., Poleszak, E., Wyska, E., Serefko, A., Wośko, S., Wlaź, A., Pieróg, M., Wróbel, A., Wlaź, P., 2016. Caffeine enhances the antidepressant-like activity of commonantidepressant drugs in the forced swim test in mice. Naunyn. Schmiedebergs Arch. Pharmacol. 389(2),211-221

- Tanra, A.J., Kagaya, A., Okamoto, Y., Muraoka, M., Motohashi, N., Yamawaki, S., 1995. TJS-010, a new prescription of oriental medicine, antagonizes tetrabenazine-induced suppression of spontaneous locomotor activity in rats. Prog. Neuropsychopharmacol. Biol. Psychiatry. 19,963–971.

- Tellez, N., Rio, J., Tintore, M., Nos, C., Galan, I., Montalban, X., 2005. Does the modified fatigue impact scale offer a more comprehensive assessment of fatigue in MS? Multiple Sclerosis. 11,198–202

- Treadway, M.T., Bossaller, N, Shelton, R.C., Zald, D.H., 2012. Effort-Based Decision-Making in Major Depressive Disorder: A Translational Model of Motivational Anhedonia. J. Abnorm. Psycho. 121(3),553-558.

- Treadway, M.T., Zald, D.H. (2011). Reconsidering anhedonia in depression: lessons from translational neuroscience. Neurosci. Biobehav. Rev. 35(3).537-555.

- Trost, A., Hauber, W., 2014. Dopamine D1/D2 receptors do not mediate the expression of conditioned place preference induced by the aftereffect of wheel running. BMC. Neurosci. 15,124.

- Tylee, A., Gastpar, M., Lepine, J.P., Mendlewicz, J., 1999. DEPRES II (Depression Research in European Society II): a patient survey of the symptoms, disability and current management of depression in the community. Int. Clin. Psychopharmacol. 14,139-151.

- Weiss, R., Laties, V. G., 1962. Enhancement of human performance by caffeine and the amphetamines. IDtiud Rmrws, 14, 1-36.

- Worden, L.T., Shahriari, M., Farrar, A.M., Sink K.S, Hockemeyer, J., Müller, C., Salamone, J.D., 2009.The adenosine A2A antagonist MSX-3 reverses the effort-related effects of dopamine blockade: differential interaction with D1 and D2 family antagonists. Psychopharmacology. 203, 489-499.

- Yger, M., Girault, J.A., 2011. DARPP-32, jack of all trades… master of which? Front Behav. Neurosci. 5,56.

- Yohn, S.E., Nunes, E.J., Randall, P.A., Baqi, Y., Müller, C.E., Correa, M., Salamone, J.D., 2012. Development of rodent models of the motivational symptoms of depression: The catecholamine depleting agent tetrabenazine affects effort-related decision making

in the T-maze barrier choice task. Program No. 923.12. 2012 Neuroscience Meeting Planner, New Orleans, LA: Society for Neuroscience. Online.

- Yohn, S.E., Thompson, C., Randall, P.A., Lee, C.A., Müller, C.E., Bagi, Y., Correa, M., Salamone, J.D., 2015 The VMAT-2 inhibitor tetrabenazine alters effort-related decision making as measured by the T-maze barrier choice task: reversal with the adenosine A2A antagonist MSX-3 and the catecholamine uptake blocker bupropion. Psychopharmacology 232,1313-1323.

- Yohn, S.E., Thompson, C., Randall, P.A., Lee, C.A., Müller, C.E., Baqi, Y., et al. (2014). The VMAT-2 inhibitor tetrabenazine alters effort-related decision making as measured by the T-maze barrier choice task: reversal with the adenosine A(2A) antagonist MSX-3 and the catecholamine uptake blocker bupropion. Psychopharmacology (Berl). 232(7),1313-1323

- Yu, G., Maskray, V., Jackson, S.H., Swift, C.G., and Tiplady, B., 1991. A comparison of the central nervous system effects of caffeine and theophylline in elderly subjects. Br. J. Clin. Pharmacol. 32,341-345.

GENERAL CONCLUSIONS

The present thesis reviews the literature and describes studies investigating the effect2 of caffeine and other selective adenosine antagonists (A_1/A_{2A}), either alone or in combination with ethanol, on different mood, memory and motivated behaviors (**Chapters 1-4**). It also reviews and evaluates the effect of caffeine on depressive symptoms, focusing on potential therapeutic effects on motivational impairments or anergia-like symptoms induced by reduced DA transmission (**Chapers 5-6**).

Chapter 1 summarized previous literature about the effects of caffeine in combination with ethanol, focusing on animal studies and behaviors related to the abuse potential of both drugs. Caffeine is consumed in combination with ethanol under the popular belief that caffeine can ameliorate the debilitating effects of ethanol. From the animal studies it can be concluded that although caffeine at low doses can in fact counteract some effects induced by ethanol, such as anxiety, sedation, narcosis, locomotion and incoordination, higher doses of caffeine can have opposite effects, impairing those behaviors even further. However, it is also evident that many important aspects of motivated behaviors regulated by ethanol are still not explored. Moreover, apparently contradictory results could be resolved if a broader range of doses of both drugs were evaluated systematically.

Caffeine can have beneficial effects on arousal, attention or mood when consumed at low to moderate doses. However, the consumption of high concentrations of caffeine is becoming popular with the introduction to the market of the so-called “energy drinks”. The results from **Chapter 2** show that caffeine at high doses can produce impairing effects on locomotion and coordination, increase anxiety, and also increase plasma corticosterone levels. Theophylline is an active metabolite of caffeine that is also present in some “energy drinks”. This methylxantine is as efficacious as caffeine at producing anxiety and locomotor suppression, and at inducing hormonal markers of stress, though caffeine seems to be more potent in this regard. Thus, both methylxantines can have undesired effects at high doses, but it is necessary the use higher doses of theophylline in order to produce those effects.

Because the highest dose of caffeine used in the previous studies produced very robust impairing effects, for subsequent studies in this thesis the highest dose used in chapter 2 was eliminated, but a broad range of caffeine doses from low to high (7.5-60 mg/kg)

was used in the following chapter. **Chapter 3** presents the effects of caffeine administered alone or in combination with ethanol on social motivation and long-term social memory. Caffeine dose-dependently decreased social interaction and impaired social preference at a range of doses that has anxiogenic effects (30-60 mg/kg). However, although the highest doses of ethanol also reduced social interaction, they did not affect preference for the conspecific. Moreover, ethanol improves social exploration at low doses. Thus, ethanol at a range of doses that has anxiolytic effects keeps social exploration mostly intact, and was able to reverse the decrease in social interaction induced by a moderate dose of caffeine. However, the results with tasks involving long-term social memory indicate that ethanol has a potent amnesic effect, even at low doses, and caffeine cannot rescue that effect. Moreover, caffeine itself also has amnesic effects at high doses.

From the studies about the role of A_1 or A_{2A} receptor involvement on social behaviors, the general conclusion is that they do not seem to mediate the effect of high doses of caffeine. It is possible that A_1 receptor antagonism could be mediating the effects of low doses, since CPT produced the same pattern of effects as the low doses of caffeine in social interaction and preference. However, MSX-3 (the selective A_{2A} receptor antagonist) potentiated social interaction keeping preference intact, thus leading us to suggest that A_{2A} receptors are not involved in the effects of caffeine on this behavior. Moreover, neither CPT nor MSX-3 produced an effect on memory, thus separating the impairing effects of high doses of caffeine on memory from its actions on A_1 and A_{2A} receptors. Only the A_{2A} antagonist had a positive effect, reducing memory impairments induced by ethanol.

Consistently in **Chapter 4,** A_{2A} receptor KO mice showed high levels of social interaction despite their anxiogenic profile, and these animals were not affected by an impairing dose of ethanol (1.0 g/kg) on social interaction. Suggesting a possible predominant role of A_{2A} receptors on attenuating ethanol-induced decreases on social interaction. And also suggesting that A_{2A} receptors could be therapeutic targets to study social impairments induced by different genetic or pharmacological manipulations. However, although MSX-3 did not impair long term social memory, KO mice spent the same amount of time exploring the familiar and novel conspecifics. This memory impairment could be due to neuroadaptations that may occur in this KO animals, or perhaps to differences in sensitivity to novelty in these animals.

In terms of the potential therapeutic effect of caffeine on activational/motivational symptoms of depression, as summarized in **Chapter 5,** caffeine has been shown to be effective in animal models of depression, reversing the effects of adenosine agonism or improving even further the effects of antidepressants on animal models such as the FST or TST. However, there are not systematic studies performed in humans, and the studies focusing on motivational symptoms (anergia or lack of motivation) in humans and in animals are scarce. The results from the T-maze barrier test demonstrate that caffeine does not improve learning in this test but it is very effective at reversing the impairing effects of TBZ on the selection of the high effort option (HD arm). In addition, caffeine reversed the anergia-like effects induced by TBZ in the T-maze that evaluates preferences for reinforcers based on effort and behavioral activation. TBZ decreased time spent interacting with the RW, but animals compensated increasing time spent in a more sedentary option (i.e. drinking sucrose). However, DA depletion did not affect sucrose intake or locomotion in the RW when evaluated in a non-choice situation, suggesting an anergia-like effect and not a non-specific effect on appetite or locomotion. Caffeine reversed the effect of TBZ suggesting a therapeutic effect on the anergia-like symptoms induced by DA depletions. At these low doses, and based on the results about DARPP-32 phosphorylation patterns, is seems reasonable to suggest that caffeine acts via adenosine receptors. Thus, TBZ increased phosphorylation of DARPP32 at Thr34 and produced an increase on pDARPP32-Thr75, logically as a consequence of the decrease in DA levels instigated by this DA depleting agent, and as demonstrated by the HPLC measurements of DA levels in ventral striatum. Caffeine decreased pDARPP-32(Thr34) expression induced by TBZ, but did not change the levels of pDARPP-32(Thr75) induced by TBZ, suggesting a predominant role of D_2-A_{2A} receptors on caffeine-TBZ interactions. These results support the idea that A_{2A} receptors play a predominant role in mediating the therapeutic actions of caffeine.

APPENDICES

APPENDIX 1:

Impact of high doses of caffeine on acute and sensitized motor activity induced by ethanol in mice.

1. 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. To determine if caffeine can actually reverse the motor stimulating and ataxic effects of ethanol, we used an open field test to evaluate the impact of caffeine (7.5, 15 and 30 mg/kg, IP) on several motor parameters affected by acute or repeated administration of ethanol (1.5 and 2.5 g/kg, IP) in adult male CD1 mice. Acutely, both caffeine and ethanol increased locomotion in a dose dependent manner. Moreover, when ethanol was administered to animals pretreated with caffeine there was an additive effect of both substances at the low dose of ethanol, and a potentiation of the stimulating effects of the high dose of ethanol in horizontal locomotion. Caffeine reversed the suppressive effect of ethanol on rearing that was supported by the wall, but was not able to reverse the ethanol-induced impairment in rearing that was not supported, which is an index of postural stability. Ethanol (1.5 g/kg) administered repeatedly in the open field produced sensitization of horizontal locomotion and rearing supported by the wall. However, acute administration of caffeine to mice previously preexposed to ethanol produced a dose dependent reduction in locomotion compared to mice preexposed to saline. On the other hand, repeated administration of caffeine (15 mg/kg) did not induced sensitization but it made animals more sensible to the stimulant effects of ethanol (1.5 g/kg). Thus, caffeine potentiated the stimulating effects of an acute dose of ethanol. The neural substrate underlying this effect could be the dopamine-adenosine functional interaction in the nucleus accumbens, a brain structure important for the regulation of locomotion, behavioral activation, and processes such as incentive salience and vigor involved in goal directed responses.

2. Methods

2.1. Subjects

Adult Swiss CD1 mice (30-45 g) were purchased from Janvier (France). Mice were housed in groups of three per cage, with standard laboratory rodent chow and tap water available *ad libitum*. They were maintained in the colony at 22 ± 1ºC with lights on from 8:00 to 20:00 hours. All experimental procedures complied with the European Community Council directive (86/609/ECC) for the use of laboratory animal subjects and with the "Guidelines for the Care and Use of Mammals in Neuroscience and Behavioral Research" (National Research Council 2003).

2.2. Drugs

Ethanol (Panreac Quimica S.A., Spain) was diluted to 20% (v/v) in physiological saline (0.9 % w/v) and administered intraperitoneally (IP) 10 minutes before testing. Caffeine (Sigma-Aldrich, Spain) was dissolved in 0.9% w/v saline and administered IP 30 minutes before testing. Saline solution was used as vehicle.

2.3. Apparatus and testing procedures.

Open Field (OF). The OF apparatus consists 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. Animals were placed in the center of the cylinder and immediately observed for 15 minutes. The behavioral test room was illuminated with a soft light, and external noise was attenuated. Horizontal and vertical locomotion in the OF were simultaneously recorded and registered manually. For horizontal locomotion an activity count was registered each time the animal crossed from one quadrant to another with all four legs. A count of vertical locomotion was registered each time the animal raised its forepaws in the air higher than its back (unsoported rear), or rested them on the wall (suported rear).

Western Blotting. Mice were deeply anaesthetized with CO2, and when the absence of reflexes was observed the animals were dislocated. The vermis was immediately removed and dissected. Cerebellar tissue samples were homogenized in icecold lysis buffer [137mM

NaCl, 20 mM Tris-HCl (pH 8.8), 1% NP40, 10 µg/ml of aprotinin, leupetin, 0.5 mM orto sodium vanadate and 0.1 mM PMSF, protease inhibitors]. Homogenates were centrifuged at 14,000 rpm for 15 minutes at 4°C. Aliquotes o supernatants were collected and used for Bradford quantification of total protein and others stored at -80ºC until analyses. Before subjected, every sample was boiled for 5 minutes. Equal amounts (50 µg) of vermis protein samples were separated by 15% SDS-PAGE and transferred to nitrocellulose membrane for 80 minutes at 30 volts. Membranes were block with 5% non-fat dry milk in TBS-Tween 0.1% for one hour and later incubated with polyclonal rabbit anti- (1:100, Santa Cruz Biotechnology) overnight at 4°C. After rinses with TBS 1% triton X-100, filters reacted with goat anti-rabbit peroxidaseconjugated secondary antibody and developed by enhanced chemiluminescence (1:20,000; Bio-Rad). Filters were probed with anti-αtubulin monoclonal antibody (1:400; Chemicon, Millipore) as an internal standard for protein quantification. The film signals were scanned and levels of the band density were blind processed and quantified by densitometry with ImageJ software. Every sample was replicated at least twice to ensure the reproducibility of the method.

Blood ethanol determinations. Additional mice (n=6 per group) were used to determine whether caffeine influenced blood ethanol levels at the same doses and times used in the behavioral studies. For that purpose animals were injected with caffeine (0 or 30 mg/kg) and with ethanol (2.5 g/kg). Trunk blood samples (20 µl) were collected 10 and 20 min after ethanol and caffeine administration respectively. Following Boehm et al. (2000), each blood sample was immediately placed in a microcentrifuge tube containing 50 µl of ice-cold 5% ZnSO4 solution. A 50-µl aliquot of 0.3 N Ba(OH)2 and 300 µl of deionized water was added. After centrifugation at 4°C (5 min, 12,000 rpm), the supernatant was removed and blood ethanol concentrations were determined by headspace gas chromatography with a flame-ionized detector (CE Instruments GC 8000, HS 850).

3. Experiments

Experiment 1: Acute administration of caffeine, ethanol or their interaction on locomotion.

Locomotor activity was evaluated in the OF. Horizontal and vertical locomotion (supported and unsupported rear) was registered manually during 10 minutes. Caffeine (0.0, 7.5, 15.0 and 30.0 mg/kg) was administered 30 minutes before testing and ethanol (0.0, 1.5, 2.5 and 3.5 g/kg) was administered 10 minutes before test. The interval time between drug administration and beginning of testing was the same for all subsequent experiments.

Experiment 2: Effect of repeated administration of ethanol on locomotion.

During 5 sessions in alternating days, animals were tested in the OF after ethanol (1.5 or 2.5 g/kg, IP) or saline administration.

Experiment 3: Effect of acute administration of caffeine (0, 15, 30 mg/kg) on ethanol (1.5 g/kg)-induced locomotor sensitization.

Two days after the last drug administration, animals in experiment 2 received an acute administration of caffeine (0, 15 or 30 mg/kg) in order to observe if there was a cross-sensitization effect.

Experiment 4: Effect of repeated administration of caffeine (0 or 15 mg/kg) on locomotion and acute chalenge with ethanol (0.0 or 1.5 g/kg).

During 5 sessions in alternating days, two new groups of animals received saline or caffeine (15 mg/kg), IP) and were tested in the OF after drug administration. Two days after the last administration, animals received an acute administration of ethanol (0.0 or 1.5 g/kg) and were tested again in the OF.

Experiment 6. Western blotting for DARPP-32.

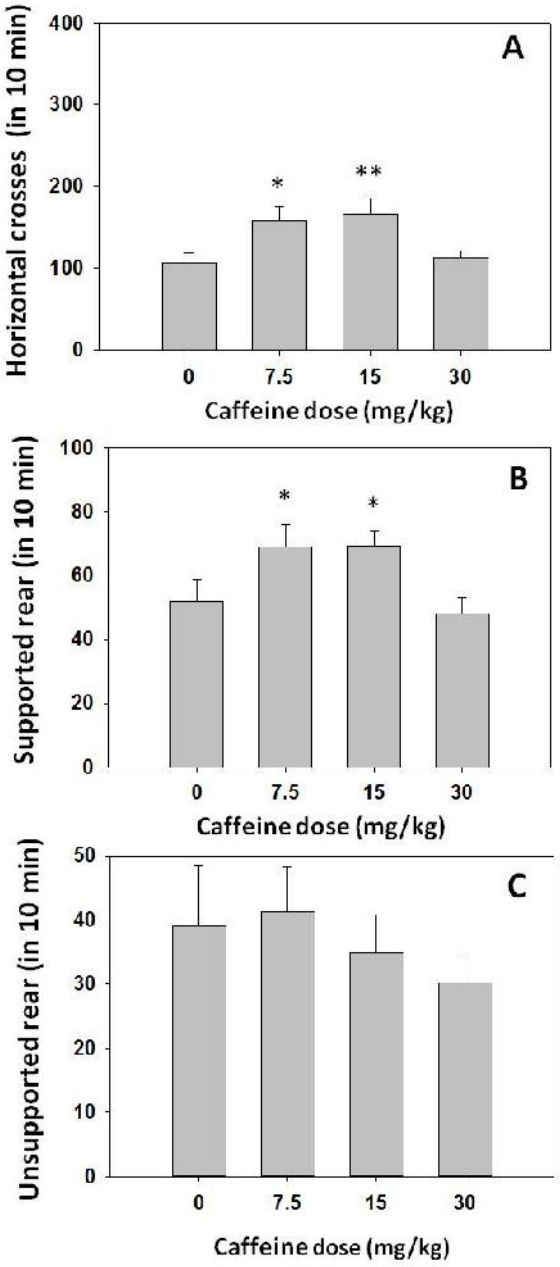
After completion of experiments 1 and 5, animals were anesthesized and brains were col.lected. Striatum samples were analyzed by western blotting for DARPP-32, pDARPP-32(Thr75), and pDARPP-32(Thr34).

Experiment 7. Blood ethanol concentration.

Additional mice were used to determine whether caffeine influenced the blood levels of ethanol at the same doses and times as those used in the behavioral studies. For that purpose animals received caffeine (0 or 30 mg/kg) and 30 min later ethanol (2.5 g/kg) was administered.

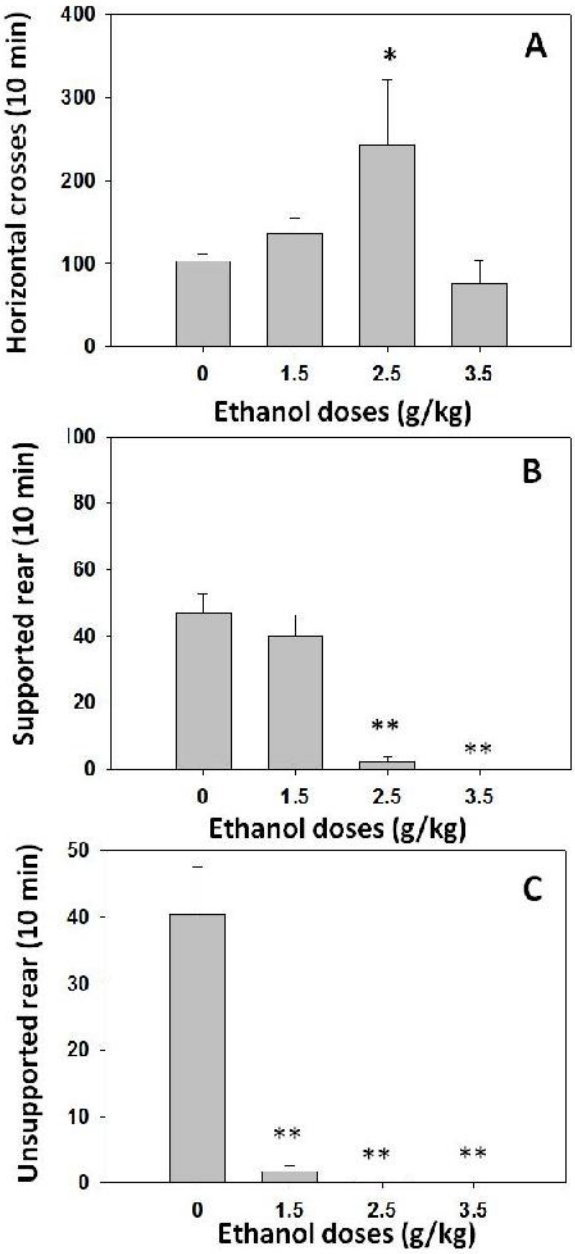
4. Preliminary results

Experiment 1: Acute administration of caffeine, ethanol or their interaction on locomotion in the open field (OF).



One way-ANOVA showed an overall effect of caffeine on horizontal crosses ($F(3,30)=4.06$, $p<0.05$), as well as on supported rear ($F(3,30)=3.48$, $p<0.05$). Planned comparisons showed that caffeine at low and moderate doses (7.5 and 15 mg/kg) significantly increased horizontal locomotion ($p<0.05$ and $p<0.01$, respectively) (**Fig 1A**). These doses of caffeine also produced significant increments in the number of unsupported rears ($p<0.05$) (**Fig 1C**). No significant effect of caffeine treatment on unsupported rear was observed ($F(3,30)=0.45$, n.s).

Figure 1. Horizontal locomotion (**A**), supported rear (**B**) and unsupported rear (**C**) in the OF after an acute administration of caffeine. Data are expressed as mean (±SEM) number of counts during 10 minutes. **p<0.01, *p<0.05 significantly different from vehicle



The effect of ethanol was also analyzed by a one-way ANOVA revealed an overall effect of ethanol treatment on horizontal crosses ($F(3,39)=3.75$, $p<0.05$), supported rear ($F(3,39)=24.11$, $p<0.01$), and unsupported rear ($F(3,39)=19.13$, $p<0.01$). Planned comparisons showed that ethanol significantly increased horizontal crosses at the dose of 2.5 g/kg ($p<0.05$) **(Fig 2A**). Supported rear was decreased by the highests doses of ethanol (2.5 and 3.5 g/kg, $p<0.01$), (**Fig 2B**). All ethanol doses significantly decreased unsupported rear ($p<0.01$) (**Fig 2C**).

Figure 2. Horizontal locomotion (**A**), supported rear (**B**) and unsupported rear (**C**) in the OF after acute administraton of ethanol. Data are expressed as mean (±SEM) number of counts during 10 minutes. **p<0.01, *p<0.05 significantly different from vehicle.

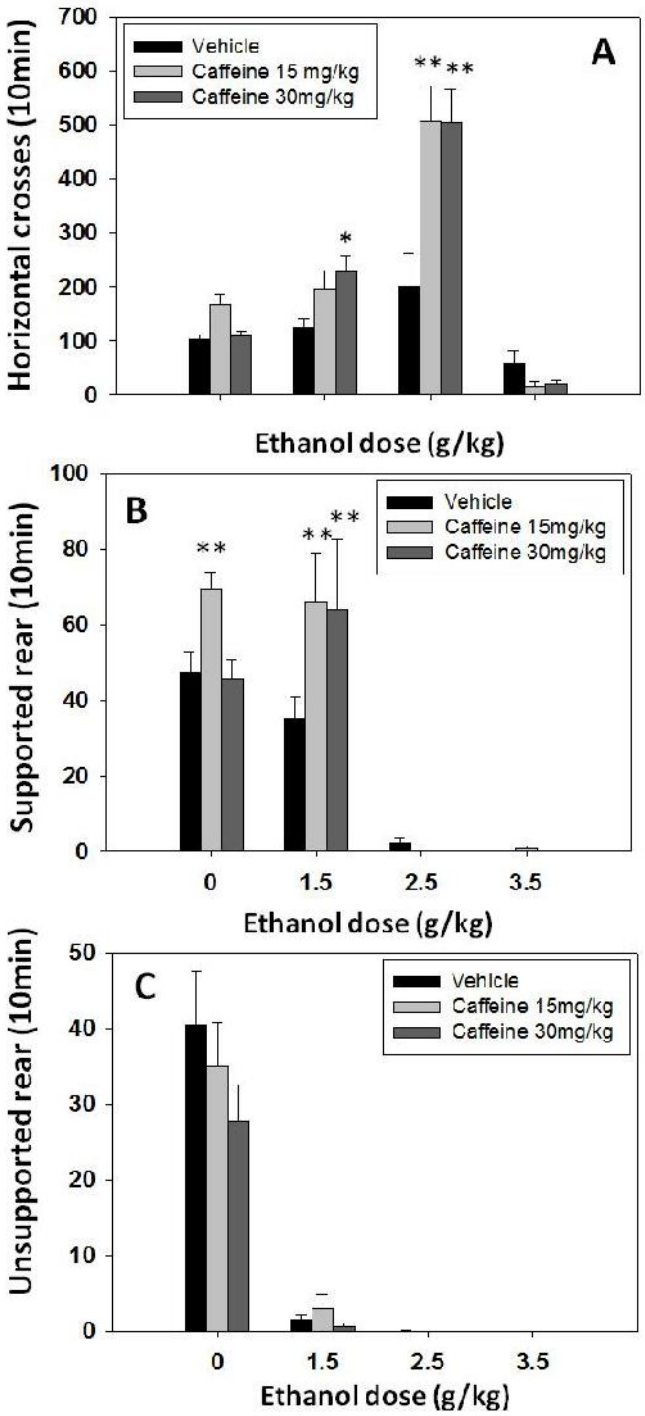


Figure 3. Horizontal locomotion (**A**), supported rear (**B**), and unsupported rear (**C**) in the OF after acute coadministration of caffeine and ethanol. Mean (±SEM) number of counts during 10 minutes. **p<0.01, *p<0.05 significantly different from 0 mg/kg caffeine in the same ethanol dose.

Factorial ANOVA (Caffeine x Ethanol) showed an overall effect of caffeine ($F(2, 112)=11.18$, $p<0.01$), ethanol ($F(3, 112)=59.35$, $p<0.01$), and caffeine-ethanol interaction ($F(6, 112)=6.64$, $p<0.01$), on horizontal crosses in the OF. Planned comparisons revealed that caffeine 30 mg/kg coadministered with ethanol 1.5 g/kg, produced a significant increase in locomotion compared with saline plus ethanol 1.5 g/kg ($p<0.05$). Caffeine 15 mg/kg plus ethanol 2.5 g/kg, increased stimulation induced by saline plus ethanol 2.5 g/kg ($p<0.01$). The same pattern of results was observed for caffeine 30 mg/kg plus ethanol 2.5 g/kg ($p<0.01$), suggesting an additive effect of caffeine on locomotion induced by low doses of ethanol, and a potentiation of the stimulation induced by ethanol at stimulant doses (2.5 g/kg). There was not a significant effect of caffeine (15 or 30 mg/kg) administered with a locomotor suppressant dose of ethanol (3.5 g/kg) or in the saline treated groups (**Fig 3A**). The factorial ANOVA (Caffeine x Ethanol) for the variable supported rear, as a measure of vertical locomotion, also showed an overall effect of caffeine [$F(2,112)=3.81$, $p<0.05$],

ethanol ($F(3,112)=62.26$, $p<0.01$), and their interaction ($F(6,112)=2.29$, $p<0.05$). Planned comparisons showed a significant increase of supported rear after caffeine at dose of 15 mg/kg ($p<0.01$). Caffeine 15 and 30 mg/kg increased supported rearing when administered with ethanol 1.5 g/kg, compared with saline in the ethanol 1.5 g/kg treated group ($p<0.01$) (**Fig 3B**). Finally, the factorial ANOVA (Caffeine x Ethanol) for unsupported rear (**Fig 3C**) showed a significant effect of ethanol treatment ($F(3,112)=66.89$, $p<0.01$). However, there was no significant effect of caffeine ($F(2,112)=0.94$, n.s.), and no significant interaction ($F(6,112)=0.83$, n.s.).

Experiment 2: Effect of repeated administration of ethanol on locomotion.

Repeated measures ANOVA showed a significant effect of treatment (saline or 1.5 g/kg ethanol) ($F(1,64)=30.87$, $p<0.01$), a significant effect of session (1 and 5) ($F(1,64)=18.15$, $p<0.01$) and treatment x session interaction ($F(1,64)=8.24$, $p<0.01$). Planned comparisons showed a locomotor stimulant effect of ethanol (1.5 g/kg) in the first session compared with saline treatment ($p<0.01$). The fifth administration (session 5) of ethanol, increased locomotion compared with its first administration (session 1) ($p<0.01$). This increase in locomotion over sessions was not observed in the saline treated group, suggesting a sensitization of locomotion induced by ethanol (**Fig 4A**). The repeated measures ANOVA showed a significant effect of treatment on supported rear ($F(1,64)=6.42$, $p<0.01$), no significant effect of session ($F(1,64)=3.32$, n.s.), but a significant effect of treatment x session interaction ($F(1,64)=4.61$, $p<0.05$) **(Fig 4B).** Planned comparisons showed that ethanol (1.5 g/kg) increased the number of supported rears when administered in session 5 compared with saline ($p<0.01$), and also compared with its administration in the session 1 ($p<0.05$). A third repeated measures ANOVA showed a significant effect of treatment on unsupported rear ($F(1,64)=127.4$, $p<0.01$), a significant effect of session ($F(1,64)=21.62$, $p<0.01$), but did not show a significant effect of treatment x session interaction ($F(1,64)=2.60$, n.s.) (**Fig 4C**).

Figure 4. Effect of repeated administration of ethanol (0.0, 1.5 or 2.5 g/kg) on horizontal locomotion (A and D), supported rear (B and E) and unsupported rear (C and F) in the OF during sessions 1 and 5. Data are expressed as mean (±SEM) number of counts during 10 minutes. **p<0.01 significantly different from session 1. ##p<0.01 significantly different from vehicle in the same session.

For the experiment in which the dose of ethanol was 2.5 g/kg, repeated measures ANOVA (Session x Treatment) for horizontal locomotion showed a significant effect of treatment ($F(1,49)=18.41$, $p<0.01$), but no significant effect of session ($F(1,49)=0.26$, n.s.), and no significant interaction ($F(1,49)=0.04$, n.s.). Thus, ethanol at this dose (2.5 g/kg) did not produced locomotor sensitization after repeated administration (**Fig 4D**). Repeated

measures ANOVA (Session x Treatment) for the variable supported rear, showed a significant effect of treatment ($F(1,49)=126.93$, $p<0.01$), but no significant effect of session ($F(1,49)=1.42$, n.s.), and no significant interaction ($F(1,49)=1.52$, n.s.) (**Fig 4E**). Finally, repeated measures ANOVA for unsupported rear, showed a significant effect of treatment ($F(1,49)=138.91$, $p<0.01$), but no significant effect of session ($F(1,49)=1.09$, n.s.) and no significant interaction ($F(1,49)=0.57$, n.s.) (**Fig 4F).** Thus, ethanol at this dose (2.5 g/kg) did not change rearing after repeated administration.

Experiment 3: Effect of acute administration of caffeine (0, 15, 30 mg/kg) on ethanol (1.5 g/kg)-induced locomotor sensitization.

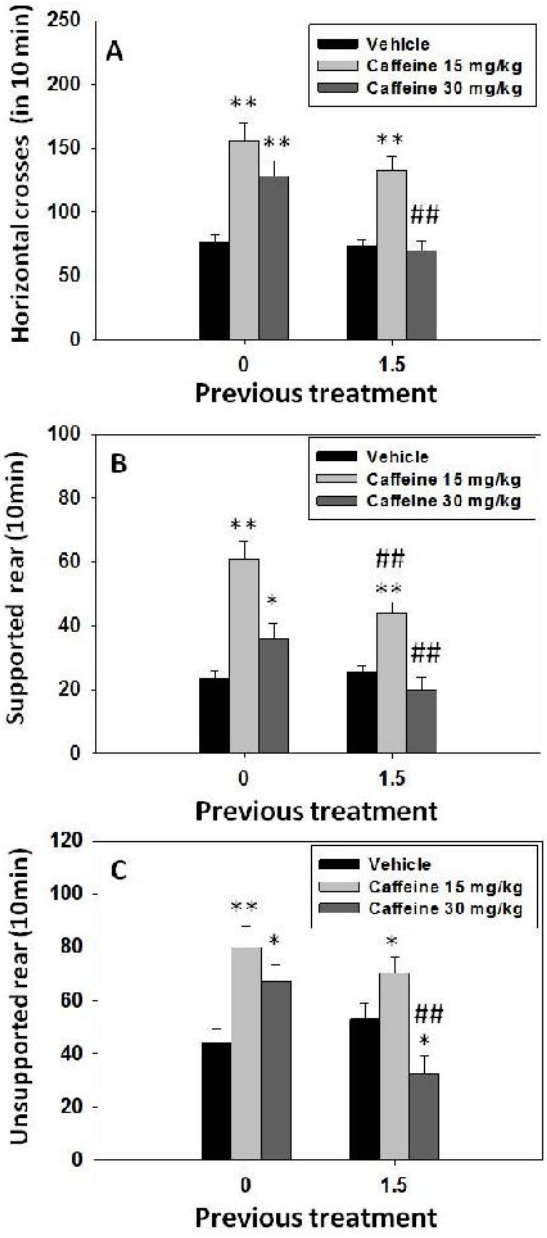


Figure 5. Effect of caffeine on horizontal locomotion (A), supported rear (B) and unsupported rear (C) in the OF in mice treated with ethanol (1.5 g/kg) in previous days. Data are expressed as mean (±SEM) number of counts during 10 minutes. **p<0.01, *p<0.05 significantly different from vehicle in the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

The factorial ANOVA; previous ethanol treatment (0.0 or 1.5 g/kg) x caffeine dose (0, 15 or 30 mg/kg) showed an overall effect of previous ethanol dose ($F1,65)=11.48$, $p<0.01$), caffeine dose ($F2,65)=25.45$, $p<0.01$), and also a significant effect of their interaction ($F1,65)=3.82$,

$p<0.05$) on horizontal locomotion **(Fig 5A).** The same patter of results were observed on supported rear **(Fig 5B)** (($F1,65)=9.66$, $p<0.01$; $(F2,65)=29.28$, $p<0.01$; $F1,65)=3.25$, $p<0.05$, respectively) and unsupported rear **(Fig 5C)** (($F1,65)=4.87$, $p<0.05$; $(F2,65)=10.90$, $p<0.01$; $F1,65)=5.36$, $p<0.01$ respectively). Planned comparison showed a stimulant effect of caffeine at both doses (15 and 30 mg/kg) in the saline pretreated group ($p<0.01$). Only the dose of 15 mg/kg of caffeine induced locomotion in the ethanol (1.5 g/kg) pretreated group ($p<0.05$). Interestingly, caffeine at the highest dose (30 mg/kg) significantly decreased locomotion in animals pretreated with ethanol (1.5 g/kg) when compared with the effect of this dose of caffeine in the vehicle pretreated group ($p<0.01$). The effect of caffeine on supported rear showed a similar pattern of effects. Caffeine increased supported rear at both doses (15 and 30 mg/kg, $p<0.01$ and $p<0.05$ respectively) in the vehicle pretreated group. However, only caffeine 15 mg/kg significantly increase this variable in the ethanol pretreated group ($p<0.01$). Caffeine 30 mg/kg decreased supported rear in the ethanol preteated group compared with its effect in the vehicle pretreated group ($p<0.01$). Finally, caffeine 15 mg/kg increased unsupported rear in the ethanol preteated group, but at the highest dose (30 mg/kg), it significantly decreased this variable ($p<0.05$). Moreover, the effect of this dose of caffeine in the ethanol pretreated group was significantly different to the effect observed in the saline pretreated group ($p<0.05$).

Experiment 4: Effect of repeated administration of caffeine (0 or 15 mg/kg) on locomotion, and acute chalenge with ethanol (0.0 or 1.5 g/kg).

The factorial ANOVA (treatment; caffeine 15 mg/kg or saline x session; 1 and 5) for horizontal locomotion, showed an overall effect of repeated treatment ($F(1,45)=7.55$, $p<0.01$), and also of session ($F(1,45)=13.64$, $p<0.01$). However, there was no significant treatment x session interaction ($F(1,45)=0.09$, n.s), suggesting no sensitization effect of repeated caffeine administration at a stimulating dose of 15 mg/kg. The same pattern of effects was observed for supported rear. The factorial ANOVA showed a significant effect of treatment ($F(1,45)=6.82$, $p<0.05$), as well as session ($F(1,45)=10.59$, $p<0.01$). But there was no significant interaction ($F(1,45)=3.12$. n.s). On the other hand, the ANOVA for the variable unsupported rear showed an overall effect of treatment ($F(1,45)=17.19$, $p<0.01$), session ($F(1,45)=10.59$, $p<0.01$), and also treatment x session interaction ($F(1,45)=39.12$, $p<0.01$). However, planned comparison on this last variable showed a significant increase

of unsupported rear in session 5 compared with session 1 only in saline treated animals ($p<0.01$) (Table 1).

	Session 1		**Session 5**	
Caffeine dose (mg/kg)	**0**	**15**	**0**	**15**
Horizontal crosses	55.2 ±4.1	80.5±7.2	71.7±6.2	100.0±11.2
Supporter rear	27.4±2.3	36.3±4.3	31.8±3.8	51.1±6.0
Unsupported rear	28.9±2.3	33.0±3.1	71.1±6.2****	31.5±3.3

Table 1. Effect of repeated administration of caffeine (0 or 15 mg/kg, IP) on horizontal locomotion, supported rear and unsupported rear in the OF during sessions 1 and 5. Data are expressed as mean (±SEM) number of counts during 10 minutes. **p<0.01 significantly different from session 1.

All these animals, after two days of no treatment or test, received a dose of 1.5 g/kg ethanol or saline. The factorial ANOVA yield no significant effect of previous treatment ($F(1,39)=0.41$, n.s), no significant effect of ethanol dose ($F(1,39)=0.11$, n.s), and no pretreatment x ethanol interaction ($F(1,39)=2.11$, n.s) (**Fig 6A**). The factorial ANOVA for variable supporter rear yield a significant effect of ethanol ($F(1,39)=6.36$, $p<0.01$), but no effect of previous treatment ($F(1,39=0.14$, n.s), and no significant interaction (1,39)=0.37, n.s) (**Fig 6B**). For unsupported rear (**Fig 6C**), caffeine pretreatment did not yield a significant effect ($F(1,39)=3.15$, n.s). However, the factor ethanol dose ($F(1,39)=108.12$, $p<0.01$), as well as the interaction ($F(1,39)=4.97$, $p<0.05$) were significant. Planned comparisons revealed significant differences between saline and ethanol (1.5 g/kg) treated animals in unsupported rear in animals previously treated with saline, and also in the groups previously treated with caffeine (15 mg/kg) ($p<0.01$). In addition, animals previously treated with caffeine (15 mg/kg), showed less number of unsupported rearings than animals treated with saline ($p<0.01$).

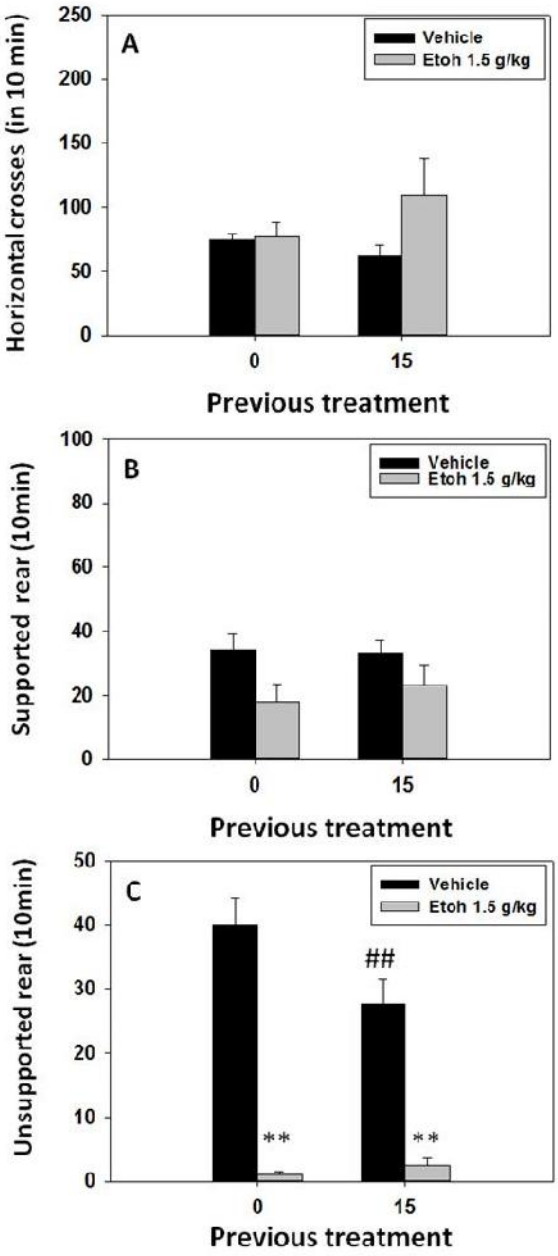
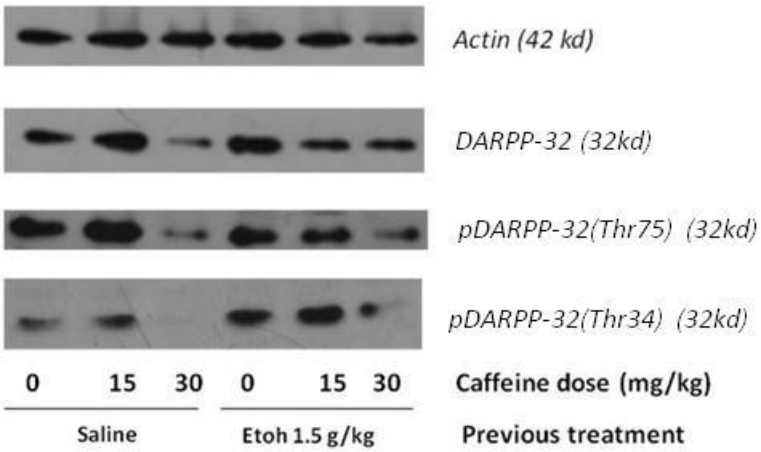


Figure 6. Effect of ethanol (1.5 g/kg) on horizontal locomotion (A), supported rear (B) and unsupported rear (C) in the OF in mice pretreated with caffeine (15 mg/kg) in previous days. Data are expressed as mean (±SEM) number of counts during 10 minutes. **p<0.01 significantly different from vehicle in the same pretreatment group. ##p<0.01 significantly different from the same dose of ethanol in animals that had received saline in previous sessions.

Further analyses, were performed for the variable locomotion. We divided animals in high and low ethanol induced locomotion using the median split among the groups that received an acute administration of ethanol 1.5 g/kg. Preliminary results demonstrate that among the low activity subgroups, there is no difference between caffeine preexposed and saline preexposed groups. However, there seems to be a clear tendency between the two high activity subgroups. Thus, a factorial ANOVA (previous treatment; caffeine vs saline x level of activity; high vs low ethanol induced activity) yielded results close to significance: Previous treatment ($F(1,20)=3.30$, $p=0.08$), level of activity ($F(1,21)=30.9$, $p<0.01$), and interaction ($F(1,31)=3.33$ $p=0.08$). The low ethanol responders subgroup preexposed to saline or caffeine (15 mg/kg) showed a horizontal locomotion average of 26.0±7.9 and

25.8±5.0 respectively, while saline or caffeine preexposed animals that showed high locomotor stimulation after ethanol administration had an average of 94.4±9.4 and 161.0±33.6 respectively.

Experiment 5. Western blotting for DARPP-32 (In progress).



Diaram showing the effect of an acute administration of caffeine at doses of 15 and 30 mg/kg in animals previously exposed to saline or ethanol (1.5 g/kg) on DARPP-32, pDARPP-32(Thr75), pDARPP-32(Thr34).

Experiment 6: Blood ethanol levels.

A two-way factorial ANOVA (ethanol x caffeine) showed a significant effect of ethanol ($F(1, 25)= 326.82$, $p<0.01$) but, no significant effect of caffeine ($F(1, 25)=0.31$, n.s.), or of the interaction ($F(1, 25)=3.39$, n.s.). These data suggest that the observed behavioral effects of ethanol coadministered with caffeine are not due to changes in blood ethanol concentration.

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

Table 1. Effect of caffeine on blood ethanol levels. Mean ± SEM (n=7-8 per group) of blood ethanol levels (in milligrams per deciliter) after acute IP administration of ethanol and caffeine at the highest doses used.

Conclusions

-Caffeine showed a bell-shaped dose response curve inducing stimulant effects on horizontal locomotion and supported rear in the OF at low doses but not at the highest dose used. Ethanol showed stimulant effects on locomotion at moderate-high (2.5 g/kg) doses but suppressed vertical locomotion; an index of motor incoordination.

- Stimulant and non-stimulant doses of caffeine (15 and 30 mg/kg) potentiated locomotion in animals treated with low and moderate doses of ethanol acutely. However, at the highest dose of ethanol (3.5 g/kg) caffeine did not reversed ethanol's suppressing effect in any of the locomotion parameters.

- Repeated administration of ethanol (1.5 g/kg) induced motor sesitization. However, there was no cross-sensitization with caffeine. Instead, a non-stimulant dose of caffeine (30 mg/kg) produced suppression in horizontal and vertical locomotion in ethanol sensitizated mice. Repeated administration of ethanol (2.5 g/kg) did not induce motor sesitization.

-Thus, caffeine potentiates locomotion at stimulant doses of ethanol but at higher doses or after suppresion of locomotion, caffeine at medium to high doses potentiates the incoordinating effects of ethanol.

- Caffeine at a stimulant dose (15 mg/kg) did not induce sesitization after 5 administrations. However, animals pretreated with caffeine showed a tendency to have a potentiated response to ethanol (1.5 g/kg) compared with saline treated animals, that was more evident in those animals that showed stimulation of locomotion.

APPENDIX 2:

Behavioral manipulations for the validation of the 3-choice running wheel T-maze

1. Abstract

One of the behavioral tests used in present dissertation for the evaluation of anergia-like effects induced by the VMAT2 inhibitor TBZ, is the 3-choice t-maze task (adapted from Correa et al., 2016). In this test animals can chose between running on a wheel (RW) vs. sedentary activities such as consuming sucrose or sniffing a neutral odor. Animals can allocate their time in reinforcing activities with different activational requirements: RW, which requires high levels of behavioral activation and effort, or with more sedentary activities shuch as eating freely available sweet pellets or as a third option sniffing through a hole where there is a cotton ball with a neutral odor. Under normal conditions, animals spent most of their time running in the RW (65%), less time eating (4%) and a few seconds sniffing the neutral odor (0.5%).

Administration of TBZ shifts behavior; decreasing time in the RW and increasing time eating, with no change interacting with the neutral odor (as observed in **chapter 6**). Thus, the 3-choice t-maze task, is sensitive to DA manipulations as observed in previous studies (Correa et al., 2016). This type of task is used for the evaluation of motivated behavior, thus it should be sensitive to factors that can change cost-benefit analyses when choosing between reinforcers such as the effort required or the value of the reinforcer (Cheeta et al., 1995; Pardo et al., 2015; Randall et al., 2011; Fisher and Mazur, 1992).

The present work explores the sensitivity of the 3-choice T-maze task to manipulations that change the value of the most preferred reinforcers used in this T-maze (RW and sweel pellets). Common manipulations of the reinforcer's value when it is palatable food or solution is the devaluation of the reinforcer changing its taste (Pickens et al., 2003; Pardo et al., 2015; Cheeta et al., 1995), prefeeding the animals with the same type of food (Pardo et al., 2012; 2015), or using drugs that induce anorexic-like effects (Randall et al., 2012). In the present experiments we devalued the food by changing the taste of the sweet pellets making them bitter and by pre-feeding the animals ad libitum with the sweet pellets. In another condition animals were deprived of standard food in order to increase food value. On the other hand, because normal mice spent most of their time running and much less time consuming sucrose we also assessed the impact of increasing wheel resistance (an effort-related challenge) on these preferences.

2. Methods

2.1. Subjects

CD1 adult male mice (N=7) purchased from Janvier, France S.A. were 15-17 weeks old (30-45 g) at the beginning of the study. Mice were housed in groups of three or four per cage, with standard laboratory rodent chow and tap water available *ad libitum*. The colony was kept at a temperature of 22±2 ºC with lights on from 08:00 to 20:00 h. All animals were under a protocol approved by the Institutional Animal Care and Use Committee of Universitat Jaume I, and all experimental procedures complied with European Community Council directive (86/609/ECC). All efforts were made to minimize animal suffering, and to reduce the number of animals used.

2.2. Testing procedures

Three-choice running wheel T-maze task. The T-maze apparatus consisted of a central corridor with two opposed arms. Each arm provided a different type of stimuli (for details, see Fig. 1). In one of them sweet pellets (TestDietTM, 50% sucrose, 45 mg each) were available, in another arm there was a RW, and in the third arm there was a hole with a cotton ball socked with a neutral non-social odor. Training as well as test sessions lasted 15 minutes. Mice were trained 5 days a week. Training phase 1: to avoid neophobia to the sweet tasting pellets, animals were enclosed in that arm with the food during 5 sessions. Training phase 2: during 4 more weeks animals were exposed, one 15 min session a day to the T-maze with free access to the three stimuli. Mice were exposed to all the conditions during consecutive weeks, day before the manipulation was considered as a baseline (BL) or normal performance.

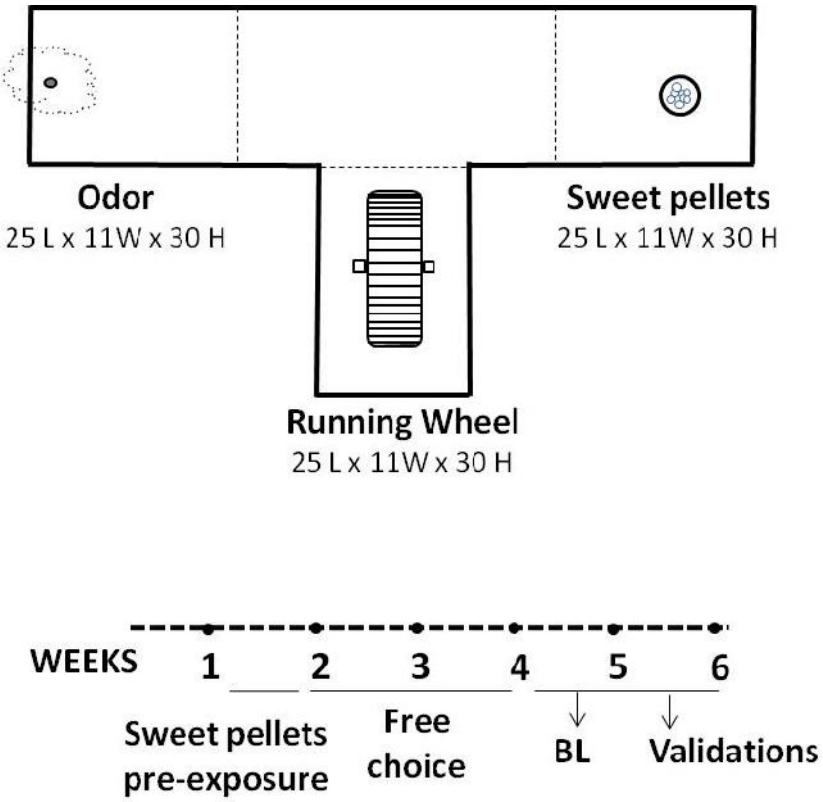


Figure 1. Schematic representation of 3 choice T-maze task.

Experiment 1. Effect of sweet food devaluation on preference in the 3-choice T-maze task.

Experiment 1.1. Change in taste: bitter pellets

Experiment 1.2. Change in appetite: Pre-feeding.

Experiment 2. Effect of food deprivation on preference in the 3-choice T-maze task.

Experiment 3. Effect of increasing RW resistance on on preference in the 3-choice T-maze task.

4. Results

Experiment 1. Effect of sweet food devaluation on preference in the 3-choice T-maze task.

Experiment 1.1. Change in taste: bitter pellets

Animals were trained as described before, and after reaching stable levels of time interacting with the three different reinforcers, a drop of caffeine (1g/L) was added to the sweet pellets in order to make them bitter. BL was assessed the day before.

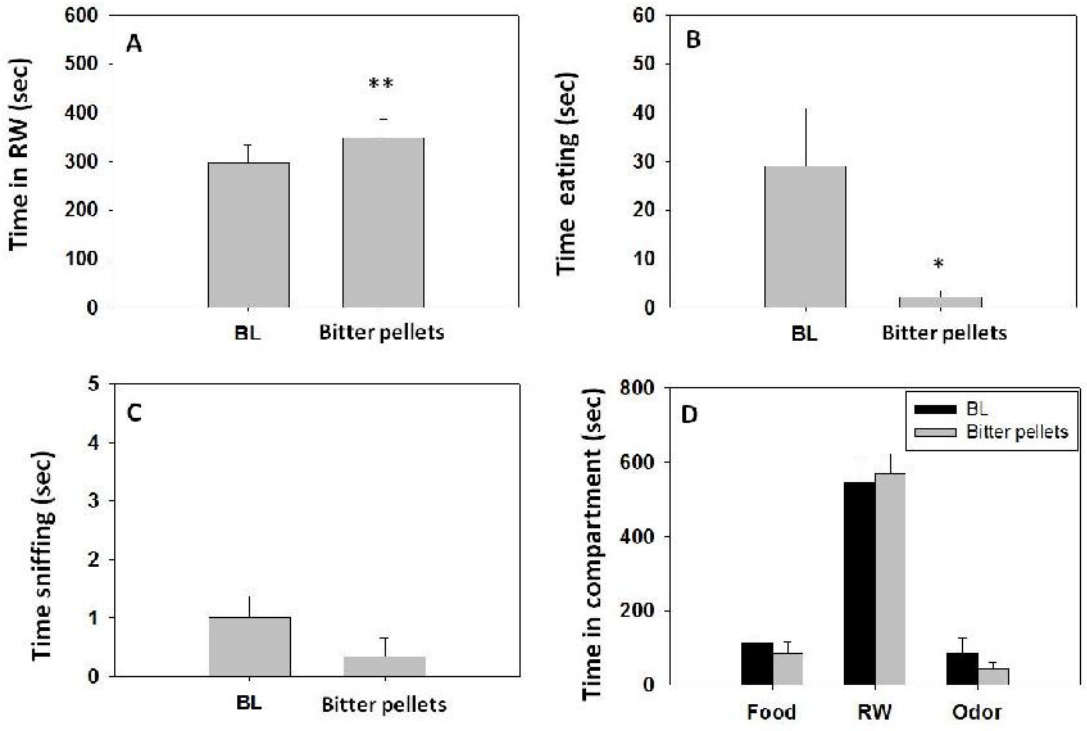


Fig 2. Effect of devaluation of pellets (bitter pellets) in the 3-choice T-maze preference task. Data are expressed as mean (±SEM) of time (seconds) spent interacting with RW **(A**), food (**B**), neutral odor (**C**) and time in different compartments (**D**) during a 15 minutes session. **p<0.01, *p<0.05 significantly different from BL.

A Student`s t-test for dependent samples showed a significant increase on time running in the RW in food devaluation condition (bitter pellets) ($t=4.56$, $p<0.01$), and a significant decreased on time spent eating compared with its BL ($t=-2.53$, $p<0.01$). However there were not differences on time sniffing the neutral odor between both conditions ($t=2.0$, n.s.) (**Fig 2 A-C).** The t-tests for dependent samples comparing time in compartments between both conditions (BL vs. food devaluation) did not showed

differences on time spent in RW compartment (t=1.13, ns), food compartment (t=-1.39, ns) and neutral odor compartment (t=1.81, ns) (**Fig 2 D).**

Experiment 1.2. Change in appetite: Pre-feeding.

After reaching stable baseline levels, animals were preexposed to sweet pellets overnight before the beginning of the experiment. The previous day was used as BL.

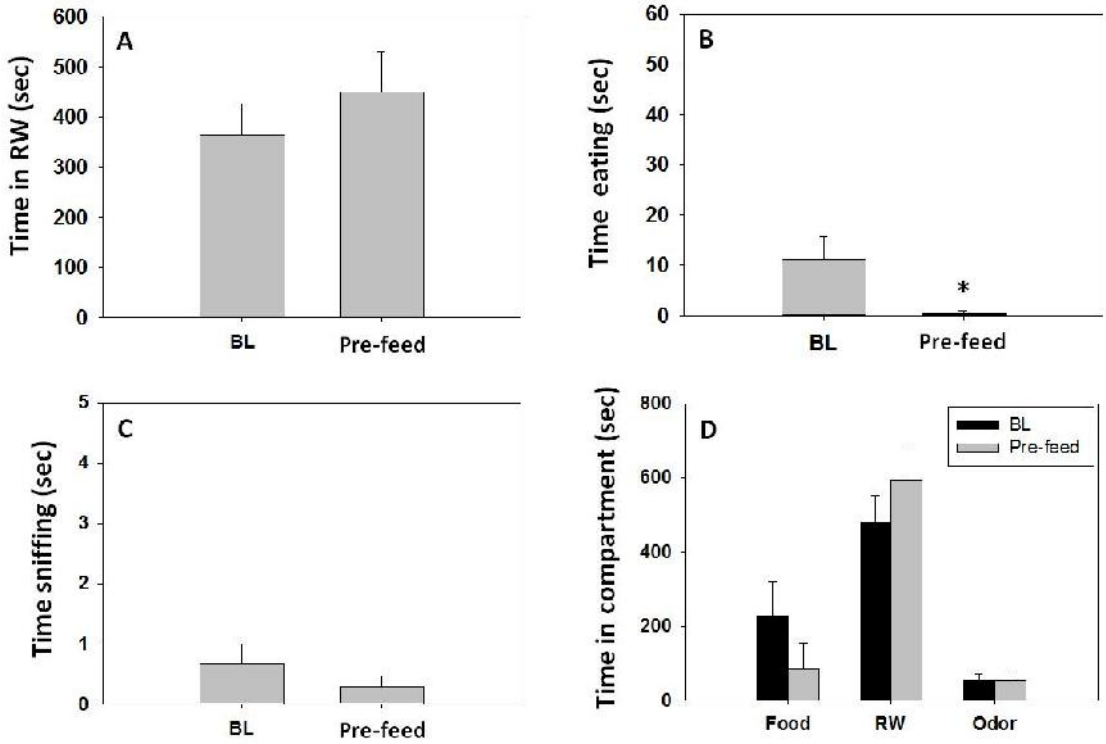


Fig 3. Effect of pre-feeding in the 3-choice T-maze preference task. Data are expressed as mean (±SEM) of time (seconds) spent interacting with RW **(A)**, food (**B**), neutral odor (**C**) and time in different compartments (**D**) during a 15 minutes session. *p<0.05 significantly different from BL.

The Student's t-test for dependent samples showed no significant effect of prefeeding on time spent RW although there was a tendency to increase (t=0.38, ns), and no significant differences between conditions on time spent sniffing the neutral odor (t=10, ns). However, there was a significant decrease on time eating in the pre-feed condition compared with BL (t=2.6, p<0.05) (**Fig 3 A-C)**. Student's t-tests for dependent samples for the variable time in compartments did not show significant differences between

conditions on time spent in RW compartment (t=0.60, ns), food compartment (t=1.48, ns) or neutral odor compartment (t=1.03, ns) (**Fig 3. D**).

Experiment 2. Effect of food deprivation on preference in the 3-choice T-maze task.

Animals were retrained and, after reaching stable levels, they were food deprived the night before the test in order to increase their appetite. The BL data correspond to the day before test.

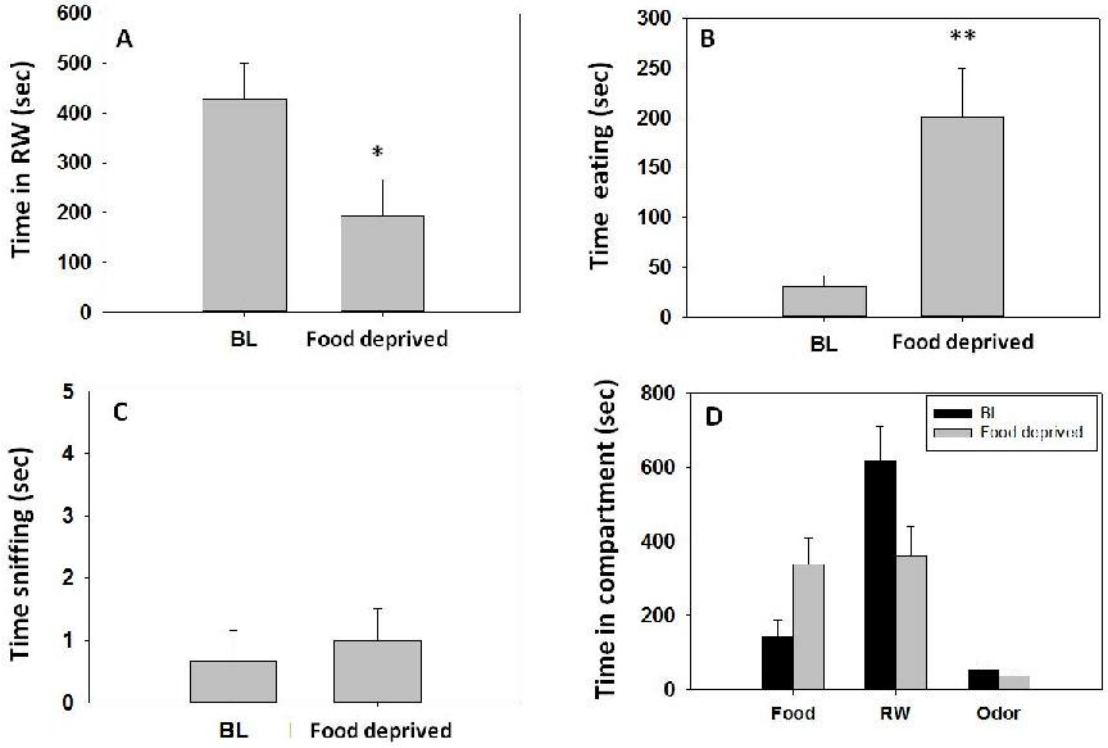


Fig 4. Effect of food deprivation in the 3-choice T-maze preference task. Data are expressed as mean (±SEM) of time (seconds) spent interacting with RW **(A)**, food (**B**), neutral odor (**C**) and time in different compartments (**D**) during a 15 minutes session. **p<0.01, *p<0.05 significantly different from BL.

The food deprivation condition significantly decreased time in RW, and significantly increased time eating compared with BL condition as showed by the Student's t-test for dependent samples (t=-3.47, p<0.05; t=4.04, p<0.01 respectively). However, there was no significant difference between conditions on time spent sniffing the neutral odor

(t=0.54, ns) (**Fig 4. A-C**). Student's t-tests for time in compartments, did not show significant differences between both conditions on time spent in food compartment (t=2.49, ns), RW compartment (t=-2.28, ns), or odor compartment (t=-1.20, ns) (**Fig 4. D**).

Experiment 3. Effect of increasing RW resistance on preference in the 3-choice T-maze task.

Mice were trained as in the previous experiments and after reaching stable levels of BL level of performance, the RW resistance was increased during two consecutive days. Test was performed during 3 consecutive days: in the first day animals had a wheel with the standard resistance (0%). For the second day weights were attached to the wheel so that the resistance increased 75%, and for the third day additional weights increased resistance to 95%.

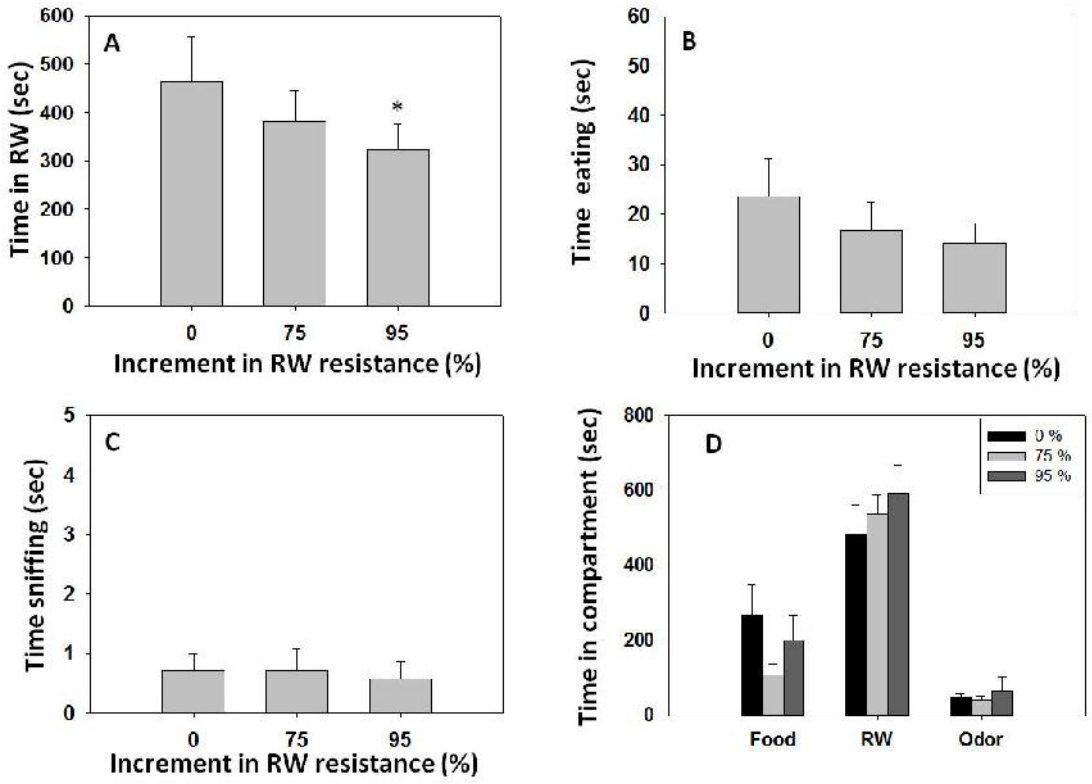


Fig 5. Effect of increasing RW resistance in the 3-choice T-maze preference task. Data are expressed as mean (±SEM) of time (seconds) spent interacting with RW **(A)**, food (**B**), neutral odor (**C**) and time in different compartments (**D**) during a 15 minutes session., *p<0.05 significant different from 0% (normal RW).

Repeated measures ANOVA showed an overall effect of RW resistance on time running in the RW ($F(2,12)=3.75$, $p<0.05$). Planned comparison analysis showed a significant decrease in time running when RW resistance was increased to 95% ($p<0.01$) (**Fig 5.A**). There was not an overall effect of RW resistance on the variables time eating ($F(2,12)=1.12$, ns.), or time sniffing ($F(2,12)=0.07$, ns) (**Fig 5. B and C**).

Repeated measures ANOVA did not show an overall effect of RW resistance on time in food compartment ($F(2,12)=3.28$, ns), RW compartment ($F(2,12)=3.04$, ns), or odor compartment ($F(2,12)=0.34$ ns) (**Fig 5. D**).

5. Discussion

In single trial situations, when several reinforcers are presented concurrently, animals distribute their time taking into account different levels of preference. However, a low preferred option can act as a good reinforcer and elicit high levels or response when it is presented alone (Franciso et al., 2008), or can be selected when the value of a more preferred reinforcer is modified.

In experiment 1 food devaluation was been tested, and animals showed a decrease on time eating but compensated increasing even more the time in the most preferred reinforcer; the RW, specially in the bitter pellets condition. Conversely in experiment 2, increasing the value of food after food deprivation, there was a significant increase on time spent eating and a compensatory decrease on time running in the RW compared to BL. This condition increased 800% the time spent eating in relation to BL, but only decreased 50% time in RW indicating that the animals were still engaged in the RW.

In experiment 3, attaching weights to the RW and thus increasing the effort required to run, reduced time in the RW, but there was no compensatory increase in sucrose preference. Time spent in the RW compartment is not reduced, suggesting that although the total time running is lower, maybe the resting times were longer than under BL conditions. Devaluation of RW motivational value by RW pre-exposure, has been tried on a previous study (Correa et al., 2016). However, the parameters used (duration of preexposure) were not enough to induce “satiation”, possibly because running in the wheel becames habitual and therefore relatively insensitive to devaluation. The neutral option was not affected by changing conditions in one of the other two reinforcers,

under any of the present set of manipulations, confirming that this is a neutral stimuly. Further studies should use manipulations of this stimuly to increase its value.

Thus, the shifts in preference induced by DA D2 antagonism (Correa et al., 2016) and by TBZ in Chapter 6 did not follow the same pattern of results as observed when running became more difficult after attaching weights to the RW (experiment 3). Thus, the effects of DA antagonism or depletion on redirecting preferences away from RW activity do not seem to mimic the effects of moderate increases in muscle exertion in the same paradigm, and do not seem to be due to an inability to run in the RW. However, we cannot rule out the possibility that those pharmacological manipulations increase appetite, thus increasing food consumption and reducing time in the RW, although the magnitude of the effect observed in the food deprivation experiment is much higher than the one observed after DA antagonism or depletion.

In summary, we confirm that changes on preference are related with changes of the reinforcer's value, showing that the 3-choice T-maze task is sensitive to motivational manipulations and mice allocate their behavior from one stimulus to another depending on their motivational relevance. Thus, this paradigm could be also sensitive to motivational impairments induced by drugs.

6. References.

- Cheeta S, Brooks S, Willner P. (1995). Effects of reinforcer sweetness and the D2/D3 antagonist raclopride on progressive ratio operant performance. Behav Pharmacol. 6(2):127-132.

- Correa M, Pardo M, Bayarri P, López-Cruz L, San Miguel N, Valverde O, Ledent C, Salamone JD. (2016). Choosing voluntary exercise over sucrose consumption depends upon dopamine transmission: effects of haloperidol in wild type and adenosine A2AKO mice. Psychopharmacology (Berl). 233(3): 393-404.

- Francisco MT, Borrero JC, Sy JR. (2008). Evaluation of Absolute and Relative Reinforcer Value Using Progressive-Ratio Schedules. Roane H, ed. Journal of Applied Behavior Analysis. 41(2):189-202. doi:10.1901/jaba.2008.41-189.

- Pardo M, López-Cruz L, San Miguel N, Salamone JD, Correa M. (2015). Selection of sucrose concentration depends on the effort required to obtain it: studies using tetrabenazine, D1, D2, and D3 receptor antagonists. Psychopharmacology (Berl). 232(13):2377-91. doi: 10.1007/s00213-015-3872-7.

- Pardo, M., Lopez-Cruz, L., Valverde, O., Ledent, C., Baqi, Y,. Müller, C.E., Salamone, J.D., and Correa, M. (2012). Adenosine A2A receptor antagonism and genetic deletion attenuate the effects of dopamine D2 antagonism on effort-based decision making in mice. Neuropharmacology. 62, 2068-2077. doi: 10.1016/j.neuropharm.2011.12.033.

- Pickens CL, Saddoris MP, Setlow B, Gallagher M, Holland PC, Schoenbaum G. (2003). Different roles for orbitofrontal cortex and basolateral amygdala in a reinforce devaluation task. J Neurosci. 23(35):11078-11084.

- Randall PA, Pardo M, Nunes EJ, López Cruz L, Vemuri VK, Makriyannis A, Baqi Y, Müller CE, Correa M, Salamone JD. (2012). Dopaminergic modulation of effort-relate0 choice behavior as assessed by a progressive ratio chow feeding choice task: pharmacological studies and the role of individual differences. PLoS One. 7(10):e47934. doi: 10.1371/journal.pone.0047934.

APPENDIX 3:

Dopamine depletion reduces preference for activity-based reinforcers in mice with low but not high experience of exercise: relation to DARPP-32 phosphorylation paterns.

1. Abstract.

Organisms frequently make cost/benefit analyses in which they weigh the value of rewards vs. the costs involved in procuring them. These decision-making processes include assessments of effort-related costs and other factors, and can involve cognitive as well as physical effort. The mesolimbic dopamine (DA) system plays a critical role in behavioral activation, exertion of effort, and effort-based decision-making, and DA antagonism and depletion in this system has been shown to induce anergia in effort-based decision tasks. Exercise has been demonstrated to have protective effects in animal models of pathologies characterized by motor disturbances such as Parkinson's disease, which involves DA loss in the nigrostriatal system. However, the beneficial effects of physical activity on symptoms such as mental fatigue or anergia, present in many psychiatric and neurological pathologies, also need to be explored. To assess DAergic involvement in the activational component of motivation, and in effort based decision-making when multiple reinforcers are available, mice received injections of tetrabenazine (TBZ), a VMAT-2 inhibitor that produces a reversible DA depletion. Mice were tested in a 3-choice-T-maze task developed for the assessment of preference between physical activity (wheel running) in one arm vs. sedentary reinforcers such as a freely available sucrose pellets in another arm, as well as a non-social (neutral) odor in the third arm. Additionally, to study the protective effects of physical exercise, different groups of animals were exposed to a daily session of forced exercise during 9 weeks (5 days a week). Under standard conditions, mice spent more time running and less consuming sucrose or sniffing. TBZ produced a shift in the relative preference; it reduced the choice of the reinforcer that involved vigorous activity, but increased consumption of a reinforcer that required little effort (sucrose). On the contrary, mice that were extensively exposed to exercise did not show TBZ-induced shifts in preference towards low-effort reinforcers such as sucrose or olfactory stimuli. These results suggest that exercise could act as a preventive therapy for the anergia-inducing effects of DA depletion. Thus, DA depletion produced effects indicative of anergia (lack of energy), but did not impair the primary rewarding effects of sucrose.

2. **Methods.**

Subjects.

CD1 male mice weighed 24-28 g at the beginning of the study (Janvier). All mice were housed in groups of 3 or 4 animals per cage with tap water available ad libitum, and were food-restricted to reach 85% freefeeding body weight throughout the study. The colony was kept at a temperature of 22± 2 ºC with lights on from 08:00 to 20:00 h. All animals were under a protocol approved by the Institutional Animal Care and Use Committee of Universitat Jaume I, and all experimental procedures complied with European Community Council directive (86/609/ECC). All efforts were made to minimise animal suffering, and toreduce the number of animals used.

Drugs

Tetrabenazine (TBZ) [(*R*,*R*)-3-Isobutyl-9,10-dimethoxy-1,3,4,6,7,11b-hexahydro-pyrido[2,1-*a*]isoquinolin-2-one] (CIMYT Quimica SL, Spain), was dissolved in a 20% dimethylsulfoxide (DMSO) solution mixed with saline and pH adjusted with 1 N HCl to bring the final solution to pH 5.5. DMSO (20%v/v) was used as its control. TBZ was administered 120 min before testing.

Testing procedures and apparatus

Forced running wheels (RW). Mice (4 weeks old at the beginning of the training) were trained in the Mouse Forced Exercise Talking Wheel System (Model 80800[a], Lafayette Intrument ©). Animals were divided in two groups. One group of mice were exercised daily, 5 days per week, for 9 weeks, beginning at the start of the dark cycle (10.00 h). Training consisted in 2 cycles of 15 min at 5 rpm, and 2 cicles of 15 min at 7 rpm with one minute of rest between cycles. The total distance travelled was 360 meters in 1 hour. The second group of mice was used as control group and they were enclosed in the Forced RW but blocked in order to be exposed to the same conditions but with non exercise. These two groups of mice constituted the “Forced” and “Blocked” conditions.

Locomotion in the open field arena (OF). The OF 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. The behavioral test room was illuminated with a soft light, and external noise was attenuated. Horizontal and vertical locomotion was registered manually during 60 min. For horizontal locomotion an activity count was registered each time the animal crossed from one quadrant to another with all four legs. A count of vertical locomotion was registered each time the animal raised its forepaws in the air higher than its back, or rested them on the wall. Animals were testes in OF the last week of training (**Figure 1**)

Anxiety in the elevated plus maze (EPM*).* The 8th week of forced training animals from Forced condition and Blocked condition were evaluated in an EPM (**Figure 1**). This paradigm consists of two open and two enclosed arms arranged in a plus configuration. This anxiety paradigm measures the avoidance that rodents show to elevated open spaces. Animals were placed in the central platform and assessed during 5 minutes. A trained observer registered time spent in the open arms, ratio of entries in the open arms to total arm entries, latency to enter the open arms and total entries in the 4 arms as an index of locomotion. An entry into an arm was recorded when the animal crossed the line that connected that arm with the central platform with all four legs.

Three-choice running wheel T-maze task. The T-maze apparatus consisted of a central corridor with two opposed arms. Each arm provided a different type of stimuli (for details, see Fig. 1). In one of them sweet pellets (TestDietTM, 50% sucrose, 45 mg each) were available, in another arm there was a RW, and in the third arm there was a hole with a cotton ball soaked with a neutral non-social odor. Training as well as test sessions lasted 15 minutes. Mice were trained 5 days a week. Training phase 1: to avoid neophobia to the sweet tasting pellets, animals were enclosed in that arm with the food during 5 sessions. Training phase 2: during 2 more weeks animals were exposed, one 15 min session a day to the T-maze with free access to the three stimuli. Test phase: This phase lasted during 3 weeks more (one week per TBZ dose). Animals started the t-maze procedure after being trained 9 weeks in the forced RW.

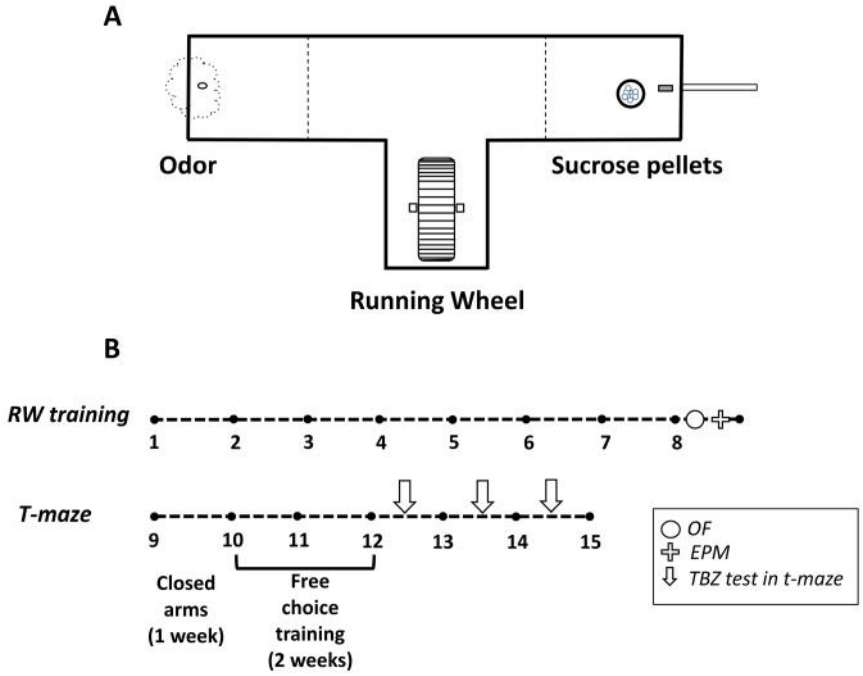


Figure 1. Schematic representation of the 3-choice RW T-maze task settings and experiments timeline.

High Performance Liquid Chromatography (HPLC) for DA level determination. Brain samples were extracted after 120 minutes of TBZ (0 and 2 mg/kg) administration. Mice were anesthetized with carbon dioxide for 30 s and decapitated. Brains were quickly removed and frozen on a Leitz Wetzlar microtome. Coronal sections 750 µm thick were cut through the striatum. A 16-gauge stainless-steel tube was used to dissect bilateral cylindrical samples from the ventral striatum and anterior cingulated (Acg). These tissue samples were then placed in 200 µl of 0.1 N perchloric acid, and then homogenized, centrifuged, and frozen. The supernatant was subsequently analyzed for DA content using HPLC with electrochemical detection (ESA Coulochem II system). The electrochemical parameters were as follows: channel 1= − 100 mV, channel 2 = +200 mV, and guard cell = +350 mV. Each liter of mobile phase contained 27.6 g sodium phosphate monobasic, 8.0% of methanol 750 µl of 0.1M EDTA, and 2875 µl of 0.4M sodium octyl sulfate dissolved in deionized ultrapure H_2O with a final pH of 4.5. The flow rate was 1.0 ml/min.

Plasma corticosterone determination. 60 minutes after being trained in Forced RW animals were sacrificed by decapitation under deep anesthesia. Animals were anesthetized with a 1.0 ml/kg IP injection of a cocktail solution containing 10.0 ml of

100 mg/ml ketamine plus 0.75 ml of 20.0 mg/ml xylazine (both from Phoenix Scientific, Inc., St. Joseph, MO, USA), 30 minutes before decapitation. Blood samples were collected in heparinized (15 units/ml of blood) Eppendorf tubes and centrifuged at 4000 rpm for 10 min. Supernatant was taken and stored at -20°C until corticosterone determination. Plasma corticosterone levels were measured spectrophotometrically using a commercially available enzymatic immunoassay kit (Rodents Corticosterone Enzyme Immunoassay System, OCTEIA Corticosterone; Immunodiagnostic Systems LTD, Boldon, England). Blood corticosterone concentration (ng/ml) was determined using a logarithmic adjustment of the standard curve.

DARPP-32 immunohistochemistry. After 120 min of TBZ (0 or 2 mg/kg) administration animals trained in Forced RW (Forced and Blocked) were anesthetized with CO_2 and transcardially perfused with 0.9% physiological saline with heparine (0.06%) for 5 min, followed by perfusion with 3.7% formaldehyde for 5 min. Brains were fixed for 24 h by immersion in 3.7% formaldehyde and then transferred into a 30% sucrose solution and stored at 4°C before brain sectioning. Free floating coronal sections of brains (40 µm) were serially cut using a Cryostat 9 (Thermo Fisher) and rinsed in 0.01 M phosphate buffer (PBS). 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.

Statistical analysis

All the behavioral experiments followed a within-subjects design, and were analyzed with 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 treatment with the vehicle control group (Keppel, 1991). For these comparisons, α level was kept at 0.05 because the number of comparisons was restricted to the number of treatments minus one. Biochemical studies were analyzed with one-way ANOVA or with a non-parametric Mann Whitney-U test in the HPLC experiment . All data were expressed as mean ± SEM, and significance was set at $p<0.05$. STATISTICA 7 software was used for statistical analyses of the data.

3. Experiments

Experiment 1. Effect of Forced RW and Blocked RW training on body weight, food intake, and stress-related parameters.

During the 9 weeks of exercise training different variables that can be sensitive to stress were registered:

- Total amount of lab chow comsumption in home cage was registered twice a week.

- Mice were weighted twice a week before during RW training.

- Defecation has been used as a measure of emotionality in rats (Hall, 1934; Sanberg, 1989), the number of fecal pellets deposited by mice in each group (forced vs. blocked RW) was recorded after their daily training session.

- Plasma corticosterone levels were quantified after the last session of training.

Experiment 2: Effect of previous RW training on EPM performance.

Experiment 3: Exploratory locomotion in a novel OF.

Experiment 4: Effect of TBZ on 3-choice T-maze performance

Experiment 5: Experiment 3. Effect of TBZ on DA tissue levels in striatum and Anterior Cingulate.

Experiment 6: Effect of TBZ on pDARPP-32(Thr34) and pDARPP-32(Thr75) in mice after completion of RW training.

4. Results

Experiment 1. Effect of Forced RW and Blocked RW training on body weight, food intake, and stress-related parameters.

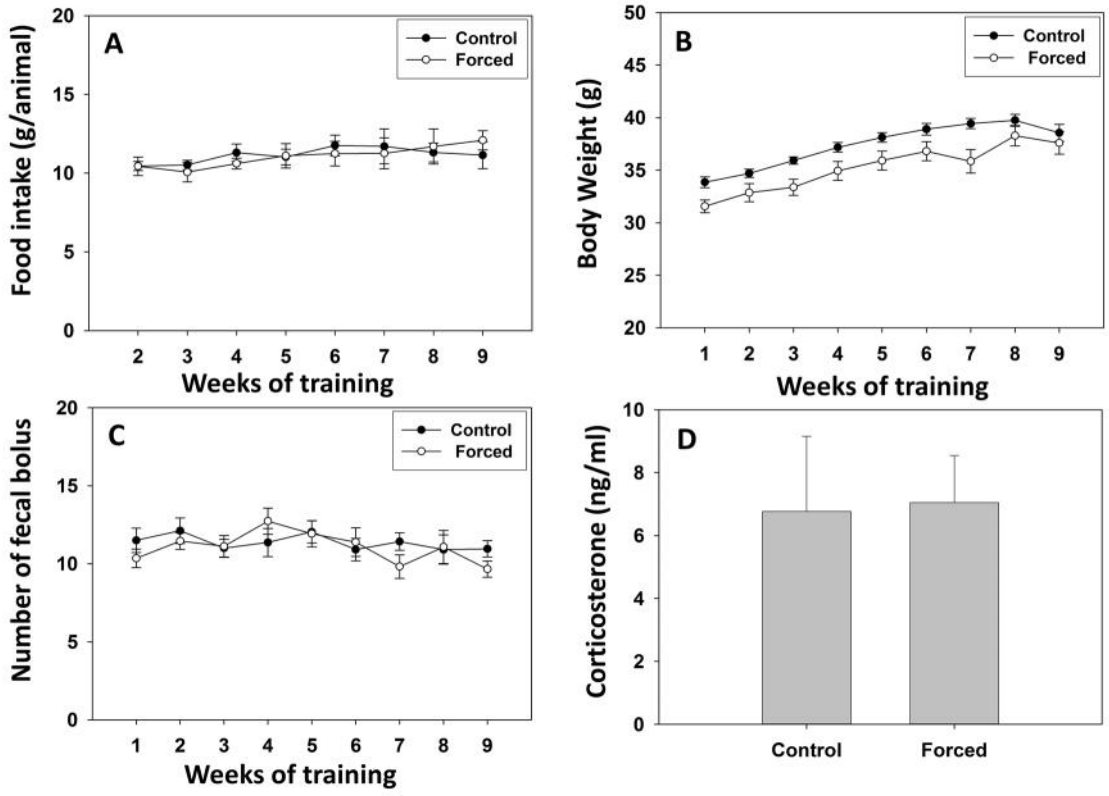


Figure 2. Body weight, food consumption, fecal bolus, and corticosterone levels after training. Mean (±SEM) body weight in grams (A), standard food consumed in home cage in grams (B), number of fecal bolus excreted during training session (C), and plasmatic corticosterone levels after the training session in nanograms per mililiter of blood (D).

Factorial ANOVA for the variable food consumption (chow consumed in their home cages during 8 weeks of training), did not showed an overall effect of condition (Control or Forced) ($F(1,72)=0.01$, n.s.), week of training ($F(7,42)=1.92$, n.s.) or interaction ($F(7,42)=0.56$, n.s) (**Fig 2A**). The factorial ANOVA for body weight showed a significant effect of training ($F(1,22)=7.71$, $p<0.05$), and a significant effect of week of training ($F(8,176)=27.02$, $p<0.01$), but no significant interaction ($F(8,176)=0.71$, n.s.) (**Fig 2B**). The ANOVA on the impact of training condition and week of training on

number of fecal bolus during training session showed no effect of training (F(1,22)=0.10, n.s.), week of training (F(8,176)=1.66, n.s.), or condition x week interaction (F(8,176)=1.10, n.s.) (**Fig 2C**). All these results indicate that this schedule of force training has no signifficant impact on amount of food consumed or excretion and body mass. In addition, forced training condition did not increased plasma corticosterone levels as showed by a t-test for independent samples (t=-0.10, n.s). Thus, there were no significant differences between control and forced animals after training on this neuroendocrine parameter of stress (**Fig 2D**).

Experiment 2: Effect of previous RW training on EPM performance.

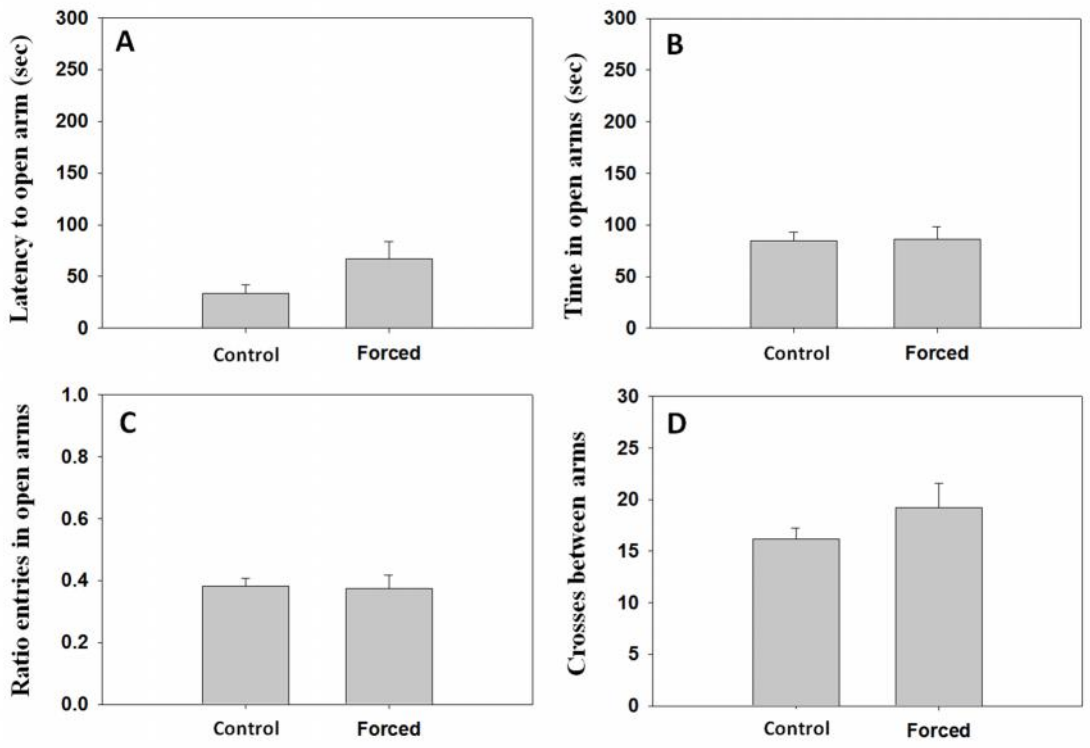


Figure 3. Anxiety measures in the elevated plus maze. Mean (±SEM) latency in seconds to enter an open arm for the first time (A), time spent in open arms in seconds (B), ratio entries into open arms compared to total entries in all arms (C), and total number of entries in the four arms as a measure of locomotion (D).

Training condition did not affect any of the parameters recorded in the EMP. Student's t-test for independent samples did not show significant differences between both training conditions in latency to enter into the open arm for the first time (t=-1.78, n.s.),

time in open arms (t=-0.10, n.s.), and ratio of entries in open arms (t=0.13, n.s.). Suggesting that animals trained in forced RW did not have an anxiogenic-like pattern of behavior in comparison with control group. There were not differences between both conditions on total crosses either (t=-1.21, n.s.).

Experiment 3: Exploratory locomotion in a novel OF.

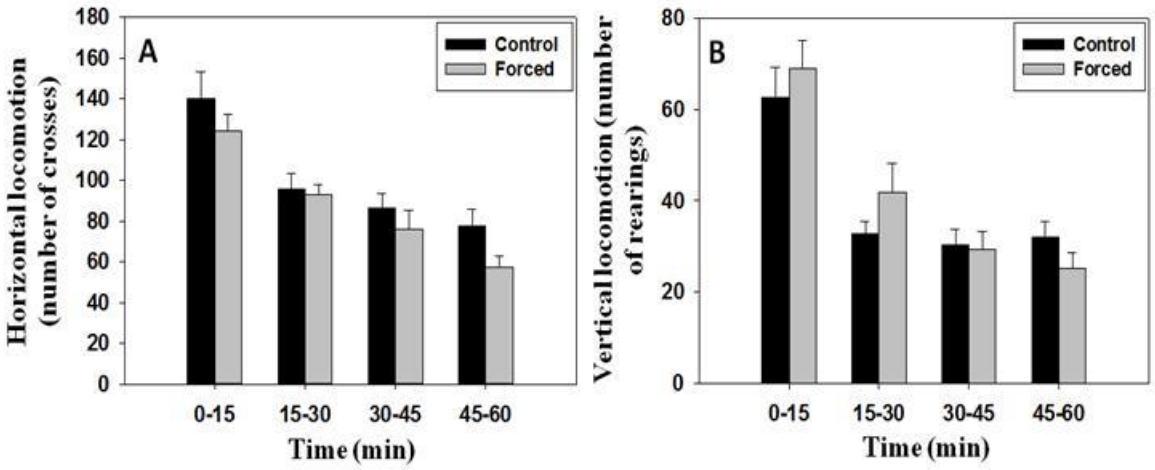


Figure 4. Exploration in novel open field. Mean (±SEM) of number of crosses between quadrants (A) and number of rearings (B).

Factorial ANOVA showed a significant effect of time in the OF on both groups of animals ($F(3,42)=29.0$, $p<0.01$). However, there was not a significant effect of training condition on horizontal locomotion $F(1,14)=0.07$, n.s.), and no significant interaction ($F(3,42)=1.00$, n.s) (**Fig 4A**). The same pattern of results was observed for vertical locomotion. Factorial ANOVA did not show a significant effect of training condition ($F(1,15)=0.44$, n.s.), there was a significant effect of time in the OF ($F3,42)=35.47$, $p<0.01$), but no significant effect of time x condition interaction ($F(3,42)=1.64$, n.s.).

Experiment 4: Effect of TBZ on 3-choice T-maze performance

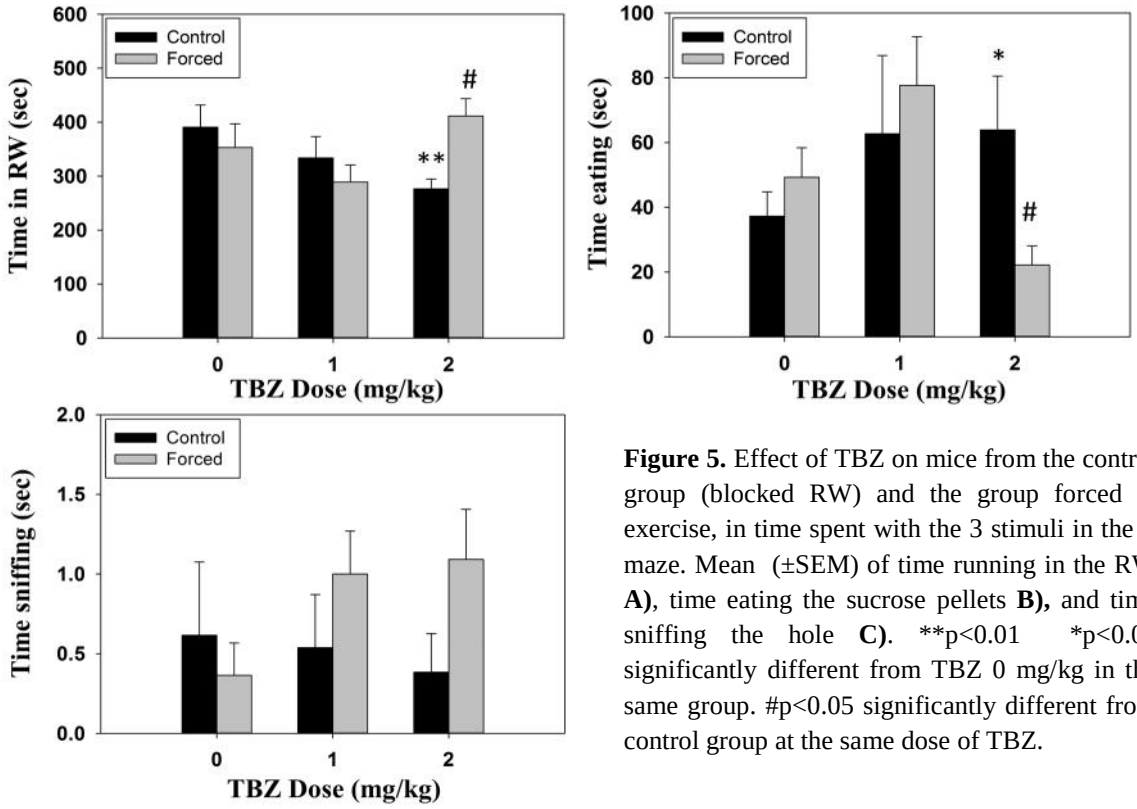


Figure 5. Effect of TBZ on mice from the control group (blocked RW) and the group forced to exercise, in time spent with the 3 stimuli in the T maze. Mean (±SEM) of time running in the RW **A)**, time eating the sucrose pellets **B),** and time sniffing the hole **C)**. **p<0.01 *p<0.05 significantly different from TBZ 0 mg/kg in the same group. #p<0.05 significantly different from control group at the same dose of TBZ.

Factorial ANOVA (condition x treatment) did not showed an effect of condition ($F(1,22)=0.20$, n.s) or dose ($F(2,44)=2.39$, n.s) on time spent running in the RW. However there was a significant effect of condition x treatment interaction ($F(2,44)=6.76$, $p<0.01$). Planned comparisons showed a significant decrease on time in the RW after TBZ administration at a dose of 2 mg/kg in the control group ($p<0.01$), but not in animals trained in the forced RW. On the other hand, the ANOVA for the sucrose consumption did not show a significant effect of condition ($F(1,22)=0.11$, n.s), treatment ($F(2,44)=2.76$, n.s), or interaction ($F(2,44)=2.88$, n.s) on time spent eating. However a tendency to increase time eating was observed in the control group. There was no significant effect of condition ($F(1,22)=1.49$, n.s), treatment ($F(2,44)=2.24$, n.s), and condition x treatment interaction $F(2,44)=0.64$, n.s) on the variable time sniffing the neutral odor.

Experiment 5: Effect of TBZ on DA tissue levels in Striatum and Anterior Cingulate.

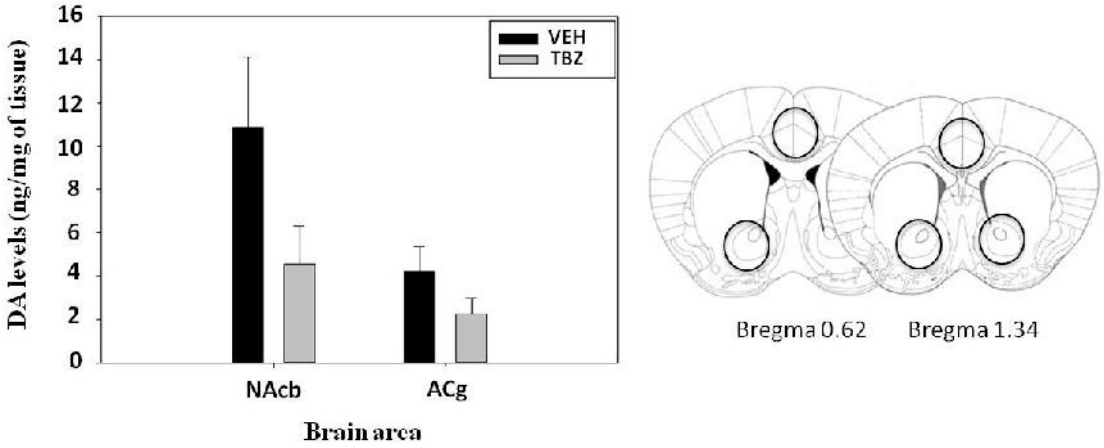


Figure 6. DA tissue levels in ventral striatum and in anterior cingulate cortex as measured by HPLC. Mean (±SEM) nanograms of DA per milligram of brain tissue.

Factorial ANOVA (brain region x treatment) showed an overall effect of brain region ($F(12,36)=1.18$, $p<0.05$), and treatment ($F(1,18)=4.38$, $p<0.05$) on DA levels, but no significant effect of brain region x treatment interaction ($F(1,18)=1.32$, n.s.).

Experiment 6: Effect of TBZ on pDARPP-32(Thr34) and pDARPP-32(Thr75) in mice after completion of RW training.

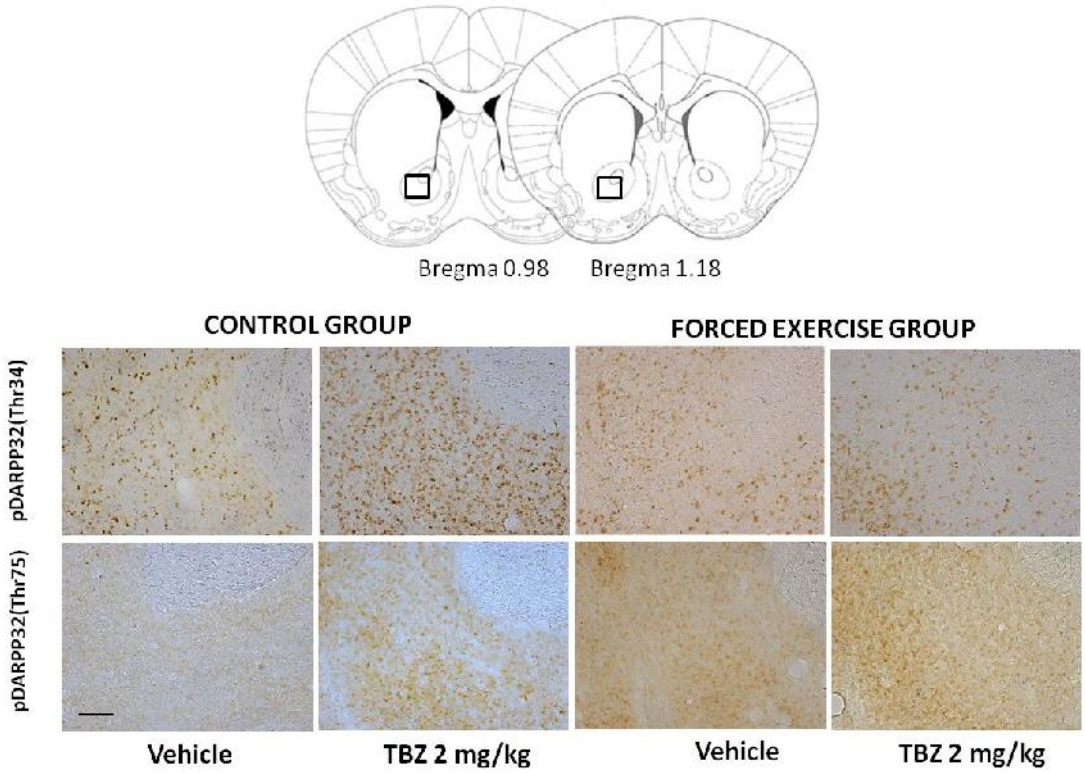


Figure 7. Upper part: Diagram of coronal sections with bregma coordinates (0.97 and 1.18) taken from Franklin and Paxinos (2007), showing location of the brain areas for pDARPP-32(Thr34) and pDARPP-32(Thr75) counting. Lower part: Photomicrographs of pDARPP-32(Thr34) and pDARPP-32(Thr75) immunoreactivity staining in nucleus Accumbens Core from representative animals. Images at 20x, scale bar = 100 μm.

Factorial ANOVA (treatment x condition) revealed a significant effect of treatment ($F(1,15)=4.25$, $p<0.05$), condition ($F(1,15)=11.35$, $p<0.01$) and treatment x condition interaction ($F(1,15)=4.61$, $p<0.05$) on DARPP32-thr34 levels in nAcb core. Planned comparisons analysis showed a significant increase on DARPP32-Thr34 after TBZ (2 mg/kg) administration in control group ($p<0.05$) but not in forced group.

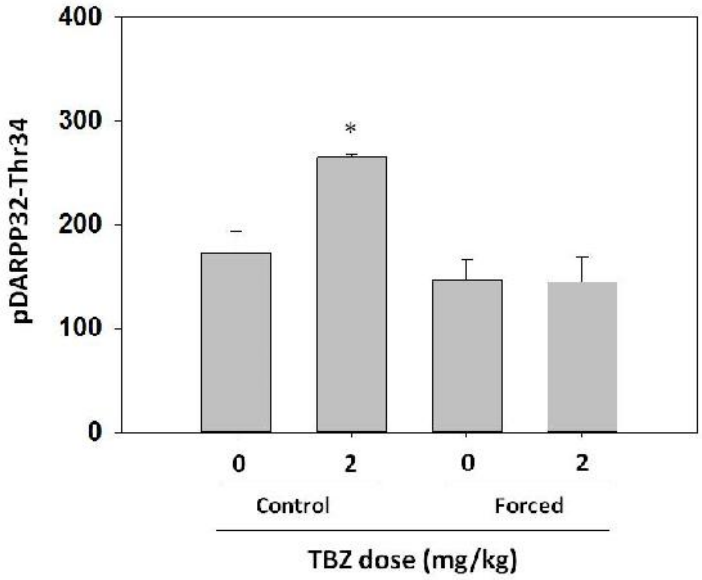


Figure 8. Effects of TBZ on pDARPP-32(Thr34) levels on nucleus Accumbens Core core in control and forced exercised animals. Mean (±SEM) of number of pDARPP-32(Thr34) staining in 300 μm^2 ROI. *p<0.05 significant differences from vehicle treatment in the same condition.

5. Discussion

TBZ produced a shift in the relative preference; it reduced the choice of the reinforcer that involved vigorous activity, but increased consumption of a reinforcer that required little effort (sucrose). In previous studies we have demonstrated in mice and rats (Nunes et al., 2013; López-Cruz et al., 2014), that none of these doses of TBZ (1 or 2 mg/kg) significantly reduced RW performance or free sucrose consumption when they were not presented concurrently. Thus, the possibility of choosing changed the impact of the drug. However, mice that were extensively exposed to exercise did not show TBZ-induced shifts in preference towards low-effort reinforcers such as sucrose or olfactory stimuli.

TBZ did not significantly decrease DA levels in striatum although a tendency was observed. However, it increased levels of the phosphorilated form of DARPP-32(Thr34) in control animals but not in animals previously trained in the forced RW.

These results suggest that exercise could act as a preventive therapy for the anergia-inducing effects of DA depletion. Thus, DA depletion produced effects indicative of anergia (lack of energy), but did not impair the primary effect of sucrose.

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