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MODULATORY EFFECTS OF ADENOSINE ON THE ACTIVATIONAL COMPONENT OF MOTIVATED BEHAVIOR REGULATED BY DOPAMINE: BEHAVIORAL AND IMMUNOHISTOCHEMICAL STUDIES

Doctoral Thesis

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ABSTRACT

For many years, it has been suggested that drugs that interfere with dopamine (DA) transmission alter the "rewarding" impact of primary reinforcers such as food. Research and theory related to the functions of mesolimbic DA are undergoing a substantial conceptual restructuring, with the traditional emphasis on hedonia and primary reward yielding to other concepts and lines of inquiry. The present research is focused upon the involvement of nucleus accumbens DA in effort-related choice behavior. Viewed from the framework of behavioral economics, the effects of accumbens DA depletions and antagonism on food-reinforced behavior are highly dependent upon the work requirements of the instrumental task, and DA depleted rats show a heightened sensitivity to response costs, especially ratio requirements. Moreover, interference with accumbens DA transmission exerts a powerful influence over effort-related choice behavior. Rats with accumbens DA depletions or antagonism reallocate their instrumental behavior away from food-reinforced tasks that have high response requirements, and show increased selection of low reinforcement/low cost options. Nucleus accumbens DA and adenosine interact in the regulation of effort-related functions, and other brain structures (anterior cingulate cortex, amygdala, ventral pallidum) also are involved. Studies of the brain systems regulating effort-based processes may have implications for understanding drug abuse, as well as symptoms such as psychomotor slowing, fatigue or anergia in depression and other neurological disorders.

RESUMEN

Durante muchos años, se ha sugerido que drogas que interfieren con la transmisión de dopamina (DA) estarían alterando el impacto "gratificante" de los reforzadores primarios como la comida. Investigación experimental y aproximaciones teóricas respecto al estudio de las funciones de la DA en la vía mesolímbica están dando lugar a una restructuración conceptual sustancial en este campo, de manera que el énfasis tradicional en la hedonia y en el reforzamiento primario han quedado apartados dado paso a una nueva conceptualización y renovadas líneas de investigación.

La presente investigación se centra en la implicación de la DA en el núcleo accumbens (Nacb) en tareas de elección basadas en el esfuerzo. Abordando esta cuestión desde el marco conceptual de la economía comportamental, los efectos de la depleción de DA en el Nacb y el antagonismo DAérgico sobre la conducta reforzada por comida dependen en gran medida de los requisitos de trabajo de la tarea. La depleción DAérgica en ratas ha demostrado causar una mayor sensibilidad a los costes de respuesta, en especial en tareas de elección con diferentes requerimientos de respuesta. Por otra parte, la alteración de la transmisión Daérgica en el Nacb ejerce una poderoso efecto sobre la conducta de elección basada en el esfuerzo. Las ratas con antagonismo o depleción DAérgica en el Nacb redirigen su conducta instrumental lejos de los alimentos percibidos como reforzantes pero que van asociados a las tareas que implican altos requerimientos de respuesta, mostrando mayor selección sobre opciones con menor requerimiento o coste de respuesta. Además de la implicación de otras estructuras cerebrales (corteza cingulada anterior, la amígdala, el área tegmental ventral) en estas funciones, la DA en el Nacb y el neuromodulador adenosina

interactúan en la regulación de las funciones relacionadas con el esfuerzo. Los estudios de los sistemas cerebrales que regulan los procesos de elección basado en el esfuerzo, pueden tener implicaciones para mejorar la comprensión del abuso de drogas, así como síntomas tales como retardo psicomotor, fatiga o anergia presentes en la depresión y otros trastornos neurológicos.

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GENERAL INTRODUCTION

In order to survive, organisms must gain access to significant stimuli such as food, water, sex and other conditions. The processes involved in such behavioral activities are varied and complex, and the brain mechanism related to these processes are a subject of considerable research activity. Instrumental learning processes involving reinforcement and punishment lead to the acquisition of behaviors that regulate the probability, proximity and availability of significant stimuli. But even when such responses are already acquired, multiple factors contribute to the selection of particular instrumental behaviors in a given environmental context. For example, in a complex environment, organisms typically have access to multiple reinforcers, which can vary in regards to their quality, quantity, and temporal characteristics. In addition, distinct instrumental actions can be associated with particular reinforcers, and these actions can vary widely in topography, and in terms of the quantitative features of the response requirements. Several areas of inquiry in behavioral science, including research on response-reinforcement matching, optimal foraging theory, and behavioral economics, have emerged in order to characterize the choice behavior observed in these complex environments (Baum, 1974; Allison, 1981, 1993; Salamone, 1987; Williams, 1988; Hursh et al., Tustin, 1995; 1988; Aparicio, 2001, 2007; Vuchinich and Heather, 2003; Hengeveld et al., 2009). This research has provided approaches for understanding how reinforcement value, as well as response requirements, influence the relative allocation of instrumental behavior across multiple options.

This perspectives article will provide an overview of recent research on the behavioral pharmacology of a specific aspect of these broader issues. One response-related factor that profoundly influences instrumental behavior is work-related response costs (Staddon 1979; Kaufman 1980; Kaufman et al. 1980; Salamone, 1986, 1987, 1992; Hursh et al., 1988; Foltin 1991; Tustin, 1995). The present review will focus upon the effects of drugs and neurochemical manipulations that affect dopamine (DA) transmission, and how these effects interact with the response requirements, particularly ratio requirements, imposed upon food-reinforced instrumental behavior. In addition, the article will review the literature on the role of DA in effort-related choice behavior, with a particular emphasis upon DA in a brain area known as the nucleus accumbens. Finally, the interactions between nucleus accumbens DA and other neurotransmitters and brain areas will be discussed, and the broader relevance of these findings will be considered.

HYPOTHESIZED ACTIONS OF DA ANTAGONISTS: THE "REWARD" HYPOTHESIS OF DA FUNCTION

There have been substantial theoretical developments in the last few years related to the hypothesized behavioral functions of DA, particularly nucleus accumbens DA. In order to consider the involvement of DA in work-related aspects of instrumental response allocation, one should place these ideas into a historical context relative to other hypothesized functions of DA. A few decades ago, it became common in the behavioral neuroscience literature to label DA as a "reward" transmitter, which was said to produce feelings of subjective pleasure or motivational appetites that mediate or drive positive reinforcement phenomena. However, it has become evident to many

investigators that there are conceptual limitations and empirical problems with the traditional DA hypothesis of "reward" (Salamone et al. 1997; 2005, 2007, 2009, 2010; Barbano & Cador 2007; Baldo & Kelley 2007), not the least of which is the use of the term "reward" itself (Cannon & Bseikri 2004; Salamone et al. 2005; Salamone 2006; Sanchis-Segura & Spanagel 2006; Yin et al. 2008). The term "reward" is rarely defined by researchers when they are using it to describe a behavioral process. Some use the term as though it were a synonym for "reinforcement", while others use it in reference to "appetite" or "primary motivation". Still others employ this term as a thinly veiled label for "pleasure". In many cases, the word "reward" seems to be used as a rather monolithic, all-encompassing term that refers globally to all aspects of reinforcement learning, motivation and emotion, whether conditioned or unconditioned. If used in this manner, the term reward is so broad as to be practically meaningless. It should be evident that it is difficult to test a hypothesis which maintains that a neurotransmitter mediates such an ill-defined set of functions. Thus, it has been suggested that it is advantageous to maintain the distinction between the terms reward and reinforcement; with this usage, reinforcement refers more directly to instrumental learning mechanisms (Wise 2004; Sanchis-Segura and Spanagel 2006), while reward tends to connote the primary motivational and emotional effects of reinforcing stimuli (Salamone & Correa, 2002; Salamone et al. 2005, 2007; Everitt & Robbins 2005).

In addition to these lexicographical and conceptual issues, there also is a substantial body of empirical evidence that has been accumulated in recent years, which fails to support the various forms of the DA hypothesis of "reward". One ironic observation is that the processes most directly linked to

the use of the term reward (i.e., subjective pleasure, primary motivation) are ones that have been demonstrated to be most problematic in terms of demonstrating the involvement of DA systems (Salamone et al. 2007). For example, the idea that nucleus accumbens DA mediates the subjectively reported pleasure associated with positive reinforcers has been strongly challenged (Salamone et al. 2007; Berridge 2007; Berridge & Kringlebach 2008). Interference with accumbens DA transmission does not impair appetitive taste reactivity for sucrose (Berridge 2007; Berridge & Kringlebach 2008), which is a frequently used behavioral marker of hedonic reactivity in rodents. Human studies have reported that DA antagonists failed to blunt the subjectively rated euphoria produced by drugs of abuse (Gawin 1986; Brauer & De Wit 1997; Haney et al. 2001; Nann-Vernotica et al. 2001; Wachtel et al. 2002). Moreover, the potential role of DA systems in instrumental behavior or learning is not limited to situations involving positive reinforcement. There is considerable evidence that striatal mechanisms in general, and nucleus accumbens DA in particular, also participate in aspects of aversive learning, punishment, and responsiveness to aversive stimuli (Salamone 1994; Munro & Kokkinidis 1997; Blazquez et al. 2002; Pezze & Feldon, 2004; Delgado et al. 2008; Faure et al. 2008; Martinez et al. 2008). Although human imaging studies often are used to support the idea that nucleus accumbens mediates subjective pleasure (e.g. Sarchiapone et al.2006; Wacker et al. 2009), this is grossly oversimplified; indeed, research employing various imaging methods has demonstrated that the human nucleus accumbens also responds to stress, aversion and hyperarousal/irritability (Liberzon et al. 1999; Pavic 2003; Jensen et al. 2003; Phan et al. 2004; Pruessner et al., 2004; Levita et al. 2009; Delgado et al., 2008,

2011). Neurochemical and physiological studies in animals clearly indicate that DA neuron activity is not simply tied to the delivery of primary positive reinforcers. Rather, DA neuron activity and DA release can be activated by a number of different aversive (e.g. footshock, tailshock, tail pinch, restraint stress, aversive conditioned stimuli, aversive drugs, social defeat stress) and appetitive conditions (McCullough & Salamone 1992; McCullough et al. 1993; Guarraci and Kapp 1999; Young, 2004; Marinelli et al. 2005; Anstrom & Woodward 2005; Broom & Yamamoto 2005; Schultz 2007a, 2007b; Brischoux et al. 2009). These neurochemical changes are seen across varying time horizons (including tonic, slow phasic and fast phasic changes; Salamone 1996; Roitman et al., 2004; Schultz 2007a, 2007b; Salamone et al. 2007; Hauber 2010; Segovia et al., 2011). Studies involving learning indicate that DA systems in general and nucleus accumbens in particular are not only involved in learning related to reinforcement (e.g. Wise, 2004), but also are involved in learning related to punishment (Shoenbaum and Setlow, 2003; Salamone et al., 2007). Thus, it has been suggested that the term "instrumental learning" would be more broadly applicable than "reinforcement learning" for describing the hypothesized role of DA in learning processes (Salamone et al., 2007).

If DA antagonism is actually interfering with the fundamental characteristics of reinforcing stimuli, this prompts one to inquire as to what those characteristics are. Of course, reinforcement refers to behavioral contingencies that act to strengthen a particular behavior; positive reinforcement refers to a process by which a response is followed by the presentation of stimulus that typically is contingent upon that response, and these events are followed by an increase in the probability of the occurrence of that response in the future.

However, it is worthwhile to consider what properties enable a stimulus to act as a reinforcer. As is often noted, Skinner did not often discuss the critical characteristics of stimuli that allow them to act as reinforcers. Nevertheless, Skinner did, on occasion, consider the role of motivational variables such as food deprivation in the process of reinforcement. For example, Skinner (1953, p 149) stated "Reinforcement thus brings behavior under the control of an appropriate deprivation. After we have conditioned a pigeon to stretch its neck by reinforcing with food, the variable which controls the neck-stretching is food deprivation." Many other investigators have offered their own perspectives on this issue, and it has been argued that there are some common characteristics that are evident across different researchers (Salamone & Correa, 2002). A large number of investigators who have written about the fundamental characteristics of reinforcing stimuli have arrived at the conclusion that stimuli that act as positive reinforcers tend to be relatively preferred, or to elicit approach behavior, and that these effects are a fundamental aspect of positive reinforcement. For example, Tapp (1969; p 173) stated "At the simplest level, reinforcers have the capacity to direct an organism's behavior. Those stimuli that are approached are regarded as positively reinforcing". Reinforcers have been described as a commodity that is in demand, or a stimulus that is being approached, self-administered, attained or preserved; they also have been described as activities that are preferred, deprived or in some way being regulated (Premack, 1959; Lea 1978; Hursh, 1988; Staddon & Ettinger, 1989; Timberlake, 1993; Dickenson and Balleine, 1994; Tustin, 1995; Salamone & Correa, 2002). According to the behavioral economic analysis offered by Hursh (1993; p 166) “responding is

regarded as a secondary dependent variable that is important because it is instrumental in controlling consumption".

For these reasons, it is important to note that low doses of DA antagonists that suppress food-reinforced instrumental behavior typically have been shown to leave behavior directed towards the acquisition and consumption of food (Salamone et al., 1991); these manipulations have little effect on food intake (Rolls et al., 1974; Fibiger et al., 1976; Salamone et al., 1991; Rusk & Cooper, 1994; Ikemoto & Panksepp, 1996), discrimination and preference based upon food reinforcement magnitude (Martin-Iversen et al., 1987; Salamone et al., 1994), and simple approach responses reinforced by food delivery (Ettenberg et al. 1981; Salamone 1986; Mekarski 1988). Although it is well known that whole forebrain DA depletions can produce aphagia (i.e., lack of eating), it is DA depletions in sensorimotor and motor-related areas of the lateral or ventrolateral caudate/putamen that have been most conclusively linked to this effect, rather than the nucleus accumbens (Ungerstedt 1971; Dunnett & Iversen 1982; Salamone et al. 1993a). In contrast, nucleus accumbens DA depletion and antagonism have been shown repeatedly not to substantially impair food intake (Ungerstedt 1971; Koob et al. 1978; Bakshi & Kelley 1991; Salamone et al. 1993a; Baldo et al., 2002; Kelley et al., 2005). Moreover, the effects of DA antagonists or accumbens DA depletions on food-reinforced instrumental behavior do not closely resemble the effects of pre-feeding or appetite suppressant drugs (Salamone et al., 1991, 2002; Aberman & Salamone 1999; Sink et al., 2008).

Although it has been suggested that the "reward-related" actions of low doses of DA antagonists or nucleus accumbens DA depletions should produce

effects that closely resemble extinction, this has not generally been observed in the literature (Faustman & Fowler, 1981, 1982; Gramling et al., 1984, 1987; Evenden & Robbins, 1983; Asin & Fibiger, 1984; Salamone 1986; Wirtschafter & Asin, 1985; Spivak and Amit, 1986; Willner et al., 1988; Feldon and Winer, 1991; Salamone et al. 1995, 1997; Rick et al. 2006). One example from this literature is Salamone (1986). Although 0.1 mg/kg of the DA antagonist haloperidol severely reduced responding on a fixed ratio (FR) 20 schedule of lever pressing, a dose 4 times that size had no effect on the reinforced response of simply being in proximity to the food dish on a fixed interval 30 sec schedule (Salamone 1986). The lack of effect of DA antagonism on this simple food-reinforced response stands in marked contrast to the effect of extinction, which substantially suppressed the instrumental response (see Figure 1). In this experiment (Salamone 1986), schedule-induced locomotor activity also was recorded in parallel with the instrumental response of being in proximity to the food dish. Despite the fact that 0.4 mg/kg haloperidol did not affect the reinforced response, it did suppress the motor activity induced by scheduled presentation of food. In combination with other studies, these results highlight several important features of the effects of DA antagonism. First, the effects of DA antagonism do not closely resemble the effects of extinction across a broad range of conditions (Salamone et al., 1997). Second, DA antagonism suppressed schedule-induced motor activity, which is consistent with other studies focusing on the effects of DA antagonism or accumbens DA depletions (Robbins & Koob, 1980; Robbins et al., 1983; Wallace et al., 1983; Salamone 1988; McCullough & Salamone, 1992; Robbins & Everitt, 2007). Finally, these results were consistent with the growing body of evidence indicating that the effects of

DA antagonists on instrumental behavior interact powerfully with the instrumental response requirement (Ettenberg et al., 1981; Mekarski, 1989).

THE EFFECTS OF DA ANTAGONISM AND ACCUMBENS DA DEPLETION INTERACT WITH THE INSTRUMENTAL RESPONSE REQUIREMENTS

In parallel with the historical developments described above, during the 1970s to the 1990s there was an emerging emphasis in the behavioral literature on effort, response costs or constraints, and economic models of operant behavior. Several investigators emphasized how response costs or constraints affected operant response output (Staddon 1979; Kaufman 1980; Kaufman et al. 1980; Foltin 1991; Tustin, 1995). Work requirements, such as the number of lever presses necessary for obtaining food, were shown to act as determinants of instrumental response output, and also to affect food consumption (Collier & Jennings, 1969; Johnson & Collier 1987). Behavioral economic models stress how a number of factors, including not only reinforcement value, but also conditions related to the characteristics of the instrumental response, can determine behavioral output (Lea, 1978; Allison, 1981, 1993; Bickel et al., 2000). Hursh et al. (1988) suggested that the price of food reinforcement as a commodity is a cost/benefit ratio expressed as the effort expended per unit of food value consumed.

Several lines of evidence have served to strengthen support for the hypothesis that the effects of interference with DA transmission interact powerfully with the instrumental response requirement. One of the ways of controlling work requirements in an operant schedule is to use various ratio

schedules. Caul & Brindle (2001) observed that the effects of the DA antagonist haloperidol on food-reinforced behavior were dependent upon the ratio requirement, with a FR1 schedule being less sensitive than a progressive ratio. One can deplete accumbens DA by local injections of a neurotoxic substance such as 6-hydroxydopamine, and several studies have used this approach. Aberman & Salamone (1999) employed a range of ratio schedules (FR1, 4, 16 and 64) to assess the effects of accumbens DA depletions. While FR1 performance was not affected by DA depletion (see also Ishiwari et al., 2004), and FR4 responding showed only a mild and transient suppression, the FR16 and FR64 schedules were much more impaired. This pattern indicated that accumbens DA depletions promoted the induction of ratio strain, i.e., rats with accumbens DA depletions were much more sensitive to the size of the ratio requirement. This pattern can be described as reflecting an increase in the elasticity of demand for food reinforcement (Salamone et al. 1997, 2009; Aberman & Salamone 1999). If the ratio requirement is analogous to the price of the commodity (reinforcement pellets), it appears that rats with accumbens DA depletions are more sensitive than control animals to the price of the food reinforcers. Needless to say, rats do not use currency to purchase operant pellets. Instead, it has been suggested that an operant procedure is more of a barter system, in which the rat trades its work (or reductions in leisure) for a commodity (Tustin, 1995; Rachlin 2003). Thus, rats with accumbens DA depletions are more sensitive than control animals to work-related response costs, and less likely to trade high levels of ratio output for food. In a subsequent experiment, Salamone et al., (2001) reported that increased sensitivity to larger ratio requirements in rats with accumbens DA depletions

were observed when rats were tested across a broader range of ratio schedules as high as FR300, even when the overall relation between lever pressing and food delivered per lever press was kept constant (i.e., an FR50 density of reinforcement, with 1 pellet every 50 responses; 2 pellets every 100 responses on the FR100; 4 pellets for every 200 responses on the FR200; and 6 pellets every 300 responses for the FR300). These results showed that both the magnitude and the organization of the ratio requirement appear to be critical determinants of the sensitivity of an operant schedule to the effects of accumbens DA depletions.

Additional experiments examined the effects of accumbens DA depletions on tandem schedules, in which a ratio requirement was attached to an interval requirement. This was done in order to ensure that the results by Aberman & Salamone (1999) and Salamone et al. (2001) reflected the influence of ratio size, as opposed to other variables such as time. Research employing tandem VI/FR schedules with varying combinations (e.g. VI 30 sec/FR5, VI 60 sec/FR10, VI 120 sec/FR10) has yielded a consistent pattern; accumbens DA depletions did not suppress overall response output in rats responding on the conventional VI schedules (i.e., those requiring only one response after the interval), but did substantially reduce responding on the corresponding VI schedule with the higher ratio requirement attached (Correa et al. 2002; Mingote et al. 2005). These findings are consistent with research showing that accumbens DA antagonism did not impair performance on a progressive interval task (Wakabayashi et al. 2004), and suggest that interval requirements *per se* do not pose a severe constraint to rats with compromised DA transmission in nucleus accumbens. These studies emphasize that, over and above any effect of

intermittence or time, ratio requirements provide a work-related challenge that is very disruptive to rats with accumbens DA depletions or antagonism.

Taking all these results together, nucleus accumbens DA depletions appear to have two major effects on ratio responding: 1) they reduce the response-enhancing effects that moderate-size ratio requirements have on operant responding (i.e., the ascending limb of the inverted-u-shaped function relating ratio requirement to response output), and 2) they enhance the response-suppressing effects that very large ratios have on operant respnding (i.e., the descending limb of the function; enhancement of ratio strain; Salamone & Correa 2002; Salamone et al., 2007, 2009). In addition, more molecular behavioral analyses indicate that accumbens DA depletions produce a slight reduction in the local rate of responding, as indicated by the distribution of interresponse times (Salamone et al. 1993b, 1999; Mingote et al. 2005), as well as an increase in pausing (Salamone et al. 1993b; Mingote et al. 2005; see also Nicola, 2010). Computational approaches have been used to characterize these effects of accumbens DA depletions on response rate on ratio schedules (e.g. Niv et al. 2007; Phillips et al. 2007). According to Phillips et al., (2007), DA release in nucleus accumbens appears to provide a window of opportunistic drive during which the threshold cost expenditure to obtain the reward is decreased (Phillips et al. 2007).

In the context of this discussion of the effects of dopaminergic drugs on ratio performance, it is useful to consider the term “reinforcement efficacy”, which is sometimes used to describe the effects of drug manipulations on ratio performance. With progressive ratio schedules, the ratio requirement increases as successive ratios are completed, and the “break point” is said to occur at the

point at which the animal stops responding. One can operationally define reinforcement efficacy in terms of the break point in a progressive ratio schedule, or by measuring ratio strain in rats responding across different FR schedules. The determination of reinforcement efficacy can be a very useful tool for characterizing the actions of drugs that are self-administered, and for comparing self-administration behavior across different substances or drug classes (e.g., Marinelli et al. 1998; Woolverton and Rinaldi, 2002; Morgan et al., 2002; Ward et al., 2005). Nevertheless, given the terminological difficulties discussed above, it is useful to stress that the term "reinforcement efficacy" should not be used simply as a replacement for "reward", and that progressive ratio breakpoints should not be viewed as necessarily providing some direct and unambiguous measure related to the subjective pleasure produced by the stimulus (Salamone, 2006; Salamone et al., 2009). Drug-induced changes in progressive ratio break points can reflect actions on several different behavioral and neurochemical processes (Arnold & Roberts, 1997; Hamill et al., 1998; Lack et al., 2008). For example, changing the response requirements by increasing the height of the lever decreased progressive ratio break points (Skjoldager et al., 1993; Schmelzeis & Mittleman 1996). Although some researchers have maintained that the break point provides a direct measure of the appetitive motivational characteristics of a stimulus, it is, as stated in a landmark review by Stewart (1974), more directly a measure of how much work the organism will do in order to obtain that stimulus. The animal is making a cost/benefit choice about whether or not to continue to respond, based partly on factors related to the reinforcer itself, but also upon the work-related response costs and time constraints imposed by the ratio schedule. For these reasons,

interpretations of the actions of drugs or lesions on progressive ratio break points should be done with caution, as should be the case for any individual task. A drug that alters the break point could do so for many different reasons. For example, recent studies have shown that the DA antagonist haloperidol can suppress food-reinforced progressive ratio responding, and lower break points, but nevertheless leave intact the consumption of a concurrently available but less preferred food source (Randall et al. 2011b; Pardo et al., 2011). These actions of haloperidol on this task differed markedly from those produced by pre-feeding and appetite suppressant drugs (Randall et al. 2011b; Pardo et al., 2011).

DA ANTAGONISM AND NUCLEUS ACCUMBENS DA DEPLETIONS AFFECT THE RELATIVE ALLOCATION OF INSTRUMENTAL RESPONDING IN EFFORT-RELATED CHOICE TASKS

As noted above, animals must make choices in complex environments that present multiple opportunities for obtaining significant stimuli, and several paths for accessing them (Williams, 1988; Aparicio, 2001, 2007). The variables that influence these choices are complex and multidimensional, and they include not only reinforcement value, but also response-related factors. Among the most important are those factors involving cost/benefit interactions based upon effort and reinforcement value (Neill & Justice, 1981; Hursh et al., 1988; Salamone & Correa 2002; Salamone et al. 2003, 2005, 2007; van den Bos et al. 2006; Walton et al. 2006; Salamone, 2010). Considerable evidence indicates that low systemic doses of DA antagonists, as well as local disruption of nucleus accumbens DA transmission, affect the relative allocation of behavior in animals responding on

tasks that assess effort-based choice behavior (Salamone et al. 2003, 2005, 2007; Floresco et al. 2008a,b; Hauber & Sommer 2009).

One task that has been used to assess the effects of dopaminergic manipulations on response allocation is a procedure that offers rats the option of lever pressing reinforced by delivery of a relatively preferred food (e.g. Bioserve pellets; usually obtained on a FR5 schedule), or approaching and consuming a less preferred food (lab chow) that is concurrently available in the chamber (Salamone et al., 1991). Trained rats under baseline or control conditions get most of their food by lever pressing, and consume only small quantities of chow. Low-to-moderate doses of DA antagonists, which block either D_1 or D_2 family receptor subtypes (cis-flupenthixol, haloperidol, raclopride, eticlopride, SCH 23390, SKF83566, ecopipam), produce a substantial alteration of response allocation in rats performing on this task; they decrease food-reinforced lever pressing but substantially increase intake of the concurrently available chow (Salamone et al. 1991, 1996, 2002; Cousins et al., 1994; Koch et al., 2000; Sink et al. 2008; Worden et al. 2009). The use of this task for assessing effort-related choice behavior has been validated in many ways. Doses of DA antagonists that produce the shift from lever pressing to chow intake do not affect total food intake or alter preference between these two specific foods in free-feeding choice tests (Salamone et al. 1991; Koch et al., 2000). In contrast, appetite suppressants from different classes, including amphetamine (Cousins et al. 1994), fenfluramine (Salamone et al., 2002) and cannabinoid CB1 antagonists (Sink et al. 2008), failed to increase chow intake at doses that suppressed lever pressing. Similarly, pre-feeding reduced both lever pressing and chow intake (Salamone et al. 1991). Furthermore, with higher ratio requirements (up to FR

20, or progressive ratios), animals that are not drug treated shift from lever pressing to chow intake

(Salamone et al. 1997; Randall et al., 2011b; Pardo et al., 2011), indicating that this task is sensitive to work load. These results indicate that interference with DA transmission does not simply reduce food intake, but instead acts to alter response allocation between alternative sources of food that can be obtained through different instrumental responses.

The shift from lever pressing to chow intake in rats performing on this task is associated with DA depletions in nucleus accumbens; decreases in lever pressing and increases in chow intake occur as a result of accumbens DA depletions, as well as local injections of D_1 or D_2 family antagonists into either the core or shell subregions of nucleus accumbens (Salamone et al. 1991; Cousins et al. 1993; Cousins & Salamone 1994; Sokolowski & Salamone 1998; Koch et al. 2000; Nowend et al., 2001; Farrar et al., 2010). Thus, although lever pressing is decreased by accumbens DA antagonism or depletions, these rats show a compensatory reallocation of behavior and select a new path to an alternative food source.

Salamone et al. (1994) also developed a T-maze procedure, in which the two choice arms of the maze lead to different reinforcement densities (e.g. 4 vs. 2 food pellets, or 4 vs. 0); under some conditions, a barrier can be placed in the arm with the higher density of food reinforcement to present an effort-related challenge. When the high density arm has the barrier in place, and the arm without the barrier contains fewer reinforcers, DA depletions or antagonism decrease choice for the high density arm, and increase selection of the low

density arm with no barrier (Salamone et al., 1994; Cousins et al., 1996; Denk et al. 2005; Mott et al., 2009; Pardo et al., 2012). Like the operant concurrent choice task, this T-maze task also has undergone considerable behavioral validation and evaluation (Salamone et al., 1994; Cousins et al., 1996; van den Bos et al., 2006; Pardo et al., 2012). For example, when there is no barrier in the maze, rodents overwhelmingly prefer the high reinforcement density arm, and neither haloperidol nor accumbens DA depletion alters their response choice (Salamone et al., 1994). When the arm with the barrier contained 4 pellets, but the other arm contained no pellets, rats with accumbens DA depletions still managed to choose the high density arm, climb the barrier, and consume the pellets (Cousins et al., 1996). In a recent T-maze study with mice, while haloperidol reduced choice of the arm with the barrier, this drug had no effect on choice when both arms had a barrier in place (Pardo et al., 2012). Thus, dopaminergic manipulations do not alter the preference for the high density of food reward over the low density, and did not affect discrimination, memory or instrumental learning processes related to arm preference. The results of the T-maze studies in rodents, together with the findings from the FR5/chow concurrent choice studies reviewed above, indicate that low doses of DA antagonists and accumbens DA depletions cause animals to reallocate their instrumental response selection based upon the response requirements of the task, and select lower cost alternatives for obtaining reinforcers (see reviews by Salamone et al., 2003, 2005, 2007; Floresco et al. 2008a).

Effort discounting procedures also have been employed to study the effects of dopaminergic manipulations. Floresco et al. (2008b) demonstrated that the DA antagonist haloperidol altered effort discounting even when the

effects of time delay were controlled for (Floresco et al. 2008b). Bardgett et al. (2009) recently developed a T-maze effort discounting task, in which the amount of food in the high density arm of the maze was diminished each trial on which the rats selected that arm (i.e., an "adjusting-amount" discounting variant of the T-maze procedures, which allows for the determination an indifference point for each rat). Effort discounting was altered by the D_1 family antagonist SCH23390 and the D_2 family antagonist haloperidol; these drugs made it more likely that rats would choose the low reinforcement/low cost arm. Increasing DA transmission by administration of amphetamine blocked the effects of SCH23390 and haloperidol, and also biased rats towards choosing the high reinforcement/high cost arm, which is consistent with operant choice studies using DA transporter knockdown mice (Cagniard et al., 2006). Together with other results, the findings reported by Bardgett et al. (2009) and Floresco et al. (2008b) support the suggestion that, across a variety of conditions, DA transmission exerts a bidirectional influence over effort-related choice behavior.

DA INTERACTS WITH OTHER TRANSMITTERS TO INFLUENCE EFFORT-RELATED CHOICE BEHAVIOR

As reviewed above, DA antagonists and accumbens DA depletions affect instrumental response output, response allocation, and effort-related choice behavior. Obviously, no single brain area or neurotransmitter participates in a behavioral process in isolation to other structures or chemicals; for that reason it is important to review how other brain areas and neurotransmitters interact with dopaminergic mechanisms. Over the last several years, several laboratories have begun to characterize the role that multiple brain structures (e.g. amygdala, anterior cingulate cortex, ventral pallidum) and neurotransmitters (adenosine,

GABA) play in effort-related choice behavior (Walton et al., 2002, 2003; Denk et al., 2005; Schweimer and Hauber, 2006; van den Bos et al. 2006; Floresco and Ghods-Sharifi 2007; Floresco et al., 2008a; Hauber & Sommer 2009; Farrar et al., 2008; Mott et al. 2009; Pardo et al., 2012).

Within the last few years, considerable emphasis has been placed upon DA/adenosine interactions. Caffeine and other methylxanthines, which are nonselective adenosine antagonists, act as minor stimulants (Ferré et al. 2008; Randall et al., 2011a). DA-rich brain areas, including the neostriatum and the nucleus accumbens, have a very high degree of adenosine A_{2A} receptor expression (Schiffmann et al. 1991; DeMet and Chicz-DeMet, 2002; Ferré et al. 2004). There is considerable evidence of cellular interactions between DA D_2 and adenosine A_{2A} receptors (Fink et al. 1992; Ferré 1997; Hillion et al. 2002; Fuxe et al. 2003). This interaction frequently has been studied in regard to neostriatal motor functions related to Parkinsonism (Ferré et al. 1997, 2001; Hauber & Munkel 1997; Svenningsson 1999; Hauber et al. 2001; Wardas et al. 2001; Morelli and Pinna 2002; Correa et al. 2004; Pinna et al. 2005; Ishiwari et al. 2007; Salamone et al. 2008a, 2008b). However, several reports also have characterized aspects of adenosine A_{2A} receptor function related to learning (Takahashi et al. 2008), anxiety (Correa and Font 2008), and instrumental responding (Font et al., 2008; Mingote et al. 2008).

Drugs that act upon adenosine A_{2A} receptors profoundly affect instrumental response output and effort-related choice behavior (Farrar et al. 2007, 2010; Mingote et al. 2008; Font et al. 2008; Worden et al. 2009; Mott et al. 2009; Pardo et al. 2012). Intra-accumbens injections of the adenosine A_{2A} agonist CGS 21680 reduced responding on a variable interval 60 sec schedule

with a FR10 requirement attached, but did not impair performance on a conventional variable interval 60 sec schedule (Mingote et al. 2008), a pattern similar to that previously shown with accumbens DA depletions (Mingote et al. 2005). In rats responding on the FR5/chow feeding concurrent choice procedure, injections of CGS 21680 into the accumbens decreased lever pressing and increased chow intake (Font et al. 2008). This effect was site specific, because injections of CGS 21680 into a control site dorsal to the accumbens had no effect.

It also has been demonstrated that adenosine A_{2A} receptor antagonists can reverse the effects of systemically administered DA D_2 antagonists in rats tested on the FR5/chow feeding concurrent choice task (Farrar et al. 2007; Worden et al. 2009; Salamone et al. 2009; Nunes et al., 2010). Moreover, systemic or intra-accumbens injections of the adenosine A_{2A} antagonist MSX-3 were able to block the effects of intra-accumbens injections of the D_2 antagonist eticlopride in rats responding on the FR5/chow concurrent choice task (Farrar et al., 2010). In studies using the T-maze barrier procedure, adenosine A_{2A} antagonists have been shown to reverse the effects of DA D_2 antagonism in rats (Mott et al., 2009) and mice (Pardo et al., 2012). Furthermore, adenosine A_{2A} receptor knockout mice are resistant to the effects of haloperidol on selection of the high reinforcement/high cost arm of the T-maze (Pardo et al., 2012).

The pattern of effects seen in these studies depends upon which specific receptor subtypes are being acted upon by the drugs being administered. Although the adenosine A_{2A} receptor antagonists MSX-3 and KW 6002 reliably and substantially attenuate the effects of D_2 antagonists such as haloperidol and eticlopride in rats responding on the FR5/chow concurrent choice procedure

(Farrar et al. 2007; Worden et al. 2009; Salamone et al. 2009; Nunes et al., 2010), they produce only a mild reversal of the effects of the D_1 antagonist ecopipam (SCH 39166; Worden et al. 2009; Nunes et al., 2010). In addition, the highly selective adenosine A_1 receptor antagonist was completely ineffective at reversing the effects of DA D_1 or D_2 antagonism (Salamone et al. 2009; Nunes et al., 2010). Similar results were obtained with rats and mice responding on the T-maze barrier choice task; while MSX-3 was able to reverse the effect of the D_2 antagonist haloperidol on selection of the high reinforcement/high cost arm, the A_1 antagonists DPCPX and CPT were not (Mott et al. 2009; Pardo et al., 2012). These results indicate that there is a relatively selective interaction between drugs that act upon DA D_2 and adenosine A_{2A} receptor subtypes. Based upon anatomical studies, it appears that this is likely to be due to the pattern of cellular localization of adenosine A_1 and A_{2A} receptors in striatal areas, including the nucleus accumbens (Fink et al. 1992; Ferré 1997; Svenningsson et al. 1999; Hillion et al. 2002; Fuxe et al. 2003). Adenosine A_{2A} receptors are typically co-localized on striatal and accumbens enkephalin-positive medium spiny neurons with DA D_2 family receptors, and both receptors converge onto the same intracellular signaling pathways. Thus, adenosine A_{2A} receptor antagonists may be so effective in reversing the actions of D_2 antagonists because of direct interactions between DA D_2 and adenosine A_{2A} receptors located on the same neurons (Farrar et al., 2010; Salamone et al., 2009, 2010).

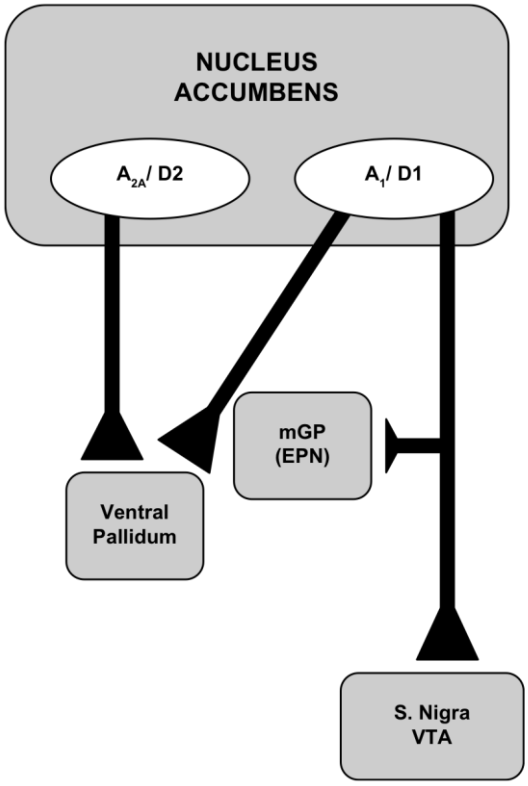

FIGURE 2 | Anatomical diagram depicting the pattern of DA and adenosine receptor localization in nucleus accumbens. See text for details (see also Ferré, 1997; Hillion et al., 2002; Fuxe et al., 2003). mGP, medial globus pallidus; epn, entopeduncular nucleus; s. nigra, substantia nigra; VTA, ventral tegmental area.

SUMMARY AND CONCLUSIONS: IMPLICATIONS FOR BEHAVIOR ANALYSIS AND PSYCHOPATHOLOGY

One possible contribution that the fields of psychopharmacology and behavioral neuroscience can make to behavioral analysis and theory is to use manipulations, such as drugs and lesions, in order to dissociate complex behavioral processes into components (Salamone et al. 2007, 2009). A measure derived from observations of behavior, or a parameter that is generated from curve-fitting analyses has many factors that contribute to it, and pharmacological research often can dissociate between these factors, because a drug can severely

affect one while leaving another basically intact. A useful example of this principle is the progressive ratio break point, which, as discussed above, is influenced by several factors (Randall et al., 2011b; Pardo et al., 2011). Another case in which this point is highly relevant is the measurement of intracranial self-stimulation thresholds. Such measures often are viewed as providing "rate-free" indices of "reward", or even "hedonia", nevertheless, they are influenced by lever pressing ratio requirements as well as the electrical current level (Fouriezos et al. 1990). Recent studies with intracranial self-stimulation thresholds indicate that dopaminergic modulation of self-stimulation thresholds does not affect reward value per se, but instead alters the tendency to pay response costs (Hernandez et al. 2010). Response-reinforcement matching also has been used in some research related to behavioral economics, reinforcer value, and the functions of DA systems (e.g. Heyman & Monaghan 1987; Aparicio 2007). Matching equations have been employed to describe the results of studies with both conventional and concurrent VI schedules, and one of the parameters (R_e) can be used to represent reinforcement value (e.g., Herrnstein 1974; see equation below for single-lever conventional VI schedules, in which B represents response rate, R represents reinforcement density, *k* is the constant for maximal responding, and Re represents the reinforcement level that generates 50% of maximum responding; $B = k\, R/(R + R_e)$). However, used in this way, R_e does not selectively represent the reinforcement value of food *per se*; actually, it reflects the relative value of entire activity of lever pressing for and consuming the food reinforcer compared to the reinforcing value of all other stimuli and responses available (Williams, 1988; Salamone et al. 1997, 2009). Several factors can contribute to this composite measure, which is one of the reasons

why other matching equations have been developed that account for deviations from matching by allowing for estimates of reinforcer sensitivity, as well as response preference or bias (Baum 1974; Williams 1988; Aparicio 2001). Clearly, a drug or lesion manipulation could yield apparent effects on "reinforcement value" that actually reflect changes in response-related factors (Salamone 1987; Salamone et al. 1997, 2009).

In view of these points, it is useful to consider how terms such as value are used in behavioral economics and neuroeconomics research. The aggregate reinforcement value of an instrumental activity (e.g. lever pressing for and consuming food) should probably be viewed as a composite measure that includes both the reinforcing value of the reinforcer itself, and also any net value or costs associated with the instrumental response that is required to obtain the reinforcer. Viewed in this manner, the effects of DA antagonists or depletions on effort-related choice behavior could be described in terms of actions upon the response costs associated with the particular instrumental response, rather than the reinforcing value of the food stimulus itself. Although the effects of haloperidol on bias may be minimal when two levers that are relatively similar are used (e.g. Aparicio 2007), they may be much larger when substantially different responses are compared (e.g. lever pressing vs. nose poking or sniffing; lever pressing vs. unrestricted access to food; barrier climbing vs. locomotion to a location containing food).

In addition to providing insights into aspects of instrumental behavior seen in the laboratory, research on effort-related choice behavior also has clinical implications. Addiction is characterized by a re-organization of the preference structure of the person, and also by a dramatic change in the

allocation of behavioral resources towards the addictive substance. Typically, there is a heightened tendency to engage in drug-reinforced instrumental behavior, and drug consumption, often at the expense of other behavioral activities. Thus, drug –reinforced instrumental behavior in humans involves many processes, including exertion of effort. Addicts will go to great lengths to obtain their preferred drug, overcoming numerous obstacles and constraints.

As well as being related to aspects of drug taking and addiction, research on effort-related choice behavior has implications for understanding the neural basis of psychiatric symptoms such as psychomotor slowing, anergia, fatigue and apathy, which are seen in depression as well as other psychiatric or neurological conditions (Salamone et al. 2006, 2007). These symptoms, which can have devastating behavioral manifestations (Stahl 2002; Demyttenaere et al. 2005), essentially represent impairments in aspects of instrumental behavior, exertion of effort and effort-related choice, which can lead to difficulties in the workplace, as well as limitations in terms of life function, interaction with the environment, and responsiveness to treatment. There is considerable overlap between the neural circuitry involved in effort-related functions in animals and the brain systems that have been implicated in psychomotor slowing and anergia in depression (Salamone et al. 2006, 2007, 2009, 2010). Thus, research on effort-related behavioral processes, and their neural regulation, could have substantial impact on clinical research related to addiction, depression, and other disorders.

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GENERAL HYPOTHESIS

DA is implicated in effort-related processes, and is essential for the activational aspect of motivation. We hypothesize that DA antagonism or DA depletion would affect effort based decision making to obtain a reward, as well as the activational effects of conditioned stimuli associated to a reinforcer while, leaving intact the consummatory or directional aspect of motivation, such as the intake of different reinforcers with no effort demands.

Furthermore, adenosine has been described as a new target for the treatment of the symptoms seen on these processes due to its co-localization with DA. We hypothesize that antagonists acting on adenosine receptors would be able to attenuate DA antagonist effects on behavioral activation, and effort based decision making. More specifically, A_{2A} receptor antagonism would reverse the effects of DA D_2 receptor antagonism.

Lastly, as Nacb has been described as an important brain area directly related to the regulation of some aspects of motivation and motor control, we hypothesized that DA antagonists would increase the signal of different cellular markers on this area and, adenosine antagonists would be able to attenuate or reverse those effects.

SPECIFIC HYPOTHESIS:

The **specific hypothesis** for each group of experiments in the present work, were the following:

Chapter 1: Selection of sucrose concentration after DA depletion and selective DA antagonists depends on the effort required by the instrumental response: studies using tetrabenazine and D_1, D_2 and D_3 antagonists.

- DA antagonists impair effort expenditure depending on the particular subtype of DA that is antagonized. Lever pressing for 5% sucrose is expected to be decreased after DA antagonism, producing a shift towards the intake of free 0.3% sucrose. However, no effect of DA antagonism is expected if no effort demand is required to obtain sucrose.

- It is expected that DA antagonism does not resemble the effect of pre-exposing animals to both sucrose solutions.

- It is expected that non selective adenosine antagonists reverse DA antagonism effects on behavior due to DA/adenosine receptor interactions.

- On behavioral activation, it is expected that DA antagonists decrease horizontal and vertical locomotion. Furthermore, non selective adenosine antagonists are expected to increase locomotion due to their psychostimulant properties.

Chapter 2: Individual differences in work output relate to DA dependent signal transduction mechanisms in Nacb: Studies of DA D_2 antagonism, adenosine A_{2A} antagonism, cannabinoid CB1 antagonism and pre-feeding on effort-related choice behavior as assessed by a progressive ratio/chow feeding choice task.

- It is expected that under a novel variant procedure that utilizes a progressive ratio (PROG15)/chow feeding task, normal animals will press the lever to obtain the more preferred pellets, to a certain break point.

- Haloperidol would affect PROG responding in a manner that was not dependent upon decreases in primary food motivation or appetite, and thus would decrease PROG lever pressing but leave chow intake intact.

- MSX-3, an A_{2A} adenosine antagonist, would produce a behavioral effect that is generally opposite to that produced by haloperidol; it is expected to increase lever presses and highest ratio achieved, decreasing chow consumption.

- DA antagonism is not expected to resemble effects of the appetite suppressants and the effects of the reinforcer devaluation provided by pre-feeding. Due to the putative appetite suppressant effects of interfering with cannabinoid CB1 receptor transmission, it was expected that AM251, as well as pre-feeding, would decrease both lever pressing and chow consumption.

- Due to the increasing work requirements and individual differences on this tasks, DARPP-32 immunoreactivity in Nacb would be greater in animals with high baseline levels of lever pressing (i.e., "high responders") than in rats with low levels of lever pressing.

Chapter 3: Effect of subtype-selective adenosine receptor antagonists on basal or haloperidol-regulated striatal function: studies of c-Fos expression and motor activities in outbred and A_{2A}R KO mice.

- D2 antagonist haloperidol is expected to decrease locomotion.

- Adenosine antagonists are expected to attenuate the effects of DA antagonism. Theophylline, a non selective adenosine antagonist, is expected to reverse haloperidol effects due to its action on A_{2A} receptors. CPT, a selective A_1 antagonist, is not expected to attenuate D_2 antagonism effects while A_{2A} selective antagonism, using MSX-3, is expected to reverse it.

- KO mice for the A_{2A} receptor are expected to be resistant to haloperidol effects.

- On c-Fos expression, A_{2A} receptor antagonists are expected to reverse c-Fos induction after D_2 antagonism.

Chapter 4: Adenosine A_{2A} receptor antagonism and genetic deletion attenuate the effects of dopamine D_2 antagonism on effort-based decision making in mice: Studies using a T-maze with barrier.

- The D_2 antagonist haloperidol is expected to produce a shift on the behavior towards the option with lower effort demands.

- Haloperidol effects are expected to be different compared devaluation of the reinforcer by pre-feeding. Satiated animals are expected to decrease the number of HD arm selection, increasing the number of omissions.

-Adenosine antagonists are expected to reverse DA antagonism depending on the adenosine receptor. Theophylline is expected to reverse haloperidol effects due to its action on A_{2A} receptors, increasing HD arm selections. CPT, a selective A_1 antagonist, is not expected to attenuate D_2 antagonism, while A_{2A} selective antagonism, using MSX-3, is expected to redirect the behavior towards the HD arm.

- A_{2A} receptor KO mice are expected to be resistant to D_2 antagonist haloperidol effect on this task.

-On c-Fos expression, the same pattern of adenosine-Da interactions are expected; theophylline and MSX-3 are expected to decrease c-Fos induction after D_2 antagonism.

Chapter 5: Dopamine D_2 receptor antagonism modulates the preference for primary reinforcers based on their effort requirements: studies using running wheels and sucrose consumption in mice.

- D_2 antagonism at medium doses is expected to decrease counts in the running wheel.

- In a maze where two reinforcers with different effort demands attached are present, control animals are expected to spend more time running on the wheel, exerting an effort instead of consuming sucrose.

- Haloperidol, a D_2 selective antagonist, is expected to produce a shift on behavior towards the less demanding reinforcer option. Animals are expected to increase their interaction with the reinforcer that implies lower effort demands.

- Haloperidol effects are expected not to be similar to the devaluation of the reinforcer, differing then from the condition of pre-exposure.

Chapter 6: Impact of DA D_2 receptor antagonism on the activational effects produced by olfactory conditioned stimuli associated to voluntary sucrose consumption.

- Neutral stimuli such as an odor, are expected to acquire similar activational properties after been pairing with a natural reinforcer such as 10% sucrose solution.

- The D_2 antagonist, haloperidol, is expected not to have an effect on direct sucrose intake, but is expected to dose dependently affect locomotion.

- Conditioned stimuli associated to sucrose are expected to enhance locomotion on a novel environment that does not have the unconditioned stimuli present, effect that is expected to be blocked by low doses of haloperidol.

CHAPTER 1:

SELECTION OF SUCROSE CONCENTRATION AFTER DOPAMINE DEPLETION AND SELECTIVE DOPAMINE RECEPTOR ANTAGONISTS DEPENDS ON THE EFFORT REQUIRED BY THE INSTRUMENTAL RESPONSE: STUDIES USING TETRABENAZINE AND D_1, D_2 AND D_3 ANTAGONISTS.

Abstract

Mesolimbic dopamine (DA) is involved in behavioral activation and effort-related processes. Rats with impaired DA transmission reallocate their instrumental behavior away from food-reinforced tasks with high response requirements, and instead select less effortful food-seeking behaviors. The present experiments were undertaken to study the impact of DA depletion and the individual contribution of different DA receptors on effort-based decision making. Because studies in the literature argued for a reduction in the hedonic value of sucrose after DA antagonism, the present experiments study the impact of DA antagonism on the activational and directional components of motivated behaviors using sucrose as the reinforcer. The present work systematically compared the effect of DA depletion with tetrabenozine, as well as DA D_1, D_2 and D_3 antagonists on a concurrent lever pressing/drinking choice task and on a free choice-non operant task. In the operant procedure, rats can choose between responding on a fixed ratio (FR) 7 lever-pressing schedule for a high sucrose concentration (i.e., 5%) vs. approaching and consuming a less concentrated one

(0.3%). DA depletion produced by tetrabenazine (0.75 and 1.0 mg/kg) decreased lever pressing for 5% sucrose but increased free 0.3% sucrose intake, thus inducing a shift in the choice towards a less effortful behavior. The D_1 antagonist ecopipam (0.2 mg/kg IP) and the D_2 antagonist haloperidol (0.1 mg/kg IP) altered choice behavior, reducing lever pressing and increasing free sucrose intake. D_3 antagonism did not produce any effect. The same pharmacological manipulations in rats choosing between these two solutions under free access conditions do not change preference for the high sucrose concentration and do not reduce total sucrose intake. Functional interaction between adenosine and DA receptors in the regulation of effort-based decision making was studied because they play an integral part in the regulation of striatal areas, including Nacb. Co-administration of the adenosine non-selective receptor antagonist theophylline (20 mg/kg IP) reversed the effects of the D_2 antagonist but not the D_1 antagonist. Caffeine was not able to reverse D_1 antagonism either. These results may have implications for understanding phenomena related to motivation and energy-related disorders such as psychomotor slowing or anergia in depression.

INTRODUCTION

Organisms are capable of making vigorous instrumental responses in order to gain access to significant stimuli. The behavior of animals can reflect a selection process, in which the value of a stimulus (e.g. taste of a food) relative to the cost of obtaining it (e.g. nature of the instrumental response) is an important determinant of behavioral output. According to that, it has been accepted the differentiation of at least two major functions in motivated behaviors; the directional aspect that guides behavior to specific ends and the activational aspect making emphasis in the vigor or persistence to obtain it (Cofer and Appley, 1964; Duffy, 1963; Salamone, 1988, 1991, 1992; Salamone et al., 1997).

There is increasing evidence showing that interference with dopamine (DA) transmission alters some of these aspects of motivated behavior leaving others unaffected (Ungerstedt 1971; Rolls et al., 1974, Fibiger et al., 1976; Koob et al. 1978; Salamone et al., 1991, 1993, Baldo et al. 2002). Several lines of evidence implicate DA, particularly in nucleus accumbens (Nacb), as a critical component of the brain circuitry regulating behavioral activation and effort-related processes (Salamone et al., 1991, 2003, 2005, 2007; Vezina et al., 2002; Zhang et al., 2003; Wakabayashi et al., 2004; Barbano and Cador, 2006, 2007; Cagniard et al., 2006; Phillips et al., 2007; Floresco et al., 2008; Salamone, 2010). Thus, in operant tasks, under control conditions when animals need to press a lever to obtain preferred food there is release and metabolism of DA in Nacb and striatum (Church et al. 1987; Salamone et al. 1989; McCullough et al. 1993; Segovia et al., 2011). On the contrary, rats with Nacb DA depletions or DA receptor blockade reduce lever pressing for food and show alterations in

response allocation on tasks that measure effort-related choice behavior (Salamone et al. 1991, 1997, 2003, 2005, 2006, 2007).

Studies of effort-related choice behavior typically offer animals alternative paths to obtain reinforcement, which involve cost/benefit trade-offs related to the work requirements for obtaining the reinforcer. The concurrent lever-pressing/chow-feeding procedure has been widely used for the study of DA implication in effort-based decision-making (Salamone et al., 1991). Under a fixed-ratio 5 (FR5) schedule, animals learn how to press the lever to obtain high amounts of preferred food instead of consuming the no-cost available lab chow (Salamone et al., 1991). Previous work has shown that, lesions of Nacb by 6-hydroxidopamine (6-OHDA), peripherally or intra-Nacb non selective DA receptor antagonists, as well as D_1 and D_2 -family receptor antagonists (Cousins et al. 1994; Nowend et al. 2001; Sink et al., 2008; Worden et al., 2009; Nunes et al., 2010; Salamone et al. 1991, 1996, 2002; 2009; Cousins et al. 1994; Koch et al. 2000), decrease lever pressing and increase chow consumption on the food concurrent choice task. However, by reducing food motivation after prefeeding the animals or administering appetite suppressant drugs a different pattern of response was seen; lever pressing and chow intake were both suppressed (Salamone et al., 1991).

Natural palatable rewards such as food and fluids as well as drugs of abuse have been used to study the DArgic response in the Nacb (Westerink et al., 1997; Roitman et al., 2004**).** Many studies have shown that sweet taste stimulation can act as a potent natural reward (Levine et al., 2003; Yamamoto, 2003). Thus, by using fluids containing different sucrose or saccharine concentrations researchers have assessed the role of DA in motivational and

emotional processes (Ikemoto and Panksepp, 1996; Treit and Berridge, 1990; Cannon and Palmiter, 2003; Cannon and Bseikri, 2004). Taste reactivity after oral administration of sucrose is a widely used measure of the emotional reactions to sucrose (Treit and Berridge, 1990; Peciña et al., 1997; see also Berridge and Robinson, 1998), and numerous studies have demonstrated that Nacb DA is not involved in the regulation of these emotional reactions (Ikemoto and Panksepp, 1996; Baldo et al., 2002; Treit and Berridge, 1990; Cannon and Palmiter, 2003; Cannon and Bseikri, 2004; Martinez-Hernandez et al., 2012). The directional or consummatory component in motivated behaviors also involves emotional aspects, but the nature of the measurements in these tasks is not pure measures of emotion. Thus, behavioral variables such as preference between solutions and amount of sucrose consumed can indicate if animals are oriented towards the reinforcer, but also can be influenced by the instrumental task that is required. This particular aspect of motivation has demonstrated not to be dependent on Nacb DA, but is influenced by manipulations of other neural systems (such as GABA/benzodiazepine systems in the brainstem, ventral pallidal systems where lesions produce aversion, or opioid systems in the Nacb shell (Berridge, 1996; Berridge and Peciña, 1995; Cromwell and Berridge, 1994; Peciña and Berridge, 1996a,b).

Although mesolimbic DA is a critical component of the brain circuitry regulating effort-related choice behavior (Salamone and Correa 2002; Salamone et al. 2003, 2005, 2006), other transmitters also are involved (Salamone et al. 2007; Farrar et al. 2008; Hauber and Sommer, 2009). The purine nucleoside adenosine has been involved in this type of function (Salamone and Correa 2009; Mingote et al., 2008; Font et al., 2008). Striatal areas, including

neostriatum as well as Nacb, have a high concentration of adenosine A_{2A} receptors (Jarvis and Williams 1989; Schiffmann et al. 1991; DeMet and Chicz-DeMet 2002; Ferré et al. 2004), and there is a functional interaction between DA D_2 and adenosine A_{2A} receptors, which are co-localized on enkephalin-containing medium spiny neurons (Fink et al. 1992; Ferré 1997; Ferré et al., 1997, 2008b; Hillion et al. 2002; Fuxe et al. 2003). Furthermore, adenosine A_1 and DA D_1 receptors tend to be co-localized on the same substance P- containing striatal neurons (Ferré 1997, 2008; Ferré et al. 1997, 2005). Thus, non receptor selective adenosine antagonists such as caffeine and theophylline can act on both subpopulations of neurons (Armentero et al., 2011).

The present work was undertaken to examine the role of DA in effort-related choice behavior using an adaptation of the concurrent lever pressing/ chow food procedure originally developed by Salamone et al., (1991). Most of the previous research has been focused on food as the reinforcer. However, in the present set of experiments we evaluated selection of palatable fluid concentrations of sucrose (5% versus 0.3% w/v). In the present conditions, animals need to press the lever under a FR7 schedule to have access to the 5% sucrose solution while having free access to the 0.3% sucrose solution during the session. The same sucrose concentrations were also used in parallel experiments in which both solutions were given to animals under free access-no lever pressing requirements. These free access experiments are a way to evaluate DA involvement in the directional aspect of motivation under minimal or no effort demanding conditions and can provide information about possible palatability alterations after DA antagonism. On these two different tasks we evaluated the effect of the DA depleting agent tetrabenazine, a selective vesicular monoamine

transporter-inhibitor for VMAT-2 (Zheng et al., 2006; Fasano and Bentivoglio, 2009). The following experiments evaluated DA selective antagonists with different affinity profiles for the DA receptors subtypes; D_1, D_2 and D_3 receptors. Previous research has focused mostly on D_1 and D_2 receptors, but less is known about the D_3 role in effort based decision making. As control experiments we evaluated the impact of letting animals to satiate of both sucrose solutions before the experimental sessions started. Furthermore, two experiments were also conducted to determine the ability of non-selective adenosine antagonists to reverse DA antagonism effects on choice. Thus, theophylline and caffeine were used to reverse the behavioral effects of D_1 and D_2 antagonists.

MATERIALS AND METHODS

Animals

Adult male, Sprague-Dawley rats (Janvier, France) were housed in pairs in a colony maintained at 23ºC with 12-h light/dark cycles (lights on at 8:00 h). Rats (N=256) weighed 190-240 g at the beginning of the study. Rats were initially water restricted, after the first day of training and then, they were fed supplemental water to maintain the water restriction throughout the study (20 ml/day/rat), with chow available *ad libitum* in the home cages. Despite water restriction, rats gained weight normally throughout the experiment. All animals were under a protocol approved by the Institutional Animal Care and Use Committee of Universitat Jaume I, and all experimental procedures complied with European Community Council directive (86/609/EEC). All efforts were made to minimize animal suffering, and to reduce the number of animals used.

Pharmacological agents

Sucrose (Sigma Quimica C.O) solutions were dissolved in tap water for oral consumption. All drugs were administered intraperitoneally (IP). Tetrabenazine (Tocris Bioscience) was dissolved and sonicated in 20% DMSO which was dissolved in 0.9% saline (pH=4.5). SCH39166 (ecopipam; (6aS-trans)-11-Chloro-6,6a,7,8,9, 13b-hexahydro-7-methyl-5H-benzo[d] aphtha[2,1-b]azepin- 12-ol hydrobromide), (Tocris Bioscience), a highly selective D_1 receptors antagonist (Alburgues et al., 1992), was dissolved in a 0.2% tartaric acid solution (pH=4.0), which was also used as the vehicle control. Haloperidol (Sigma Quimica C.O), a relatively selective DA D_2 receptor antagonist, was dissolved in 0.2% tartaric acid solution (pH=4.0), which also was used as the vehicle control. GR103691 (Tocris Bioscience), a DA antagonist with high affinity to D_3 receptor (Audinot et al, 1998), was dissolved and sonicated in 20% DMSO which was dissolved in 0.9% saline (pH=4.5). Theophylline and Caffeine (Tocris Bioscience), nonselective adenosine antagonists, were dissolved in distilled water (pH=7.4).

Doses of tetrabenozine, ecopipam, haloperidol, theophylline and caffeine used for the experiments were based upon previous research (Sink et al., 2008; Worden et al., 2009; Randall et al., 2011) and on pilot studies. The specific doses of each drug were selected in order to be high enough to produce a robust shift from lever pressing to free intake, but low enough not to produce a general disruption of behavior. The dose range chosen for D3 antagonist was based upon doses listed in published behavioral studies involving IP administration in rats (Gerlach et al., 2011; Clifford and Waddington, 1998) and higher doses.

Apparatus and testing procedures

Operant chambers (28 cm x 23 cm x 23 cm; Med Associates Inc., St. Albans, VT) were used for the concurrent FR7/free sucrose procedure experiments. These chambers were equipped with a retractable lever that was located on the right side of the wall (2 cm above the floor), which triggered the entry of a retractable graduated cylinder tube with rubber stopper and a stainless steel sipper spout with double ball bearings to prevent leakage, on the same wall (5 cm above the grid floor) when pressed. This tube contained 5% w/v sucrose. Lever pressing also activated an interior chamber light during retractable drinking disposal. The opposite wall contained a drinking spout (0.3% w/v sucrose), not retractable. All chambers were housed in sound attenuated enclosures with exhaust fans that masked external noise. Electrical inputs/outputs of each chamber were controlled by an IBM compatible PC (Med-Associates software).

Locomotion chamber. The open field was 80 cm x 60 cm x 52 cm. The behavioral test room was illuminated with a soft light, and external noise was attenuated. Animals were habituated to the open field during 10 minutes 24 hours before the test session. Locomotor activity was registered manually. For horizontal locomotion an activity count was registered each time the animal crossed from a 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.

Concurrent FR7/free sucrose. Operant sessions occurred once a day for 5 days/week. Animals were trained to lever press for access to a 5% sucrose (g/l)

solution. Rats were initially trained during 4 days to lever press on a FR1 reinforcement schedule: during 3 days sessions lasted 30-min with the 5% sucrose dispenser available during 30, 15 and 5 seconds progressively every time a ration was completed. On the 4rth day the session was reduced for the rest of the experiment to 15 minutes and the 5% sucrose dispenser was available for 5 seconds. On the second phase rats were shifted for 2 days to a FR5 schedule. Finally rats were shifted to FR7 (5 days/week, 2 weeks). Rats were then trained on the concurrent FR7/sucrose 0.3% procedure. With this task, 0.3% sucrose was freely available on the opposite side of the chamber during the FR7 sessions. At the end of the session, rats were immediately removed from the chamber, and sucrose intake was determined by measuring the remaining fluids. Rats were trained until they attained stable levels of baseline lever pressing and free 0.3% sucrose intake (i.e consistent responding over 200 lever presses per 15 min during the last 5 days), after which drug testing began. Every day rats received supplemental water (20 ml /animal) in the home cage.

Free two-bottle sucrose drinking paradigm. Animals were individually placed during 15 minutes in new home cages (20 cm x 45 cm x 25 cm) where two bottles containing 0.3% and 5% sucrose drinking solutions were placed separated 10 cm apart for 5 days/week. To control for possible side preferences, the left-right positions of the bottles were randomly assigned to different rats. In order to train these groups in a similar way to the operant groups, rats were initially exposed to the 5% sucrose concentration (30 min, for 3 days) after which 0.3% and 5% sucrose were concurrently present during 15 minutes sessions for 3 weeks before testing started. At the end of the session, rats were immediately removed from the chamber, and sucrose intake was determined by

measuring the remaining fluid. Rats received supplemental water (20 ml/day/rat) in the home cage.

For the pre-exposure condition, animals were trained as described above, and the day before test, they had *ad libitum* access to 5%, 0.3% sucrose and water, for 24 hours in their home cage. Then, animals were exposed to an operant session where sucrose intake and lever presses were registered. Additional groups were exposed to free two-bottles sessions.

Locomotion experiment. Different groups of animals were tested with the higher dose of every drug used in the drinking experiments.

Experiments

Each rat received all drug doses in their particular experiment in a randomly varied order (one treatment per week, with none of the treatment sequences repeated across different animals in the same experiment). Baseline (i.e. non-drug) sessions were conducted 4 additional days per week. The specific treatments and testing times for each experiment are listed below.

Experiment 1: *Effect of introducing free low sucrose concentration concurrently available in the operant chamber on lever pressing behavior***.** As described above, animals were trained under a FR7 schedule until a stable baseline on lever pressing was achieved. The low free access sucrose 0.3% concentration was introduced and training proceeded. Lever pressing for the 5% concentration was registered before and after introducing the alternative fluid (N= 30, same animals as in experiments 3, 4 and 8B).

Experiment 2: *Effect of the DA depleting agent tetrabenazine on selection of different sucrose concentrations in operant and free intake procedures.*

A). Effect of tetrabenazine on concurrent FR7/free sucrose choice. On the test day, trained rats (N=9) received the following tetrabenazine doses: 0.0, 0.5, 0.75 and 1.0 mg/kg (90 minutes before testing) and lever pressing and sucrose intake of 5% or 0.3% concentration were assessed.

B). Effect of tetrabenazine on free access two-bottle sucrose choice. On the test day, trained rats (N=10) received the following tetrabenazine doses: 0.0, 0.5, 0.75 and 1.0 mg/kg (90 minutes before testing) and free sucrose intake of 5% or 0.3% concentration were assessed.

Experiment 3: *Effect of the D_1 antagonist ecopipam (SCH 39166) on selection of different sucrose concentrations in operant and free intake procedures.*

A). Effect of different doses of the D_1 antagonist ecopipam (SCH-39166) on concurrent FR7/free sucrose choice. On the test day, trained rats (N=10) received the following ecopipam doses: 0.0, 0.05, 0.1 and 0.2 mg/kg (30 minutes before testing).

B). Effect of different doses of the D_1 antagonist ecopipam (SCH-39166) on free access two-bottle sucrose choice. On the test day, trained rats (N=10) received the following ecopipam doses: 0.0, 0.05, 0.1 and 0.2 mg/kg (30 minutes before testing).

Experiment 4: *Effect of the D_2 antagonist haloperidol on selection of different sucrose concentrations in operant and free intake procedures.*

A). Effect of different doses of the D_2 antagonist haloperidol on FR7/free sucrose choice. On the test day, trained rats (N=10) received the following haloperidol doses: 0.0, 0.025, 0.05 and 0.1 mg/kg (50 minutes before testing).

B). Effect of different doses of the D_2 antagonist haloperidol on free access two-bottle sucrose choice. On the test day, trained rats (N=10) received the following haloperidol doses: 0.0, 0.025, 0.05 and 0.1 mg/kg (50 minutes before testing).

Experiment 5: *Effect of the D_3 antagonist GR103691 on selection of different sucrose concentrations in operant and free intake procedures.*

A). Effect of different doses of the D_3 antagonist GR103691 on FR7/free sucrose choice. On the test day, trained rats (N=10) received the following GR103691 doses: 0.0, 0.5, 1.0 and 2.0 mg/kg (30 minutes before testing).

B). Effect of different doses of the D_3 antagonist GR103691 on free access two-bottle sucrose choice. On the test day, trained rats (N=10) received the following GR103691 doses: 0.0, 0.5, 1.0 and 2.0 mg/kg (30 minutes before testing).

Experiment 6: *Effect of pre-exposure to sucrose solutions on selection of different sucrose concentrations in operant and free intake procedures.*

A). Effect of pre-exposure to sucrose on FR7/free sucrose choice. Animals (N=8) had *ad libitum* water, 5% and 0.3% sucrose in their home cages during 24 hours previous to being tested in the FR7/free sucrose choice. After

the operant session, lever pressing and sucrose intake of 5% or 0.3% concentration were assessed.

B). Effect of pre-exposure to sucrose on free access two-bottle sucrose choice. Animals (N=9) had *ad libitum* water, 5% and 0.3% sucrose in their home cages during 24 hours previous to being tested in the free choice paradigm. Sucrose intake of 5% or 0.3% concentration were assessed after the session ended.

Experiment 7: *Effect of reducing sucrose concentration of the free fluid in the operant choice procedure: study using the D_2 antagonist haloperidol.* Initial experiments on free consumption demonstrated that animals (N=20) did not differentiated between 0.1 and 0.2% (t (1,18)=1.03, n.s.), but they did differentiated between 0.2 and 0.3% sucrose solutions (t (1,18)=43.59, $p<0.01$) (see table 1). Thus, in the present experiment we assessed the impact of haloperidol when the free access solution concurrently available on the operant choice procedure was lower than the one used for all previous experiments. Animals were trained as described above and the free access sucrose solution was 0.2% rather than 0.3%. On the test day, trained rats (N=10) received the following haloperidol doses: 0.0, 0.025, 0.05 and 0.1 mg/kg (50 minutes before testing), and lever pressing and sucrose intake of 5% or 0.2% concentration were assessed.

		Volume consumed (ml)
Group 1	0.3% sucrose	6.7±0.5
	0.2% sucrose	2.5±0.4**
Group 2	0.2% sucrose	4.2±0.9
	0.1% sucrose	3.1±0.5

Table 1. Preference for two different concentrations of sucrose in different groups of animals under a free choice paradigm. Mean (±SEM) ml consumed in 15 min.

Experiment 8: *Ability of non-selective adenosine antagonists theophylline and caffeine to reverse the effects of D_1 and D_2 antagonists on selection of different sucrose concentrations in operant and free intake procedures.*

A). Effect of theophylline and caffeine after ecopipam (SCH39166) administration on FR7/free sucrose choice. Different groups of animals were used for the present experiments (N=60). On the test day, trained rats received the following treatments: 0.0 or 0.2 mg/kg ecopipam (30 minutes before testing) plus 0 or 20 mg/kg theophylline or 0 or 20 mg/kg caffeine (20 minutes before testing). Lever pressing and sucrose intake of 5% or 0.3% concentration were assessed at the end of the session.

B). Effect of theophylline after haloperidol administration on FR7/free sucrose choice. For this experiment, we used animals that had a baseline level below the criterion established for the operant experiments (over 200 lever presses for 5 days before testing started) to see if theophylline improved the

response of the low performers. On the test day, trained rats (N=10) received the following treatments: 0 or 0.1 mg/kg haloperidol (50 minutes before testing) plus 0 or 20 mg/kg theophylline (20 minutes before testing). Lever pressing and sucrose intake of 5% or 0.3% concentration were assessed at the end of the session.

C). Effect of theophylline and caffeine on two-bottle sucrose drinking paradigm. Different groups of animals were used for the present experiments (N=30). On the test day, trained rats received the following treatments: 0.0 or 20 mg/kg theophylline or 20 mg/kg caffeine (20 minutes before test). Sucrose intake of 5% or 0.3% concentration was assessed at the end of the session.

Experiment 9: *Effect of tetrabenazine, ecopipam, haloperidol, GR103691, theophylline and caffeine on locomotor activity***.** Different groups of animals were used for the present experiments (N=40). The different vehicles used in the previous experiments were used for the locomotion experiment. Because there were no differences between them in locomotion (data not shown), all these data were considered the vehicle group. On the test day, rats received vehicle or one of the following treatments: 1.0 mg/kg of tetrabenazine, 0.2 mg/kg ecopipam, 0.1 mg/kg haloperidol, 2.0 mg/kg GR103691, 20 mg/kg theophylline or 20 mg/kg caffeine. Horizontal and vertical locomotion were simultaneously recorded during 10 minutes.

Statistical analyses

The dependent variables total number of lever presses and ml of sucrose intake from the 15 min sessions were analyzed with repeated measures of analysis of variance (ANOVA) except for experiments 8 A and C on which a 2

way-factorial ANOVA was applied. 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. Locomotion data were analyzed using between-groups ANOVA. STATISTICA 7 software was used for statistical analysis of the data. All data were expressed as mean ±SEM, and significance was set at $p<0.05$.

RESULTS

Experiment 1: *Effect of introducing free low sucrose concentration concurrently available in the operant chamber on lever pressing behavior.* Repeated measures ANOVA for the factor day of training showed a significant effect on lever pressing ($F(10,290)=7.33$, $p<0.01$). Planned comparisons yield significant differences between the last day of FR7 alone and the following two days of free 0.3% sucrose concurrently available ($p<0.01$) (see Fig. 1). As expected, repeated measures ANOVA for the factor day of training on 5% sucrose intake was also significant ($F(10,290)=4.1$, $p<0.01$). Planned comparisons showed significant differences between last day of FR7 alone and the following two days of free 0.3% sucrose introduction ($p<0.05$). Thus, the presence of a new sucrose source in the operant cage produced a temporal shift in behavior that disappeared by the third day.

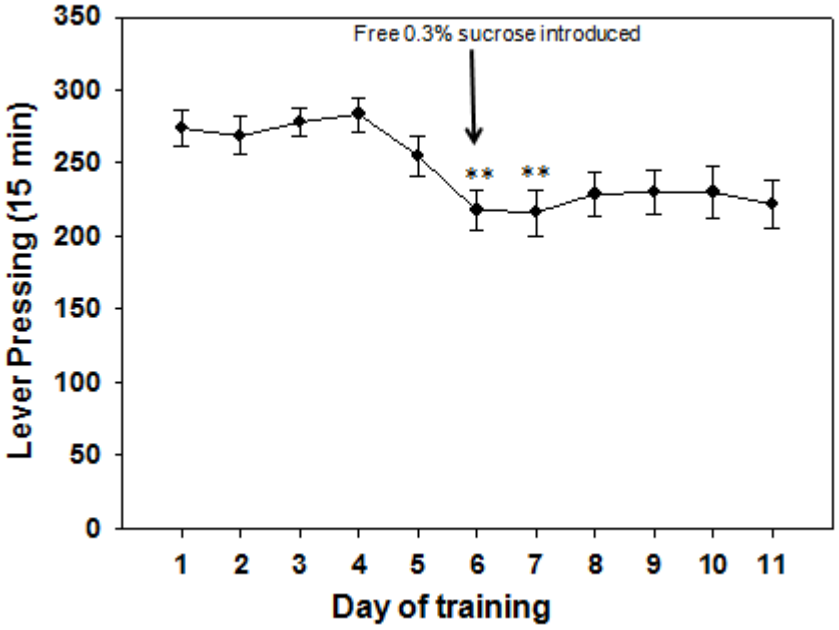

Fig. 1. Effect of introducing a spout providing free sucrose on the operant performance. Mean (±SEM) number of lever presses in 15 min. **p<0.01 significantly different from the last day with no concurrent 0.3% sucrose available.

Experiment 2: *Effect of the DA depleting agent tetrabenazine on selection of different sucrose concentrations in operant and free intake procedures.*

A). Effects of tetrabenazine in the operant procedure are shown in figure 2A, 2B and 2C. The ANOVA for repeated meassures indicated that tetrabenazine produced a significant effect on lever pressing (F(3, 24)=5.15, p<0.01), on 5% sucrose intake (F(3, 24)=6.88, p<0.05) and on 0.3% sucrose intake (F(3,24)=6.79, p<0.01). Planned comparisons showed that tetrabenazine significantly reduced lever pressing at the two highest doses, 0.75 mg/kg (p<0.05) and 1 mg/kg (p<0.01) compared to vehicle, as well as reducing 5%

sucrose intake (0.75 mg/kg, $p<0.05$, and 1 mg/kg, $p<0.01$) compared to vehicle, and significantly increased 0.3% sucrose intake at all doses tested ($p<0.01$).

B).The effect of tetrabenazine on free access sucrose intake is shown in fig. 2D and 2E. Repeated measures ANOVA yielded no effect of the factor dose on 5% sucrose intake ($F(3,27)=1.11$, n.s.) or 0.3% sucrose intake ($F(3,27)=0.09$, n.s.).

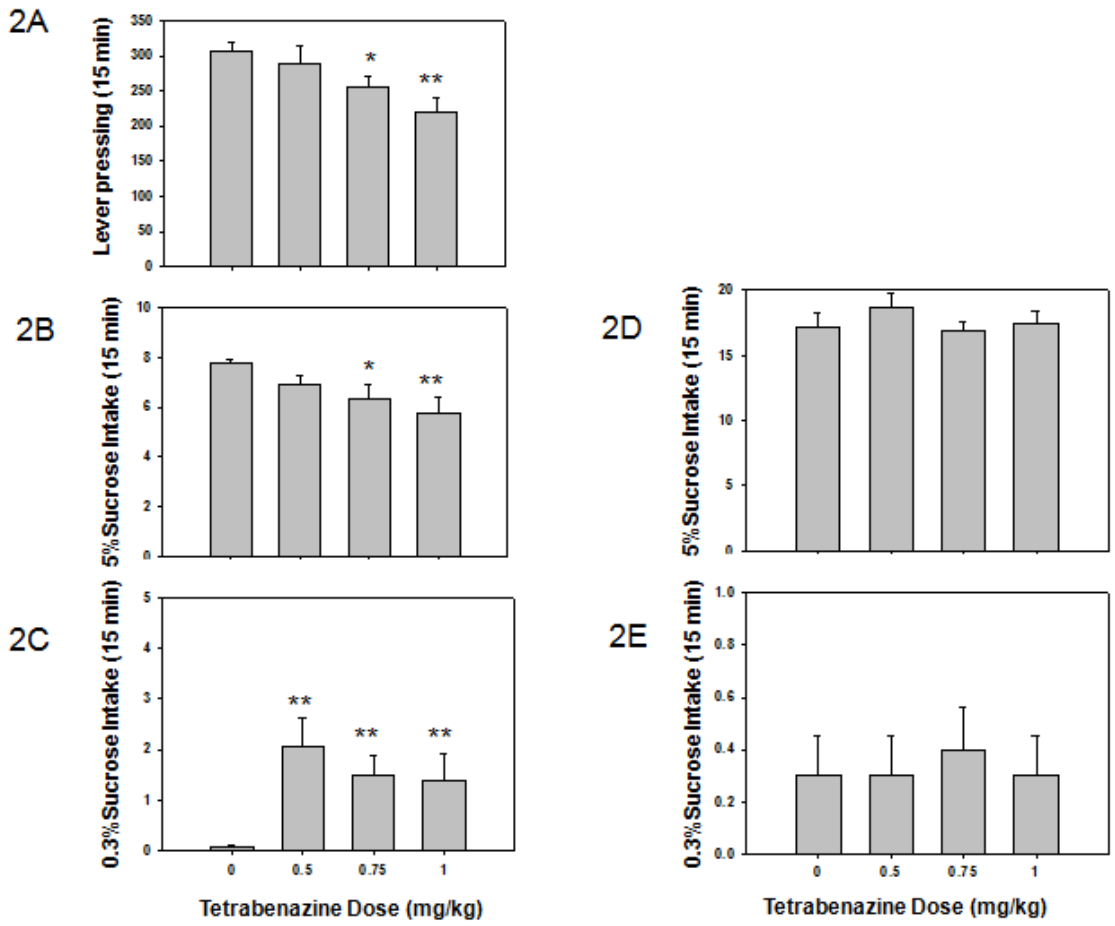

Fig. 2. Effect of tetrabenazine (0.0, 0.5, 0.75 and 1 mg/kg) in the operant choice paradigm A) lever presses, B) 5% sucrose intake, and C) 0.3% sucrose intake, and in the free choice paradigm D) 5% free sucrose intake, and E) 0.3 free sucrose intake. Mean (±SEM) number of lever presses or ml consumed in 15 min. *$p<0.05$ **$p<0.01$ significantly different from vehicle.

Experiment 3: *Effect of the D_1 antagonist ecopipam (SCH 39166) on selection of different sucrose concentrations in operant and free intake procedures.*

A). The effect of ecopipam in the operant task is shown in fig. 3A, 3B and 3C. The repeated measures ANOVA indicated that ecopipam produced a significant effect on lever pressing (F (3, 27)=10.05, p<0.01), on 5% sucrose intake (F (3,27)= 15.04, p<0.01) and on 0.3% sucrose intake (F (3, 27)= 6.22, p<0.01). Planned comparisons showed that ecopipam significantly reduced lever pressing at the doses of 0.1 mg/kg (p<0.05) and 0.2 mg/kg (p<0.01) compared to vehicle, as well as reducing 5% sucrose intake at the same doses (p<0.01) compared to vehicle and, significantly increased 0.3% sucrose intake at the doses of 0.1 and 0.2 mg/kg (p<0.01).

B). Figures 3D and 3E show the effect of ecopipam on free access sucrose intake. Repeated measures ANOVA yielded no effect on 5% sucrose intake (F (3,27)=0.40, n.s.) or 0.3% sucrose intake (F (3,27)=0.40, n.s.).

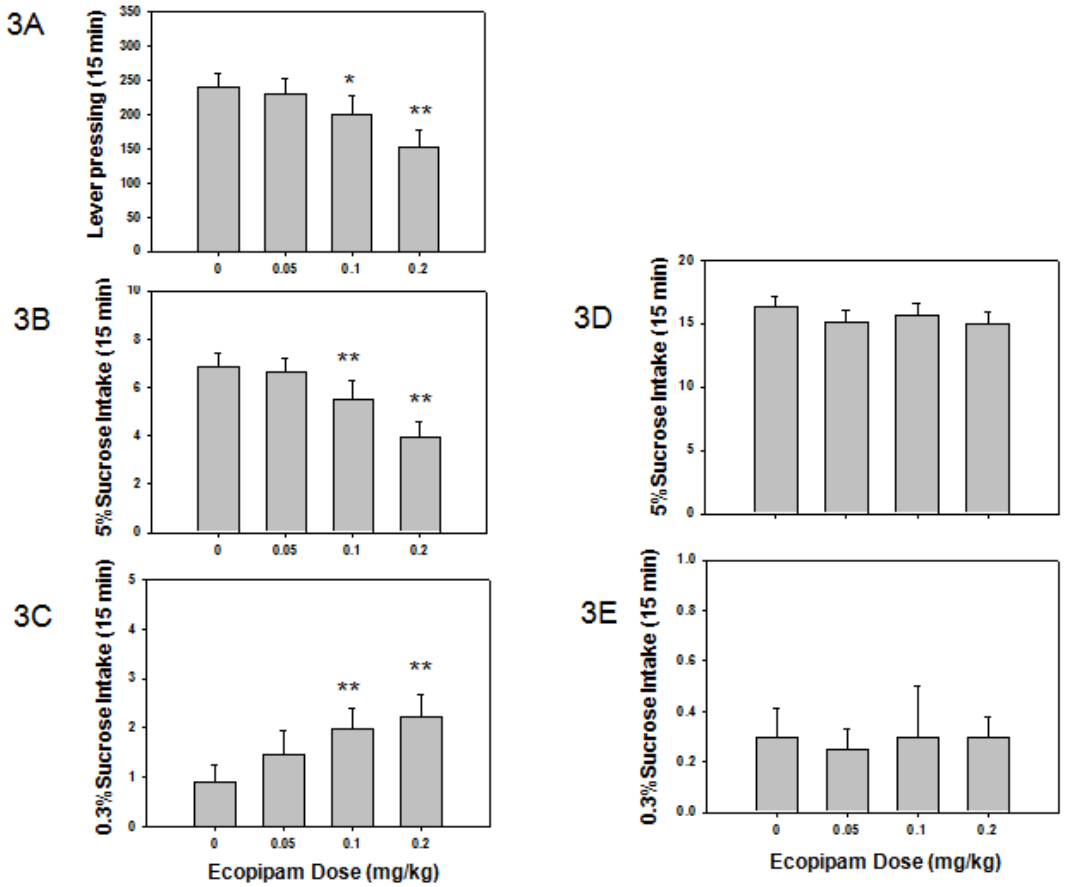

Fig. 3. Effect of ecopipam (0.0, 0.05, 0.1 and 0.2 mg/kg) in the operant choice paradigm A) lever presses, B) 5% sucrose intake, C) 0.3% sucrose intake, and in the free choice paradigm D) 5% free sucrose intake, and E) 0.3% free sucrose intake. *p<0.05 **p<0.01 significantly different from vehicle. Mean (±SEM) number of lever presses or ml consumed in 15 min.

Experiment 4: *Effect of the D_2 antagonist haloperidol on selection of different sucrose concentrations in operant and free intake procedures.*

A). Effects of haloperidol in the operant procedure are shown in fig. 4A, 4B and 4C. The ANOVA for the main factor haloperidol showed that this drug produced a significant effect on lever pressing ($F(3, 27)=17.98$, $p<0.01$), on 5% sucrose intake ($F(3,27)=16.35$, $p<0.01$) and on 0.3% sucrose intake ($F(3, 27)=6.22$, $p<0.01$). Planned comparisons revealed significant differences

between 0.0 mg/kg and the two highest doses of haloperidol (0.05 and 0.1 mg/kg, $p<0.01$) on lever pressing, on 5% sucrose intake ($p<0.05$ and $p<0.01$ respectively), and a significant difference between 0.0 mg/kg and 0.1 mg/kg ($p<0.01$) on 0.3% sucrose intake.

B). Figures 4D and 4E show the effects of haloperidol on free access sucrose intake. Repeated measures ANOVA yielded no effect of the factor dose on free access 5% sucrose intake ($F(3,27)=0.41$, n.s.), 0.3% sucrose intake ($F(3,27)=0.64$, n.s.).

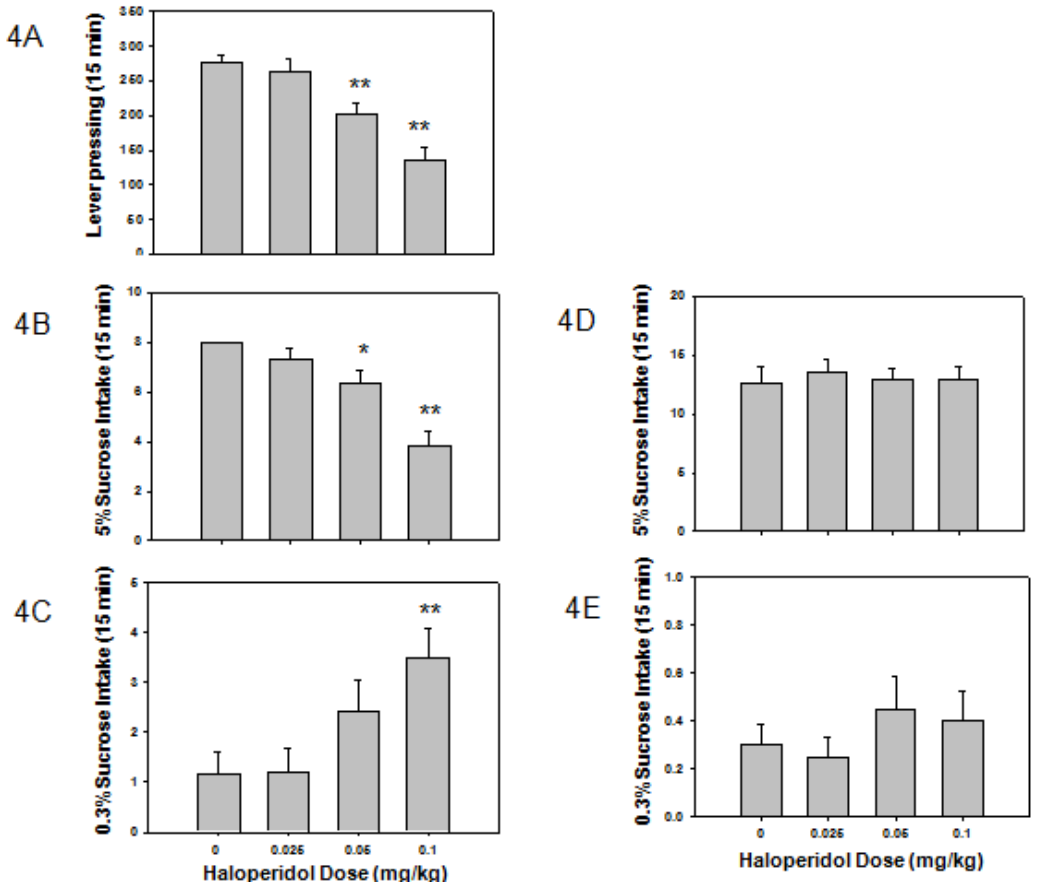

Fig. 4. Effect of haloperidol (0.0, 0.025, 0.05 and 0.1 mg/kg) in the operant choice paradigm A) lever presses, B) 5% sucrose intake, and C) 0.3% sucrose intake, and in the free choice paradigm, D) 5% free sucrose intake, and E) 0.3% free sucrose intake. Mean (±SEM) number of lever presses or ml consumed in 15 min. *p<0.05 **p<0.01 significantly different from vehicle.

Experiment 5: *Effect of the D_3 antagonist GR103691 on selection of different sucrose concentrations in operant and free intake procedures.*

A). The effect of different doses of the D_3 antagonist GR103691 on the operant procedure are shown in fig. 5A, 5B and 5C. Repeated measures ANOVA for the factor GR103691 dose yielded no significant effect on lever pressing ($F(3,27)=1.98$, n.s.), on 5% sucrose intake ($F(3,27)=1.21$, n.s.) and on 0.3% sucrose intake ($F(3,27)=2.19$, n.s.).

B). The effect of GR103691 on free sucrose intake is shown in fig. 5D and 5E. Repeated measures ANOVA yielded no effect of the factor dose on 5% sucrose intake ($F(3,27)=0.91$, n.s.) or 0.3% sucrose intake ($F(3,27)=0.42$, n.s.).

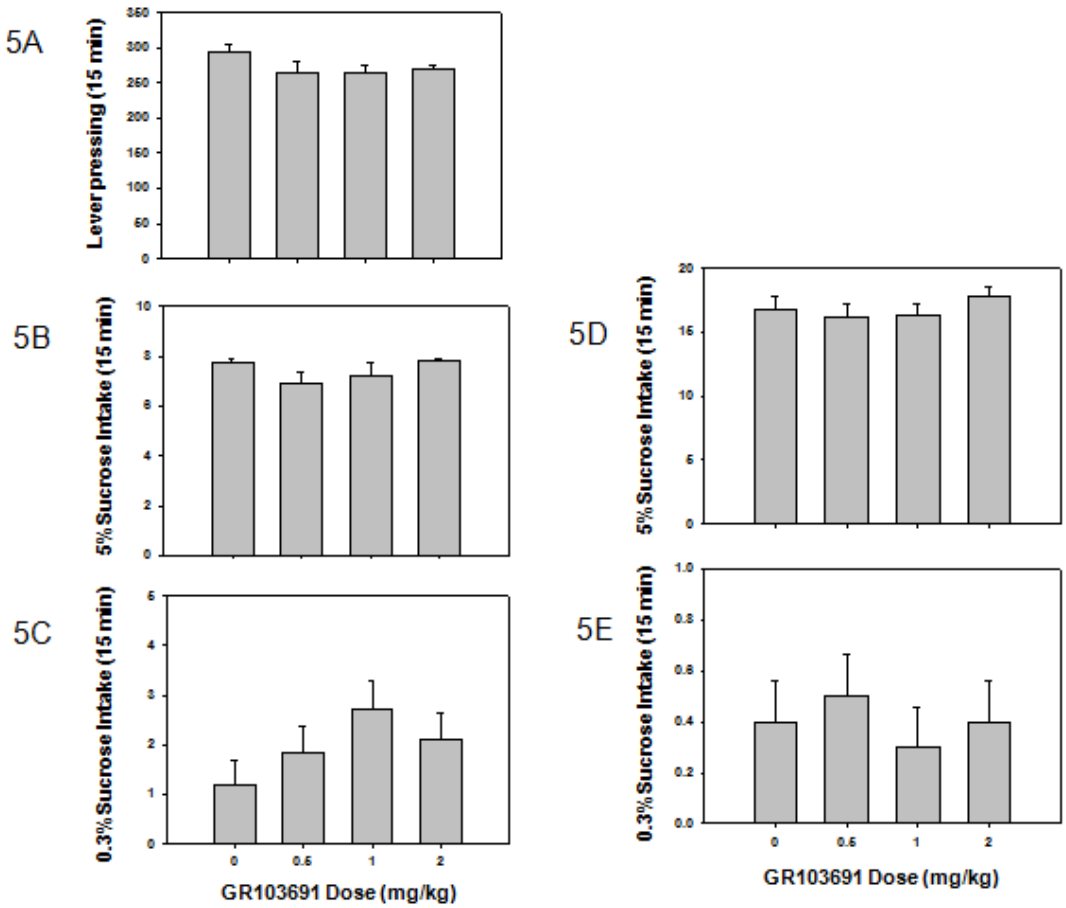

Fig. 5. Effect of GR103691 (0.0, 0.5 1 and 2 mg/kg) in the operant choice paradigm, A) lever presses, B) 5% sucrose intake, and C) 0.3% sucrose intake, and in the free choice paradigm, D) 5% free sucrose intake, and E) 0.3% free sucrose intake. Mean (±SEM) number of lever presses or ml consumed in 15 min.

Experiment 6: *Effect of pre-exposure to sucrose solutions on selection of different sucrose concentrations in operant and free intake procedures.*

A). Figures 6A, 6B and 6C show the effect of pre-exposing animals to both concentrations of sucrose 24 hours before the test was performed. The repeated measures ANOVA indicated that to pre-expose the animals produced a significant effect on lever pressing ($F(1,7)=10.63$, $p<0.05$), and on 5% sucrose intake ($F(1,7)= 18.40$, $p<0.01$). Although the significance was close ($F(1,7)=4.84$, $p=0.064$), 0.3% sucrose intake was not affected.

B). The effect of pre-exposing the animals to reduce sucrose motivation, on free access sucrose intake is shown in fig. 6D and 6E. The ANOVA indicated that to pre-expose the animals produced a significant effect on 5% sucrose intake ($F(1,8)= 64$, $p<0.01$), and no effect on 0.3% sucrose intake ($F(1,8)=0.00$, n.s.) since the level of intake was already very low.

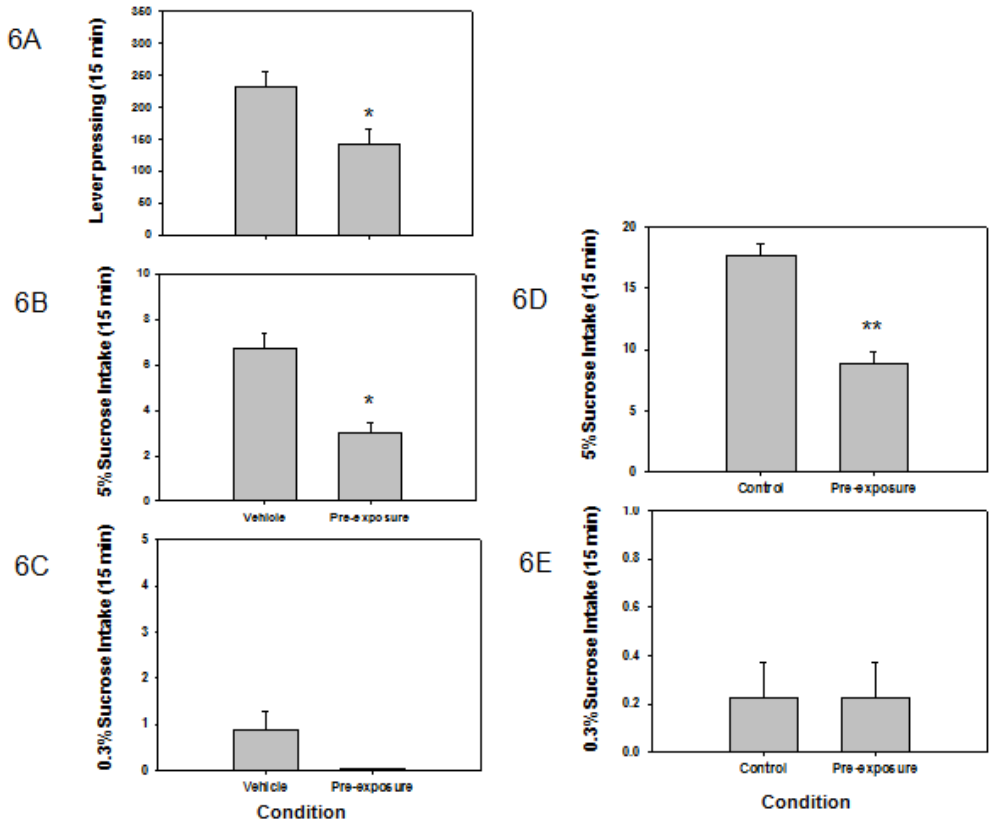

Fig. 6. Effect of sucrose pre-exposure in the operant choice paradigm, A) lever presses, B) 5% sucrose intake, and C) 0.3% sucrose intake, and in the free choice paradigm. D) 5% free sucrose intake and E) 0.3% free sucrose intake. Mean (±SEM) number of lever presses or ml consumed in 15 min. *p<0.05, **p<0.01 significantly different from control condition.

Experiment 7: *Effect of reducing sucrose concentration of the free fluid in the operant choice procedure: study using the D_2 antagonist haloperidol.*

The effects of haloperidol in the operant procedure when the free fluid is a concentration of 0.2% sucrose are shown in fig. 7A, 7B and 7C. The ANOVA indicated that haloperidol produced a significant effect on lever pressing (F(3, 27)=4.46, p<0.05), on 5% sucrose intake (F(3,27)=5.87, p<0.01), but no effect on 0.2% sucrose intake (F(3,27)=0.20, n.s.). Planned comparisons revealed that haloperidol significantly reduced lever pressing at the highest dose compared to

vehicle (p<0.05) as well as reducing 5% sucrose intake (p<0.05) compared to vehicle. These data indicate that the compensation in sucrose intake towards the low concentration fluid is only produced when the animals perceived the fluid as sweet but not because there was a fluid intake deficiency.

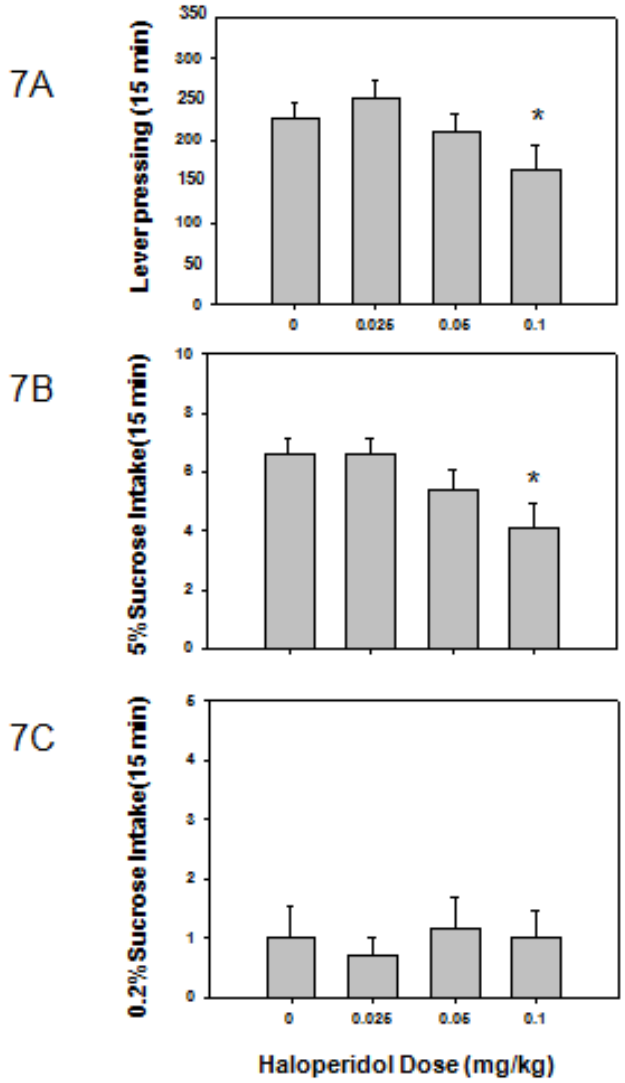

Fig. 7. Effect of haloperidol (0.0, 0.025, 0.05 and 0.1 mg/kg) in the operant choice paradigm, A) lever presses, B) 5% sucrose intake, and C) 0.2% sucrose intake. Mean (±SEM) number of lever presses or ml consumed in 15 min. *p<0.05 significantly different from vehicle.

Experiment 8: *Ability of non-selective adenosine antagonists, theophylline and caffeine to reverse the effects of D_1 and D_2 antagonists on selection of different sucrose concentrations in operant and free intake procedures.*

A). A two-way (ecopipam dose x xantine treatment) factorial ANOVA was performed. Figure 8A shows the effects on lever pressing. There was a significant effect of ecopipam ($F(1,54)=17.04$, $p<0.01$), but no significant effect of the factor xantines ($F(2,54)=2.18$, n.s.) and no significant ecopipam x xantine interaction ($F(2,54)=0.22$, n.s.). The same pattern was observed for 5% sucrose intake (Fig. 8B). There was a significant effect of ecopipam ($F(1,54)=28.55$, $p<0.01$), but no significant effect of xantines ($F(2,54)=1.93$, n.s.) and no significant ecopipam x xantine interaction ($F(2,54)=0.46$, n.s.). Finally in figure 8C is shown the effect on 0.3% free sucrose intake. There was a significant effect of ecopipam dose ($F(1,54)=7.17$, $p<0.01$), a significant effect of xantine dose ($F(2,54)= 10.28$, $p<0.01$), but no significant ecopipam x xantine interaction ($F(2,54)=1.71$, n.s.). Thus, neither theophylline nor caffeine was able to reverse the impact of ecopipam on operant performance and shift in sucrose consumption towards the free available fluid.

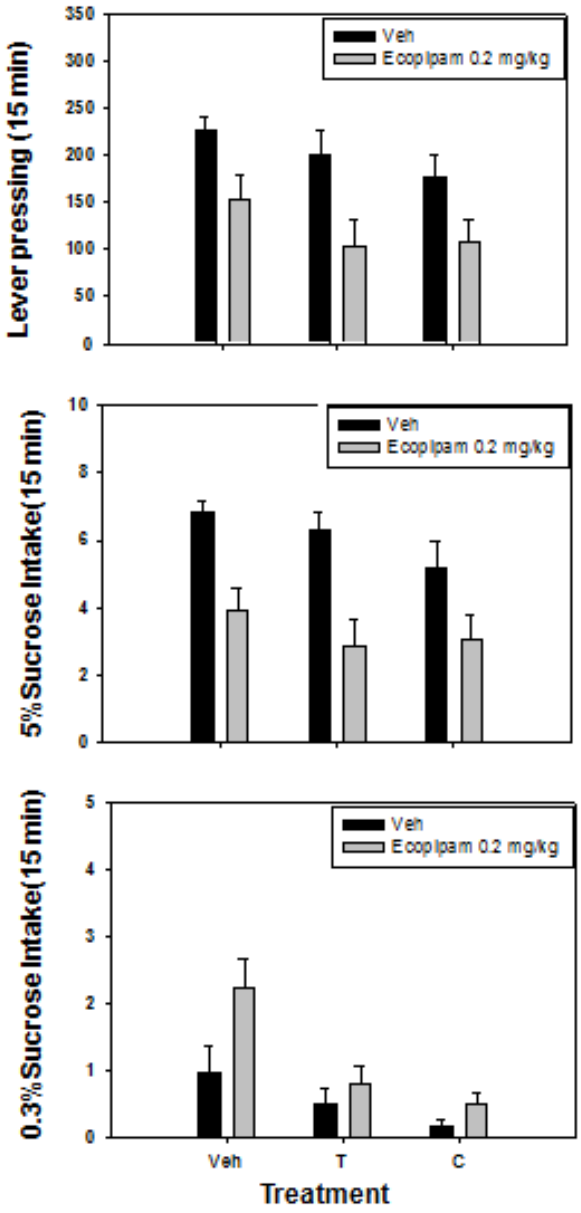

Fig. 8. Effect of nonselective adenosine antagonists caffeine and theophylline (0 and 20 mg/kg) in ecopipam (0 and 0.2 mg/kg) treated animals in the operant choice paradigm. A) lever presses B) 5% sucrose intake, and C) 0.3% sucrose intake. Mean (±SEM) number of lever presses or ml consumed in 15 min.

B). Figure 9A, 9B and 9C depict the effects of theophylline on haloperidol actions in the operant procedures in animals that had a low baseline level of lever pressing before the testing phase started. Repeated measures ANOVA across conditions indicated a significant overall treatment effect on

lever pressing ($F(3, 27)=15.04$, $p<0.01$), on 5% sucrose intake ($F(3, 27)=15.88$, $p<0.01$) and on 0.3% sucrose intake ($F(3,27)= 5.00$, $p<0.01$. Planned comparisons showed that HP/Veh was significantly different from the Veh/Veh control condition ($p<0.01$) for all the dependent variables. In addition, co-administration of theophylline and haloperidol (HP/T) significantly increased lever presses and 5% sucrose intake as well as decreasing free 0.3% sucrose intake ($p<0.01$) compared to the HP/Veh condition, indicating an attenuation of the haloperidol effects. Thus, although theophylline on its own did not produce significant changes it did produced a significant reversal of the effects of haloperidol on all measures.

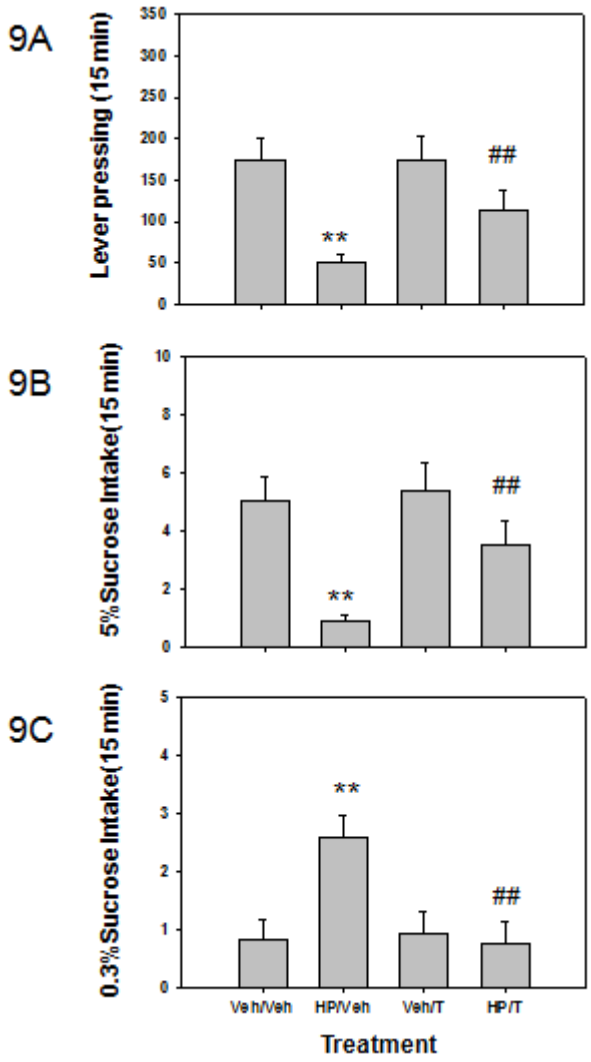

Fig. 9. Effect of the nonselective adenosine antagonist theophylline (0 and 20 mg/kg) in haloperidol (0 and 0.1 mg/kg) treated animals in the operant choice paradigm. A) lever presses B) 5% sucrose intake, and C) 0.3% sucrose intake. Mean (±SEM) number of lever presses or ml consumed in 15 min. **p<0.01 significantly different from Veh/Veh. ##p<0.01 significantly different from Veh/HP.

C). The effect of caffeine and theophylline on free access sucrose intake is shown in table 2. One-way ANOVA for the factor treatment (Vehicle / Theophylline 20 mg/ Caffeine 20 mg) yielded no significant effect neither for 5% sucrose intake ($F(2,27)=0.55$, n.s.) nor for 0.3% sucrose intake ($F(2,27)=0.10$, n.s.).

Treatment	5% sucrose	0.3% sucrose
Vehicle	14.8 ± 1.7	0.2 ± 0.1
Theophylline	12.9 ± 1.2	0.2 ± 0.1
Caffeine	13.8 ± 1.0	0.2 ± 0.1

Table 2. Effect of theophylline and caffeine (0.0 and 20 mg/kg) on free 5% sucrose intake and free 0.3% sucrose intake. Mean (±SEM) ml consumed (average in 15 min).

Experiment 9: *Effect of tetrabenazine, ecopipam, haloperidol, GR103691, theophylline and caffeine on locomotor activity.* Independent student's t tests were done for every drug comparing it to vehicle. Results for horizontal and vertical locomotion are shown in Table 4. The *t-tests* yielded no significant effect of tetrabenazine on horizontal (horizontal (t=0.02, df=13, n.s.)

neither vertical locomotion (t=1.60, df=13, n.s.) Same results were found for ecopipam on horizontal (t=2.65, df=14, n.s.) and vertical locomotion (t=3.33, df=14, n.s.), and for GR103691 on horizontal (t=1.95, df=13, n.s.) and on vertical locomotion (t=1.42, df=13, n.s.). Haloperidol was the only DA receptor antagonist that yielded significant effects on horizontal (t=4.93, df=13, p<0.05) and vertical locomotion (t=6.32, df=13, p<0.05) compared to vehicle. Furthermore, theophylline did not alter significantly horizontal (t=2.53, df=13, n.s.), nor vertical locomotion (t=1.61, df=13, n.s.), while caffeine increased significantly both measures, horizontal (t=9.22, df=12, p<0.01), and vertical locomotion (t=7.80, df=12, p<0.05), compared to vehicle group.

Treatment	Horizontal Locomotion	Vertical Locomotion
Vehicle	115.9 ± 19.1	46.0 ± 8.1
Tetrabenazine	112.0 ± 18.9	33.1 ± 5.7
Ecopipam	80.4 ± 12.2	27.9 ± 5.8
Haloperidol	72.0 ± 3.8*	22.3 ± 4.0*
GR103691	147.6 ± 12.3	35.0 ± 3.4
Theophylline	160.1 ± 21.4	59.1 ± 6.1
Caffeine	202.3 ± 22.4**	76.2 ± 6.2*

Table 3. Effect of treatment (vehicle, tetrabenazine (1 mg/kg), ecopipam (0.2 mg/kg), haloperidol (0.1 mg/kg), GR103691 (2 mg/kg), theophylline (20 mg/kg) and caffeine (20 mg/kg) on horizontal and vertical locomotion. Mean (±SEM) number of counts in the open field. *p<0.05, **p<0.01 significantly different from vehicle.

DISCUSSION

The present experiments evaluated the involvement of DA in the regulation of motivated responses and relative preference for sweet drinks. A

concurrent lever-pressing/intake task was adapted from previous procedures (Salamone et al., 1991). These paradigm allows animals to choose between lever pressing in an operant FR schedule for a "better" reward (in this case 5% sucrose) versus consuming freely a "less preferred" reward (0.3% sucrose), thus allowing to separately study the neurobehavioral bases of reward seeking and of reward taking. Sucrose has been extensively used for the study of emotional reactivity and for the study of the hedonic value of rewards (Peciña et al., 1997; Martinez-Hernandez et al., 2012). However, very few studies have used this reward for the study of effort-based decision-making. Additionally, separated experiments assessed the preference and consumption of the high and low sucrose concentrations in no effort choice situations.

We have used pharmacological tools to study the impact of DA storage depletion, and selective DA receptor antagonists for the D_1, D_2 and D_3 subtypes. Moreover, in control experiments a reduced motivational state was established by allowing the animals to get satiated from both type of sucrose concentrations or by devaluating even more the low cost-less preferred sucrose concentration. Thus, DA depletion by the selective VMAT-2 blocker tetrabenazine dose dependently decreased lever pressing for 5% sucrose while increasing the volume of the concurrently available 0.3% sucrose solution consumed in the operant choice paradigm. Animals redirected the behavior towards the less effortful option and drunk higher amounts of the less preferred solution. However, no dose of tetrabenazine modified the preference or the volume consumed of these sucrose concentrations in the non-operant concurrently available procedure, indicating that tetrabenazine leaves intact the palatability of sucrose. In previous studies, the neurotoxic agent 6-OHDA injected in Nacb

produced long lasting (days or weeks) DAergic depletions that have demonstrated to decrease lever pressing for palatable food but increase freely available chow consumption in a very similar paradigm (Cousins et al., 1994). Tetrabenazine has preferential effects on VMAT-2 in DA containing terminals, as shown by imaging studies that utilize the radiotracer ^{11}C-DTBZ as a stable structural marker of DA neurons (Blesa et al. 2010; Okamura et al. 2010; Kilbourn et al. 2010; Tong et al. 2008). This drug is commonly used in humans to treat hyperkinectic disorders, but common side effects include fatigue and anergia (Astin and Gumpert 1974; Kingston 1979; Jankovic and Beach 1997; Kenney et al. 2007).

To identify which family or DA subtype receptor's is implicated on the observed results after DA depletion in this paradigm, selective antagonists acting on D_1, D_2 and D_3 receptors were also tested. Ecopipam (SCH39166, D_1 antagonist) as well as haloperidol (D_2 antagonist) dose dependently decreased lever pressing for 5% sucrose intake, producing a shift on the animal's choice towards consuming greater amounts of 0.3% sucrose solution. Previous work has shown that peripherally or intra-accumbens D_1-family antagonists SCH23390, SKF83566 and ecopipam (Cousins et al. 1994; Nowend et al. 2001; Salamone et al. 2002; Sink et al., 2008; Worden et al., 2009; Nunes et al., 2010) as well as the D_2 DA antagonists haloperidol, raclopride and eticlopride (Salamone et al. 1991, 1996, 2009; Cousins et al. 1994; Koch et al. 2000), decrease lever pressing and increase chow consumption on the food concurrent choice task.

However, the D_3 antagonist GR103691 did not produce changes in any of the dependent variables evaluated, neither in the operant procedure nor in the

free choice situation. There are not many data on the behavioral effects of this D_3 antagonist. When injected directly into the basolateral amygdala it produced anxiolytic effects at doses that did not affected locomotion or rearing (Diaz et al., 2011). In a range of doses from 0.008 to 1.0 mg/kg IP, GR103691 did not affect parameters such as locomotion, rearing, grooming, sniffing or eating in rats (Clifford and Waddington 1998). The range of doses used in the present experiments (0.5-2.0 mg/kg, IP) expands the range of doses, but still no effect was obtained. The only other study that has assessed the role of D_3 antagonist on effort based decision making has used a single dose of U99194, and has find no alteration of choice in a T maze paradigm (Bardgett et al., 2009).

From all these data it seems clear that DA implication on effort-related processes is mediated by D_1 and D_2 receptors, but not D_3 receptors. Although, the use of higher doses and different D_3 antagonists would be necessary to clearly conclude that this type of receptors are not involved in the regulation of effort based decision-making. D_1 and D_2 family antagonists have consistently shown to produce similar effects on response allocation as measured by the concurrent FR5 lever pressing/chow feeding task (Salamone et al., 1991). This effort-related choice has been linked to DA function in Nacb (Salamone et al., 1997, 2007, 2009a, 2010). Neurons of the Nacb not only express D_1 and D_2 receptors, but they express D_3 receptors as well. In fact, Nacb is one of the few brain areas where this type of receptor is highly expressed (Diaz et al., 1995; Le Moine and Bloch, 1996). D_3 receptors seem to be co-localized with both type of receptors (Le Moine and Bloch, 1996), thus is possible that the D_3 contribution to Nacb function regulation is dependent on D_1 or D_2 activation.

Thus, under this new operant task in which animals have a choice between pressing a lever under FR7 schedule to obtain 5% sucrose solution and freely drinking from 0.3% sucrose, they chose to press the lever under control condition but, DA depletion and DA antagonism on D_1 and D_2 receptor produces a reorganization of behavior, increasing low free access sucrose intake, essentially selecting a new "path" to obtain the sucrose solution, although at the same time leaving intact the palatability and preference for 5% sucrose when there is no effort attached to this high concentration. The use of this task as a measure of effort-related choice behavior has been validated in several ways. For instance, the above described pattern of effects was not produced by pre-feeding to reduce sucrose motivation. After reducing motivation by allowing free consumption of 5% and 0.3 % sucrose solutions, satiated animals showed a decrease on lever pressing and 5% sucrose consumption but they did not shifted behavior towards the other source of sucrose. In fact, if anything, the rats reduce their intake of the low concentrated sucrose to almost 0 ml. Thus from both pre-feeding experiments (operant and free) we can conclude that the animals still chose to drink some 5% sucrose solution although significantly less than control non-satiated animals, and drink negligible amounts of 0.3% sucrose solution. These results are in accordance with experiments using food as the reinforcer in the operant situation (Salamone et al., 1991). Moreover, all the pharmacological conditions that produce the shift in the operant situation did not alter sucrose intake or preference in free-drinking choice tests, which indicates that DAergic manipulations were not simply changing sucrose palatability. Additionally, lowering the concentration of the already low concentrated solution (from 0.3 to 0.2%), thus, devaluating the free fluid sucrose concentration, animals behavior

resemble the pre-exposure condition; haloperidol decreased lever pressing and 5% sucrose solution, but there was no shift towards the consumption of the 0.2% sucrose solution. The 0.2% sucrose is a concentration that, in most of our animals, seems not to be perceived as a reinforcer since 100% of animals significantly consumed much more 0.3% over 0.2%, but when 0.2% was offered concurrently to 0.1% there was not a significant difference between them.

DA does not participate in effort-related processes in isolation. The interaction between DA and adenosine has also been studied based on the co-localization between DA/adenosine receptors in medium-spiny neurons of the striatum (Fink et al. 1992; Ferré 1997; Ferré et al. 1997, 2008b; Hettinger et al. 2001; Chen et al. 2001; Hillion et al. 2002; Fuxe et al. 2003). This fact has guided previous studies of motivation, behavioral activation and effort-related processes (Farrar et al., 2007; Font et al., 2008; Mingote et al., 2008; Mott et al., 2009; Salamone et al., 2007; Worden et al., 2009). Non selective adenosine antagonists such as caffeine and theophylline act as minor stimulants that enhance motor activity in rodents and humans (Lopez-Cruz et al., 2011; Garret and Holtzman, 1994; Daly and Fredholm, 1998; Karcz-Kubicha et al., 2003; Antoniou et al., 2005, Kuribara et al., 1992) and are commonly consumed by humans for their "energetic effects" (Antoniou et al., 2005; Ferré, 2008; Reissig et al., 2009). The present study examined the ability of these two non selective adenosine antagonists that, acting on A_1 as well as A_{2A} receptors, can produce effects in both D_1 and D_2 containing neurons. In the present studies, caffeine and theophylline, did not affect preference and volume of 5% and 0.3% sucrose consumed in the non-operant situation, showing no impaired palatability after adenosine antagonism. However, they were not able to reverse the effects of D_1

antagonism in the operant choice task, at a dose that significantly stimulated FR20 performance in an operant task for food (Randall et al., 2011). In previous studies it has been demonstrated that the effect of D_1 antagonism on effort based decision making is not reversed either by selective A_1 antagonists and is minimally reversed by A_{2A} antagonists (Nunes et al., 2010). In general, the effects of D_1 antagonist ecopipam were harder to reverse with selective adenosine antagonists that the effects of the D_2 antagonist haloperidol (Nunes et al., 2010). Because in rats caffeine has demonstrated to reverse the effects on food choice behavior modulated by haloperidol (Salamone et al., 2009), in our next experiment, theophylline was used to reverse the effect of haloperidol in a group of rats that had a low baseline performance in lever pressing (below the median split from all the other groups; 241 lever presses), and did not reach criterion. In these animals theophylline (20 mg/kg) significantly reversed the effects of haloperidol in all three variables. Thus, non-selective adenosine antagonists have potential utility for the treatment of energy-related dysfunctions (such as anergia) that can affect the activational component of motivation.

Sucrose drinking has been used to measure the putative reward functions of DA (Berridgem 2000; Berridge and Robinson, 1998; Nowend et al., 2001; Schneider et al., 1992; Smith, 1995). It has been reported that high doses of DA antagonists decrease sucrose consumption (Xenakis and Sclafani, 1982; Schneider et al., 1992; Hsiao and Smith, 1995; Hajnal et al., 2007). However, although after lower doses of D1 and D2 antagonists there is no effect on sucrose intake (Hajnal et al., 2007), the results of high doses have been used to state that DA antagonists blunt the ‘hedonic’ or ‘reward’ value of natural reinforcers such as sucrose, or suppress the appetite for them, or impair the

unconditioned responses to them.

The term “hedonic” refers to an emotion (Ribot 1989). Thus, the “anhedonic” effects of DA antagonists focus in the unconditioned emotional response to sucrose as well as in the role of DA in directing behavior towards the acquisition and consumption of sucrose. In relation to the first point, previous work has concluded that DA transmission interference, produced by DA antagonists or reducing DA levels directly on Nacb or striatum, does not modify gustative reactivity to sucrose a test developed for the evaluation of unconditioned emotional responses in rodents and other animals including humans (Berridge, 2000; Berridge and Robinson, 1998). As for the second point, measures of the directional component or motivated behaviors or the appetite to consume a reward are voluntary intake and preference tests (Berridge and Robinson, 1998; Salamone and Correa 2002). With these measurements, it has been demonstrated that DA antagonists injected into Nacb in low doses that impaired learning or suppressed locomotion did not suppress food or water consumption (Baldo et al., 2002). In our results haloperidol reduced locomotion but it did not affect non-effort consumption and preference for the high concentration of sucrose. Consistently, flupentixol injected in Nacb reduced speed to approach 20% sucrose at the end of a corridor, but did not affect final sucrose intake (Ikemoto and Panksepp, 1996).

DA antagonists can be producing many different processes that are not related to “hedonics”. The literature on DAergic involvement in sucrose drinking, like the literature on chow consumption, demonstrates that conditions that suppress sucrose drinking are accompanied by signs of motor dysfunction, specifically orofacial functions (see Fowler and Mortell, 1992; Das and Fowler,

1996). Several motor parameters related to licking are impaired by DA antagonists, including lap volume and tongue extension, lick force, lick duration, and lick efficiency (Fowler and Das, 1994; Fowler and Mortell, 1992; Gramling and Fowler, 1985; Hsiao and Chen, 1995; Schneinder et al., 1990). As for food high doses of DA antagonists affect rate of feeding (Blundell, 1987; Salamone et al., 1990, 1993). These motor effects of high doses of DA antagonists, therefore, place additional emphasis on studies that employ low doses of these substances. Low-to-moderate doses of DA antagonists are not acting as appetite suppressants that generally blunt primary food motivation, but instead are acting on other processes (e.g., behavioral activation, instrumental response output, response allocation, effort-related processes; Salamone et al., 1991, 1997, 2002, 2003, 2005, 2007; Kelley et al., 2005; Baldo and Kelley, 2007; Barbano and Cador, 2007; Niv et al., 2007; Phillips et al., 2007; Floresco et al., 2008; Sink et al,. 2008; Nunes et al., 2010). Salamone (1988) suggested that moderate interference with accumbens or striatal DA systems affects activational aspects of motivated behavior, but has little direct effect on directional aspects. Fundamental aspects of motivation, including perception and discrimination of the reward magnitude, and appetite, are left intact after DA antagonists or Nacb DA depletions (Martin-Iverson et a., 1987; Salamone et al., 1990, 1994; Treit and Berridge, 1990; Cousins et al., 1996; Aparicio, 1998; Berridge and Robinson, 1998; Berridge, 2000). If interference with DA systems blunted primary food motivation, then the effects of DA antagonists or DA depletions should closely mimic the effects of pre-feeding to reduce food motivation. In fact, several studies have demonstrated that the effects of DA antagonists or DA depletions differ substantially from the effects of pre-feeding. In terms of food

intake, the effects of haloperidol or forebrain DA depletions on the patterns of eating (e.g. rate of feeding, time spent feeding) differ substantially from the effects of pre-feeding (Salamone et al., 1991). As we have observed in present and previous experiments with sucrose and food, in the concurrent lever pressing/ free drinking task, pre-exposure to the sucrose solutions suppressed both lever pressing and free consumption, while DA antagonists and Nacb DA depletions decreased lever pressing but increased free consumption (Salamone et al., 1991).

Taking all these data together, under control conditions, rats press the lever to obtain the higher concentration of sucrose even if a low concentration of sucrose is freely available in the chamber. This preference for the high concentration sucrose was consistent also among the non-operant free concurrent access situation. However, if low doses of DA antagonists suppress lever pressing for natural reinforcers because they produce a general reduction in motivation (activational and directional components), or appetite, or the primary reinforcing or incentive properties of sucrose, then reductions of sucrose intake should be evident in the same dose range as the suppression of lever pressing. Yet, as it has been demonstrated in the present set of results, that is not the case. However, interference with DA transmission interacts powerfully with the work requirements of instrumental tasks, enhancing the costs associated with instrumental actions that have high work requirements. Rats with impaired Nacb DA transmission are less likely to work for a reinforcer, and in choice tasks, they are biased towards alternative paths to reinforcers that have lower work-related response costs (see Salamone et al. 2007, 2009b for review). Thus, in an FR8 task to obtain 60 microliters of 10% sucrose solution, the number of rewards

earned is reduced by injection of D_1 or D_2 receptor antagonists into the Nacb core, an effect that was not observed in an FR1 task (Nicola 2010). The D_1 antagonists SCH23390 injected into VTA failed to alter a single response requirement (RR) of 20 or RR1 lever presses for 20 minutes access to 2% sucrose fluid (Czachowsi et al., 2012). Both of these RR schedules require a very low rate of response in 30 minutes (animals typically lever press 50 times or 8 time respectively) compared to the 200 lever presses in 15 minutes required in the present experiment. Hsiao and Chen (1995) demonstrated that the response requirement (i.e. height of the spout) was an important determinant of the effect of the D_2 antagonist pimozide on 2% sucrose drinking. Thus DA antagonist shifted the behavior of water deprived animals from high effort sucrose consumption towards low effort (ease reachable water spout) less preferred fluid (tap water). Rats remained fully capable of assuming the standing posture for sustained and efficient licking of sucrose if no option was available. The authors define the behavior of DA antagonist treated rats as "indolent" (Hsiao and Chen, 1995).

In the present studies interference with DA transmission is not lowering the reinforcing value of sucrose, but rather, is lowering the reinforcing value of the instrumental actions required to obtain sucrose. In summary, DA depleting agents and DA antagonism using low-to-moderate doses of DA antagonists, reduce the tendency to work for a reinforcer while leaving intact appetite or primary food or sucrose motivation.

Moreover, within the last few years, we have determined that adenosine interacts with DA in the regulation of these effort-related functions. The DA antagonism effect has been reversed or attenuated by adenosine antagonists,

giving support to the notion that link DA and adenosine systems in Nacb in the regulation of instrumental response output and effort-related choice behavior (Farrar et al., 2007; Font et al., 2008; Mingote et al., 2008; Mott et al., 2009; Salamone et al., 2007, 2009; Worden et al., 2009). Further understanding on these processes can help to approach pathologies related to behavioral activation and effort (Salamone et al., 2007). Symptoms as anergia, psychomotor slowing and fatigue can be seen in depression and other disorders (Demyttenaere et al., 2005; Salamone et al., 2007) and there is increasing evidence proposing adenosine antagonists as new targets for treating them (Salamone et al., 2007).

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CHAPTER 2:

DOPAMINERGIC MODULATION OF EFFORT-RELATED CHOICE BEHAVIOR AS ASSESSED BY A PROGRESSIVE RATIO CHOW FEEDING CHOICE TASK: PHARMACOLOGICAL STUDIES AND THE ROLE OF INDIVIDUAL DIFFERENCES.

Abstract

Mesolimbic dopamine (DA) is involved in behavioral activation and effort-related processes. Rats with impaired DA transmission reallocate their instrumental behavior away from food-reinforced tasks with high response requirements, and instead select less effortful food-seeking behaviors. In the present study, the effects of the DA D_2 antagonist haloperidol, as well as other treatments, were assessed using a progressive ratio (PROG)/chow feeding concurrent choice task. With this task, rats can lever press on a PROG schedule reinforced by a preferred high-carbohydrate food pellet, or alternatively approach and consume the less-preferred laboratory chow that was concurrently available. Rats pass through each ratio level by completing 15 ratios, after which the ratio requirement is incremented by one additional response (e.g. FR1 15 times, FR2 15 times, etc.). Haloperidol (0.025-0.1 mg/kg) reduced number of lever presses and highest ratio achieved but did not significantly affect chow intake. In contrast, the adenosine A_{2A} antagonist MSX-3 increased lever presses and highest ratio achieved, but decreased chow consumption. The cannabinoid CB1 inverse agonist and putative appetite suppressant AM251 decreased lever

presses, highest ratio achieved, and chow intake; this effect was similar to that produced by pre-feeding. In the final study, DA-related signal transduction activity (pDARPP-32(Thr34) expression) was greater in high responders (i.e., rats with high lever pressing output) compared to low responders. Thus, the effects of DA antagonism differed greatly from those produced by reduced food motivation or decreases in CB1 transmission. It appears unlikely that haloperidol is reducing PROG responding because of a general reduction in the valuation of food reinforcement. Furthermore, nucleus accumbens core signal transduction activity is related to individual differences in work output.

INTRODUCTION

Nucleus accumbens dopamine (DA) plays an important role in regulating activational aspects of motivated behaviors (i.e., vigor, persistence, exertion of effort), which enable organisms to overcome work-related response costs in order to gain access to significant stimuli (Salamone, 1992; Salamone et al., 1991, 1997, 2003, 2007; Salamone and Correa, 2002; Van den Bos et al., 2006). The increased activity induced by scheduled presentation of food reinforcement pellets is accompanied by increases in accumbens DA release, and is reduced by DA antagonism and accumbens DA depletions (Salamone 1986, 1988; McCullough and Salamone 1992). Rats with accumbens DA depletions are very sensitive to ratio requirements in operant lever pressing schedules (Sokolowski and Salamone, 1998; Aberman and Salamone, 1999; Correa et al., 2002; Mingote et al., 2005). Moreover, DA antagonism or interference with accumbens DA transmission alters response allocation in tasks that measure effort-related choice behavior (Salamone et al., 1991, 1997, 2003, 2005, 2006, 2007).

Several behavioral tasks have been used to investigate the role of DA in effort-related choice. Some studies have used the T-maze barrier task in which the animal must choose to either climb a barrier to receive a high density of food reward or instead choose the arm of the maze with no barrier that leads to a lower density of food reward; in these studies, interference with DA transmission decreased selection of the barrier arm and increased choice of the low effort arm with no barrier (Salamone et al., 1994; Cousins et al., 1996; Denk et al. 2005; Mott et al., 2009; Pardo et al. 2012; Mai et al. 2012). T-maze and lever pressing versions of effort discounting tasks also have demonstrated that

DA antagonism shifts choice behavior of rats towards low effort alternatives (Floresco et al. 2008; Bardgett et al. 2009). Another task that has been used is the concurrent fixed-ratio 5 (FR5)/chow feeding procedure in which rats can either lever press on a FR5 schedule for a preferred high-carbohydrate food, or approach and consume less-preferred rodent chow that is freely available in the chamber (Salamone et al., 1991, 2002, 2003, 2007). Under baseline or control conditions, rats tested with this procedure typically obtain most of their food by lever pressing while consuming very little of the chow. Systemic or intra-accumbens administration of DA antagonists, as well as accumbens DA depletions, have been shown to produce a shift in response allocation such that lever pressing is decreased but chow intake is substantially increased (Salamone et al., 1991, 1996, 2002; Cousins and Salamone, 1994; Cousins et al., 1994; Koch et al. 2000; Nowend et al. 2001; Sink et al., 2008; Farrar et al., 2010). This effect is not due to drug-induced changes in food preference or consumption (Salamone et al. 1991; Koch et al. 2000). Moreover, the effects induced by DA antagonism or depletion differ substantially from those seen following pre-feeding (Salamone et al., 1991) or treatment with appetite suppressant drugs such as fenfluramine (Salamone et al., 2002) or cannabinoid CB1 antagonists/ inverse agonists (Sink et al., 2008); these appetite-related manipulations failed to increase chow intake at doses that suppress lever pressing.

In addition to nucleus accumbens DA, there is a body of research implicating adenosine in behavioral activation and effort-related processes (Farrar et al., 2007; Font et al., 2008; Mingote et al., 2008). Adenosine A_{2A} receptors are primarily localized in striatal areas, including both neostriatum and

nucleus accumbens (Jarvis and Williams, 1989; Schiffmann et al., 1991; DeMet and Chicz-DeMet, 2002; Ferre et al., 2004). Furthermore, there is a functional interaction between DA D2 and adenosine A_{2A} receptors (Fink et al., 1992; Ferre, 1997; Hillion et al., 2002; Fuxe et al., 2003). Intra-accumbens injections of the adenosine A_{2A} agonist CGS 21680 decreased locomotor activity (Barraco et al. 1993) and lever pressing on a ratio schedule (Mingote et al. 2008), and also produced changes in effort-related choice behavior similar to the effects of DA antagonism (Font et al. 2008). In contrast, adenosine A_{2A} antagonists have been shown to stimulate locomotor activity (Collins et al. 2010), and increase fixed interval response rate (Randall et al. 2011). Furthermore, several studies have shown that adenosine A_{2A} antagonists are capable of reversing the effects of DA D_2 antagonists on tests of effort-related choice behavior (Farrar et al., 2007; Worden et al., 2009; Mott et al. 2009; Salamone et al., 2009; Nunes et al. 2010; Farrar et al. 2010; Pardo et al. 2012).

The present studies were undertaken to investigate a novel variant of the operant choice procedure that utilizes a progressive ratio (PROG) work requirement. Similar to the FR5/chow feeding choice task, rats tested with this PROG/chow feeding procedure have the choice of either responding on the lever reinforced by presentation of the more preferred operant pellets vs. approaching and consuming the less preferred rodent chow. To assess potential differences in behavioral effects across a variety of conditions, four separate experiments were conducted using the PROG/chow feeding choice task to study the effects of the DA D_2 antagonist haloperidol, the adenosine A_{2A} antagonist MSX-3, the cannabinoid CB1 antagonist/inverse agonist and putative appetite suppressant AM251, and the reinforcer devaluation provided by pre-feeding. In addition to

behavioral pharmacology, DARPP-32-Thr34 immunohistochemistry was utilized to further investigate signal transduction activity in 4 specific regions of interest. Because the nucleus accumbens is implicated in effort-related decision making, both the core and shell divisions were selected for analysis. Furthermore, Schweimer and Hauber (2005) demonstrated the importance of DA signaling in the anterior cingulate cortex in effort-related decision making, therefore the CG1 and CG2 divisions of the cingulate cortex were analyzed for DA activity following a PROG/Choice behavioral session.

It was hypothesized that haloperidol would affect PROG responding in a manner that was not dependent upon decreases in primary food motivation or appetite, and thus would decrease PROG lever pressing but leave chow intake intact. In addition, it was hypothesized that MSX-3 would produce a behavioral effect that was generally opposite to that produced by haloperidol (i.e., increases in PROG lever pressing and decreases in chow intake). Due to the putative appetite suppressant effects of interfering with cannabinoid CB1 receptor transmission (McLaughlin et al. 2003; Salamone et al. 2007; Sink et al. 2008; Randall et al. 2010), it was expected that AM251, as well as pre-feeding, would decrease both lever pressing and chow consumption. Finally, it was hypothesized that DARPP-32 immunoreactivity in nucleus accumbens would be greater in animals with high baseline levels of lever pressing (i.e., "high responders") than in rats with low levels of lever pressing.

MATERIALS AND METHODS

Animals

48 adult male Sprague-Dawley rats (Harlan, Indianapolis, IN, USA) were housed in a colony maintained at 23 °C with 12-h light/dark cycles (lights on at 0:700 h). Rats weighed 300–350 g at the beginning of the study, and were initially food deprived to 85% of their free-feeding body weight for operant training. Rats were fed supplemental chow to maintain the food restriction throughout the study, with water available ad libitum in the home cages. Despite food restriction, rats were allowed modest weight gain throughout the experiment. All animal protocols were approved by the University of Connecticut Institutional Animal Care and Use Committee, and followed NIH guidelines.

Pharmacological Agents

Haloperidol was obtained from Sigma-Aldrich (St. Louis, MO) and was dissolved in 0.2% tartaric acid solution. This solution also served as the vehicle control for haloperidol. MSX-3 was synthesized in the laboratory of Christa Müller (University of Bonn, Bonn, Germany). MSX-3 was dissolved in 0.9% saline solution and then pH adjusted with 1M sodium hydroxide to a final pH of 7.4.. 0.9% saline solution served as the vehicle control for MSX-3. AM251 was synthesized in the laboratory of Alex Makriyannis (Northeastern University, Boston, MA). AM251 was dissolved in dimethylsulfoxide (DMSO), Tween 80, and 0.9% saline at a ratio of 1:1:8. This solution also served as the vehicle control for AM251. All doses were selected based on previous work (Sink et al., 2008; Randall et al., 2011).

Apparatus and testing procedures

Preliminary studies were conducted to determine the optimal rate at which

the schedule progressed (i.e., number of reinforcements per ratio level and by how much the ratio requirement increased with each level). Initial studies used either 9 or 12 separate ratios at each ratio level; these schedules produced animals that received most of their food from consuming the freely available lab chow as opposed to responding on the lever (data not shown). It was found that by having to complete15 ratios at each ratio level rats generally lever pressed at higher levels before switching to chow. Behavioral sessions were conducted in operant conditioning chambers (28x23x23 cm^3; Med Associates). Rats were initially trained to lever press on a continuous reinforcement schedule (30-min sessions; 45-mg pellets, Bioserve, Frenchtown, NJ, USA) for 1 week. Animals were then were shifted to the PROG schedule (30-min sessions, 5 days/week) and trained for several additional weeks. For PROG sessions, the ratio started at FR1 and was increased by one additional response every time 15 reinforcements were obtained (FR1x15, FR2x15, FR3x15,…). Additionally, this schedule included a "time-out" feature that would deactivate the response lever, removing the option for reinforcement for the remainder of the session, if 2 minutes elapsed without a ratio being completed. Upon reaching a stable baseline of responding, rats were then trained on the concurrent PROG/chow-feeding procedure. With this task weighed amounts of laboratory chow (Laboratory Diet, 5P00 Prolab RMH 3000, Purina Mills, St. Louis, MO, USA; typically 15–20 g) were concurrently available on the floor of the chamber during the PROG sessions. At the end of the session, rats were immediately removed from the chamber, and food intake was determined by weighing the remaining food (including spillage). Rats were trained until they attained stable levels of baseline lever pressing and chow intake, after which drug testing began. For

most baseline days rats did not receive supplemental feeding, however, over weekends and after drug tests, rats received supplemental chow in the home cage. On baseline and drug treatment days, rats normally consumed all the operant pellets that were delivered from lever pressing during each session.

pDARPP32(Thr34) visualization and quantification

Free floating coronal sections (50μm) were serially cut using a cryostat freezing microtome (Weymouth, MA, USA) and rinsed in 0.01M PBS (pH 7.4). Sections for pDARPP32-Thr34 visualization were incubated in a solution of 0.1% triton-X, 5% normal donkey serum, and PBS for 30 min to block endogenous staining. pDARPP32(Thr34) sections were transferred into the primary antibody anti-pDARPP32(Thr34) at a concentration of 1:1000 (Santa Cruz Biotechnology, USA) for 48 h incubation. Following the primary antibody treatment, sections were rinsed in PBS and incubated in the secondary antibody, anti-rabbit HRP conjugate, envision plus (DAKO, Carpinteria, CA, USA) for 2 h. The immunohistochemical reaction was developed using diaminobenzidine (DAB) as the chromagen. Processed sections were then mounted to un-coated slides, air dried, and cover-slipped using Cytoseal 60 (Thermo Scientific) 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 SPOT software. Cells that were positively labeled for pDARPP32(Thr 34) were quantified with ImageJ software (v. 1.42, National Institutes of Health sponsored image analysis program) and a macro written to automate particle counting with regions of interest that correspond to pixel

intensity. The size of the "region of interest" section counted was 1000x1000μm. For each animal, cell counts were at levels that correspond to 1.70mm through 0.70mm relative to bregma (Paxinos and Watson, 1998) bilaterally from at least three sections, and counts were averaged across sides and sections. All cell counting was done by an observer who was blind to the experimental conditions.

Experiments

For experiments 1-3a and 4, the same group of animals was used (n=32), while a different group of animals was used for experiment 3b (n=16). For all experiments using drug manipulations (1,2,4), all animals were given a single vehicle injection 1 week prior to the beginning of testing to habituate them to the testing procedures. Experiments 1,2, and 3b used a within-group design in which each rat received all drug or vehicle treatments (IP) in their particular experiment in a randomly varied order (one treatment per week). Baseline training sessions (i.e., non-drug) were conducted 4 days per week.

Experiment 1: *Effects of the dopamine D_2 antagonist haloperidol on PROG/chow feeding choice performance.* To assess the effects of haloperidol, rats were trained on the PROG/chow procedure described above. On test days, animals received injections of 0.025, 0.05, 0.1 mg/kg haloperidol or vehicle, 50 minutes prior to behavioral testing.

Experiment 2: *Effects of the adenosine A_{2A} antagonist MSX-3 on PROG/chow feeding choice performance.* To assess the effects of the adenosine A_{2A} antagonist MSX-3 on progressive choice performance, the same group of animals was used. The animals were first given 1 week off from any drug testing, but continued normal baseline training. On test days, animals received

injections of 0.5, 1.0, 2.0 mg/kg MSX-3 or vehicle, 20 minutes prior to behavioral testing.

Experiment 3: *Effects of appetite manipulations on PROG/chow performance.*

3A. Effects of pre-feeding to reduce food motivation. To assess the effects of pre-feeding on progressive/choice performance, the same group of animals was again given 1 week off from testing. The night before testing, animals were taken off of food restriction and given *ad libitum* access to lab chow. On the test day, several hours before behavioral testing, animals were given *ad libitum* access to Bioserve pellets in the home cage. Performance on test day was then compared to performance on the previous baseline day.

3B: Effects of the cannabinoid CB1 inverse agonist and putative appetite suppressant AM251. To assess these effects, a new group of animals (n=16) were trained on the PROG/choice procedure described above. On test days, animals received injections of 2.0, 4.0, 8.0 mg/kg AM251 or vehicle, 30 minutes prior to behavioral testing, once per week, in a randomly varied order.

Experiment 4: *Effects of PROG/chow responding on pDARPP32-THR34 expression: high vs. low responders.*Following the conclusion of experiment 3, animals (n=32) were given 1 week to re-stabilize their baselines. During the following week, 90 minutes after a baseline training session, animals were sacrificed and perfused to obtain tissue for pDARPP32-(Thr34) immunohistochemical analysis as explained below. For statistical analysis, these animals were divided into two groups; high performers and low performers

determined by a median split of lever pressing performance on the day of perfusion.

Statistical analysis

For the behavioral pharmacology experiments, number of lever presses, maximum ratio achieved, active lever time (in seconds) and chow intake (grams) were analyzed using repeated measures analysis of variance (ANOVA). Non-orthogonal planned comparisons using the ANOVA error term (Keppel, 1991) were used to compare each treatment with the vehicle control. In addition, to provide another statistical measure of the reciprocal relationship between lever pressing and chow intake in each experiment, correlations were performed between number of lever presses and chow intake data collapsed across all conditions within the experiment (e.g., Salamone et al. 2002). For experiment 4, pDARPP32(Thr34) cell counts were analyzed for differences in expression between high and low responders (after a median split of the lever pressing data) for each of 4 regions of interest, and t-tests were performed to determine significant differences.

RESULTS

Experiment 1: *Effects of the DA D_2 antagonist haloperidol.* Haloperidol significantly decreased the number of lever presses ($F(3,93) = 4.598$, $p < 0.01$, see figure 1A). Planned comparisons revealed that there was a significant difference between 0.1 mg/kg haloperidol and vehicle conditions ($p < 0.05$). Coinciding with the decreases in raw numbers of presses, haloperidol also significantly decreased maximum ratio achieved ($F(3,93) = 8.661$, $p < 0.01$,

figure 1B), and the amount of time the lever remained active ($F(3,93) = 6.723$, $p < 0.01$, figure 1C); for both measures, planned comparisons showed a significant difference between vehicle and 0.1 mg/kg haloperidol ($p < 0.05$). Haloperidol produced no significant effects on chow consumption in the dose range tested (figure 1D). However, there was a tendency for animals that had high control rates of responding, and correspondingly low vehicle levels of chow intake, to show increases in chow intake with haloperidol; this was marked by the significant correlation between vehicle number of lever presses and the change in chow consumption from vehicle to the highest dose of haloperidol (r = 0.69, df = 30, $p < 0.05$). Collapsed across all conditions, there was a significant negative correlation between number of lever presses and chow consumption (r = -0.765, df = 126, $p < 0.05$), which demonstrated the overall inverse relationship between lever pressing and chow intake.

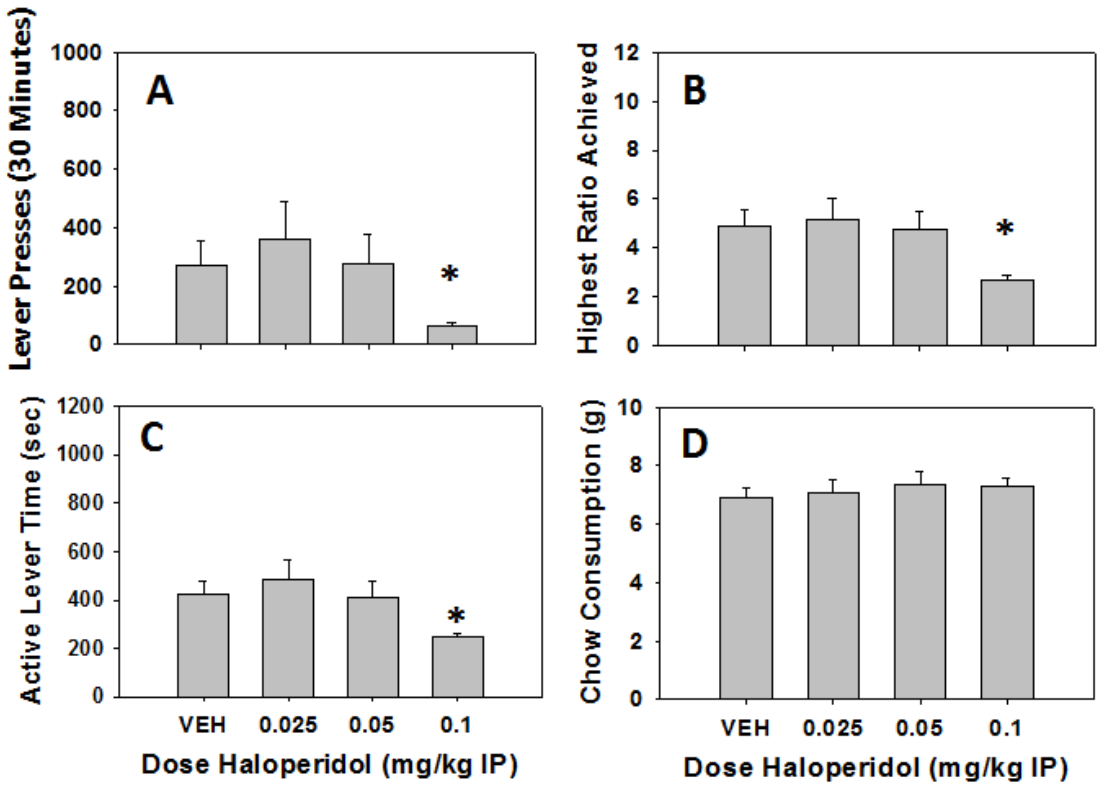

Fig 1 (A-D). Effects of Haloperidol on the concurrent PROG/chow-feeding procedure. (A): Mean (±SEM) number of lever presses in 30 minutes. (B): Mean (±SEM) maximum ratio achieved during test sessions (C): Mean (±SEM) time in seconds that the lever remained active. (D): Mean (±SEM) amount of chow intake. *p<0.05, different from vehicle (VEH).

Experiment 2*: Effects of the adenosine A_{2A} antagonist MSX-3*. MSX-3 affected all four of the observed behavioral measures. MSX-3 significantly increased number of lever presses (F(3,93) = 4.120, p < 0.01, figure 2A), and planned comparisons showed that both 1.0 and 2.0 mg/kg doses of MSX-3 increased number of lever presses compared to vehicle (p < 0.05). There also was a significant increase in maximum ratio achieved (F(3,93) = 8.206, p < 0.01, see figure 2B), with the 1.0 and 2.0 mg/kg doses of MSX-3 differing significantly from vehicle (p < 0.05). Furthermore, MSX-3 increased active lever time (F(3,93) = 3.784, p < 0.05, figure 2C). Planned comparisons showed that only the 2.0 mg/kg MSX-3 significantly affected active lever time (p < 0.05). Conversely, MSX-3 significantly decreased chow intake (F(3,93) = 8.017, p < 0.01, see figure 2D). Planned comparisons revealed that chow intake was decreased at a dose of 2.0 mg/kg MSX-3 compared to vehicle (p < 0.05). As with experiment 1, there was a significant negative correlation between lever pressing and chow intake when the data were collapsed across all conditions (r = -0.781, df = 126, p < 0.05).

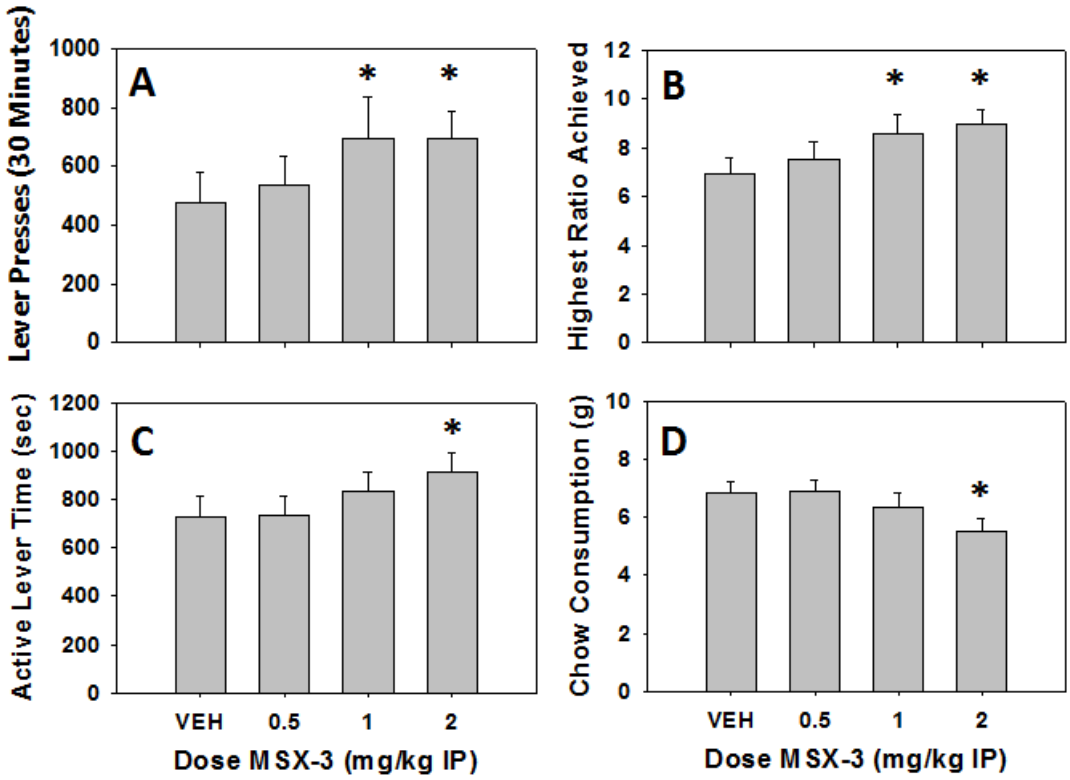

Fig 2. (A-D). Effects of MSX-3 on the concurrent PROG/chow-feeding procedure. (A): Mean (±SEM) number of lever presses in 30 minutes. (B): Mean (±SEM) maximum ratio achieved during test sessions. (C): Mean (±SEM) time in seconds that the lever remained active. (D): Mean (±SEM) amount of chow intake. *p<0.05, different from vehicle (VEH).

Experiment 3: *Effects of appetite-related manipulations on PROG/chow performance: effects of pre-feeding and the putative appetite suppressant AM251*. Experiment 3a studied the effects of pre-feeding on PROG/chow intake choice performance. Compared to the previous baseline day, pre-feeding the animals prior to the session produced marked decreases in number of lever presses ($t = 2.96$, $df = 31$, $p < 0.05$), and maximum ratio achieved ($t = 3.94$, $df = 31$, $p < 0.05$), but no significant effect on active lever time (figures 3A-C). Pre-feeding significantly decreased chow intake ($t = 13.69$, $df = 31$, $p < 0.01$)

compared to previous day baseline performance (figure 3D). There was no significant overall correlation between number of lever presses and chow consumption (r = 0.12, df = 62, n.s.).

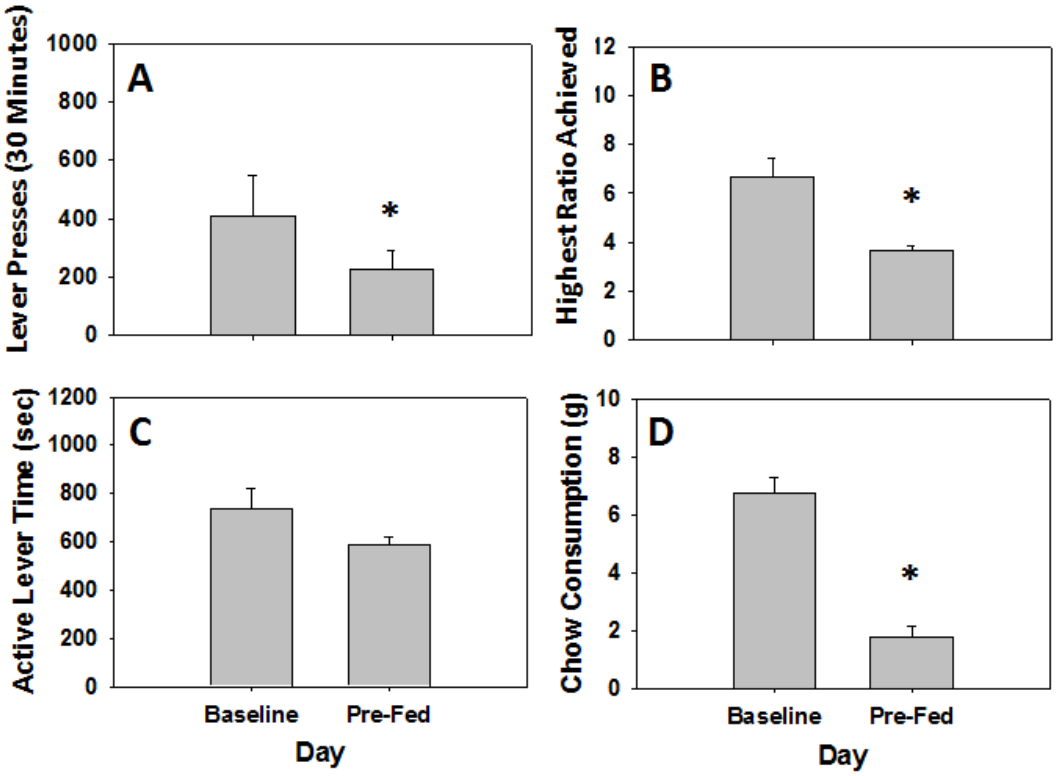

Fig 3. (A-D). Effects of AM251 on the concurrent PROG/chow-feeding procedure. (A): Mean (±SEM) number of lever presses in 30 minutes. (B): Mean (±SEM) maximum ratio achieved during test sessions. (C): Mean (±SEM) time in seconds that the lever remained active. (D): Mean (±SEM) amount of chow intake. *p<0.05, different from Baseline.

Experiment 3b studied the effects of the cannabinoid CB1 inverse agonist AM251 on PROG/chow performance. AM251 decreased the number of lever presses (F(3,45) = 3.891, p < 0.05, figure 4A), and the maximum ratio achieved (F(3,45) = 5.811, p < 0.05, see figure 4B). Planned comparisons showed that with both measures, only the highest dose of 8.0 mg/kg AM251 significantly

differed from vehicle ($p < 0.05$). AM251 did not produce any significant changes in active lever time (figure 4C), but it did produce a significant decrease in chow intake ($F(3,45) = 45.634$, $p < 0.01$, figure 4D), with all doses being significantly different from vehicle ($p < 0.05$). There was no significant overall correlation between number of lever presses and chow consumption (r = -0.05, df = 62, n.s.).

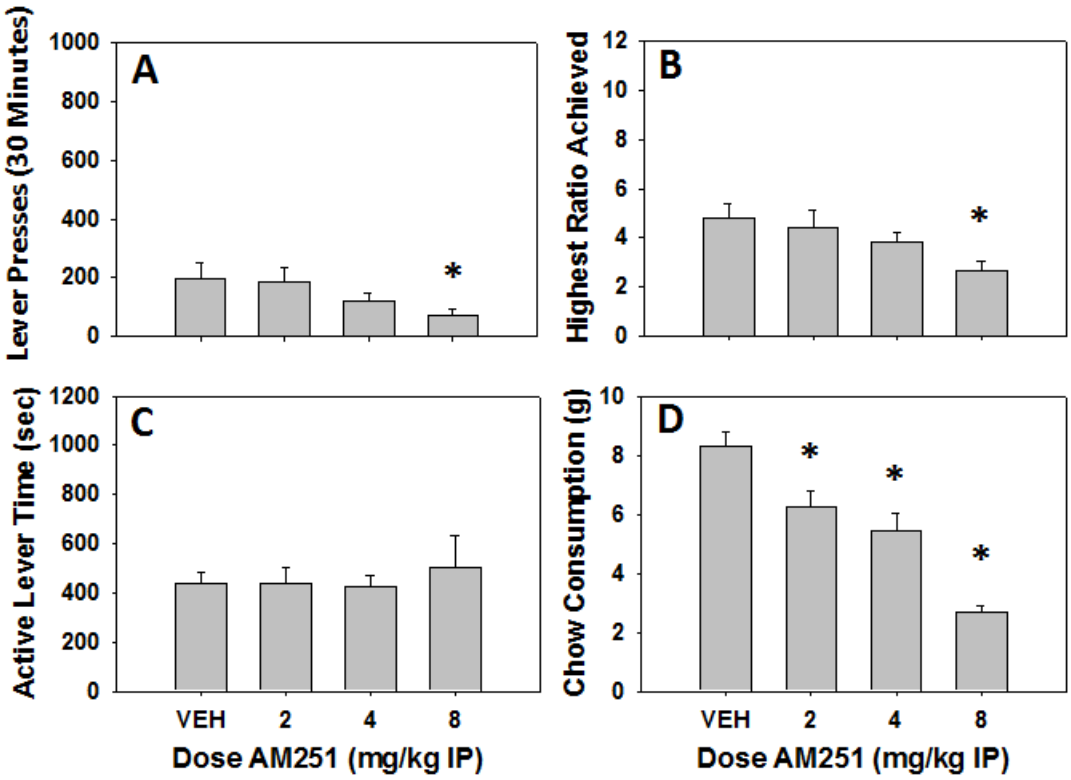

Fig 4. (A-D). Effects of Pre-Feeding on the concurrent PROG/chow-feeding procedure. (A): Mean (±SEM) number of lever presses in 30 minutes. (B): Mean (±SEM) maximum ratio achieved during test sessions. (C): Mean (±SEM) time in seconds that the lever remained active. (D): Mean (±SEM) amount of chow intake. *p<0.05, different from vehicle (VEH).

Experiment 4: *pDARPP-32(Thr34) Immunohistochemistry in high and low responders*. Performance on the progressive ratio/chow feeding choice task

was highly variable; some rats lever pressed fewer than 100 times and had high levels of chow intake, while others lever pressed more than 1000 times and consumed only small amounts of chow. This variability was seen across all the experiments described above, and in some cases was related to the drug effects seen. For example, the effects of haloperidol were more marked in rats with higher control levels of lever pressing. When a median split was done, and high and low lever pressing was used as a factor in a 2 x 4 factorial ANOVA, there was an overall effect of dose ($F(3,90) = 5.071$, $p < 0.05$) and importantly, a dose by group interaction ($F(3,90) = 4.189$, $p < 0.05$). Although the repeated measures ANOVA demonstrated that both low and high responders showed significant decreases in number of lever presses (low responders: $F(3,45) = 2.790$, $p < 0.05$; high responders: $F(3,45) = 4.638$, $p < 0.05$), analysis of effect sizes showed that the suppressive effect of haloperidol on number of lever presses was greater in high responders ($eta^2 = 0.236$) than low responders ($eta^2 = 0.157$). Similar analyses revealed differences between high and low responders in the AM251 experiment, with only the high responders showing a significant drug effect on number of lever presses. Because of this large variability, the final experiment investigated potential neurochemical differences between high and low responders, using pDARPP-32(Thr34) as a marker of signal transduction activity. To analyze the pDARPP-32(Thr34) expression data, a median split based upon behavioral performance during the final test day was performed, yielding two groups: high responders (n = 16, mean = 812.44, SEM = 201.68, range = 205–2852) and low responders (n = 16, mean = 116.31, SEM = 12.81, range = 54–190). Four regions of interest were selected for analysis: cingulate cortex CG1/CG2 and nucleus accumbens core/shell (Figures 5-6).

There was no difference in pDARPP-32(Thr34) expression between high and low responders in CG1 ($t = -0.066$, $df = 28$, n.s.) or CG2 ($t = 0.172$, $df = 25$, n.s.). When examining the nucleus accumbens, the shell showed no significant differences in pDARPP-32(Thr34) expression between high and low responders ($t = 1.415$, $df = 30$, n.s.). In contrast, nucleus accumbens core showed a significant difference in expression between high and low responders ($t = 2.703$, $df = 29$, $p < 0.05$, Figures 5-6).

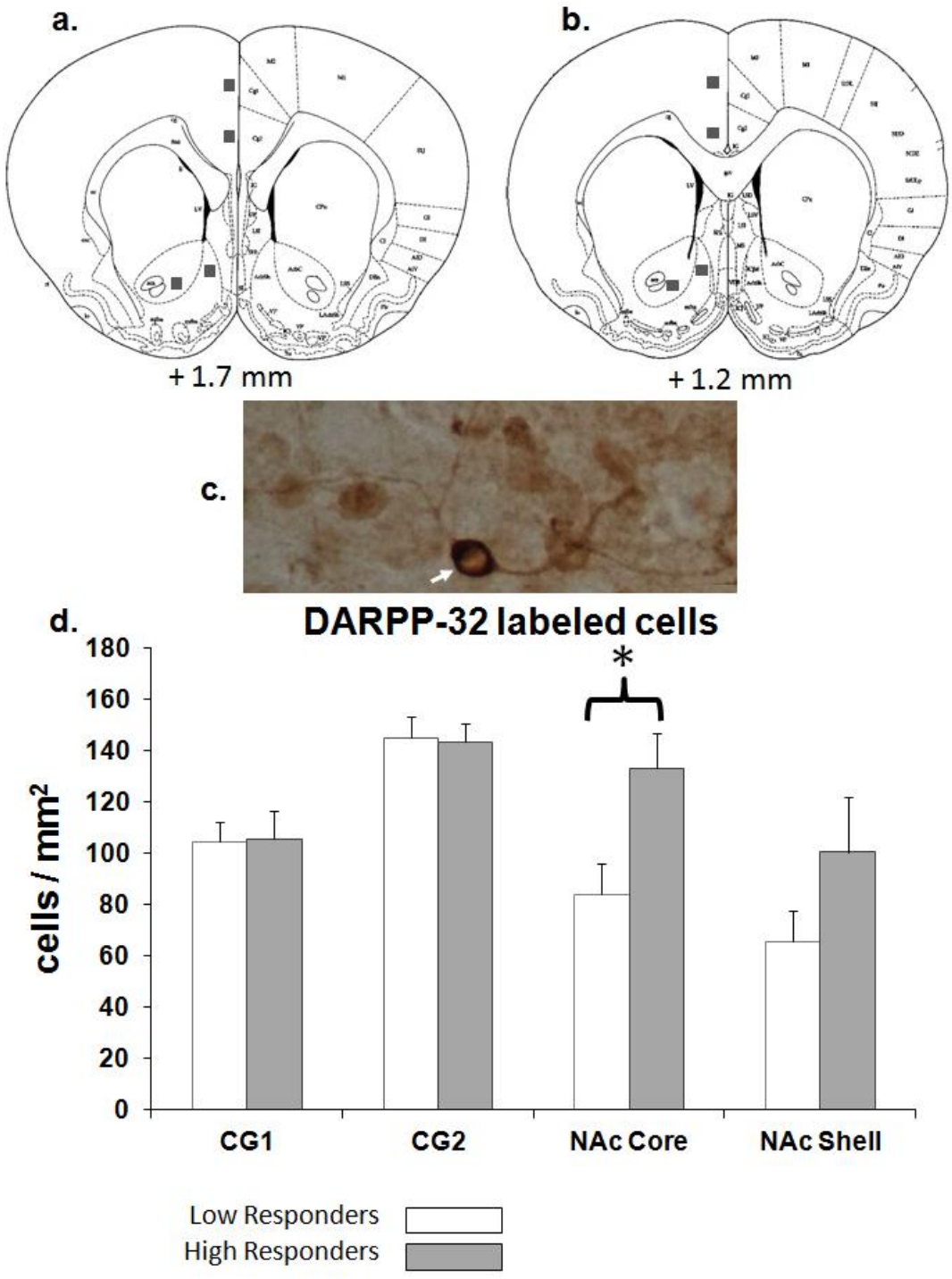

Fig 5 (A-D). pDARPP-32(Thr34) immunocytochemistry – (A and B): Atlas plates (modified from Paxinos and Watson, 1998) with regions of interest denoted by squares. (C): High magnification photomicrograph of pDARPP-32(Thr34) immunoreactive cells at 40x magnification. (D): Mean (±SEM) number of pDARPP-32(Thr34) positive cells counted in each region of interest in high performers and low performers. There were significantly more pDARPP-

32(Thr34) positive cells counted in the nucleus accumbens core of high performers compared to low performers. (* $p < 0.05$)

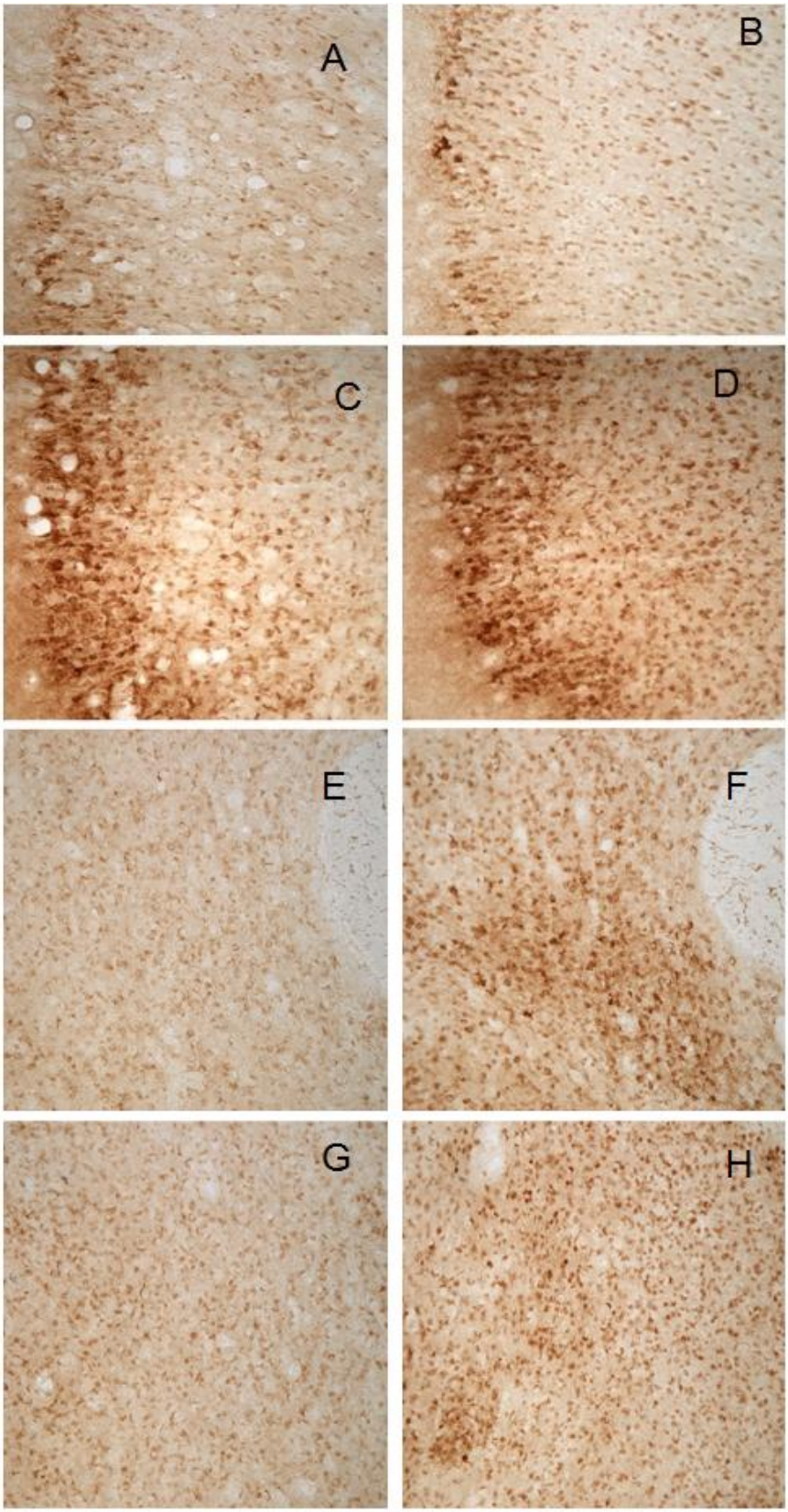

Fig 6 (A-H). Pictures showing expression of pDARPP-32-Thr34 immunoreactivity in CG1 (A-B) CG2 (C-D), Nacb core (E-F) and shell (G-H) in a representative animal of the high responders group (right side images) and of the low responders group (left side images) tested in the concurrent PROG/chow-feeding procedure. Scale bar: 50 microm.

DISCUSSION

The present studies investigated the effects of several manipulations on a concurrent progressive ratio/chow feeding task. Experiment 1 demonstrated that the DA D_2 antagonist haloperidol decreased number or lever presses, maximum ratio achieved, and active lever time (i.e., the time the PROG schedule was active). These findings are consistent with previous studies showing the ability of DA antagonists or accumbens DA depletions to reduce food-reinforced lever pressing in animals responding on the concurrent FR5/choice task (Salamone et al., 1991, 2002; Sink et al., 2008), as well as conventional operant schedules, including various versions of the progressive ratio schedule (Aberman et al., 1998; Hamill et al. 1999). Despite producing clear reductions in multiple measures of operant responding, haloperidol did not decrease chow intake, which indicates that primary food motivation was intact in haloperidol-treated rats. Moreover, previous studies have shown that 0.1 mg/kg haloperidol does not change preference for high carbohydrate food pellets relative to chow, or reduce total intake of either food type (Salamone et al. 1990, 1991). In fact, there was a slight tendency for some rats to show increased chow intake after haloperidol treatment, which was marked by the significant correlation between vehicle lever pressing and the change in chow intake between vehicle and the highest dose of haloperidol. In other words, animals that were high lever press

responders under the vehicle condition, and therefore had correspondingly low levels of chow intake, showed greater increases in chow consumption on haloperidol than low responders did. In fact, the four rats with the highest level of lever pressing showed very substantial increases in chow intake after haloperidol injection (i.e., increases of 4-7 grams compared to baseline). Nevertheless, unlike the previous experiments using the FR5/chow choice procedure (e.g. Salamone et al. 1991, 2002; Sink et al. 2008), haloperidol did not produce an overall significant increase in chow intake. One possible explanation for this pattern is the different levels of chow intake with the two procedures. With the FR5/chow choice procedure, baseline or control levels of lever pressing are relatively high, while chow intake is relatively low (i.e., 1-2 grams), making it possible to observe an increase in chow intake with administration of a DA antagonist. In contrast, baseline or control levels of chow intake are much higher with the PROG/chow choice procedure (i.e., 7-8 grams), and are near ceiling levels of chow intake for a 30 minute period without water being available. For example, Randall et al. (2010) demonstrated that food-restricted rats in a free feeding study consume approximately 8 grams of chow in a 30-minute period. Thus, with the PROG/chow choice procedure, it is difficult to observe drug-induced increases in chow intake in animals that are already eating chow at maximal or near maximal levels.

Experiment 2 showed that the adenosine A_{2A} antagonist MSX-3 produced effects that were opposite to those of haloperidol; MSX-3 increased number of lever presses and maximum ratio achieved, and also increased the amount of time that animals kept the lever active during the session. This is consistent with previous work showing that adenosine A_{2A} antagonists have

stimulant-like properties. For example, the adenosine A_{2A} antagonists MSX-3 and istradefylline were both shown to increase lever pressing on a fixed interval 4-minute schedule, which generates a relatively low baseline rate of responding (Randall et al., 2011). Nevertheless, the present results are the first to demonstrate that an adenosine A_{2A} antagonist can increase lever presses and break point on a progressive ratio schedule. In addition, the PROG/chow feeding choice procedure allowed for parallel assessment of food intake, and MSX-3 was observed to decrease chow consumption at the highest dose. Interestingly, although MSX-3 and haloperidol produced opposite effects on measures of PROG lever pressing and chow intake, in both experiments, the reciprocal relation between lever pressing and chow intake was preserved, as indicated by the high negative correlations between lever pressing and chow intake across all treatments (-0.76 and -0.78). This inverse correlation between lever pressing and chow intake has been reported in previous experiments studying the effects of DA antagonists or depletions on FR5/chow feeding choice performance (Cousins et al. 1993; Salamone et al. 2002; Sink et al. 2008).

Experiment 3 was conducted to determine the effect of appetite-related manipulations on PROG/chow feeding choice performance, in order to provide a contrast with the effects of haloperidol. Two different appetite manipulations were employed: pre-feeding, and administration of a cannabinoid CB1 receptor antagonist/inverse agonist. Pre-feeding animals prior to their test session, which was used to reduce food motivation and thereby devalue the food reinforcement (Salamone et al. 1990; Aberman and Salamone 1999), produced marked decreases in number of lever presses and highest ratio achieved. But, unlike the effects of haloperidol, pre-feeding also substantially reduced chow consumption.

In experiment 3b, the CB1 receptor antagonist/inverse agonist AM251 produced similar effects to those resulting from pre-feeding. CB1 antagonists/inverse agonists are putative appetite suppressant drugs that have been shown to decrease food intake in animals (Chen et al., 2004; Colombo et al., 1998; Shearman et al., 2003; McLaughlin et al., 2003, 2005, 2006; Sink et al., 2008, Randall et al., 2010) and humans (Pi-Sunyer et al., 2006; Despres et al., 2005; Van Gaal et al., 2005). On the PROG/chow feeding choice task, AM251 decreased number of lever presses, maximum ratio achieved, and chow consumption. Thus, the pattern of effects on lever pressing and chow intake produced by pre-feeding and AM251 differed markedly from those produced by haloperidol. Moreover, while there was a high inverse correlation between lever pressing and chow intake in the haloperidol experiment, there were no significant correlations between these measures in the pre-feeding and AM251 experiments. This analysis shows that the inverse relation between lever pressing and chow intake, which is evident under baseline conditions and also in the haloperidol experiment, is not shown when primary food motivation is reduced by pre-feeding or drugs, because under appetite-related manipulations rats show decreases in both food reinforced lever pressing and chow consumption (Salamone et al. 2002; Sink et al. 2008). Taken together, these results demonstrate that it is exceedingly unlikely that haloperidol is decreasing PROG lever pressing because of a reduction in primary food motivation or reinforcement. Clearly, in the absence of parallel measures of food intake, progressive ratio break points should not be used as markers of food "reward", or hedonic reactivity to food.

An important aspect of the PROG/choice procedure is that performance is characterized by substantial individual variability. While some rats lever pressed relatively little (i.e., < 100 times) and had high levels of chow intake, others lever pressed much more (i.e., up to > 2800 responses) and ate relatively little chow. Analysis of the first experiment showed that the effects of haloperidol on lever pressing were greater in rats with higher control levels of lever pressing. Experiment 4 employed pDARPP-32-(Thr34) immunohistochemistry to determine if there were neurochemical differences between high responders and low responders. The entire group of animals was divided in two by a median split based upon numbers of lever presses, and DARPP-32 expression was determined in four regions of interest. High responders did not differ from low responders in terms of DARPP-32 expression in CG1 or CG2 regions of anterior cingulate cortex, or in nucleus accumbens shell. However, high responders did show greater DARPP-32 expression in nucleus accumbens core than low responders. DARPP-32 immunoreactivity was used to provide a signal transduction marker of neural activity, and evidence indicates that DA acting through the D_1 receptor and the G proteins (G_s/G_{olf}) activates adenylate cyclase activity, thereby stimulating PKA-mediated phosphorylation of DARPP-32 at the Thr34 site (Nishi et al. 2000; Kuroiwa et al. 2008; Bateup et al. 2008; Yger and Girault 2011). DARPP-32 expression has been used to study of drug action (Bateup et al. 2008; Yger and Girault 2011), and a few studies have focused on changes in DARPP-32 immunoreactivity associated with behavioral manipulations. Danielli et al. (2010) demonstrated that DARPP-32 showed increased expression in nucleus accumbens shell during the first exposure to a novel food. Recently, Segovia et al. (2012) reported that

pDARPP-32(Thr34) expression in nucleus accumbens shell and core was increased in animals undergoing FR5 operant training. Although several neurochemical factors can influence pDARPP-32(Thr34) production, it is reasonable to suggest that the higher level of pDARPP-32(Thr34) expression in high responders relative to low responders could reflect greater DA transmission in the animals working harder on the lever pressing component of the task (Segovia et al. 2011, 2012). If so, this could indicate that individual differences in work output are related to increased DA transmission in ventral striatum, as recently shown in a human imaging study (Treadway et al. 2012).

In summary, the DA antagonist haloperidol reduced the number of lever presses and highest ratio achieved but did not significantly affect chow intake. In contrast, the adenosine A_{2A} antagonist and minor stimulant MSX-3 increased lever presses and highest ratio achieved, but decreased chow consumption. Pre-feeding and administration of the cannabinoid CB1 antagonist/inverse agonist AM251 decreased lever presses, highest ratio achieved, and chow intake. Thus, the effects of DA antagonism differed greatly from those produced by reduced food motivation or decreases in CB1 transmission. It appears unlikely that haloperidol is reducing PROG responding because of a general reduction in the valuation of food reinforcement. Furthermore, DA-related signal transduction activity (pDARPP-32(Thr34) expression) was greater in high responders (i.e., rats with high lever pressing output) compared to low responders, indicating that nucleus accumbens core signal transduction activity is related to individual differences in work output. Future studies should compare the effects of DA D_1 and D_2 antagonists, and should determine if adenosine A_{2A} antagonism is capable of reversing the effects of DA antagonism. Studies comparing

cannabinoid CB1 inverse agonists with neutral antagonists (e.g. Sink et al. 2008; Randall et al. 2010) would be useful for further exploration of the role of CB1 receptor signaling in performance on this procedure. Finally, additional neurochemical correlates should be investigated for their possible relation to lever pressing output on this task, including microdialysis studies of DA release (Segovia et al. 2011), and other markers of signal transduction activity (e.g. c-Fos, pDARPP-32(Thr75)) in different striatal cell types (e.g. encephalin or substance P positive neurons; Segovia et al. 2012).

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CHAPTER 3:

EFFECT OF SUBTYPE –SELECTIVE ADENOSINE RECEPTOR ANTAGONISTS ON BASAL OR HALOPERIDOL-REGULATED STRIATAL FUNCTION: STUDIES OF C-FOS EXPRESSION AND MOTOR ACTIVITIES IN OUTBRED AND A_{2A}R KO MICE.

Abstract

Dopamine (DA) regulates behavioral activation, and typical antipsychotic drugs produce psychomotor slowing. In contrast, minor stimulants that act on adenosine can facilitate behavioral activation at low doses. We studied the locomotor stimulating properties of adenosine antagonists with different selectivity profiles for adenosine receptors, and their impact on the impaired locomotion produced by the DA D_2 receptor antagonist haloperidol, as well as c-Fos expression in 5 striatal subregions. Additionally we assessed the impact of haloperidol on locomotion in adenosine A_{2A} receptor knockout (A_{2A}RKO) mice. Male CD1 and A_{2A}RKO mice were evaluated for horizontal and vertical locomotion in an open field. Theophylline (5.0-15.0 mg/kg) and the A_{2A} antagonist MSX-3 (2.0 mg/kg) increased horizontal locomotion. The A_1 antagonist CPT did not. Haloperidol (0.05-0.1 mg/kg) produced a dose dependent decrease in both measures of locomotion. Co-administration of theophylline (10.0-15.0 mg/kg), MSX-3 (1.0-3.0 mg/kg) and CPT (9.0 mg/kg) reversed haloperidol effects. A_{2A}RKO mice were resistant to the effects of haloperidol. Although adenosine antagonists did not increase c-Fos expression

on their own, theophylline and MSX-3, but not CPT, attenuated haloperidol induction of c-Fos expression. Our results indicate that D_2 and A_{2A} receptors interact to regulate exploratory behaviors, and c-Fos immunoreactivity in all striatal subregions.

INTRODUCTION

In rodents, locomotor activity is an innate exploratory behavior (Kelley, 1993) regulated by a complex cascade of neurochemical interactions involving the basal ganglia and related brain areas. Locomotion in rodents can be used as a measure of motor function, but also as a marker of behavioral activation in response to environmental events such as novelty (Hooks and Kalivas, 1995) or scheduled presentation of food pellets to food-deprived animals (Salamone, 1988). Dopamine (DA) is a key neurotransmitter in the regulation of behavioral activation, and it is well known that dopaminergic mechanisms play an important role in regulating locomotor activity (Fishman et al., 1983). In particular, nucleus accumbens (Nacb) DA has been clearly implicated in the regulation of spontaneous, novelty-induced, food-induced, and drug-induced locomotion (Kelley and Iversen, 1976; Koob et al., 1978; Ahlenius et al., 1987; McCullough and Salamone, 1992; Correa et al., 2002, 2004). Administration of DA antagonists decreases a variety of activities, including horizontal and vertical locomotion (Janssen et al., 1966; Salamone, 1987). Locomotion in the open field has been shown to be effectively suppressed by both D_1 and D_2 antagonists (Janssen et al., 1966; Beninger, 1983; Fishman et al., 1983; Molloy et al., 1986). Decreases in locomotion induced by DA D_2 antagonists could be related to the psychomotor slowing that is induced in humans treated with these drugs (Heinz et al., 1998). In contrast, psychostimulant drugs by potentiating DA function, either directly or indirectly, can facilitate behavioral activation (Antoniou et al., 1998; Karcz-Kubicha et al., 2003; Quarta et al., 2004), although at high doses they can induce stereotypies that translate into reduced interaction with the environment (Antoniou et al., 1998).

Within the last few years, evidence has begun to emerge indicating that brain adenosine plays an important role in regulating the behavioral functions of the basal ganglia (Ferré et al., 1997; Hauber, 1998; Svenningsson et al., 1999). Striatal areas that are rich in DA, including neostriatum and Nacb, also have a high concentration of adenosine receptors (Jarvis and Williams, 1989; Schiffmann et al., 1991; DeMet and Chicz-DeMet, 2002; Ferré et al., 2004). Several subtypes of adenosine receptors are expressed in the brain, of which the A_1 and A_{2A} adenosine receptor subtypes are most prevalent in the basal ganglia. Moreover, A_{2A} receptors are expressed at very high levels in the striatum and Nacb (Ferré et al., 1993; Svenningsson et al., 1997; Tanganelli et al., 2004; Pinna et al., 2005), while A_1 receptors are expressed throughout the brain (Fastbom et al., 1986, 1987a,b; Svenningsson et al., 1997). There is considerable interest in the behavioral actions of drugs that modulate adenosine receptor function. Nonselective adenosine receptor antagonists such as caffeine act as minor stimulants and are commonly consumed by humans to produce activation, providing "energy" and alertness (Antoniou et al., 2005; Ferré, 2008; Ferré et al., 2008b; Reissig et al., 2009). Consistent with its profile as a minor stimulant, caffeine has been shown repeatedly to enhance locomotor activity in rodents (Garrett and Holtzman, 1994; Daly and Fredholm, 1998; Karcz-Kubicha et al., 2003; Antoniou et al., 2005). Moreover, pharmacological modulation of adenosine A_{2A} receptors has a profound influence on motor control. Thus, while A_{2A} receptor stimulation exerts a suppressive effect on motor function (Barraco et al., 1993, 1994), adenosine A_{2A} antagonists can increase locomotion (Popoli et al., 1998; Antoniou et al., 2005; Collins et al., 2010). The locomotor stimulant effects of A_1 antagonists appear to be more variable and may depend upon the

selectivity of the particular drug used (Marston et al., 1998; Popoli et al., 1998; Karcz-Kubicha et al., 2003; Collins et al., 2010). Previous evidence indicates that adenosine A_{2A} receptors in Nacb may be important for mediating the locomotor effects of A_{2A} antagonists. Thus, when injected directly into the Nacb adenosine A_{2A} agonists have been shown to suppress locomotion (Barraco et al., 1993, 1994; Hauber and Munkle 1997) and A_{2A} antagonists produce a dose-related increase in locomotor activity (Nagel et al., 2003).

In neostriatum and Nacb, DA D_2 receptors are reported to interact with high affinity with the adenosine subtype A_{2A} receptors on the enkephalin-positive striatopalllidal neurons (Schiffmann et al., 1991; Fink et al., 1992; Ferré, 1997; Rosin et al., 1998; Svenningsson et al., 1999; Hettinger et al., 2001; Chen et al., 2001; Hillion et al., 2002; Fuxe et al., 2003; Ferré et al., 2008a). Thus, adenosine A_{2A} antagonists are being intensively studied for their potential antiparkinsonian effects (Malec, 1997; Hauber et al., 2001; Moo-Puc et al., 2003; Correa et al., 2004; Antoniou et al., 2005; Ishiwari et al., 2007; Salamone et al., 2008a,b; Varty et al., 2008) and also for the treatment of anergic symptoms such as psychomotor slowing and fatigue that are seen in patients with depression and other disorders (Farrar et al., 2007, 2010; Salamone et al., 2007, 2009, 2010; Nunes et al., 2010).

Because of the interest in the neurochemical interactions involved in psychomotor slowing and motor control, as well as the interest in identifying novel treatments for effort-related symptoms of depression, and non-dopaminergic treatments for parkinsonism, it is important to characterize the effects of adenosine antagonists in both human clinical trials and animal models. Most of the preclinical studies of DA/adenosine interactions have been

conducted in rats. However, studies of these pharmacological interactions in mice are important for establishing generalizations across multiple species (McKerchar and Fowler, 2005). Moreover, genetic knockout models circumvent the intrinsic limitations of pharmacological agents with partial specificity, and these tools are widely available in mice. In the present work we studied the locomotor stimulating properties of adenosine antagonists with different selectivity profiles for adenosine receptors, and their impact on the locomotor suppression induced by the DA D_2 receptor antagonist haloperidol. Thus, we characterized the locomotor effects of low doses of the non-selective adenosine antagonist theophylline, the A_1 antagonist CPT, and the selective A_{2A} antagonist MSX-3, either alone or in combination with haloperidol. We also determined if adenosine A_{2A}R KO mice are resistant to the locomotor effects of haloperidol. Finally the impact of all these pharmacological manipulations on striatal and Nacb areas was studied using c-Fos expression as a marker of neural activity.

MATERIALS AND METHODS

Animals

A total of 226 CD1 adult male mice (n=7-10 per group) purchased from Harlan-Interfauna Ibérica S.A. (Barcelona, Spain) were 6 weeks old (25-30 g) at the beginning of the study. Male mice lacking the A_{2A} adenosine receptor type and wild-type (WT) littermates weighed 25–30 g at the beginning of the study (Universite Libre de Bruxelles, Brussels, Belgium), and were generated as previously reported (Ledent et al., 1997; Soria et al., 2006) from a CD1 background. 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 ± 2 ºC with 12-h light/dark cycles (lights on at 13:00 hours). 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).

Pharmacological agents

All drugs were administered intraperitoneally (IP). Theophylline (TOCRIS Bioscience) was dissolved in 0.9% w/v saline (pH=7.4). MSX-3 ((*E*)-phosphoric acid mono-[3-[8-[2-(3- methoxphenyl)vinyl]-7-methyl-2,6-dioxo-1-prop-2-ynyl-1,2,6,7-tetrahydropurin-3-yl]propyl] ester disodium salt) was synthesized at the laboratory of Dr. Christa Müller at the Pharmazeutisches Institut, Universität Bonn, in Bonn, Germany (Hockemeyer et al., 2004). MSX-3 (free acid) was dissolved in 0.9% w/v saline (pH=7.4). CPT (8-cyclopentyltheophylline) (Sigma Química C.O), was dissolved in 0.9% w/v saline (pH=7.4). Haloperidol (Sigma Química C.O), a relatively selective DA D_2 family receptor antagonist, selected because it is a widely prescribed antipsychotic drug, was dissolved in a 0.3% tartaric acid solution in water (pH=4.0), which also was used as the vehicle control. Doses of all four drugs were taken from previous mouse and rat studies from our laboratories (Ishiwari et al., 2007; Pardo et al., 2012).

Apparatus and testing procedures

Open-field Locomotion. Mice were handled and weighed twice a week during 10 weeks after arriving to the laboratory. However, the animals were not pre-exposed to the behavioral paradigm. Testing was performed in an open-field, which consisted of a Plexiglas cylinder with translucent walls (30 cm in

diameter and 30 cm high) and an opaque floor divided into four equal quadrants by two intersecting lines. On the test day, treatments were administered acutely IP; haloperidol 50 minutes, and CPT, MSX-3 or theophylline, 20 minutes before the open field test started. After these time intervals, 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. Locomotor activity was registered manually. For horizontal locomotion an activity count was registered each time the animal crossed from a 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.

c-Fos visualization and quantification. Free floating coronal sections (40 um) 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 (H_2O_2) for 30 min to block endogenous staining. Sections were then rinsed in PBS (3 × for 5 min) and transferred into the primary antibody, anti-c-Fos (Calbiochem, Germany) for a 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 2 h. The immunohistochemical reaction was developed using diaminobenzidine (DAB) as the chromagen. Processed sections were then mounted to gelatin-coated slides, air dried, and cover-slipped using Cytoseal 60 (Thermo Scientific) 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

(Nacb core, Nacb shell, DLS) were magnified at 20X and captured digitally using SPOT 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 was used for statistical analysis.

Experiments

A total of seven experiments were performed in this study. All experiments used a between-groups design.

Experiment 1: *Effect of haloperidol on locomotor activity in the open-field.* Mice (N=30) received one injection of haloperidol (0.0, 0.025, 0.05 or 0.1 mg/kg) 50 min before being tested in the open field for 15 min. Horizontal (Fig. 1A) and vertical (Fig. 1B) locomotion were simultaneously recorded.

Experiment 2. *A. Effect of theophylline on locomotor activity in the open field.* Mice (N=32) received one dose of theophylline (0.0, 5.0, 10.0 or 15.0 mg/kg) 20 minutes before the open field test started. Horizontal (Fig. 2A) and vertical (Fig. 3A) locomotion were simultaneously recorded.

Experiment 2. B. *Effect of theophylline on haloperidol reduction of locomotion in the open field.* Mice (N=32) received one injection of haloperidol (0.1 mg/kg) 30 minutes before receiving one injection of theophylline (0.0, 5.0, 10.0 or 15.0 mg/kg). Twenty minutes after the second injection animals were introduced in the open field and testing started. Horizontal (Fig. 2B) and vertical (Fig. 3B) locomotion were simultaneously recorded.

Experiment 3. A. *Effect of CPT on locomotor activity in the open field.* Mice (N=34) were tested during 15 minutes in the open field after an IP

injection of CPT (0.0, 3.0, 6.0 or 9.0 mg/kg) 20 minutes before testing. Horizontal (Fig. 4A) and vertical (Fig. 5A) locomotion were simultaneously recorded.

Experiment 3. B. *Effect of CPT on haloperidol reduction of locomotion in the open field.* Mice (N=34) received one haloperidol injection (0.1 mg/kg) plus a CPT (0.0, 3.0, 6.0 or 9.0 mg/kg) injection at the same time intervals as in experiment 2.B. Horizontal (Fig. 4B) and vertical (Fig. 5B) locomotion were simultaneously recorded.

Experiment 4. A. *Effect of MSX-3 on locomotor activity in the open field.* Mice (N=32) received an injection of MSX-3 (0.0, 1.0, 2.0 or 3.0 mg/kg) 20 minutes before being tested during 15 minutes in the open field. Horizontal (Fig. 6A) and vertical (Fig. 7A) locomotion were simultaneously recorded.

Experiment 4. B. *Effect of MSX-3 on haloperidol reduction of locomotion in the open field.* Mice (N=32) were treated with haloperidol (0.1 mg/kg) and MSX-3 (0.0, 1.0, 2.0 or 3.0 mg/kg) and tested as described in experiment 2.B. Horizontal (Fig. 6B) and vertical (Fig. 7B) locomotion were simultaneously recorded.

Experiment 5: *Effect of haloperidol on A_{2A}R KO mice in the open-field.* Mice (WT N=19 and KO N=20) received one injection of haloperidol (0.0 or 0.1 mg/kg) 50 min before being tested in the open field for 15 min. Horizontal (Fig. 8A) and vertical (Fig. 8B) locomotion were simultaneously recorded.

Experiment 6: *Effect of Theophylline, CPT or MSX-3 on c-Fos immunoreactivity in different areas of the Nacb and striatum.* After completion of the open field session, mice (N=25) were anesthesized and perfused, and

brain sections were stained for c-Fos immunoreactivity as described above. Thus, mice received treatments of either vehicle, 15.0 mg/kg theophylline, 9.0 mg/kg CPT, or 3.0 mg/kg MSX-3, 140 minutes before anesthesia.

Experiment 7: *Effect of theophylline, CPT or MSX-3 on c-Fos immunoreactivity after haloperidol administration in different areas of the Nacb and striatum.* After completion of the open field session, mice (N=36) were anesthesized and perfused, and brain sections were stained for c-Fos immunoreactivity as described above. All mice were treated with tartaric acid vehicle or haloperidol (0.1 mg/kg) 140 min before anesthesia, and then 30 min after the first injection they received treatments of either saline vehicle, 15.0 mg/kg theophylline, 9.0 mg/kg CPT, or 3.0 mg/kg MSX-3.

Statistical analyses

Number of horizontal and vertical locomotion counts were analyzed separately in all the experiments. With the exception of experiment 5, all the other experiments were analyzed using a one way between-groups simple ANOVA followed by non-orthogonal planned comparisons using the overall error term, comparing vehicle to the other doses in experiments with no haloperidol and the haloperidol plus vehicle treatment with each of the other treatment conditions (including the vehicle alone group) in the reversal studies (Keppel, 1991). Since the behavior of animals receiving a single injection of vehicle or two separate injections of vehicle were not statistically different, and in order to reduce the total number of animals, only one vehicle group (represented in the graphs as a discontinuous line) was used for experiments A and B. In experiment 5 a two-way ANOVA and Tukey *post hoc* test were used.

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

RESULTS

Experiment 1: *Effect of haloperidol on locomotor activity in the open-field.* One way ANOVA for the haloperidol dose factor (0.0, 0.025, 0.05 or 0.1 mg/kg) showed a significant effect of dose on horizontal locomotion ($F(3,36)=12.42$, $p<0.01$; Fig. 1A). Planned comparisons yielded significant differences between vehicle and the two highest doses of haloperidol ($p<0.01$). The ANOVA for the vertical locomotion data (Fig. 1B) showed a significant effect of the factor haloperidol dose ($F(3,36)=10.28$, $p<0.01$). Planned comparisons revealed significant differences between vehicle and the lowest ($p<0.05$) and highest ($p<0.01$) doses of haloperidol. Because the 0.1 mg/kg dose of haloperidol consistently reduced horizontal and vertical locomotion, for the following experiments we used this dose to study the potential reversal effects of adenosine antagonists on haloperidol-induced suppression of locomotion.

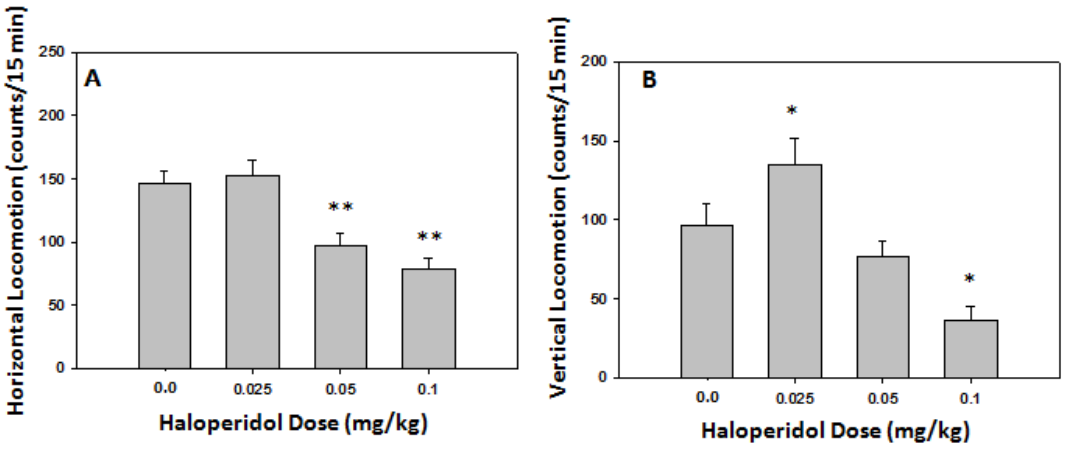

Fig. 1 A and B. Effect of haloperidol (0.0, 0.025, 0.05 or 0.1 mg/kg) on A) horizontal locomotion and B) vertical locomotion. Mean (±SEM) number of counts in the open field. *p<0.05 **p<0.01 significantly different from vehicle.

Experiment 2A. *Effect of theophylline on locomotor activity in the open field.* One-way ANOVA for the theophylline dose factor (0, 5, 10 or 15 mg/kg) yielded a significant effect on horizontal locomotion (Fig. 2A; $F(3,28)=4.2$, $p<0.05$). Planned comparisons showed that all doses were different from control condition, 5.0, 15.0 mg/kg ($p<0.05$) and 10.0 mg/kg ($p<0.01$). However, the one-way ANOVA for the theophylline dose factor showed no significant effect on vertical locomotion (Fig. 3A).

Experiment 2B. *Effect of theophylline on haloperidol reduction of locomotion in the open field*. The one-way ANOVA for the drug treatment factor with 5 levels (Veh-Veh, HP-Veh, HP-5, HP-10 or HP-15) showed a significant effect of the treatment on horizontal locomotion ($F(4,35)=25.25$, $p<0.01$). Planned comparisons demonstrated that haloperidol reduced horizontal locomotion compared to vehicle ($p<0.01$), and that theophylline at all doses significantly increased open-field locomotor activity relative to haloperidol (Fig. 2B; $p<0.01$). The ANOVA for the drug treatment factor on the vertical locomotion activity showed also a significant effect ($F(4,35)=6.26$, $p<0.01$). Planned comparisons revealed significant differences between animals treated with haloperidol compared to animals treated with vehicle ($p<0.01$). Moreover, the two highest doses of theophylline, 10.0 and 15.0 mg/kg ($p<0.05$), reversed the suppression on locomotion produced by haloperidol. These results can be seen in Fig. 3B.

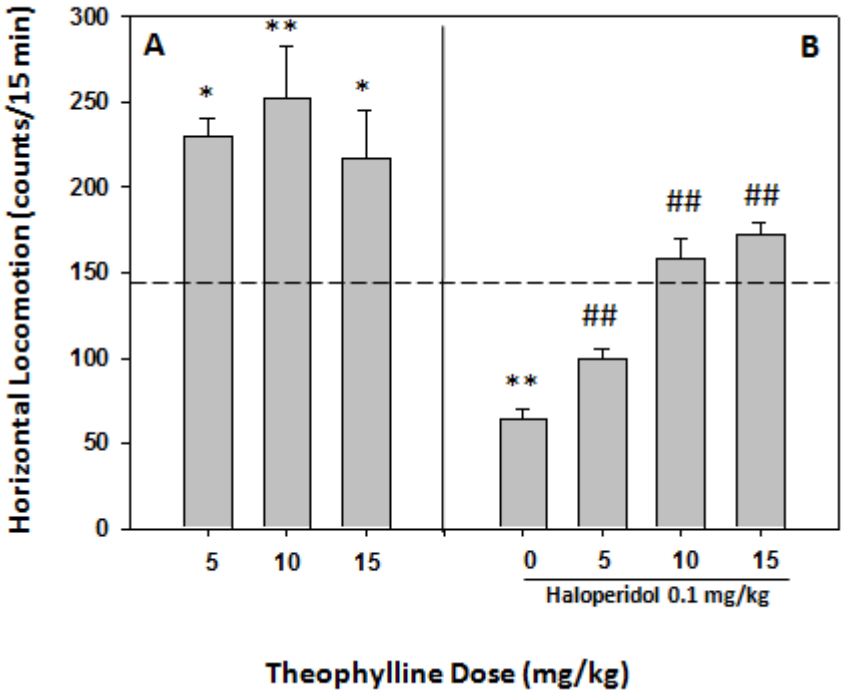

Fig. 2 A and B. Effects of different doses of theophylline alone (A), or in combination with haloperidol (0.1 mg/kg) (B) on mice horizontal locomotion. The discontinuous horizontal line represents the mean value for the vehicle group. Mean (±SEM) number of counts in the open field. *p<0.05, **p<0.01 significantly different from vehicle. ##p<0.01 significantly different from HP/Veh.

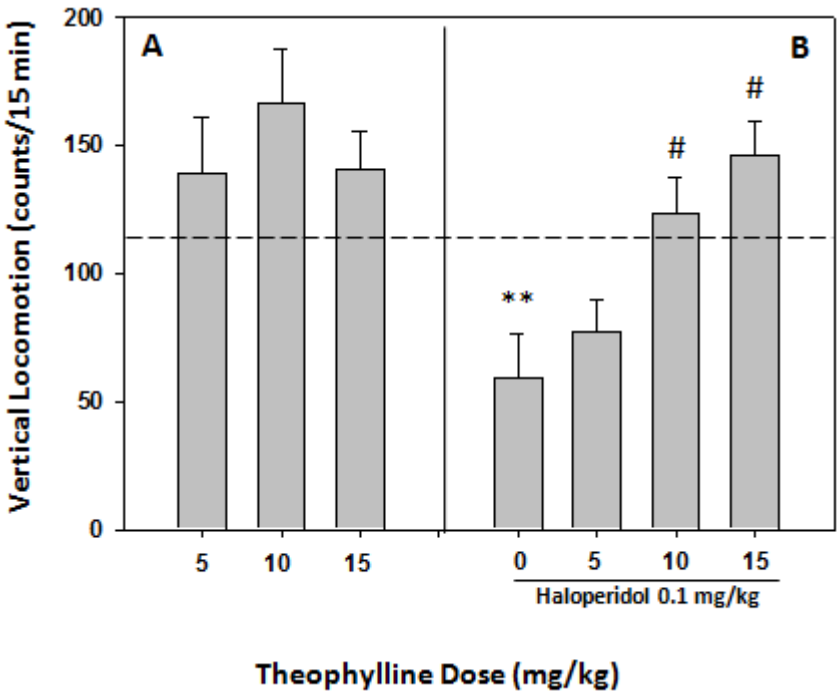

Fig. 3 A and B. Effects of different doses of theophylline alone (A), or in combination with haloperidol (0.1 mg/kg) (B) on mice vertical locomotion. The discontinuous horizontal line represents the mean value for the vehicle group. Mean (±SEM) number of counts in the open field. **p<0.01 significantly different from vehicle. #p<0.05 significantly different from HP/Veh.

Experiment 3A. *Effect of CPT on locomotor activity in the open field.* The one-way ANOVA for the factor CPT dose (0.0, 3.0, 6.0 or 9.0 mg/kg) did not yield statistical significance, for either horizontal locomotion (Fig. 4A), or vertical locomotion (Fig. 5A).

Experiment 3B. *Effect of CPT on haloperidol reduction of locomotion in the open field.* The ANOVA for the factor drug treatment (Veh-Veh, HP-Veh, HP-3, HP-6 or HP-9) yielded a significant effect on horizontal locomotion ($F(4,37)=7.52$, $p<0.01$; Fig. 4B). Planned comparisons indicated that haloperidol suppressed locomotion compared to vehicle ($p<0.01$). The group that received

haloperidol plus 9.0 mg/kg of CPT and the group haloperidol plus vehicle were significantly different (p<0.05). The one-way ANOVA for the vertical locomotion data showed a significant effect of the treatment (F(4,37)=11.48, p<0.01; Fig. 5B). Planned comparisons indicated that haloperidol suppressed rearing compared to vehicle (p<0.01). However, none of the doses of CPT reversed the effects of haloperidol on vertical locomotion.

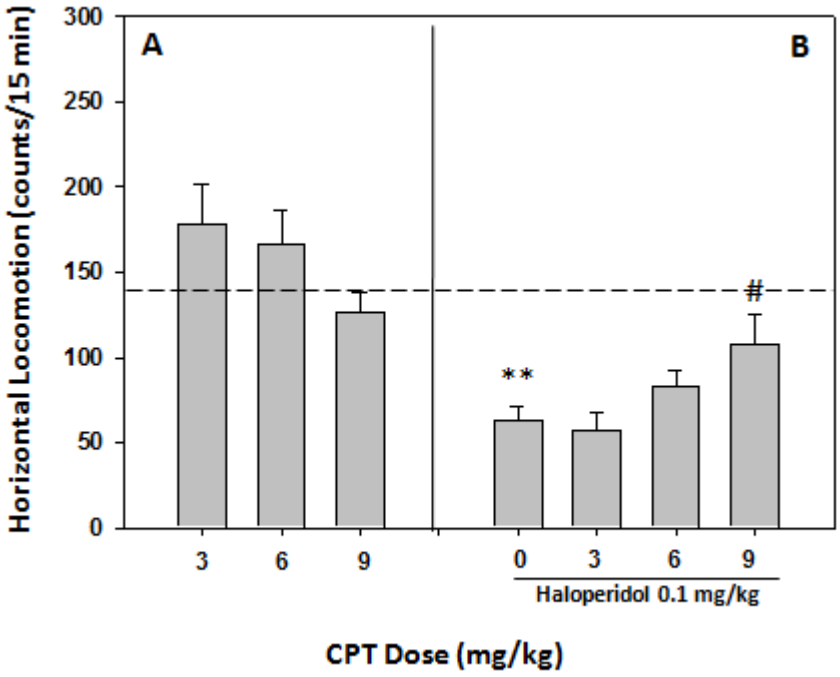

Fig. 4 A and B. Effects of different doses of CPT alone (A), or in combination with haloperidol (0.1 mg/kg) (B) on mice horizontal locomotion. The discontinuous horizontal line represents the mean value for the vehicle group. Mean (±SEM) number of counts in the open field. **p<0.01 significantly different from vehicle. #p<0.05 significantly different from HP/Veh.

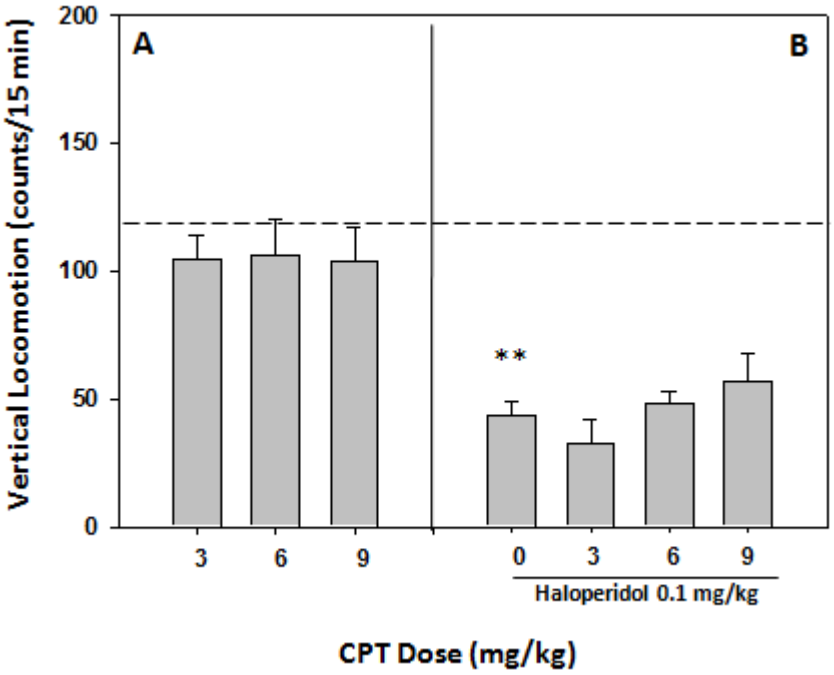

Fig. 5 A and B. Effects of different doses of CPT alone (A), or in combination with haloperidol (0.1 mg/kg) (B) on mice vertical locomotion. The discontinuous horizontal line represents the mean value for the vehicle group. Mean (±SEM) number of counts in the open field. **p<0.01 significantly different from vehicle.

Experiment 4A. *Effect of MSX-3 on locomotor activity in the open field.* The one-way ANOVA for the factor MSX-3 dose (00, 1.0, 2.0 or 3.0 mg/kg) showed a significant effect of dose on horizontal locomotion ($F(3,28)=3.77$, $p<0.05$). Planned comparisons revealed significant differences ($p<0.05$) between animals treated with vehicle and the group treated with 2.0 mg/kg (Fig. 6A). Analysis of the vertical locomotion data showed a statistically significant effect of MSX-3 ($F(3,28)=5.9$, $p<0.01$), and the planned comparisons showed that the MSX-3 dose 2.0 mg/kg was again significantly different ($p<0.01$) from the control group (Fig. 7A).

Experiment 4B. *Effect of MSX-3 on haloperidol reduction of locomotion in the open field.* An ANOVA for the factor drug treatment (Veh-Veh, HP-Veh, HP-1, HP-2 or HP-3) yielded a significant effect of MSX-3 doses on horizontal locomotion ($F(4,35)=11.14$, $p<0.01$). Planned comparisons showed that all MSX-3 doses were different from the haloperidol plus vehicle group ($p<0.01$; Fig. 6B). The one way ANOVA also yielded a significant effect of treatment on vertical locomotion ($F(4,35)= 4.3$; $p<0.01$); again all MSX-3 doses were different from haloperidol plus vehicle (1.0 mg/kg, $p<0.05$ and 2.0, 3.0 mg /kg, $p<0.01$; Fig. 7B).

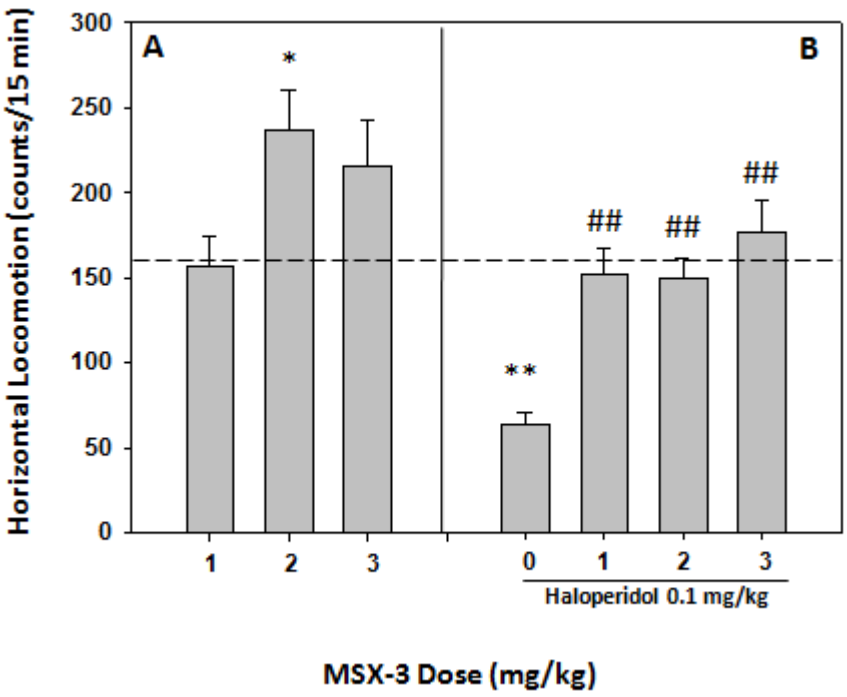

Fig. 6 A and B. Effects of different doses of MSX-3 alone (A), or in combination with haloperidol (0.1 mg/kg) (B) on mice horizontal locomotion. The discontinuous horizontal line represents the mean value for the vehicle group. Mean (±SEM) number of counts in the open field. *p<0.05, **p<0.01 significantly different from vehicle. ##p<0.01 significantly different from HP/Veh.

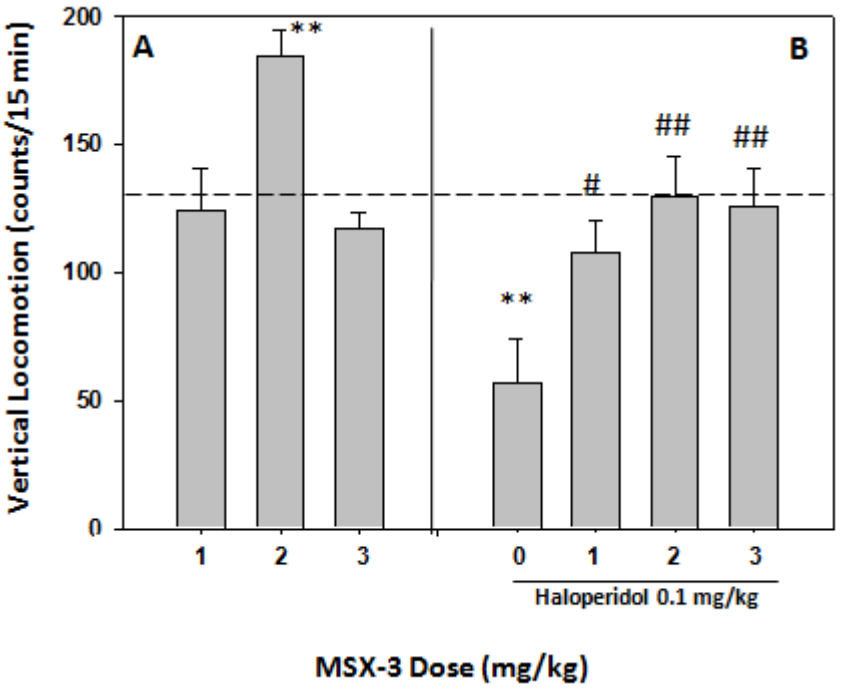

Fig. 7 A and B. Effects of different doses of MSX-3 alone (A), or in combination with haloperidol (0.1 mg/kg) (B) on mice vertical locomotion. The discontinuous horizontal line represents the mean value for the vehicle group. Mean (±SEM) number of counts in the open field. **p<0.01 significantly different from vehicle. #p<0.05, ##p<0.01 significantly different from HP/Veh.

Experiment 5: *Effect of haloperidol on locomotor activity in the open-field in $A_{2A}R$ KO mice.* Two-way ANOVA of the horizontal locomotion data (strain factor (WT vs. KO) and haloperidol treatment factor (0.0 or 0.1 mg/kg)) showed no significant effect of the strain factor, but a statistically significant effect of the haloperidol dose ($F(1,35)= 13.73$, $p<0.01$), and a significant strain x haloperidol treatment interaction ($F(1,35)=8.03$, $p<0.01$). The post hoc tests revealed that under vehicle conditions motor activity was significantly different between WT and KO mice ($p<0.05$), and that haloperidol 0.1 mg/kg significantly reduced horizontal locomotion only in WT animals ($p<0.01$) but

not in KO mice (Fig. 8A). The two-way ANOVA for the vertical locomotion data (Fig. 8B) resulted in a significant overall strain difference ($F(1,35)=6.42$, $p<0.05$), a significant effect of haloperidol treatment ($F(1,35)=11.30$, $p<0.01$), and also a significant interaction ($F(1,35)=13.01$, $p<0.01$). The post hoc tests yielded significant differences between WT and KO mice after receiving vehicle injections ($p<0.01$), and haloperidol 0.1 mg/kg significantly reduced vertical locomotion only in WT animals ($p<0.01$) but not in KO mice. These results indicate that $A_{2A}R$ KO mice were resistant to the suppressing effects of this dose of haloperidol on both forms of locomotion.

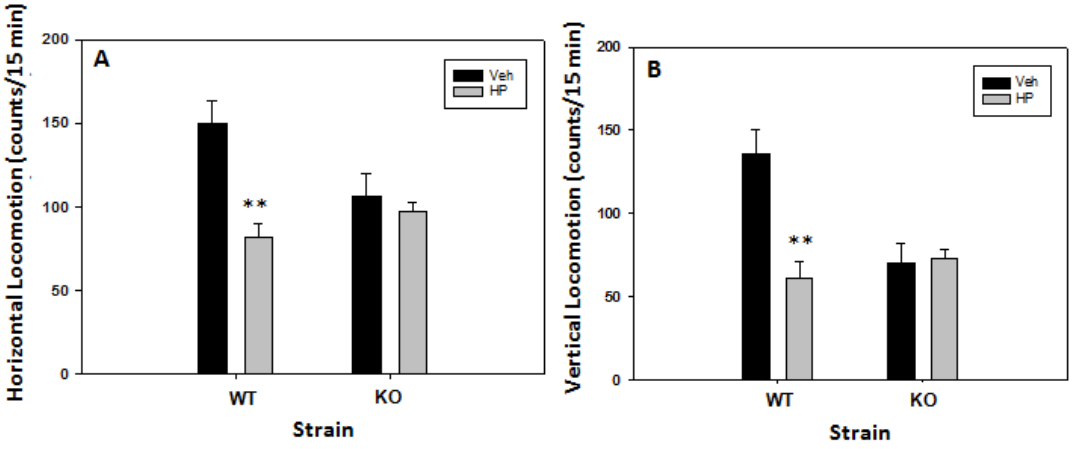

Fig. 8 A and B. Effect of haloperidol (0 or 0.1 mg/kg) in WT and $A_{2A}R$ KO mice on (A) horizontal and (B) vertical locomotion. Mean (±SEM) counts in the open field. **p<0.01 significantly different from vehicle in the corresponding substrain group.

Experiment 6: *Effect of Theophylline, CPT or MSX-3 on c-Fos immunoreactivity in different areas of the Nacb and striatum.* The c-Fos counts in different brain areas (see Fig. 9) were analyzed by two-way (treatment x brain area) factorial ANOVA. There was not an effect of the drug treatment factor, but

a significant difference between brain areas were found ($F(4,103)=14.99$, $p<0.01$), and no significant treatment x brain area interaction. Even though there was not an interaction, we analyzed every brain area separately, and again found that the corresponding one-way ANOVA for the drug treatment factor (Vehicle, theophylline 15.0 mg/kg, CPT 9.0 mg/kg and MSX-3 3.0 mg/kg) on c-Fos positive cells in the different brain areas was not significant for any of them. Thus, none of the adenosine antagonist at these doses had an effect on c-Fos expression by themselves.

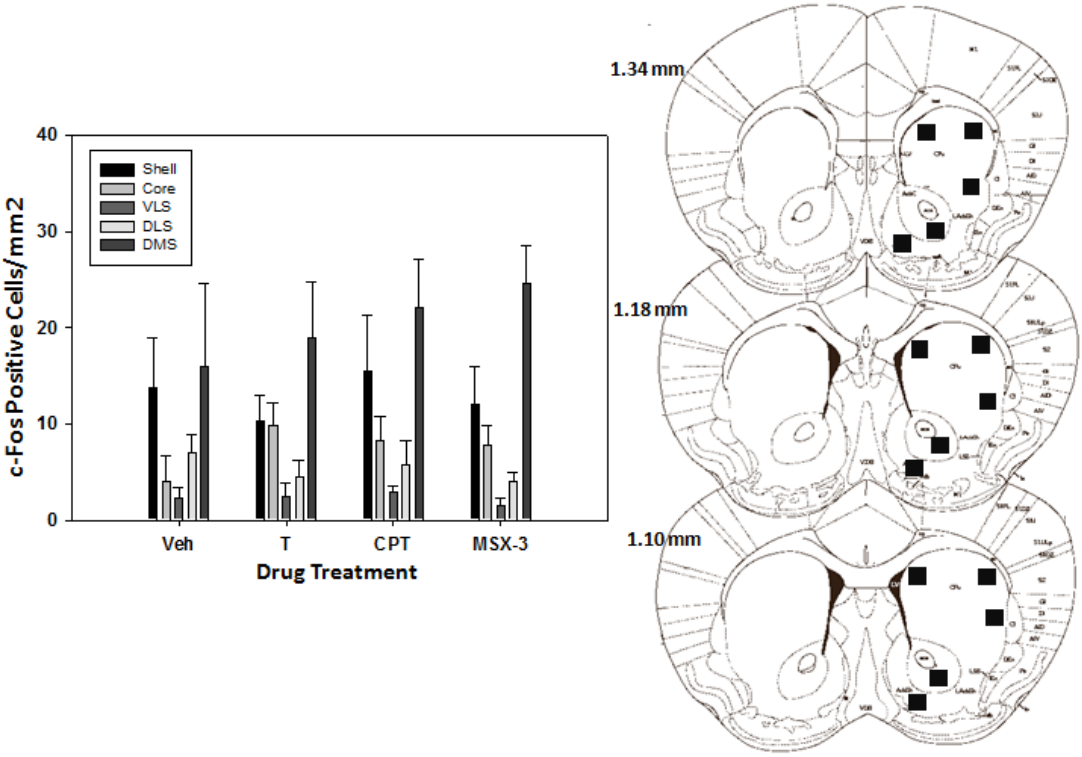

Fig. 9. Left: Effect of different treatments; vehicle, theophylline 15 mg/kg, CPT 9 mg/kg, and MSX-3 3 mg/kg on c-Fos expression in the Nacb Core, Nacb Shell, ventrolateral striatum (VLS), dorsolateral striatum (DLS) and dorsomedial striatum (DMS). Mean (±SEM) number of c-Fos positive cells per mm^2. Right: Diagram of coronal sections with bregma coordinates from Franklin and Paxinos 2007, showing location of the brain areas for c-Fos counting.

Experiment 7: *Effect of theophylline, CPT or MSX-3 on c-Fos immunoreactivity after haloperidol administration in different areas of the Nacb and striatum.* The c-Fos counts in different brain areas (see Fig. 10) were analyzed by a two-way (treatment x brain area) factorial ANOVA. There was a significant overall treatment effect ($F(4,153)=28.06$; $p<0.01$), but no significant difference between brain areas, and no significant treatment x brain area interaction. Planned comparisons on the data collapsed across brain areas indicated that haloperidol produced a significant overall induction of c-Fos expression compared to vehicle-vehicle ($p<0.01$), which was attenuated by co-administration of MSX-3 and theophylline ($p<0.01$).

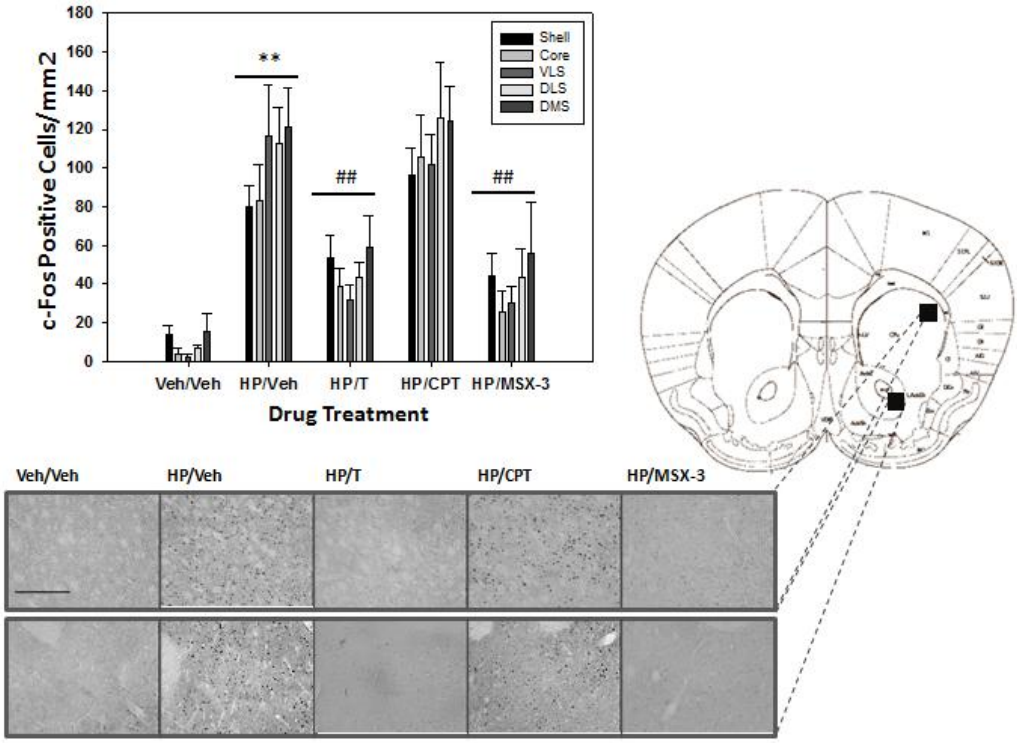

Fig. 10. Left upper part: Effect of theophylline 15 mg/kg, CPT 9 mg/kg, and MSX-3 3 mg/kg on c-Fos immunoreactivity after haloperidol (0.1 mg/kg) administration in the Nacb Core and Shell, VLS, DLS, and DMS. Mean (±SEM) number of c-Fos positive cells per mm^2. **p<0.01 significantly different from Veh/Veh; ##p<0.01 significantly different from HP/Veh. Right and left lower

part: Diagram of coronal section with bregma coordinates from Franklin and Paxinos 2007, showing location of two brain areas for c-Fos counting. Photomicrographs of c-Fos staining in DLS and Nacb Core from representative animals in each treatment group. Low power images (20x). Scale bar=250 um.

DISCUSSION

The present experiments were conducted to characterize and compare the impact of adenosine antagonists with different selectivity profiles on several aspects of exploratory behavior in mice. Moreover, we studied the interaction between DA D_2 and adenosine A_{2A} receptors in the regulation of horizontal and vertical locomotion in WT as well as genetically modified mice. To do so we used the same range of doses that, in one of our previous studies in mice, was able to reverse haloperidol-induced impairments in effort-related choice behavior in a T-maze paradigm (Pardo et al., 2012).

Acute administration of haloperidol suppresses locomotion in rats (Ishiwari et al., 2004) and mice (Satrr and Starr 1986; Fujiwara, 1992). The present results show that haloperidol produced a decrease in horizontal locomotion at the two highest doses employed 0.05 and 0.1 mg/kg dose, an effect that was also observed for vertical locomotion at the highest dose. The low dose of haloperidol (0.025 mg/kg) did not suppress locomotion, and in fact, it actually increased rearing. Thus, for the remaining experiments we used the highest dose (0.1 mg/kg) to study the impact of the adenosine antagonists on haloperidol-induced suppression of horizontal and vertical locomotion.

In experiment 2 we studied the impact of the non-selective adenosine antagonist theophylline on spontaneous locomotion, and in a second study we

evaluated its potential to reverse haloperidol-induced locomotor suppression. When administered alone, theophylline enhanced horizontal locomotion at all doses tested (5.0-15.0 mg/kg), but did not significantly induce rearing. Theophylline and caffeine are methylxanthines that act as minor stimulants that potentiate locomotion over a broad range of doses (Malec and Poleszak, 2006; Zhang et al., 2011). We selected doses in the lower range to induce exploration but to minimize the possible appearance of stereotypes that can reduce exploratory behavior at higher doses. Thus, with these low doses, haloperidol-induced behavioral impairments were restored by co-administration of theophylline at all doses in the case of horizontal locomotion, and at the two highest doses for the rearing. These data are in accordance with previous studies showing an attenuation of haloperidol-induced motor deficits after theophylline treatment in rats (Bishnoi et al., 2007). In the present results, behavior was restored to control levels with the two highest doses of theophylline.

In experiment 3, the adenosine A_1 antagonist CPT was used because this drug has been shown to stimulate locomotion in rats (Karcz-Kubicha et al., 2003). In the present study CPT administered alone did not produce any significant effect on horizontal or vertical locomotion at the doses tested (3.0-9.0 mg/kg). Our doses in mice are very similar to doses used in rats (1.0-10.0 mg/kg; Karcz-Kubicha et al., 2003) in which CPT did increase locomotion. This discrepancy may be due to the fact that two different type of species (mice vs. rats) and procedures (non-habituated vs. habituated animals) were used. Previous studies in mice have also found that CPT does not have any stimulant properties by itself (1.2-6.0 mg/kg; Dall'Igna et al., 2001; Malec and Poleszak, 2006). Moreover, low doses (1.0 mg/kg) of CPT in mice suppressed locomotion (Florio

et al., 1997; Dall'Igna et al., 2001). However, in the present work CPT reversed haloperidol-induced suppression of horizontal locomotion at the highest dose (9.0 mg/kg), although no effect was found on vertical locomotion. In rats, CPT (12.0 mg/kg) also appears to produce a mild reversal of the effects of D_2 antagonists on motor function. Thus, CPT produced a partial reversal of eticlopride-induced suppression of locomotion (Collins et al., 2010), as well as haloperidol-induced catalepsy (Trevitt et al., 2009). Thus, CPT in mice seems not to have clear stimulatory actions, but can minimally reverse neuroleptic actions on motor performance. CPT shows lower A_1 vs. A_{2A} binding selectivity than other A_1 antagonists (Maemoto et al., 1997). Thus, it is possible that the minimal reversal of D_2 antagonist effects observed in the present study and also in previous results (Trevitt et al., 2009; Collins et al., 2010) was due to some activity of CPT on A_{2A} receptors interacting with D_2 receptors.

In experiment 4 the results show an induction of both vertical and horizontal locomotion after administration of the intermediate (2.0 mg/kg) dose of the selective A_{2A} antagonist MSX-3. Previous results in mice also show a diversity of results on A_{2A} effects on spontaneous locomotion. MSX-3 (5.0 mg/kg) SCH58261 (2.0-6.0 mg/kg), and SCH442416 (3.0 mg/kg) were shown to induce locomotion (Hsu et al., 2009; Marcellino et al., 2010; Lerner et al., 2010). However, DMPX (1.0-6.0 mg/kg) and KW 6002 (0.3 mg/kg) were not able to increase motor activity (Dall'Igna et al., 2001; Kachroo et al., 2005). In the present results, MSX-3 restored the normal pattern of horizontal as well as vertical locomotion in haloperidol-treated mice at all doses used (1.0, 2.0 and 3.0 mg/kg). Our results are in accordance with genetic and pharmacological models of parkinsonian bradykinesia. Thus, in mice with a genetically induced

progressive depletion of DA, MSX-3 (5.0 mg/kg) reversed impaired locomotion (Marcellino et al., 2010). In rats, systemic injections of MSX-3 (0.5-10.0 mg/kg) were able to reverse the locomotor suppression produced by haloperidol (0.5 mg/kg; Ishiwari et al., 2007), and eticlopride (Collins et al., 2010), an effect that was also achieved after local MSX-3 administration into the Nacb core (Ishiwari et al., 2007).

In summary, although methylxanthines seem to clearly have stimulant properties both in habituated and non-habituated mice across a broad range of doses (El Yacoubi et al., 2000; Pastor et al., 2005; present studies), the picture for selective A_1 and A_{2A} antagonists seems less clear. In the literature A_{2A} antagonists appear to be more likely to exhibit motor activating effects than A_1 antagonists, and our results comparing selective and non-selective adenosine antagonists indicate that A_{2A} receptor antagonism seems more relevant for the stimulant properties of methylxanthines. This conclusion has been suggested in previous studies based on the lack of stimulation by caffeine in A_{2A}R KO mice but not in A_1R KO (El Yacoubi et al., 2000; Yang et al., 2009; Lazarus et al., 2011), and in studies of operant behavior in rats (Randall et al., 2011). Moreover, a large number of studies demonstrate that A_{2A} antagonism can reverse the effects of D_2 antagonism on behavioral tests in mice as well as rats. Thus, as predicted, the antagonism of adenosine A_{2A} receptors was more effective than A_1 antagonism in restoring the behavior impaired by DA D_2 antagonism. It is likely that this pattern of results in drug interaction studies is due to the co-localization of A_{2A} receptors with D_2 receptors (Mott et al., 2009; Salamone et al., 2009; Nunes et al., 2010). Adenosine A_{2A} receptors are located on striatal GABAergic enkephalin-positive neurons that also express DA D_2

receptors (Fink et al., 1992; Ferré et al., 1997; Svenningsson et al., 1999; Chen et al., 2001). DA D_2 and adenosine A_{2A} receptors converge onto the same signal transduction mechanisms and show the capacity to form heteromers (Fink et al., 1992; Ferré et al., 1997, 2004, 2008a; Svenningsson et al., 1999; Fuxe et al., 2003). A_{2A} receptors, through their coupling to G_{olf} proteins, can stimulate adenylyl-cyclase activity and activate the cAMP-PKA signaling pathway, with phosphorylation of several PKA substrates, such as DARPP-32 and CREB and the consequent increase in the expression of different genes, such as *c-fos* or *preproenkephalin* in the GABAergic enkephalinergic neuron (Ferré et al., 2008a). The tonic activation of D_2 receptors blocks the ability of A_{2A} receptors to signal through the cAMP-PKA signaling pathway. Administration of D_2 receptor antagonists produces a significant increase in the PKA-dependent phosphorylation of DARPP-32 and an increase in the expression of *c-fos* and *preproenkephalin* genes, which depends on the ability of D_2 receptor blockade to liberate A_{2A} receptor signaling activated by endogenous adenosine. Thus, the neural effects of DA D_2 receptor antagonists can be counteracted by co-administration of A_{2A} receptor antagonists (Ferré et al., 2008a).

Taking all these results into consideration, in experiment 5, we decided to investigate spontaneous locomotion and the impact of D_2 antagonism on WT and adenosine A_{2A}R KO mice. Our results showed that adenosine A_{2A}R KO mice had reduced levels of spontaneous activity, which is consistent with previous studies (Ledent et al., 1997; Chen et al., 2001). However, after receiving 0.1 mg/kg of haloperidol, A_{2A}R KO mice did not show suppression of either horizontal or vertical locomotion, thus showing resistance to the effects of a dose of haloperidol that significantly suppressed both types of locomotion in WT

mice. A_{2A}R KO mice have been demonstrated to be more resistant than WT animals to the cataleptic effect of DA antagonists like haloperidol or SCH 23390 (Chen et al., 2001; El Yacoubi et al., 2001). Moreover, in a previous study these KO mice have been shown to be more resistant to haloperidol-induced impairments in effort-related decision making (Pardo et al., 2012). There are a few possible mechanisms that could underlie the lack of effect of haloperidol on A_{2A}R KO mice. First, it is possible that genetic deletion of striatal A_{2A} receptors could alter striatal D_2 receptor function. There is evidence of antagonistic intramembrane A_{2A}– D_2 interactions, by which stimulation of adenosine A_{2A} receptors decreases the ability of DA to displace a D_2 antagonist from binding to D_2 receptors (Ferré et al., 1999). Thus, A_{2A}R KO could be enhancing the ability of endogenous DA to compete with haloperidol for binding to DA receptors. In addition, because adenosine A_{2A} and DA D_2 receptors converge onto the same adenylyl cyclase-related signal transduction cascade (Ferré et al., 1997, 2008a), deletion of A_{2A} receptors could be altering the signal transduction effects of D_2 receptor blockade.

Finally, c-Fos expression was quantified in experiments 6-7. Although previous studies have reported that very high doses of adenosine antagonists could affect c-Fos expression (Nakajima et al., 1989; Le et al., 1992; Johansson et al., 1992, 1994; Svenningsson et al., 1995; Dassesse et al., 1999), the results of experiment 6 showed that none of the adenosine antagonists studied had any effects on c-Fos expression when administered alone at the doses used in the behavioral experiments. In experiment 7, c-Fos expression was evaluated in mice that had been exposed to the open field, and also had received injections of a D_2 antagonist. Because D_2 and adenosine A_{2A} receptor stimulation has opposite

effects on stimulation of cAMP-related pathways, it was hypothesized that adenosine A_{2A} antagonism would blunt the ability of the D_2 antagonist to affect transcription of immediate early genes and induce formation of Fos-related proteins. Earlier reports have shown increased c-Fos expression in striatal areas, including Nacb as well as neostriatum, after systemic administration of D_2 family antagonists mainly in rats (Betz et al., 2009; Farrar et al., 2010; Hussain et al., 2002; Pinna et al., 1999; Svenningsson et al., 1999). Also, it has been previously observed that the increases in c-Fos expression induced by D_2 antagonists in striatal areas can be attenuated by co-administration of A_{2A} receptor antagonists or by theophylline (Boegman and Vincent, 1996; Pinna et al., 1999; Ward and Dorsa, 1999; Hussain et al., 2002; Betz et al., 2009; Farrar et al., 2010; Pardo et al., 2012). The present results demonstrated that a relatively low dose of haloperidol (0.1 mg/kg), which decreased open field locomotion, also produced a parallel increase in c-Fos expression across all the striatal structures studied. Moreover, at doses that did not have an effect on their own, theophylline and MSX-3 both reversed the haloperidol-induced increase in c-Fos expression. This pattern of results is very similar to the results reported in a recent paper from our laboratory using the same range of doses for all drugs (Pardo et al., 2012). In that paper, haloperidol injections reduced high effort instrumental behaviors, and led to the selection of responses that were less demanding in terms of effort. This effect on behavior was reversed by theophylline and MSX-3, and these results were paralleled by c-Fos expression in the same animals that were performing the task (Pardo et al., 2012). In the present studies, c-Fos expression was determined in mice that also were tested in

the open field, thus providing a behaviorally relevant cellular marker of the interaction between DA D_2 and adenosine A_{2A} receptors.

In humans, antipsychotic drugs that act as D_2 antagonists induce many side effects, including parkinsonism (Marsden et al., 1975) and psychomotor slowing (Heinz et al., 1998). The present studies suggest that adenosine A_{2A} antagonists such as MSX-3 could be a useful therapeutic tool for the treatment of neuroleptic-induced parkinsonism and psychomotor slowing, since they do not have strong stimulant effects, but they nevertheless attenuate the behavioral impairments produced by antipsychotic drugs. Furthermore, our pharmacological results are supported by the finding that genetic deletion of the A_{2A} receptor makes animals resistant to the effects of haloperidol. In contrast, adenosine A_1 antagonism produced a minor reversal in the reduced exploration produced by DA D_2 antagonism. Interestingly, although the doses of theophylline employed showed that it has a clear psychostimulant profile, it also is capable of reducing haloperidol-induced impairments in several aspects of exploratory behavior and willingness to exert effort in goal directed behaviors (Pardo et al., 2012).

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CHAPTER 4:

ADENOSINE A_{2A} RECEPTOR ANTAGONISM AND GENETIC DELETION ATTENUATE THE EFFECTS OF DOPAMINE D_2 ANTAGONISM ON EFFORT-BASED DECISION MAKING IN MICE: STUDIES USING A T-MAZE WITH BARRIER

Abstract

Brain dopamine (DA) and adenosine interact in the regulation of behavioral activation and effort related processes. In the present studies, a T-maze task was developed in mice for the assessment of effort-related decision making. With this task, the two arms of the maze have different reinforcement densities, and a vertical barrier is positioned in the arm with the higher density (HD), presenting the animal with an effort-related challenge. Under control conditions mice prefer the HD arm, and climb the barrier to obtain the larger amount of food. The DA D2 receptor antagonist haloperidol decreased selection of the HD arm and increased selection of the arm with the low density of reinforcement. However, the HD arm was still the preferred choice in haloperidol-treated mice trained with barriers in both arms. Pre-feeding the mice to reduce food motivation dramatically increased omissions, an effect that was distinct from the actions of haloperidol. Co-administration of theophylline, a nonselective adenosine receptor antagonist, partially reversed the effects of haloperidol. This effect seems to be mediated by the A2A receptor but not the A1 receptor, since the A2A antagonist MSX-3, but not the A1 antagonist CPT, dose

dependently reversed the effects of haloperidol on effort-related choice and on c-Fos expression in the dorsal striatum and nucleus accumbens. In addition, adenosine A2A receptor knockout mice were resistant to the effects of haloperidol on effort-related choice in the maze. These results indicate that DA D2 and adenosine A2A receptors interact to regulate effort-related decision making and effort expenditure in mice.

INTRODUCTION

Vigor, persistence, and high work output are fundamental features of motivated behavior (Salamone, 2010). These activational aspects of motivation are highly adaptive because they enable organisms to overcome obstacles or work related response costs that separate them from significant stimuli (Salamone, 2010; Salamone et al., 1994, 2003, 2007; Salamone and Correa, 2002; van den Bos et al., 2006). An important feature of adaptive behavior in the face of work-related challenges is effort-related decision making. Organisms frequently must make cost/benefit analyses in which they weigh the value of available rewards vs. the costs involved in procuring them (Day et al., 2011; Phillips et al., 2007; Salamone and Correa, 2002; Walton et al., 2006).

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 (Barbano and Cador, 2006; Cagniard et al., 2006; Floresco et al., 2008; Phillips et al., 2007; Salamone, 2010; Salamone et al., 1991, 2003, 2005, 2007, 2009). Interference with DA transmission by administering DA antagonists or DA depleting agents typically biases rats towards low effort alternatives for obtaining access to food (Salamone et al., 2007, 2009). Considerable evidence indicates that brain adenosine receptor mechanisms interact with DA systems in the regulation of effort-related choice behavior (Salamone and Correa, 2009). In addition to Nacb DA, other transmitters (GABA, glutamate, adenosine) and brain areas (prefrontal/anterior cingulate cortex, basolateral amygdala, ventral pallidum) also are involved (Farrar et al., 2008; Floresco et al., 2008; Ghods-Sharifi et al.,

2009; Hauber and Sommer, 2009; Salamone et al., 2007, 2009; Walton et al., 2002, 2003). Nacb and caudate/putamen have a high concentration of adenosine A_{2A} receptors (DeMet and Chicz-DeMet, 2002; Ferré et al., 2004; Jarvis and Williams, 1989). DA D_2 and adenosine A_{2A} receptors are co-localized on enkephalin-containing medium spiny neurons, and these receptors interact by forming heteromers, and by convergence on to the same signal transduction pathways (Ferré, 2008; Ferré et al., 1997, 2008; Fink et al., 1992; Fuxe et al., 2003). Recent evidence indicates that DA D_2 and adenosine A_{2A} receptors interact to regulate effort-related functions (Font et al., 2008; Mingote et al., 2008; Salamone and Correa, 2009). Microinjections of the adenosine A_{2A} agonist CGS 21680 into the Nacb produced effects on instrumental behavior and effort-related choice that resembled those produced by Nacb DA antagonism or depletion (Font et al., 2008; Mingote et al., 2008). Furthermore, adenosine A_{2A} antagonists such as MSX-3 and istradefylline (KW6002) were able to reverse the effects of the DA D_2 antagonists haloperidol and eticlopride on effort-related choice behavior, under conditions that employed a concurrent FR5/feeding choice procedure (Farrar et al., 2007, 2010; Nunes et al., 2010; Salamone et al., 2009; Worden et al., 2009), and a T-maze barrier choice task (Mott et al., 2009).

One shortcoming of this work is that virtually all of it has been conducted in rats; only a few studies of effort-related choice have been performed in mice (Beeler et al., 2010; Cagniard et al., 2006), and there have been no mouse studies focused upon the DA/adenosine interactions involved in this aspect of motivation. It is critical to study mice as well as rats to establish generalizations across multiple species (McKerchar and Fowler, 2005). Moreover, genetic knockout models circumvent the intrinsic limitations of pharmacological agents

with partial specificity, and these tools are widely available in mice. Thus, studies of motivated behavior involving mice with DA transport knockdown (Beeler et al., 2010; Cagniard et al., 2006) or DA deficiency (Robinson et al., 2005, 2006, 2007) have contributed substantially to our understanding of the behavioral functions of brain DA. Moreover, genetic deletion of the adenosine A_{2A} receptor in mice has been shown to alter the locomotor response to adenosine antagonists (El Yacoubi et al., 2000; Halldner et al., 2004; Yu et al., 2008; Lazarus et al., 2011), and to affect amphetamine sensitization (Chen et al., 2003) and self-administration of cocaine and MDMA (Ruiz-Medina et al., 2011; Soria et al., 2006), as well as aspects of cognition and motor function (Wei and Chen, 2011; Xiao et al., 2011).

The present studies were undertaken to develop and validate a mouse test of effort-related choice behavior (experiments 1-3) using a variant of the T-maze barrier task developed originally for rats (Cousins et al., 1996; Mott et al., 2009; Salamone et al., 1994), to assess the ability of adenosine antagonists with different selectivity profiles (theophylline, MSX-3, and CPT) to attenuate the effects of the DA D_2 antagonist haloperidol (experiments 4-6) or to exert actions when administered alone (experiment 7), and to determine if adenosine A_{2A} receptor knockout (A_{2A}R KO) mice are resistant to the effects of haloperidol on T-maze performance (experiment 8). Finally, evaluations of the impact of these pharmacological manipulations on the expression of c-Fos in Nacb core and shell, as well as the dorsolateral neostriatum (DLS), were conducted (experiment 9) in order to provide a cellular marker of the interaction between DA D_2 and adenosine A_{2A} receptor antagonists in mice performing in the T maze.

MATERIALS AND METHODS

Animals

CD1 male mice (N= 73) weighed 24-28 g at the beginning of the study (Harlan-Interfauna Ibérica S.A., Barcelona, Spain). Male mice lacking the A_{2A} adenosine receptor type and wild-type (WT) littermates (N=7 and 9 respectively) weighed 25–30 g at the beginning of the study (Universite Libre de Bruxelles, Brussels, Belgium), and were generated as previously reported (Ledent et al., 1997; Soria et al., 2006) from a CD1 background. 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% free-feeding body weight throughout the study. The colony was kept at a temperature of 22 $\pm$ 2 ºC with lights on from 0800 to 2000 hours. 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.

Pharmacological agents

All drugs were administered intraperitoneally (IP). Haloperidol (Sigma Química C.O), a relatively selective DA D_2 family receptor antagonist, was dissolved in a 0.3% tartaric acid solution (pH=4.0), which also was used as the vehicle control. Haloperidol was selected because this widely prescribed antipsychotic drug has been used in previous T-maze experiments in rats (Salamone et al., 1994; Denk et al., 2005; Mott et al., 2009), and therefore could provide a useful cross-species validation. Theophylline (TOCRIS Bioscience),

CPT (8-cyclopentyltheophylline) (Sigma Química C.O), and MSX-3 ((*E*)-phosphoric acid mono-[3-[8-[2-(3-methoxphenyl)vinyl]-7-methyl-2,6-dioxo-1-prop-2-ynyl-1,2,6,7-tetrahydropurin- 3-yl] propyl] ester disodium salt; synthesized at the laboratory of Dr. Christa E. Müller at the Pharmazeutisches Institut, Universität Bonn, in Bonn, Germany), were dissolved in 0.9% w/v saline (pH 7.4). MSX-3 is a prodrug that is cleaved *in vivo* to the pharmacologically active compound MSX-2 (Hockemeyer et al., 2004).

Apparatus and testing procedures

The T-maze apparatus consisted of a central corridor with two opposed arms (see Fig. 1). Each arm provided a different density of food: 2 pellets (15 mg each) were in the high density (HD) arm and 1 pellet was in the low density (LD) arm. For most experiments, the HD arm contained 14 cm vertical barrier that provided the effort-related challenge. Pellets (Bioserve, Frenchtown, NJ, USA) were located in dishes placed near the far walls of the maze arms. Half the mice had the HD arm with the barrier consistently located on the left side, while half the mice had the HD arm and barrier on the right side.

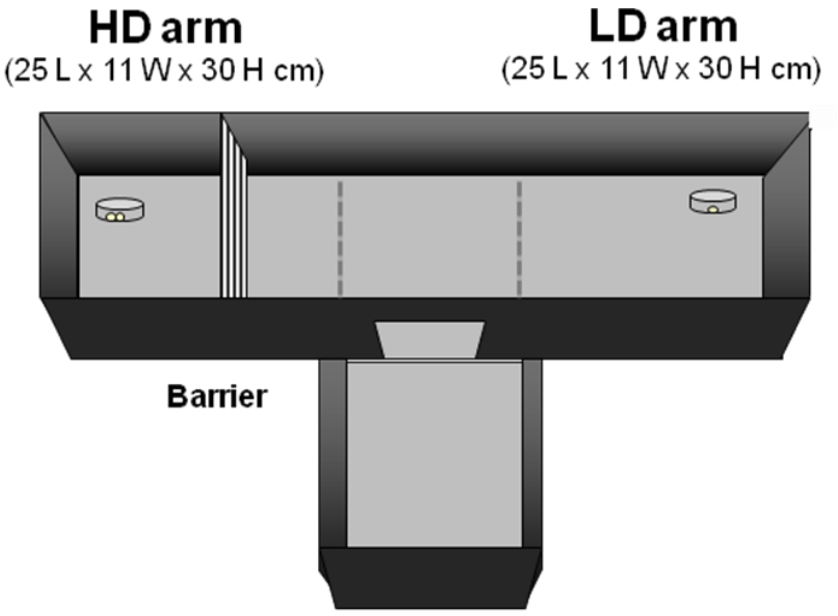
Training phases: During the first training phase no barrier was present. The first 2 days of the initial training, mice had free access to both arms of the T-maze upon exiting the start arm, and were allowed to consume all pellets in both HD and LD arms of the maze before being returned to the start arm. Upon completion of this initial training, mice were then trained to select between the HD or the LD arm, with no barrier in place. For this and all subsequent training and testing procedures, the animals were only allowed to choose one arm of the maze; after the initial arm choice, the other arm was blocked. The criterion

before a new learning phase started was set so that animals had to choose the HD arm at least 85% of the time for 2 consecutive days. In the second training phase a small barrier (5.5 cm high) was introduced in the HD arm. In the third phase a medium barrier (12 cm high) replaced the short one. Finally, in the fourth training phase and for the rest of the experiment, the high barrier (14 cm high) was used. The average training sessions for the CD1 animals in the 4 phases across experiments was 36 total sessions: Phase 1: 12 sessions; Phase 2: 6 sessions; Phase 3: 7 sessions; and Phase 4: 11 sessions. For the KO and WT animals was 34 total sessions: Phase 1: 10 sessions; Phase 2: 5 sessions; Phase 3: 5 sessions; and Phase 4: 14 sessions. A total of five animals that did not consistently reach the 85% criterion before the drug testing phase started were removed from experiments 1, 5 and 6.

High Barrier Training and Test Sessions: Each trial started when the gate in the start arm was opened. Latency measurements marked the time from the opening of the start arm until the initiation of food consumption in the selected arm. In each trial every mouse had the option of going to any of the two choice arms to consume the pellets. When the mice entered into one arm the other arm was blocked. Immediately after the animals finished consuming the food, they climbed back across the barrier and returned by themselves to the start arm where they were briefly held for the inter-trial interval (2-3 seconds), during which the food pellets that had been consumed were replaced. This procedure was repeated for 30 trials during each daily training session. Sessions started two hours after the colony lights where on. Animals had one training session per day, 5 days a week. Baseline training under these conditions continued for several weeks, until stable performance was achieved. During the drug testing phase there was one

drug treatment day and 4 baseline days before the next drug day.

Additional Behavioral Validation Procedures: In experiment 2, when the mice completed the fourth phase with one barrier in the HD arm, one additional training week was conducted with a high barrier in the HD arm and an additional high barrier in the LD arm, after which drug testing was conducted. In experiment 3, mice were trained as in experiment 1, but on the day of the pre-feeding test, animals had *ad libitum* access to food for 24 hrs in their home cages the day before the test session. During the test session the number of HD or LD selections, or omissions (no arm selection for 40 seconds), were recorded.



Starting arm with retractable gate.
(25 L x 11 W x 30 H cm)

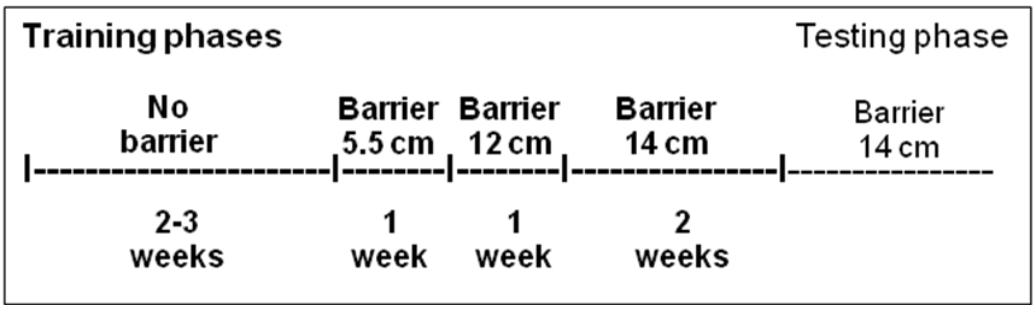


Fig.1. Top view of the mouse T-maze apparatus used in the present study. All the surfaces and the doorway were constructed out of Plexiglas, and the top of all arms were open. The barrier (depicted in the high density arm, to the left) was constructed of wire mesh. The HD arm contained 2 food pellets, and the LD arm contained 1 food pellet.

c-Fos visualization and quantification

Free floating coronal sections (50 □m) 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 (H_2O_2) for 30 min to block endogenous staining. Sections were then rinsed in PBS (3 × for 5 min) and transferred into the primary antibody, anti-c-Fos (Calbiochem, Germany) for a 48 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 2 h. The immunohistochemical reaction was developed using diaminobenzidine (DAB) as the chromagen. Processed sections were then mounted to gelatin-coated slides, air dried, and cover-slipped using Cytoseal 60 (Thermo Scientific) 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 (Nacb core, Nacb shell, DLS) were magnified at 20X and captured digitally using SPOT 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 was used for statistical analysis.

Experiments

Experiments used a within-groups design, in which each mouse received all treatments in a random order over consecutive weeks, once a week (no treatment sequence was repeated across different animals in any of the experiments). During the test session the number of HD or LD selections and the latency to reach the food was recorded by an observer who was unaware of the experimental condition. All T-maze experiments were conducted with a single barrier (in the HD arm) except for experiment 2.

Experiment 1: *Effect of different doses of haloperidol on performance in the T-maze*. Mice (N=7) received injections of tartaric acid vehicle, 0.025, 0.05 or 0.1 mg/kg haloperidol 50 min before the test.

Experiment 2: *Effect of 0.1 mg/kg of haloperidol on performance in the T-maze with two barriers.* Mice (N=12) received injections of either tartaric acid or haloperidol 0.1 mg/kg 50 min before the test.

Experiment 3: *Effect of pre-feeding on performance in the T-maze*. Mice (N=12) were tested under three experimental conditions: pre-feeding, tartaric acid or haloperidol (0.1 mg/kg).

Experiment 4: *Effect of theophylline on T-maze performance after haloperidol administration*. Mice (N=12) received injections of tartaric acid vehicle or haloperidol (0.1 mg/kg), which were administered 50 min before test. Saline vehicle or theophylline (5.0, 10.0 and 15.0 mg/kg) was administered 20 min before testing.

Experiment 5: *Effect of CPT on T-maze performance after haloperidol administration*. Mice (N=10) received the following treatments: tartaric acid vehicle or haloperidol (0.1 mg/kg), which were administered 50 min before test. Saline vehicle or CPT (3.0, 6.0 and 9.0 mg/kg) was administered 20 min before testing.

Experiment 6: *Effect of MSX-3 on T-maze performance after haloperidol administration.* Mice (N=8) were injected with tartaric acid vehicle or haloperidol (0.1 mg/kg) 50 min before testing began. Saline vehicle or MSX-3 (1.0, 2.0 and 3.0 mg/kg) was administered 20 min before testing.

Experiment 7: *Effect of theophylline, CPT and MSX-3 alone on T-maze performance.* Mice (N=12) received injections of either saline vehicle, theophylline (15.0 mg/kg), CPT (9.0 mg/kg), or MSX-3 (3.0 mg/kg), 20 min before testing.

Experiment 8: *Effect of haloperidol on T-maze performance in A_{2A}R KO and WT mice*. Adenosine A_{2A}R KO mice and WT control animals (N=7 and 9, respectively) received the following treatments: tartaric acid vehicle, 0.05 or 0.1 mg/kg haloperidol, 50 min before the test.

Experiment 9: *Effect of theophylline, CPT or MSX-3 on c-Fos immunoreactivity after haloperidol administration.* After completion of the T-maze session in experiments 4, 5 and 6, mice (n=30) were anesthesized and perfused, and brain sections were stained for c-Fos immunoreactivity as described above. All mice were treated with tartaric acid vehicle or haloperidol (0.1 mg/kg) 140 min before anesthesia, and then 30 min after the first injection

they received treatments of either saline vehicle, 15.0 mg/kg theophylline, 9.0 mg/kg CPT, or 3.0 mg/kg MSX-3, 30 min later.

Statistical analysis

The total numbers of HD arm selections and the latencies to reach the food across treatments were analyzed using repeated measures ANOVA, followed by Tukey HSD post hoc test. Data for experiment 8 were analyzed using a two way-factorial ANOVA. STATISTICA 7 software was used for statistical analysis of the data. All data were expressed as mean ±SEM, and significance was set at $p<0.05$.

RESULTS

Experiment 1: *Effect of different doses of haloperidol on performance in the T-maze*. Repeated measures ANOVA indicated a significant effect of haloperidol treatment ($F(3,18)=7.90$; $p<0.01$) on HD choice (Fig. 2 left panel). Post hoc analyses with the Tukey test revealed significant differences between 0.0 mg/kg and the two highest doses of haloperidol, 0.05 mg/kg and 0.1 mg/kg ($p<0.05$ and 0.01 respectively). These results indicate a dose-related effect of haloperidol on HD choice behavior, with the lowest dose not producing a significant effect and the two highest doses significantly reducing the selection of the HD arm that had the barrier. With the latency measure repeated measures ANOVA revealed a significant effect of haloperidol treatment ($F(3,18)=3.24$; $p<0.05$). Mean $\pm$ SEM latencies (seconds) were as follows: Veh=2.9 $\pm$ 0.3 / HP (0.025mg/kg)= 2.6 $\pm$ 0.2 / HP (0.05mg/kg)=3.2 $\pm$ 0.4 / HP (0.1mg/kg)=3.5 $\pm$ 0.4. However, the Tukey test did not reveal significant differences between vehicle

treatment and any dose of haloperidol; the only significant difference was between the 0.025 mg/kg dose and the 0.1 mg/kg dose ($p<0.05$).

Experiment 2: *Effect of 0.1 mg/kg of haloperidol on performance in the T-maze with two barriers.* Repeated measures ANOVA showed that when both arms had a vertical barrier, there was no significant effect of haloperidol treatment on either the HD arm choice (Fig. 2 right panel) or the latency measure (Veh=2.5 $\pm$ 0.3 seconds; HP (0.1mg/kg)=4.4 $\pm$ 1.4 seconds).

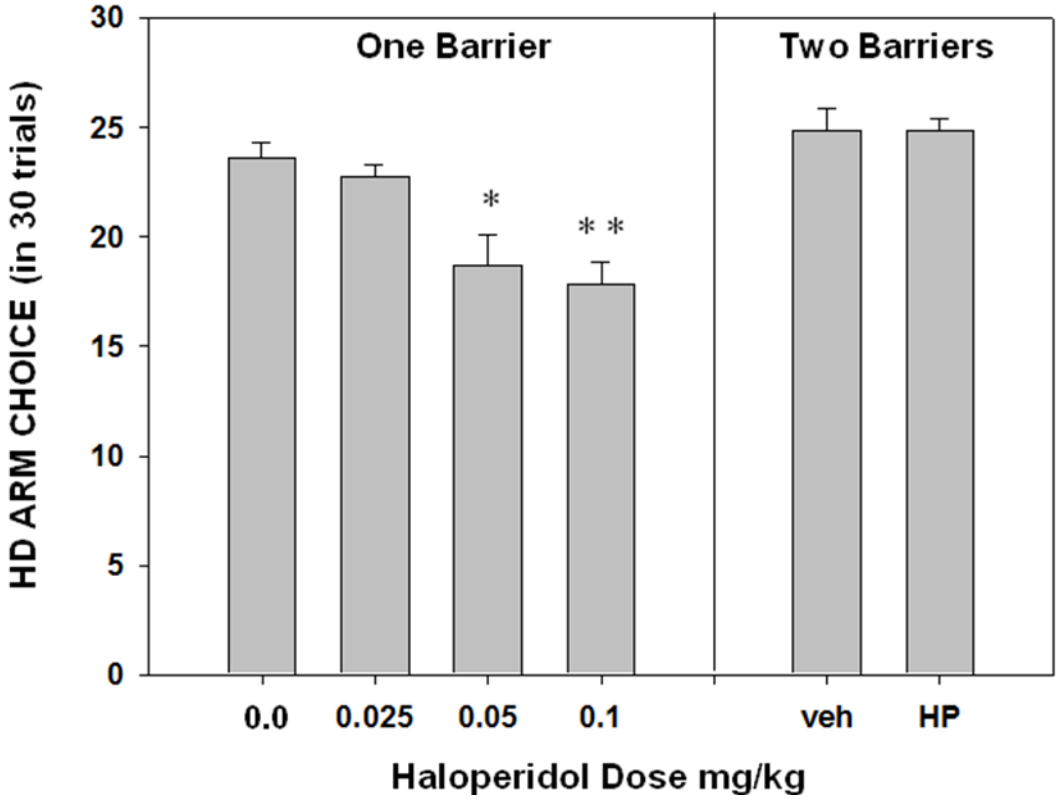


Fig. 2. Effect of haloperidol on HD arm choice in the T-maze with one barrier in the HD arm (left panel), and in the T-maze with barriers in both the HD and the LD arm (right panel). Mean (±SEM) number of HD arm choices. *$p<0.05$, **$p<0.01$ significantly different from vehicle in the corresponding experiment.

Experiment 3: *Effect of pre-feeding on performance in the T-maze*. Figure 3 depicts the results of the pre-feeding experiment. A separate ANOVA was performed for each of the three behavioral measures obtained. There was a significant treatment effect on HD arm crossings ($F(2,22)=35.46$; $p<0.01$). The Tukey test indicated that all three groups differed from each other ($p<0.01$). In addition, there was a significant effect on LD arm crossings ($F(2,22)=4.45$; $p<0.05$); the haloperidol-treated group differed from vehicle ($p<0.05$). Finally, there also was a significant effect on omissions ($F(2,22)=9.19$; $p<0.01$). On this measure, the pre-fed condition significantly differed from both the haloperidol condition and the vehicle condition ($p<0.01$).

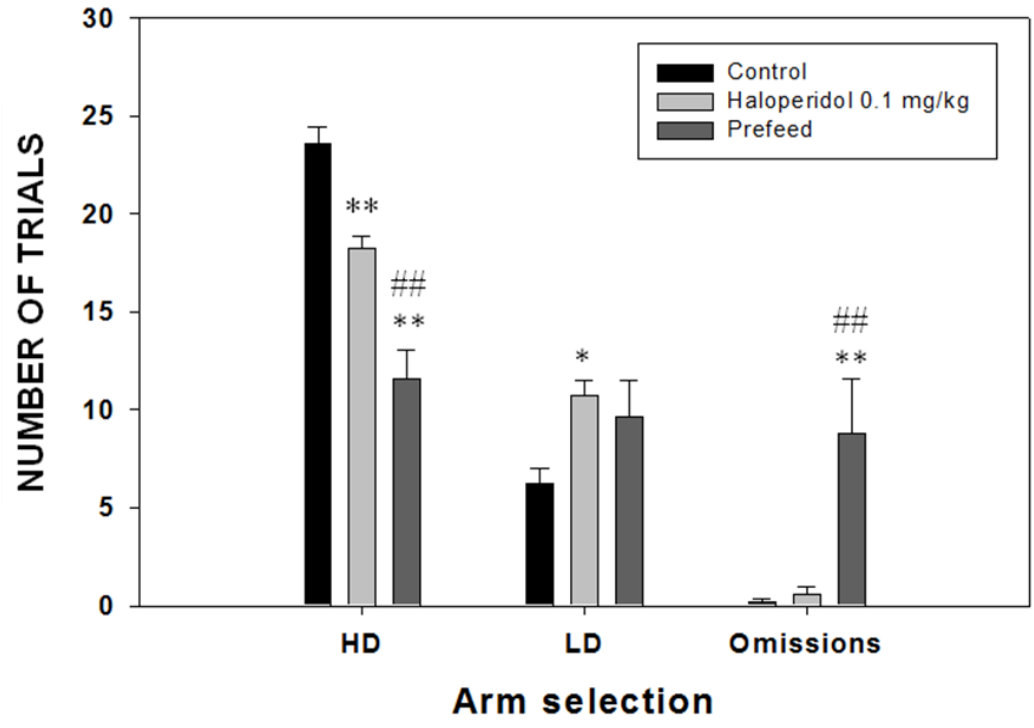


Fig. 3. Effect of different conditions (vehicle control, haloperidol treatment and pre-fed) on HD arm selection, LD arm selection, and omissions. Mean (±SEM) number of choices in the T-maze with barrier in the HD arm. *p<0.05, **p<0.01 significantly different from the vehicle control group in the same arm selection;

##p<0.01 significantly different from haloperidol in the same arm selection.

Experiment 4: *Effect of theophylline on T-maze performance after haloperidol administration*. In the theophylline reversal studies (Fig. 4 left panel), repeated measures ANOVA revealed that there was an overall effect of drug treatment ($F(4,44)=19.05$; $p<0.01$) on HD arm selection. The Tukey test revealed significant differences between control condition (Veh/Veh) and the haloperidol condition (HP/Veh) ($p<0.01$). Also, co-administration of theophylline with haloperidol significantly increased HD arm selection at the two highest doses (HP/10T and HP/15T; $p<0.01$ and $p<0.05$ respectively) compared to the HP/Veh condition, indicating an attenuation of the effect of haloperidol at these doses of theophylline. Repeated measures ANOVA yielded no significant effect on the latency measure (Mean $\pm$ SEM latencies in seconds: Veh/Veh=2.7$\pm$0.2; HP/Veh=4.0$\pm$0.3; HP/Theophylline (5.0 mg/kg)=3.8$\pm$0.6; HP/Theophylline (10.0 mg/kg)=3.2 $\pm$ 0.4; HP/Theophylline (15.0 mg/kg)=3.6 $\pm$ 0.4).

Experiment 5: *Effect of CPT on T-maze performance after haloperidol administration*. Repeated measures ANOVA across treatments showed that there was a significant overall effect of drug treatment on HD arm selection ($F(4,36)=33.38$; $p<0.01$; Fig. 4 middle panel). The Tukey test revealed a significant difference between the control condition (Veh/Veh) and haloperidol plus vehicle ($p<0.01$). However, there were no significant differences between the HP/Veh condition and any of the HP/CPT conditions, indicating that CPT did not alter the effect of haloperidol. Repeated measures ANOVA showed that there

was a significant overall treatment effect on latency to reach the HD arm ($F(4,36)=2.83$; $p<0.05$). When analyzed with the Tukey test animals treated with the highest dose of CPT (9.0 mg/kg) plus haloperidol showed significant increases in latency compared to Veh/Veh condition ($p<0.05$), indicating that CPT plus haloperidol were making the animal slower in reaching the food, thus possibly contributing to a reduced choice of the HD arm; mean $\pm$ SEM latencies (seconds) were as follows: Veh/Veh=3.4 $\pm$ 0.6; HP/Veh=6.8 $\pm$ 2.3; HP/CPT(3.0 mg/kg)=7.8 $\pm$ 2.4; HP/CPT(6.0 mg/kg)=12.0 $\pm$ 3.5; HP/CPT(9.0 mg/kg)= 12.1 $\pm$ 3.4*.

Experiment 6: *Effect of MSX-3 on T-maze performance after haloperidol administration*. MSX-3 produced a robust and significant reversal of the effects of haloperidol on HD arm selection (Fig. 4 right panel). Repeated measures ANOVA across conditions indicated a significant overall treatment effect ($F(4,28)=16.44$; $p<0.01$). Tukey test showed that the HP/Veh condition was significantly different from the Veh/Veh control condition ($p<0.01$). In addition, co-administration of MSX-3 with haloperidol significantly increased HD arm selection at all three doses (HP/M1 $p<0.05$, HP/M2 and HP/M3 $p<0.01$ respectively) compared to the HP/Veh condition, indicating an attenuation of the effect of haloperidol at all doses of MSX-3. Repeated measures ANOVA of the latency data showed no significant differences between conditions; Mean $\pm$ SEM latencies (seconds) were: Veh/Veh= 2.4 $\pm$ 0.1; HP/Veh= 3.9 $\pm$ 0.8; HP/MSX-3 (1.0 mg/kg)= 2.9 $\pm$ 0.3; HP/MSX-3 (2.0 mg/kg)= 2.7 $\pm$ 0.3; HP/MSX-3 (3.0 mg/kg)= 2.9 $\pm$ 0.4.

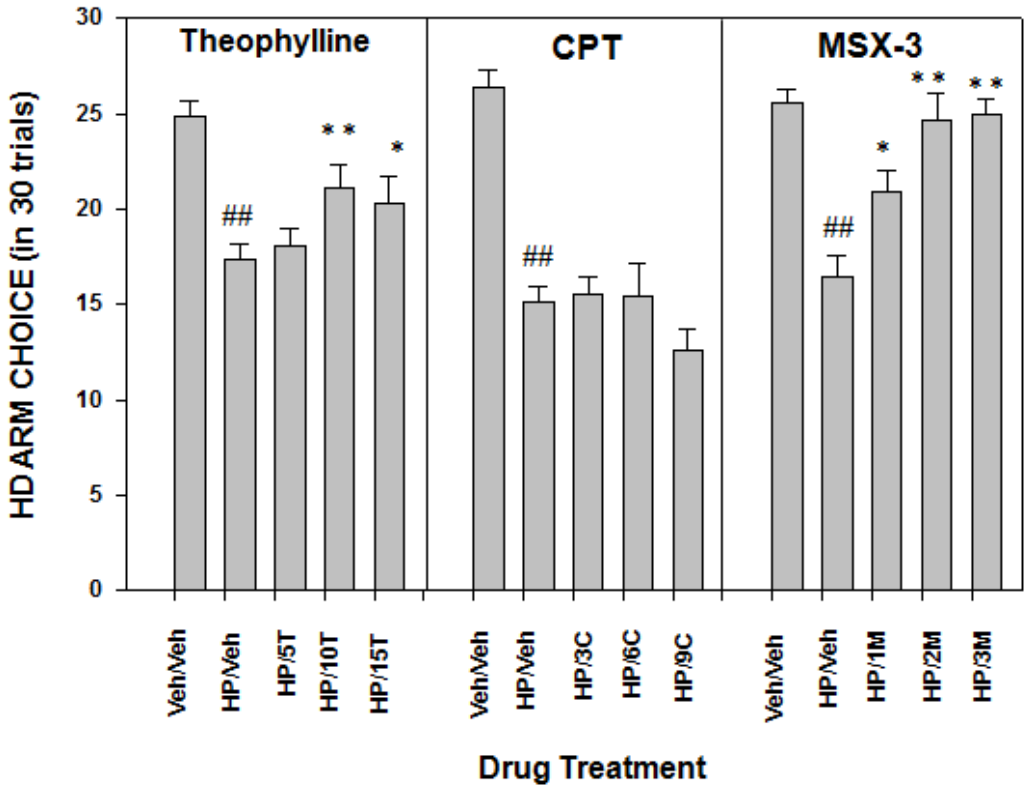


Fig. 4. Effects of theophylline (left panel), CPT (middle panel), and MSX-3 (right panel) in mice co-administered haloperidol on HD arm choice. Mean (±SEM) number of HD arm choices in the T-maze with barrier in the HD arm. ##p<0.01 significantly different from Veh/Veh in the corresponding experiment; *p<0.05, **p<0.01 significantly different from HP/Veh in the corresponding experiment.

Experiment 7: *Effect of theophylline, CPT and MSX-3 alone on T-maze performance.* The highest doses of theophylline, CPT and MSX-3 used in experiments 4, 5 and 6, were tested in the absence of haloperidol (Table 1). Repeated measures ANOVA across drug treatments (Vehicle/Theophylline/CPT/MSX-3) showed a significant effect on HD arm selections ($F(3,33)=6.84$; $p<0.01$). Tukey test analyses showed that theophylline

(p<0.05) and MSX-3 (p<0.01) significantly reduced selection of the HD arm compared to vehicle, effects that were in the opposite direction from those shown when these drugs were co-administered with haloperidol in experiments 4 and 6. Repeated measures ANOVA for the latency measure was not significant; Mean ± SEM latencies (seconds) were: Veh= 3.0 ± 0.6 / Theophylline (15.0 mg/kg)= 2.4 ± 0.3 / CPT (9.0 mg/kg)= 3.2 ± 0.5 / MSX-3 (3.0 mg/kg)= 2.3 ± 0.2.

Treatment	HD arm choice (in 30 trials)
Vehicle	24.2 ± 0.7
Theophylline (15 mg/kg)	21.1 ± 0.9*
CPT (9 mg/kg)	23.1 ± 1.0
MSX-3 (3 mg/kg)	20.2 ± 1.2**

Table 1. Effect of different treatments on HD arm choice in the T-maze. Mean (±SEM) number of HD arm choices. *p<0.05, **p<0.01 significantly different from Vehicle.

Experiment 8: *Effect of haloperidol on T-maze performance in A2A R KO and WT mice*. In figure 5, the impact of haloperidol on the two substrains of mice (A_{2A}R KO and WT) is depicted. Factorial ANOVA with a repeated measures factor (drug treatment; 0.0/0.05/0.1 mg/kg haloperidol) and a between-groups factor (group; A_{2A}R KO /WT) showed no effect of group on HD arm selection, but did yield a statistically significant effect of haloperidol treatment ($F(2,28)=29.82$; p<0.01). Importantly, the haloperidol treatment x group

interaction was also significant ($F(2,28)=11.04$; $p<0.01$), demonstrating that the effect of drug treatment differed between WT and KO mice. The Tukey post hoc test showed significant differences between both doses of haloperidol compared to vehicle in WT animals ($p<0.01$). However, among the KO mice, no dose was significantly different from vehicle. Thus, haloperidol decreased the HD arm selection in WT mice, but not in KO mice, indicating that A_{2A}R KO mice were resistant to the disruptive effect of haloperidol. ANOVA with the latency measure yielded a significant effect of the drug treatment factor ($F(2,28)=3.80$; $p<0.05$), indicating that haloperidol in general increased the latency in both groups of animals. Mean $\pm$ SEM latencies (seconds) were as follows: WT/Veh=1.8 $\pm$ 0.1; A_{2A}R KO /Veh=2.5 $\pm$ 0.2; WT/HP (0.05 mg/kg)= 2.0 $\pm$ 0.1; A_{2A}R KO /HP (0.05 mg/kg)=2.6 $\pm$ 0.3; WT/HP (0.1 mg/kg)=3.6 $\pm$ 1.1; A_{2A}R KO /HP (0.1 mg/kg)=3.6 $\pm$ 1.1. However, there was no significant effect of mouse type, nor was there an interaction between the two main factors.

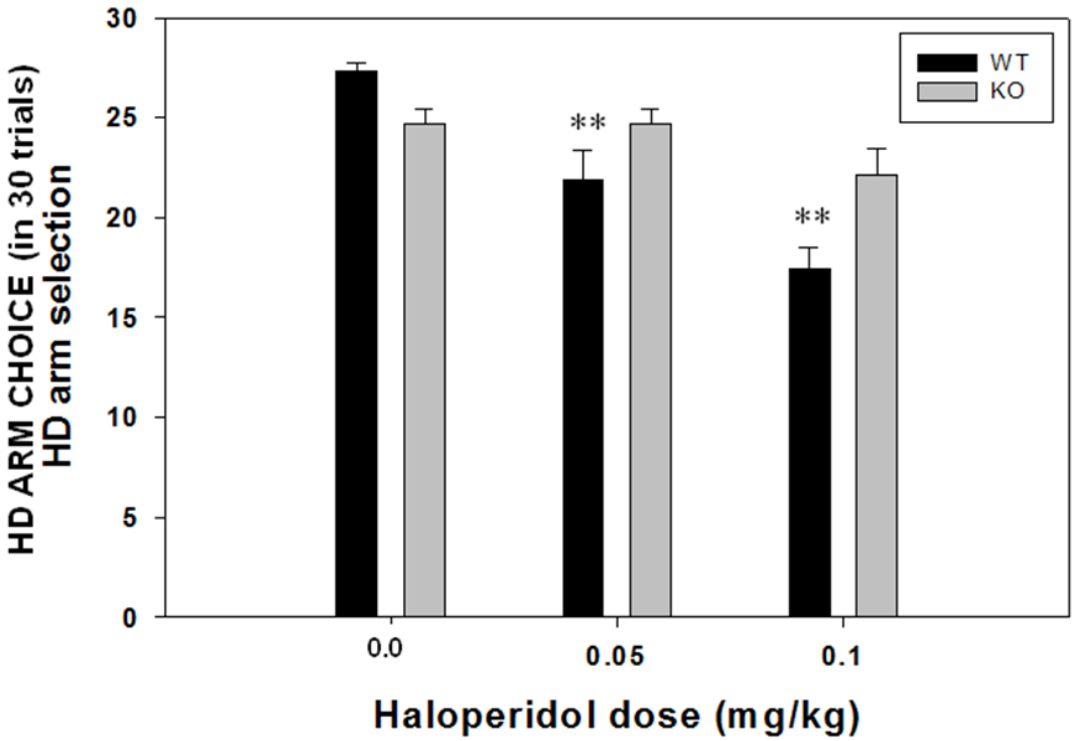


Fig. 5. Effect of haloperidol (0.0, 0.05 and 0.1 mg/kg) in WT and A_{2A}R KO mice on HD arm choice. Mean (±SEM) number of HD arm choices in the T-maze with barrier in the HD arm. **p<0.01 significantly different from vehicle in the corresponding substrain group.

Experiment 9: *Effect of theophylline, CPT or MSX-3 on c-Fos immunoreactivity after haloperidol administration.* The c-Fos counts in different brain areas (see Fig. 6) were analyzed by a two-way (treatment x brain area) factorial ANOVA. There was a significant overall treatment effect ($F(4,76)=8.34$; $p<0.01$), but no significant difference between brain areas, and no significant treatment x brain area interaction. Tukey tests on the data collapsed across brain areas indicated that haloperidol produced a significant overall induction of c-Fos expression compared to vehicle ($p<0.01$), which was attenuated by co-administration of MSX-3 ($p<0.05$).

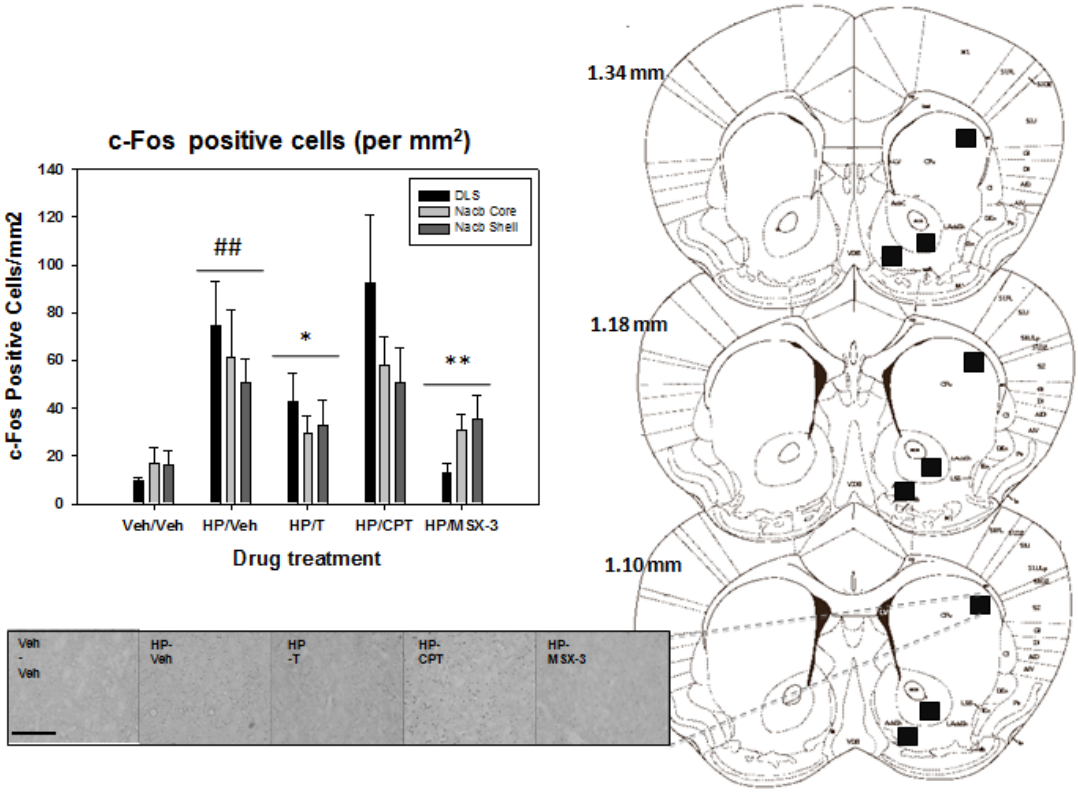


Fig. 6. Left upper part: Effect of different treatments on c-Fos expression in the nucleus accumbens (Nacb) Shell, Core and dorsolateral neostriatum (DLS). Mean (±SEM) number of c-Fos positive cells per mm^2 (vehicle plus vehicle (Veh/Veh), Haloperidol 0.1mg/kg plus Vehicle (HP/Veh), Haloperidol 0.1 mg/kg plus Theophylline 15 mg/kg (HP/T), Haloperidol 0.1 mg/kg plus CPT 9 mg/kg (HP/CPT), or Haloperidol 0.1 mg/kg plus MSX-3 3 mg/kg (HP/MSX-3). ##p<0.01 significantly different from Veh/Veh; *p<0.05, significantly different from HP/Veh. Left lower part: Photomicrographs of c-Fos staining in DLS from representative animals in each treatment group. Low power images (20x). Scale bar=250 □m. Right part: Diagram of coronal sections with bregma coordinates from Franklin and Paxinos 2007, showing location of the brain areas for c-Fos counting.

DISCUSSION

The present studies were undertaken to characterize the interaction between DA D_2 and adenosine A_{2A} receptors in the regulation of effort-based decision making in WT as well as genetically modified mice. This research employed a variant of the T-maze barrier choice paradigm originally developed for rats (Cousins et al., 1996; Salamone et al., 1994), and a number of validation tests and control experiments also were performed. In addition, c-Fos immunoreactivity studies were conducted, focusing upon striatal areas (Nacb and neostriatum) that contain a high concentration of adenosine A_{2A} receptors, and which have a high degree of D_2/A_{2A} co-localization. Taking all the results in to consideration, it is clear that the T-maze barrier choice procedure is a useful task for the exploration of effort-based decision making in mice.

In experiment 1, the results demonstrated that low doses of haloperidol redirected the behavior of CD1 mice towards the less effortful response option, substantially decreasing HD arm selection. Moreover, because vehicle and haloperidol-treated mice in experiment 1 always chose either the HD or LD arm, there was a concomitant increase in selection of the LD arm (i.e., the arm without the barrier, see Fig. 4) in haloperidol-treated mice. The shift from selection of the HD arm to the LD arm with no barrier occurred in a dose-dependent manner, with the greatest effects being seen at 0.1 mg/kg. The dose response curve observed in the present results points to a more potent effect of haloperidol in CD1 mice compared to Sprague-Dawley rats, since in mice the dose at which there was a significant effect on choice was 0.05 mg/kg, while in Sprague-Dawley rats the minimum dose was 0.1 mg/kg (Mott et al., 2009; Salamone et al., 1994) and in Lister Hooded rats it was 0.2 mg/kg (Denk et al., 2005; Walton

et al., 2005). Based upon the present results and previous findings, it is clear that despite the effects of haloperidol on maze arm selection, rodents were still able to engage in food-motivated behaviors by selecting an alternative route of food that does not require climbing a barrier (Mott et al., 2009; Salamone et al., 1994). This observation supports the idea that fundamental features of food motivation remain intact after DA antagonism at the doses used (Salamone and Correa, 2002; Salamone et al., 2007; see experiment 3 discussion below).

The results of experiment 2, in which the T-maze had a 14 cm barrier in both arms, demonstrated that when mice had no choice other than climbing a barrier to get some food, there was no effect of haloperidol (0.1 mg/kg) on arm selection. Thus, DA antagonism did not appear to reduce barrier crossings and cause animals to shift to the LD arm in experiment 1 simply because this treatment set an absolute ceiling on the number of barrier crossings the mice could perform. Furthermore, these data indicate that haloperidol-induced reductions in selection of the HD arm with the barrier in the other experiments were not due to changes in memory or discrimination between the arms, and also suggest that haloperidol was not affecting discrimination or preference of the density of reward (Martin-Iverson et al., 1987; Salamone et al., 1994). Consistent with these observations, 0.1 mg/kg IP haloperidol did not affect arm choice between HD and LD when there was no barrier present (Salamone et al., 1994). In Hooded rats, when a barrier was also placed in the LD arm, haloperidol (0.2 mg/kg, IP) treated animals continued to show a strong preference for the HD arm (Denk et al., 2005), and in Sprague Dawley rats, DA depletions in the anterior cingulate cortex (Schweimer et al., 2005) produced a shift to the LD arm in the one barrier T-maze, but had no effect on the animal's choice in the two barrier T-

maze, thus showing the same pattern as that shown in CD1 mice in the present study. Nacb DA depletions produced a shift from the HD to the LD arm when the LD arm had no barrier (Cousins et al., 1996; Salamone et al., 1994), but, as was the case with haloperidol administration, DA depletions did not affect choice when neither the HD nor the LD arm had a barrier (Salamone et al., 1994). Moreover, accumbens DA depletions had no significant effect on barrier climbing when rats had to choose between an arm with a barrier that had 4 food pellets vs. another arm that had no barrier but also no food (Cousins et al., 1996). Taken together with the present results, these studies indicate that low doses of DA antagonists, as well as Nacb DA depletions, establish a condition in which barrier crossings are reduced, so long as the rodents have an alternative path for obtaining food at low response costs.

In experiment 3, the effects of haloperidol were compared with those produced by pre-feeding to reduce food motivation. In this experiment, an additional behavioral measure was recorded (i.e., omissions: failure to make a choice after leaving the start box). The results of experiment 3 showed that there were virtually no trials in which vehicle or haloperidol-treated animals failed to choose one of the two arms of the maze. Nevertheless, pre-fed animals sowed a dramatic increase in omissions, and a relative indifference between the three options (HD selection, LD selection, omission). Thus, haloperidol did not display a pattern of effects that was consistent with a drug-induced reduction in appetite for food. These pre-feeding results are in accordance with previous studies in rats showing that the effects of DA antagonists or Nacb DA depletions on food-related tasks do not closely resemble those produced by pre-feeding or appetite suppressant drugs (Aberman and Salamone, 1999; Blundell and Thurlby, 1987;

Clifton et al., 1991; Salamone et al., 1991; Sink et al., 2008). Another choice paradigm developed for rats (Salamone et al., 1991), the concurrent chow/ fixed-ratio 5 (FR5) schedule operant task, has been used to assess the impact of pharmacological manipulations on effort-based choice. Low to moderate doses of many different DA antagonists injected IP or directly into Nacb (Cousins et al., 1994; Cousins and Salamone, 1994; Koch et al., 2000; Nowend et al., 2001; Salamone et al., 1991, 1996; Sokolowski and Salamone, 1998), produced a decrease in FR5 lever pressing for food but an increased intake of the concurrently available chow. In contrast to these data, it was seen that pre-feeding, as well as some appetite suppressants (e.g. fenfluramine, CB1 antagonists and inverse agonists) suppressed both lever pressing and chow intake (Salamone et al., 1991, 2002; Sink et al., 2008). Thus, the present results, together with previous studies, indicate that rats and mice treated with low doses of DA antagonists still allocate considerable time for food acquisition and consumption, and do not exhibit signs of satiation or reduced appetite.

The present results indicated that haloperidol did not significantly affect the overall run latency in CD1 mice. Some of these results with latency measures appear to be somewhat different from those reported in previous papers that used rats. In Sprague Dawley rats (Cousins et al., 1996; Salamone et al., 1994), 0.1 mg/kg IP of haloperidol and Nacb DA depletion significantly increased response latencies in both the barrier and no-barrier conditions. However, despite this increased latency, every animal completed the 30 trials per session. In Hooded rats, a relatively higher dose (0.2 mg/kg) of haloperidol caused a slight increase in time taken to climb the barrier, but the effect was much more robust for the latencies to go from the starting arm to the barrier than from the top of the barrier

to the food, indicating that the overall effect on latency was not specifically due to a difficulty to climb the barrier. However, this dose of haloperidol made some animals to stop completing the 10 trials per session (Denk et al., 2005). But despite these minor differences, the overall pattern of results from experiments 1-3 provide a validation of the effort-based choice T-maze paradigm as a useful way to study motivated behaviors in mice, as well as rats.

Of course, Nacb DA must participate in effort-related processes in concert with other brain structures and neurotransmitters, and for that reason the present studies investigated the ability of adenosine antagonists to reverse the effects of a D_2 antagonist on the T-maze effort based choice paradigm. The second group of experiments (experiments 4-6) studied the interaction between adenosine receptor antagonists with different profiles of selectivity and the DA D_2 family antagonist haloperidol. In particular, the interaction between DA D_2 and adenosine A_1 and A_{2A} receptors was investigated. The results showed that theophylline, a nonselective adenosine antagonist, could partially reverse the effects of haloperidol (0.1 mg/kg) on performance of the T-maze barrier choice paradigm. Theophylline produced a moderate improvement in the selection of the HD arm in haloperidol-treated animals at the two highest doses (10.0 and 15.0 mg/kg), although theophylline did not appear to completely restore control levels of HD arm selection. The effects of theophylline on the T-maze choice impairment induced by haloperidol were similar to the effects of caffeine in the concurrent chow/FR5 schedule operant task (Salamone et al., 2009). In that study, caffeine reversed the effects of haloperidol in rats, but its effect size was smaller than the effect of an A_{2A} antagonist (KW6002), and bigger than that produced by an A_1 antagonist (DPCPX; Salamone et al., 2009). Thus, experiment

5 and 6 were conducted to study the relative involvement of A_{2A} and A_1 receptors on the T-maze effort-related choice. The selective A_1 antagonist CPT, in the dose range tested, did not reverse the effects of haloperidol. Adenosine A_1 receptor antagonism failed to re-establish baseline levels of HD arm selection; animals co-administered CPT and haloperidol continued selecting the LD arm for approximately half of the trials, showing the same pattern of behavior as mice treated with haloperidol plus vehicle. This was the only case among all the pharmacological manipulations used in the present work in which the latency to reach the food was increased substantially (four-fold) after co-administration of an adenosine antagonist with haloperidol (the highest dose of CPT (9.0 mg/kg) plus haloperidol significantly differed from haloperidol alone). This result indicates some degree of motor impairment due to the combination of both haloperidol and CPT, which is consistent with a previous report indicating that the A_1 antagonist DPCPX worsened performance in haloperidol-treated rats (Mott et al., 2009). In experiment 6, co-administration of the adenosine A_{2A} antagonist MSX-3 with haloperidol restored the normal pattern of behavior and substantially reversed the effects of haloperidol. These results were obtained at the two highest doses of MSX-3 used (2.0 and 3.0 mg/kg), which reached HD choice levels comparable to the Veh/Veh control group. The lowest dose of MSX-3 (1.0 mg/kg) partially reversed the haloperidol effects. Although MSX-3 alone did not alter HD arm choices in rats (Mott et al., 2009), the present studies with mice showed that theophylline and MSX-3 administered alone could reduce HD selection. It is possible that these effects were due to the psychomotor stimulant properties of adenosine antagonists (Ferré 2008; Randall et al., 2011),

which could have led to a more random pattern of arm selection or more impulsive choice.

Previous studies with rats, using both the T-maze procedure and operant choice task, showed a similar interaction between D_2 family antagonists and A_{2A} antagonists (Farrar et al., 2007; Mott et al., 2009; Worden et al., 2009). All these data together demonstrate that A_{2A} antagonism can reverse D_2 antagonism on tasks involving effort-related processes in mice as well as rats. Thus, as predicted, the antagonism of adenosine A_{2A} receptors was more effective than A_1 antagonism in restoring the behavior impaired by DA D_2 antagonism. The moderate effects of the two nonselective antagonists, caffeine (Salamone et al., 2009) and theophylline (present results), can be explained by reports indicating that, at least in the case of caffeine, there is some degree of preference for adenosine A_1 receptors over A_{2A} receptors (Ferré, 2008). It is likely that this pattern of results in drug interaction studies is due to the co-localization of A_{2A} receptors with D_2 receptors (Mott et al., 2009; Nunes et al., 2010; Salamone et al., 2009). Adenosine A_{2A} receptors are located on striatal GABAergic enkephalin-positive neurons that also express DA D_2 receptors (Fink et al., 1992; Ferré et al., 1997; Svenningsson et al., 1999; Chen et al., 2001). DA D_2 and adenosine A_{2A} receptors converge onto the same signal transduction mechanisms and show the capacity to form heteromers (Fink et al., 1992; Ferré et al., 1997, 2004, 2008; Svenningsson 1999; Fuxe et al., 2003). A_{2A} receptors, through their coupling to G_{olf} proteins, can stimulate adenylyl-cyclase activity and activate the cAMP-PKA signaling pathway, with phosphorylation of several PKA substrates, such as DARPP-32 and CREB and the consequent increase in the expression of different genes, such as *c-fos* or *preproenkephalin* in the GABAergic

enkephalinergic neuron (Ferré et al., 2008). The tonic activation of D_2 receptors blocks the ability of A_{2A} receptors to signal through the cAMP-PKA pathway. Administration of D_2 receptor antagonists produces a significant increase in the PKA-dependent phosphorylation of DARPP-32 and an increase in the expression of *c-fos* and *preproenkephalin* genes, which depends on the ability of D_2 receptor blockade to liberate A_{2A} receptor signaling activated by endogenous adenosine. Thus, the neural effects of the D_2 receptor antagonists can be counteracted by co-administration of A_{2A} receptor antagonist (Ferré et al., 2008).

Taking all these results in to consideration, we decided to explore the impact of D_2 antagonism on WT and adenosine A_{2A}R KO mice in experiment 8. Our results showed that adenosine A_{2A}R KO mice were more resistant than WT mice to the effects of haloperidol on HD arm selection in the T-maze. Haloperidol failed to produce any change in HD arm selection in the A_{2A}R KO mice, while it significantly reduced choice of the HD arm in the WT mice at both doses. No difference was seen in the latency. Thus, genetic elimination of adenosine A_{2A} receptors blocked the shift to a less effortful option in response to haloperidol**.** A_{2A}R KO mice have been demonstrated to be more resistant than WT animals to the cataleptic effect of DA antagonists like haloperidol or SCH 23390 (Chen et al., 2001; El Yacoubi et al., 2001). In our study, A_{2A}R KO animals showed no significant learning deficit in the T-maze compared to the WT mice (see supplemental material), and previous studies with A_{2A}R KO animals have showed no impairment in memory (Wang et al., 2006) in these animals. However, A_{2A}R KO mice have been reported to have reduced spontaneous locomotion (Chen et al., 2001; Ledent et al., 1997), a factor that could be related to the non-significant tendency to reach higher latencies in the

present experiments. There are a few possible mechanisms that could underlie the lack of effect of haloperidol on A_{2A}R KO mice. First, it is possible that genetic deletion of striatal A_{2A} receptors could alter striatal D_2 receptor function. There is evidence of antagonistic intramembrane A_{2A}– D_2 interactions, by which stimulation of adenosine A_{2A} receptors decreases the ability of DA to displace a D_2 antagonist from binding to D_2 receptors (Ferré et al., 1999). Thus, A_{2A}R KO could be enhancing the ability of endogenous DA to compete with haloperidol for binding to DA receptors. In addition, because adenosine A_{2A} and DA D_2 receptors converge onto the same adenylyl cyclase-related signal transduction cascade (Ferré et al., 1997, 2008), deletion of A_{2A} receptors could be altering the signal transduction effects of D_2 receptor blockade.

Finally, c-Fos expression was quantified as a cellular marker of D_2-A_{2A} interactions in Nacb and striatum in mice trained on the T-maze task. Because D_2 and adenosine A_{2A} receptor stimulation has opposite effects on stimulation of cAMP-related pathways, it was thought that adenosine A_{2A} antagonism should blunt the ability of the D_2 antagonist to affect transcription of immediate early genes and induce formation of Fos-related proteins. Earlier reports have shown increased c-Fos expression in striatal areas, including Nacb, after systemic administration of D_2 family antagonists (Betz et al., 2009; Farrar et al., 2010; Hussain et al., 2002; Pinna et al., 1999; Svenningsson et al., 1999b). Also, it has been previously observed that the increases in c-Fos expression induced by D_2 antagonists in striatal areas, including Nacb, can be attenuated by co-administration of A_{2A} receptor antagonists and by the nonselective adenosine antagonist, theophylline (Betz et al., 2009; Boegman and Vincent, 1996; Farrar et al., 2010; Pinna et al., 1999, Ward and Dorsa, 1999). So far, all the studies have

been done in other behavioral settings (catalepsy) or in studies that mainly involved untrained rats. In our results, the pharmacological manipulations were done in mice highly trained in the T maze procedure. Potentially, the neuroadaptations that underlie the establishment of a well learned or habitual behavior could have produced a different outcome on c-Fos expression. Nevertheless, the present results demonstrated that the low dose of haloperidol 0.1 mg/kg produced a decrease in high effort selection that was parallel to the increase in c-Fos expression across the striatal structures studied. Moreover, MSX-3, an A_{2A} receptor antagonists, that in rats has been demonstrated to reduce eticlopride-induced c-Fos expression in Nacb (Farrar et al., 2010), was effective in reversing the effect of haloperidol on c-Fos expression and arm selection in the T-maze choice procedure in mice. Theophylline, which is a non-selective adenosine receptor antagonist, also tended to show the same pattern of effects, but the results did not reach statistical significance. Thus, the present results with c-Fos provide a behaviorally relevant cellular marker of the interaction between DA D_2 and adenosine A_{2A} receptors in mice trained on the T-maze task.

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CHAPTER 5:

DOPAMINE D_2 RECEPTOR ANTAGONISM MODULATES THE PREFERENCE FOR PRIMARY REINFORCERS BASED ON THEIR EFFORT REQUIREMENTS: STUDIES USING RUNNING WHEELS AND SUCROSE CONSUMPTION IN MICE.

Abstract

Organisms frequently make effort-related decisions based upon assessments of motivational value and response costs. Dopamine (DA), particularly in nucleus accumbens (Nacb), regulates effort-related processes. In the present experiments, the DA D_2 receptor antagonist haloperidol was used to study the DAergic involvement in the activational and directional components of motivated behaviors when multiple reinforcers are available. A T-maze task was developed for the assessment of preference for two different types of rewards: physical activity (i.e., running in a wheel) in one arm and 5% sucrose access in the other. In Swiss male mice tested for locomotion in a running wheel (RW) enclosed in a cage, haloperidol (0.1 and 0.2 mg/kg) decreased spontaneous locomotion. In the T-maze, under normal conditions, sucrose consumption was less preferred than the RW. However, haloperidol produced a shift in arm preference, reducing time spent on the RW, but increasing selection of sucrose. Haloperidol fragmented the pattern of RW behavior so that the animals spent less total time running but increased the number of running episodes initiated. However, time consuming sucrose and frequency of drinking bouts for sucrose

were increased. Thus, D_2 antagonism reduced the choice of a primary reinforcer that involved vigorous activity, but increased consumption of a reinforcer that could be freely obtained and required little effort. Pre-exposing animals to both reinforcers did not produce this shift towards more sucrose consumption. In summary, the present results implicate DA D_2 receptors in the regulation of behavioral activation and effort-based choice. This research allows for a better understanding of psychiatric symptoms such as psychomotor slowing, fatigue or anergia that can be observed in pathologies like depression. Moreover, it demonstrates that DA systems regulate preference for engaging in physical activities relative to other reinforcing conditions.

INTRODUCTION

Nucleus accumbens (Nacb) dopamine (DA) is an important component of the neural circuitry that regulates behavioral activation and the ability of organisms to overcome work-related response costs in motivated behaviors (Salamone et al. 2003, 2005, 2007, 2009b; Robbins and Everitt 2007). The effects of Nacb DA depletions or DA receptor antagonism on food-reinforced behavior interact powerfully with the response requirements of an instrumental task. Research with concurrent choice tasks involving distinct reinforcers that can be obtained by activities that have different work requirements has shown that rodents with accumbens DA depletions or DA receptor antagonists reallocate their instrumental behavior away from food-reinforced tasks that have high response requirements (e.g., ratio requirements, vigorous activities such as climbing), and instead select a less-effortful type of food-seeking behavior (Salamone et al. 2007; Pardo et al., 2012).

In addition to being an instrumental requirement for obtaining access to motivational stimuli, considerable research indicates that physical activities can have intrinsic motivational or reinforcing properties. In research with rodents, one of the most commonly studied voluntary physical activities is wheel running**.** Sherwin (1998) concluded that wheel running in rodents is "self-reinforcing" rather than being a redirected or substitute activity. Running appears to be motivationally regulated like other appetitive behaviors (Mueller et al., 1997). Thus, wheel running can be used as the motivational stimulus for the establishment of a conditioned place preference (Lett et al. 2000), and as an explicit reinforcer in operant conditioning procedures (Collier et al. 1990; Iversen 1993; Catania, 1966; Epling and Pierce, 1992; Premack, 1972).

Of course, the choice to engage in voluntary physical activity is always undertaken in relation to the possible selection of other alternatives, such as sedentary behaviors, drugs or food consumption. However, if a running wheel (RW) is present in a complex environment that offers other alternatives such as drugs of abuse, rats will spend a considerable amount of time engaged in running activity (McMillan et al., 1995; Kanarek et al., 1995; Cosgrove et al., 2002). Some studies have demonstrated that when given a choice between food and RW, rats often choose running over food (Epling and Pierce, 1992; Routtenberg, 1968; Symons 1973), and food consumption decreases on days that rats have access to RW (Mueller et al., 1997). However, little is known about the neural mechanisms involved in the selection of physical activity relative to other gustatory reinforcers such as sucrose.

In the present study a rodent behavioral model for investigating the decision making processes that allow for the selection of voluntary physical activity relative to other activities was developed. Thus, we employed a multi-arm maze task that allowed mice to choose between an arm that uses the opportunity to engage in wheel running as the reinforcer vs. selecting other maze arms that lead to a bottle containing 5% sucrose or an empty arm. With this paradigm we assessed the impact of DA antagonism and compared it with conditions that reduce motivation such as free access to RW and 5% sucrose previous to the test session on the activational and the directional component of motivated behavior.

MATERIALS AND METHODS

Animals

Swiss male mice (N=22) weighed 24-28 g at the beginning of the study (Janvier, France). Mice were housed in groups of three per cage; with standard laboratory rodent chow and tap water available *ad libitum* (see specific conditions for each experiment). Subjects were maintained at 22 $\pm$ 2 ºC with 12-h light/dark cycles. 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).

Pharmacological agents

Haloperidol (Sigma Quimica C.O), a DA D_2 receptor antagonist, was dissolved in a 0.3% tartaric acid solution (pH=4.0), which also was used as the vehicle control. Haloperidol was administered intraperitoneally (IP), 50 minutes before testing started. Sucrose (Sigma Quimica C.O) was dissolved in tap water (5% w/v) and used for oral self-administration.

Apparatus and testing procedures

Testing sessions started two hours after the colony lights were on. The behavioral test room was illuminated with a soft light, and external noise was attenuated.

RW locomotion. The automated RW 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. The session lasted 30 minutes.

T-Maze paradigm. The maze apparatus consisted of a central corridor with two opposed arms (25 L x 11 W x 30 H cm). Each arm provided a different type of reinforcer: A RW in one arm (this RW was not connected to a digital counter), 5% sucrose solution on the opposite arm and nothing in the central corridor. Half of the mice had the RW consistently located on the left arm, while half the mice had the RW on the right arm. Training phase 1: in pilot studies we observed a strong preference for the RW such that most animals did not even approach the sucrose arm once. Thus, for the experiment, during this first phase, the RW arm was blocked to force animals to explore the sucrose arm. In addition, for the first 4 days, mice had restricted water access in the home cage (3.5 ml/day/mice). After a total of 10 days of exposure only to sucrose, access to the RW arm was concurrently allowed. Training phase 2: Animals were trained once a day for 15 minutes, during 5 days before the drug tests began. Water in the home cage was restricted to 5 ml/day/mice. Test session: animals were introduced in the T maze and, during 15 minutes, accumulated time spent in the wheel (duration), time consuming sucrose, and number of interactions with the sucrose or the RW (bouts) were recorded.

Experiments

Experiment 1: *Effect of haloperidol on locomotor behavior in the RW.* Mice (N=12) were trained 5 days a week during 60 minutes for 3 weeks. A within-groups design was used. During the drug testing phase there was one drug treatment day and 4 baseline days before the next drug day. On the test day,

animals received one injection of haloperidol (0.0, 0.025, 0.05, 0.1 and 0.2 mg/kg) 50 min before being tested in the RW for 30 min.

Experiment 2: *Effect of haloperidol on the preference for RW versus sucrose in the T-Maze.* During the drug testing phase there was one drug treatment day per week and 4 baseline days before the next drug day. Thus, a within-groups design was used. On the test day, mice (N=10) received the following haloperidol doses: 0.0, 0.05, 0.1 and 0.2 mg/kg, 50 minutes before testing.

Experiment 3: *Effect of pre-exposure to RW and sucrose reinforcers on the preference in the T-Maze.* Once experiment 2 was completed, mice were trained one additional week and then had 24 hours of free 5% sucrose in their home cages as well as having 2 periods of 60 minutes free access to a RW in a new cage where sucrose was also concurrently available. After these pre-exposure sessions, animals were moved to their home cages 15 minutes before the test session started. In addition to the previous dependent variables, inactive time (time not engaged interacting with any of the reinforcers) was recorded during 15 minutes.

Statistical analyses

Experiments were analyzed using a one-way repeated measures ANOVA, followed by non-orthogonal planned comparisons using the overall error term, which compared vehicle to all the other doses (Keppel, 1991). Because the sucrose data were highly variable, data were square route transformed prior to performing the ANOVA. STATISTICA 7 software was used. All data were expressed as mean ± SEM, and significance was set at $p<0.05$.

RESULTS

Experiment 1: *Effect of haloperidol on locomotor behavior in the RW.* Repeated measures ANOVA yielded a significant effect of the haloperidol treatment (0.0, 0.025, 0.05, 0.1 and 0.2 mg/kg) (F (4,44)=6.71, p<0.01) on number of turns in the RW (Fig. 1). Planned comparisons revealed a dose dependent effect of the haloperidol on RW locomotion, since only the two highest doses 0.1 mg/kg (p<0.05) and 0.2 mg/kg (p<0.01) were significantly different from the 0.0 mg/kg dose.

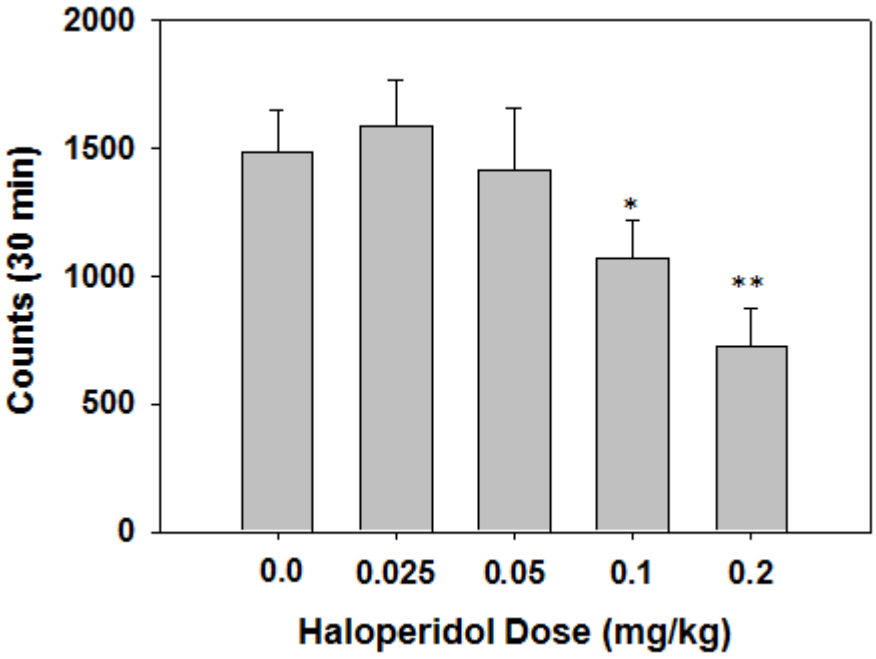

Fig. 1. Effect of haloperidol (0.0, 0.025, 0.05 or 0.1 mg/kg) on locomotion in the RW. Mean (±SEM) number of counts during 30 minutes. *p<0.05 **p<0.01 significantly different from vehicle.

Experiment 2: *Effect of haloperidol on the preference for RW versus sucrose in the T-Maze.* Independent ANOVAs were performed to analyze the time and frequency (i.e., number of bouts initiated) data for both the RW and the sucrose intake measures. Repeated measures ANOVA for the main factor haloperidol dose (0.0, 0.05, 0.1 and 0.2 mg/kg) indicated a significant effect (F (3,27)=6.32, p<0.01) on time spent in the RW (Fig. 2A). Planned comparisons revealed significant differences between 0.0 mg/kg and all doses of haloperidol, 0.05 mg/kg (p<0.05), 0.1 and 0.2 mg/kg (p<0.01). The ANOVA for the running bouts in the RW indicated also a significant effect of the haloperidol treatment (F (3, 27)=5.18, p<0.01; Fig. 2B). Significant differences between 0.0 mg/kg and 0.05 mg/kg (p<0.01), and also with 0.1 mg/kg (p<0.05) were found. The ANOVA for the time spent consuming sucrose yielded a significant effect of haloperidol treatment (F (3, 27)=3.47, p<0.05. Fig. 2C). Significant differences between 0.0 mg/kg and 0.05 mg/kg and 0.2 (p<0.05), and also with 0.1 mg/kg (p<0.01) were found, with these doses of haloperidol substantially increasing sucrose drinking. Finally, the repeated measures ANOVA indicated a significant effect of haloperidol treatment (F (3,27)=3.94, p<0.05) on sucrose drinking bouts (Fig. 2D). Planned comparisons revealed significant differences between 0.0 mg/kg and 0.05 mg/kg and 0.2 (p<0.05), and also with 0.1 mg/kg (p<0.01) were found.

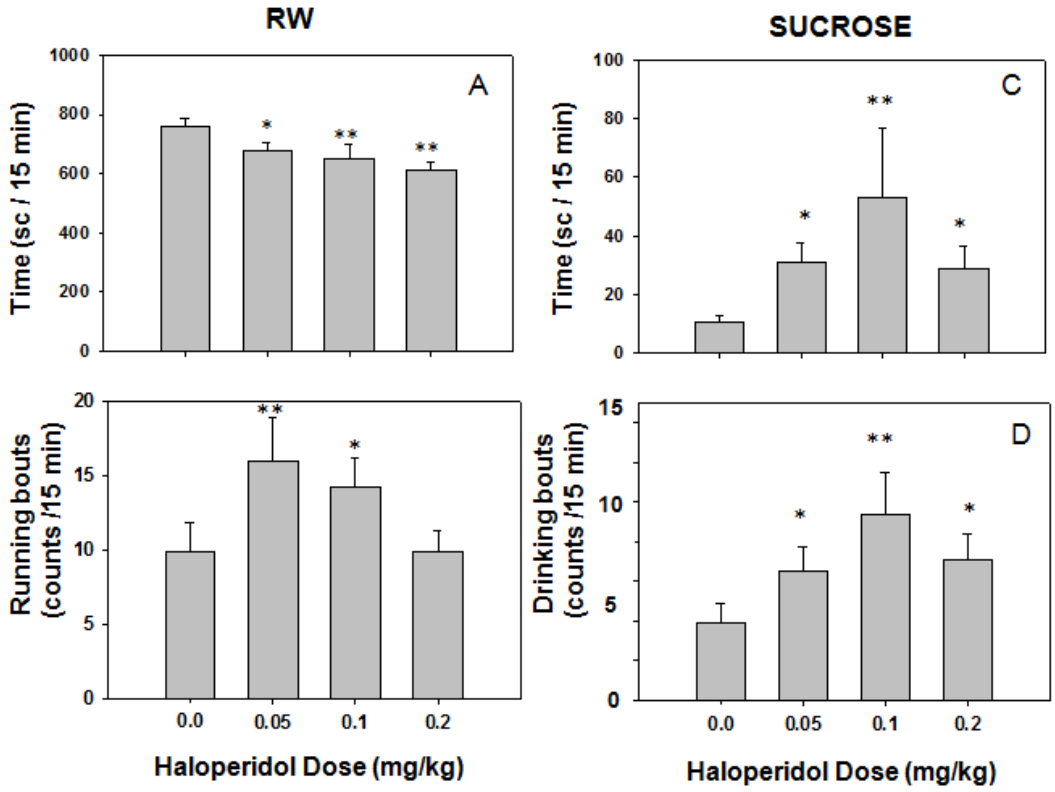

Fig. 2. Effect of haloperidol (0.0, 0.05, 0.1 and 0.2 mg/kg) on the T-Maze with different reinforcers. Mean (±SEM) on A) time spent in the RW (seconds in 15 min), B) times behavior in the RW was initiated (counts in 15 min), C) time spent consuming sucrose (seconds in 15 min) and D) times of sucrose consumption episodes (counts in 15 min). *p<0.05 **p<0.01 significantly different from vehicle.

Experiment 3: *Effect of pre-exposure to RW and sucrose reinforcers on the preference in the T-Maze.* Figure 3 depicts the results of comparing control animals with the 0.1 mg/kg haloperidol dose and the pre-exposed group on time spent running in the RW, consuming sucrose, or not interacting with any of these reinforcers. A separate ANOVA was performed for each of the three measures. There was a significant treatment effect on time spent on the RW ($F(2,18)=4.93$, $p<0.05$). Planned comparisons indicated that haloperidol and pre-

exposure conditions differed from the control condition (p<0.01 and p<0.05 respectively). In addition, there was a significant effect on number of sucrose drinking bouts (F (2,18)=10.12, p<0.01). In this case only the haloperidol condition differed from the other two groups (control condition, p<0.01, and from the pre-exposed group, p<0.05). Lastly, there also was a significant effect on inactive time (F (2, 18)=4.68, p<0.05). On this measure, both haloperidol and pre-exposure condition differed from control condition (p<0.05 and p<0.01 respectively).

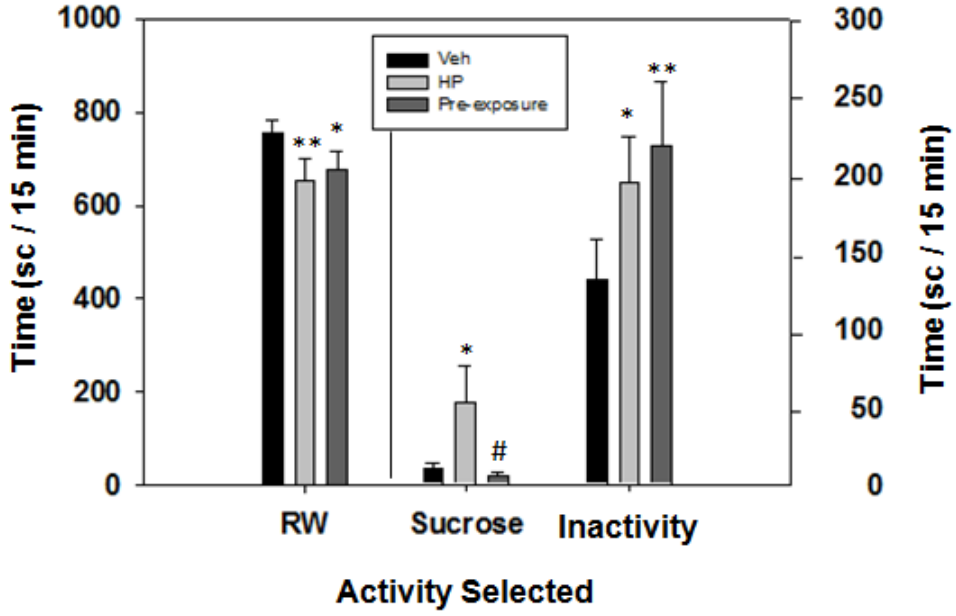

Fig. 3. Effect of different treatments (vehicle control, haloperidol 0.1 mg/kg and pre-exposure) on time spent in the RW (left axis), time consuming sucrose and inactivity (right axis) in the T-maze. Mean (±SEM) seconds in 15 minutes. *p<0.05 **p<0.01 significantly different from the vehicle control group in the same selected activity; #p<0.05 significantly different from haloperidol in the same selected activity.

DISCUSSION

The present study evaluated the impact of DA antagonism on the preference for two different types of reinforcers; a highly palatable reinforcer and an activity-based reinforcer. Normal mice have a high preference for engaging in physical activities (Epling and Pierce, 1992; Routtenberg, 1968; Symons 1973). Running on a wheel has been used as a reinforcer *per se* in rodents. Rats would press a lever (on a progressive ratio, a FR10 or a variable interval schedule) in order to gain access to a RW (Pierce et al., 1986; Collier et al. 1990; Belke and Dunbar, 1998). Even though sucrose is also a very reinforcing substance in animals (Pfaffmann, 1978; Bachmanov et al., 1997; Berridge, 2000; Steiner et al., 2001; Levine et al., 2003; Yamamoto, 2003), and is often preferred to other palatable reinforcers (saccharine, or food) and to drugs of abuse (McMillan et al., 1995; Kanarek et al., 1995; Cosgrove et al., 2002), we have observed that a very high percentage of mice exposed concurrently to RW and sucrose for the first time chose to spend most of their time interacting and running on the wheel and very few of them try the sucrose. This is a normal pattern of behavior since typically rodents display neophobia for new tastants (Amico et al., 2005; Mason et al., 1978; Minasyan et al., 2007; Stewart and Reidinger, 1984). Thus, in the initial training phase we deprived the mice of water and exposed them only to the sucrose solution in order to make sure that later on, baseline preferences were based on animals exposed to both reinforcers. Even then, non-water deprived control animals spent 84% of the time on the RW and only 1.2% of the time sniffing or drinking from the bottle spout dispensing 5% sucrose.

As shown in the first experiment, haloperidol dose dependently reduces locomotion on a computerized RW attached to a cage. The doses that were effective in reducing locomotion were 0.1 and 0.2 mg/kg. Thus, for the preference test we included these doses but also a lower dose that did not suppress locomotion. The effect of haloperidol on several measures of preference for RW or sucrose indicate that in the T maze, mice receiving haloperidol (at all doses tested) showed significantly reduced time spent on the RW, even at the dose of 0.05 mg/kg that did not affect locomotion in the caged RW. The significant effect of 0.05 mg/kg haloperidol in this case is probably due to the fact that, while in the caged RW the animal has no other significant source of sensory stimulation, in the T maze the perceived value of running on a wheel is weighed against another type of stimulus; the sucrose. Thus, after receiving a DA antagonist, and when given a choice, mice shift their preference by reducing time spent on the RW but significantly increasing time spent interacting with the less preferred sucrose reinforcer.

In addition, haloperidol fragmented the pattern of running in the wheel. Although the total interaction time with the RW was significantly less, haloperidol treated animals stop and started this behavior more times than vehicle at the two lowest doses. This fragmented pattern of behavior was similar to that previously reported for haloperidol- treated rats in a semi-naturalistic foraging environment (Salamone, 1988). In the present studies, the high dose did not produce this increase in running bouts; mice treated with 0.2 mg/kg initiate episodes of running in the wheel with the same frequency as control animals, although the total duration of these episodes was shorter. As for the sucrose, animals engaged in drinking more frequently than control animals at all

doses tested. In summary, this shift in relative preference produced by a D_2 antagonist, from a reinforcer that involves physical activity to a reinforcer that requires little energy expenditure, supports the role of DA in behavioral activation but not in the consumption of palatable reinforcers such as sucrose. Similar results were reported previously when rodents were given a choice between food and RW (Epling and Pierce, 1992; Routtenberg, 1968; Symons 1973). It also has been previously demonstrated that local blockade of D_1 and D_2 receptors the in Nacb of food deprived rats suppressed spontaneous motor activity and shifted the structure of feeding towards longer bout durations, but did not alter the total amount of food consumed (Baldo et al., 2002). Non-selective DA antagonists injected into Nacb reduced speed to approach a sucrose solution at the end of a corridor, but drug treatment did not affect final sucrose intake (Ikemoto and Panksepp, 1996). The effects obtained in the present work were obtained in the context of a choice situation. Haloperidol reduced locomotion counts and total duration of RW activity, and at the same time it did increase preference for sucrose by increasing time spent drinking and sniffing, and initiating drinking bouts. On the operant FR5/chow feeding choice procedure, low-to-moderate doses of D_1 and D_2 antagonists all produced a decreased lever pressing for food but substantially increased intake of the concurrently available chow (Salamone et al., 2010).

This pattern of effects was not produced by pre-exposing the animals to both reinforcers. After reducing motivation by allowing free sucrose consumption and free access to a RW before testing, mice reduced time spent in the RW, and, like haloperidol, also increased time not interacting with any of the reinforcers. However, differently from haloperidol, mice did not shift towards an

increase in sucrose consumption. Thus, the pattern of effects produced by haloperidol does not mimic the effect of sucrose satiation. DA antagonists or Nacb DA depletions do not produce effects that closely resemble those produced by pre-feeding or appetite suppressant drugs in concurrent operant lever pressing/chow feeding choice tasks (Salamone et al, 1991; Blundell and Thurlby, 1987; Clifton et al, 1991; Aberman and Salamone, 1999; Sink et al, 2008; Randall et al., submitted) or in a T maze with a different effort-choice based task (Pardo et al., 2012). Together with other results, these findings demonstrate that interference with DA transmission does not simply reduce appetite (Salamone and Correa 2009).

A different T-maze procedure for rats and mice that is closely related to the present study was developed previously in order to assess the effects of accumbens DA depletions on effort-related choice behavior (Salamone et al., 1994; Pardo et al., 2012). With this procedure, the two choice arms of the maze can have different food reinforcement densities, and a barrier can be placed in the arm with the higher density of food to vary task difficulty. When no barrier was present in the arm with the high reinforcement density, rodents mostly chose that arm, and neither haloperidol nor Nacb DA depletion altered their response choice (Salamone et al., 1994). When the arm with the barrier contained pellets, but the other arm was empty, rodents with Nacb DA depletions were slower than control rats, but still managed to choose the high density arm, climb the barrier, and consume the pellets (Cousins et al., 1996). Yet Nacb DA depletions and DA antagonists dramatically altered choice behavior when the high density arm had the barrier in place, and the arm without the barrier contained an alternative food source. In this case, animals with compromised DA function showed decreased

choice for the high density arm, and increased choice for the low density arm (Cousins et al., 1996; Salamone et al., 1994; Mott et al., 2010; Pardo et al., 2012). Interestingly, the same doses of haloperidol had no effect on choice when both arms were blocked by barriers (Pardo et al., 2012); this observation confirms that the haloperidol-treated mice were capable of climbing the barrier, but chose not to when there was an alternative food source available that could be obtained with less effort. Again, in the T-maze, there were virtually no trials in which vehicle or haloperidol-treated animals failed to choose one of the two arms of the maze. Nevertheless, pre-fed animals showed a dramatic increase in omissions, and a relative indifference between the three options (high density selection, low density selection, omission) (Pardo et al., 2012). Thus, haloperidol did not display a pattern of effects that was consistent with a drug-induced reduction in appetite for food.

Based on all these results a clear role for Nacb DA has been described on the activational aspect of motivation. It has been demonstrated that when animals have a choice between two options with different effort demands, they reallocate their instrumental response selection based upon the response requirements of the task, DA antagonists are able to redirect the behavior of animals towards the less effortful option, while leaving intact the orientation towards acquiring a reinforcer (see Salamone and Correa, 2002; Salamone et al., 2009). This conclusion is in accordance with the present results. In the novel RW-Sucrose T-maze procedure the choice is between a reinforcer that requires high levels of activation (running in a wheel) or the consumption of sucrose, a much less energetic option.

It has been previously reported that a number of factors can influence wheel running activity, including environmental conditions (de Rijke et al. 2005; Cabeza de Vaca et al. 2007; de Visser et al. 2007), pharmacological or lesion manipulations (Iwamoto et al. 1999; Cabeza de Vaca et al. 2007) and genetic factors (Morishima-Yamato et al. 2005; de Visser et al. 2007). It is reasonable to argue that the intrinsic reinforcing value of voluntary activities, including not only lever pressing or barrier climbing, but also activities such as wheel running, are of critical importance for understanding several aspects of motivation and decision making (Salamone et al. 2009b). For example, Nacb DA depletions, which are known to suppress several types of spontaneous, novelty-induced, and schedule-induced behaviors in rats, also were shown to suppress schedule-induced wheel running (Wallace et al. 1983).

In summary, there have been numerous advances in the last few years that have helped to characterize the neural circuitry involved in behavioral activation and effort-related processes in rodents. Furthermore, a review of this literature suggests that there is a striking similarity between the brain mechanisms involved in behavioral activation and effort-related processes in rats, and those involved in energy-related disorders such as anergia, fatigue and psychomotor slowing seen in depressed humans (Salamone et al. 2007). As noted above, research on effort-related processes in rats can offer potential clues as to the neural systems involved in the regulation of physical activities such as wheel running. In fact, some of the brain systems that are known to be involved in behavioral activation and effort-related processes in rats also have been implicated in wheel running behavior. The present results are consistent with the idea that motivational and motor processes show considerable overlap in terms

of their neural mechanisms (Mogenson et al., 1980; Salamone et al., 1992; Salamone and Correa, 2002; Salamone et al., 2006). DA is particularly involved in preparatory or instrumental behavior, and activational aspects of motivation (Salamone et al., 1991). Thus, it makes sense that running in a wheel, which requires considerable behavioral activation, is sensitive to disruption with DA depletion. In contrast, direct responses to a reinforcer that do not require much effort is not altered after DA manipulations. Our data and that of others (e.g. Cannon and Bseikri, 2004) suggest that hedonic value of reinforcers and the directional aspect of motivation are intact after DA function has been compromised.

It has been noted that the biological basis of fatigue, energy and motivational impairments in depression is still unknown, although catecholamine systems have been implicated (Stahl, 2002). As D_2 antagonism induces motor impairments as well as motivational impairments in effort-related choice procedures, it has been suggested that psychomotor slowing in depression may be functionally similar to the impairment in activational aspects of motivation that results from a suppression of DA activity in the brain (Salamone et al., 2007). Several lines of evidence illustrate the mental health relevance of engaging in physical activity. Activity-related symptoms, such as psychomotor slowing, anergia and fatigue, are fundamental and debilitating symptoms of depression (Tylee et al. 1999; Salamone et al. 2006, 2007). Moreover, there is a substantial literature indicating that exercise in humans provides a variety of physical and mental health benefits. Some studies have indicated that exercise may help alleviate fatigue or other energy-related symptoms that are sometimes seen in parkinsonian patients (Friedman 2009), and research with animal models

indicates that exercise can have neurotrophic and neuroprotective effects (Dishman et al. 2006; Zigmond et al. 2009). Furthermore, substantial evidence suggests that a lack of physical activity could contribute to the development of depression (Lambert 2006). These observations have led many basic researchers and clinicians to suggest that exercise could be used as an intervention for the prevention of disease and the treatment of various neurological or psychiatric symptoms (Dishman et al. 2006), as well as drug abuse (Smith et al. 2008; Zlebnik et al. 2010). Of course, such an intervention would require that the person complies with the exercise plan and adheres to the specific program in place (Ekkekakis et al. 2008). The choice to engage in voluntary physical activity is always undertaken in relation to the possible selection of other alternatives, such as sedentary behaviors or food consumption. For this reason, it is important to identify the factors that influence the choice to engage in physical activity.

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CHAPTER 6:

IMPACT OF DOPAMINE D_2 RECEPTOR ANTAGONISM ON THE ACTIVATIONAL EFFECTS PRODUCED BY OLFACTORY CONDITIONED STIMULI ASSOCIATED TO VOLUNTARY SUCROSE CONSUMPTION.

Abstract

The present experiments explored dopaminergic involvement in the activational effects of stimuli associated with a natural reinforcer. In the first group of experiments, the DA D_2 receptor antagonist haloperidol (0.025-0.1 mg/kg) decreased spontaneous locomotion in a novel open field (OF). However, in the same dose range, haloperidol did not suppress free intake of sucrose, or sucrose preference relative to water, in animals with different levels of baseline motivation (i.e., water restricted or *ad libitum* available water). These results support previous findings indicating that although DA is involved in the regulation of locomotion, low doses of DA antagonists do not seem affect consumption of primary reinforcers such as sucrose. In the subsequent experiments, mice were individually presented for 30 minutes per day either with a bottle containing water or with a bottle containing a solution of 10% sucrose in a random order. Concurrently, every solution was always associated with the presentation of an olfactory conditioned stimulus (CS). The olfactory CS associated with the sucrose solution (CS+) was able to enhance locomotion in mice compared to the effect produced by the presentation of the stimuli

associated with water (CS-). This enhancement was observed in animals tested in different paradigms on measures of locomotion and exploration; in a running wheel, and also in horizontal and vertical exploration in a novel OF. These results show the energizing properties acquired by initially neutral stimuli when associated with an intrinsically reinforcing stimuli. However, the activational effects of the CS+ were blunted by a dose of haloperidol (0.05 mg/kg) that had demonstrated not to affect spontaneous vertical or horizontal locomotion in the first experiment and that did not affect vertical or horizontal locomotion in animals presented with the CS- in the OF. Moreover, because the CS was presented 15 cm above the floor and hanging from the walls of one of the quadrants, vertical locomotion in the group presented with the CS+ was mainly increased in the quadrant where this stimulus was placed, pointing at a targeted increase in exploration towards the CS+ stimulus. Horizontal locomotion was significantly enhanced in the CS+ containing quadrant, but also in the rest of the quadrants. The amount of sucrose consumption during the association days was not correlated with any of the parameters of locomotion in the OF. In summary, a dose of a D_2 antagonist that did not have an effect on consummatory behavior, nor on spontaneous locomotion, reduced the invigorating properties of conditioned stimuli. This research allows for a better understanding of psychiatric symptoms such as psychomotor slowing, fatigue or anergia that can be observed in pathologies characterized by these symptoms such as depression.

INTRODUCTION

Vigorous physical activity is a fundamental aspect of motivated behavior (Salamone 2010 a,b; Salamone et al. 2007, 2009). Animals foraging in the wild often cover wide areas of space, and surmount numerous obstacles, to gain access to motivationally relevant stimuli. Because organisms are separated from these significant stimuli by environmental constraints or obstacles (i.e., response "costs"), instrumental behaviors often are characterized by a high degree of vigor, persistence and work output. Thus, motivated behaviors are said to have an energetic or activational component (Salamone et al. 2007, 2009 b; Salamone 2010 a).

Nucleus accumbens (Nacb) dopamine (DA) is an important component of the neural circuitry that regulates the ability of organisms to overcome work-related response costs in motivated behavior (Salamone et al. 2003, 2005, 2007, 2009b; Robbins and Everitt 2007). This system is involved in several behavioral processes, including aspects of motivation, motor control, and learning (Bradberry 2007; Robbins and Everitt 2007; Salamone et al. 2007). Thus, Nacb DA is involved in behavioral activation (i.e., “motivational arousal”, or “response invigoration”; Salamone et al. 2007). Moreover, Nacb DA is one of the brain areas that has been most strongly implicated in the regulation of locomotor activity. DA depletions and DA receptor antagonists suppress spontaneous, novelty-induced, schedule-induced and stimulant-induced locomotor activity (Koob et al. 1978; Robbins and Koob 1980; Cousins et al. 1993; Correa et al. 2002; Salamone et al. 2007; Collins et al., 2009; Pastor et al., 2002).

The sweet taste of sucrose is strongly rewarding for animals such as rodents and primates (Bachmanov et al., 1997; Berridge, 2000; Steiner et al., 2001; Levine et al., 2003; Yamamoto, 2003). Appetitive rewarding events are things that elicit approach reactions, serve as goals that direct voluntary behavior, and also as positive reinforcers. In appetitive learning, a primary reward is repeatedly paired with a neutral stimulus, until ultimately the conditioned stimulus (CS), reliably elicits a behavioral reaction similar to the reaction instigated by the primary reward. Thus, important components of the behavioral response are transferred from the primary reward to the conditioned, reward-predicting stimulus (Berridge and Robinson 2003). The CS induces similar preparatory approach behavior as to the primary reward itself, thus gaining some control over behavior (Flagel et al., 2007; Berridge and Robinson 2003). A CS previously paired with a stimulus perceived as a reinforcer can evoke DAergic activity (Berridge and Schulkin, 1989; Bindra, 1974; Breslin et al., 1990; Delamater et al., 1986; Rozin and Schulkin, 1990; Toates, 1985; Wise, 1982, 1985; Katner and Weiss, 1999; Weiss et al., 1993, 2000), particularly within the Nacb core (Day et al., 2007; Roitman et al., 2004). Moreover, cues paired with a preferred reward, (e.g. a CS+ paired with sucrose), have been shown to evoke greater DA responses than cues that predicted less preferred rewards (CS-, saccharin) (McCutheon et al., 2012). In addition, D_1 and D_2 receptor adaptations underlie approach behaviors directed towards signals associated to rewards (Flagel et al., 2007).

Thus, in the present study we associate odor cues to a more preferred reward (CS+, sucrose) and different odor cues to a less preferred reward (CS-, water), and we tested if this CS+ has activational properties as measured by the

induction of locomotion in an open field (OF). We also evaluate if this activation is directed towards the CS predicting reward. In addition, haloperidol (a D2 antagonist) was used to study the impact of DA manipulations on the putative activational effect of the CS+. Initially, we evaluated the impact of haloperidol, in a broad dose range, on spontaneous locomotion in the OF and in preference and consumption of sucrose. It was hypothesized that DA antagonism would affect behavioral activation while leaving the consumption of the primary reinforcer intact.

MATERIALS AND METHODS

Animals

Swiss male mice (N=149) weighed 24-28 g at the beginning of the study (Janvier, France). Mice were housed in groups of three per cage, with standard laboratory rodent chow and tap water available *ad libitum* (see specific conditions for each experiment). Subjects were maintained at 22 $\pm$ 2 ºC with 12-h light/dark cycles. All animals were covered 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).

Pharmacological agents

Haloperidol (Sigma Quimica C.O), a DA D_2 receptor antagonist, was dissolved in a 0.2% tartaric acid solution (pH=4.0), which also was used as the vehicle control. Haloperidol was administered intraperitoneally (IP), 50 minutes

before testing started. Sucrose (Sigma Quimica C.O) was dissolved in tap water and used for oral self-administration.

Apparatus and testing procedures

Testing sessions started two hours after the colony lights were on. The behavioral test room was illuminated with a soft light, and external noise was attenuated.

Water and Sucrose free consumption and preference. Different groups of mice had either *ad libitum* water or water restricted to 5.0 ml/day/mouse in their home cage. During one hour a day animals were individually exposed to a gradated tube containing 10% sucrose and another containing tap water. Each animal was exposed to these solutions for 2 weeks until after which a criterion of 1 ml of sucrose minimum was consumed over three consecutive days in order to avoid floor effects in the study of DA antagonist effects. Sucrose and water intake were measured at the end of the test session by reading the meniscus of the solutions along the gradations.

Association of olfactory stimulus with 10% sucrose and water. In the initial phase, animals were water restricted to 5.0 ml/day/mouse in their home cage during 4 weeks, after which *ad libitum* water was available in their home cages for the rest of the experiment (4 more weeks before testing session began). Conditioning sessions were performed for 30 minutes a day. Animals were individually placed in a different cage from the home cage with water or 10% sucrose access and an odorant located on the top of the cage was presented as the conditioned stimuli (CS). In order to avoid possible preferences for one of the two odors (papaya or strawberry), half of the animals had water associated with

odor A (CS-), and sucrose with odor B (CS+), while half had sucrose associated with odor A (CS+) and water with odor B (CS-). Every pair of stimuli (solution plus odor) was presented in a random order for 8 weeks, maintaining equal number of pairing sessions across weeks. The test day one of the odors was present either in the RW (experiment 3A) or in the novel OF (experiment 3B) and locomotion was assessed. Sucrose was never present during the test session.

Running wheel (RW) locomotion. The automated RW 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. The session lasted 15 minutes.

OF locomotion. The OF consisted of a Plexiglas cylinder with translucent walls (30 cm in diameter and 30 cm high) and an opaque floor divided into four equal quadrants by two intersecting lines. Locomotor activity was registered manually. Horizontal and vertical locomotion were simultaneously recorded. For horizontal locomotion an activity count was registered each time the animal crossed from a 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 placed in the center of the cylinder and immediately observed for 15 minutes. For experiment 5, an odor was placed in one wall of the OF at a height of 20 cm from the floor. This odor acted as CS+ for half of the animals (was paired consistently with sucrose) and as CS- for the rest of animals (was paired consistently with water). Separate measurements were taken either for activity the quadrant where the CS (odor) was placed vs. the other 3 quadrants in this experiment.

Experiments

Experiment 1: *Effect of haloperidol on 10% sucrose and water preference and intake.* Because water accessibility is a powerful motivational factor, and since animals in the conditioning experiment went through a period of restricted water access followed by a period of *ad libitum* access in the home cage, we evaluated these two housing conditions on preference and fluid consumption after haloperidol administration. Thus, mice (N=29, 15 for the water restricted experiment and 14 for the *ad libitum* experiment) received haloperidol (0.0, 0.025, 0.05 and 0.1 mg/kg) 50 minutes before the intake test started. A within-groups design was used. After testing began, there was one drug treatment day a week and 4 baseline days before the next drug day.

Experiment 2: *Effect of different doses of haloperidol on locomotion in the OF*. In order to make experimental conditions similar to experiment 5, mice (N=45) were handled and weighted twice a week during 10 weeks after arriving to the laboratory. Animals were not pre-exposed to the OF paradigm. On the test day animals received one injection of haloperidol (0.0, 0.025, 0.05 or 0.1 mg/kg) 50 min before being tested in the OF for 15 min. As in experiment 5, a between-groups design was used.

Experiment 3: *Effect of olfactory CS associated with sucrose or water on measures of spontaneous locomotion.*

3.A. Effect of CS on locomotion in the RW. Mice (N=9) were trained to associate odor and fluid as described above, and 3 hours later were habituated to the RW during 30 minutes. A within-groups design was performed. During the test phase, mice were introduced in the RW cage with one of the CS placed on

top of the cage and RW counts were registered during 15 minutes. Additional training was done the following days and the effect of the other CS was evaluated one week later.

3.B. Effect of CS on horizontal and vertical locomotion in the OF. Mice (N=35) were trained to associate odor and fluid as described above. No conditioned training was done the day of the testing session. To mimic experiment 4 conditions, animals were introduced in the OF with no odor, and 15 minutes later one of the odors (CS+ or CS-) was introduced in the upper part of the OF attached to the wall, and centered in one of the quadrants. A between-groups design was used.

Experiment 4: *Effect of haloperidol on horizontal and vertical locomotion in the OF in the presence of a CS***.** During the training phase, mice (N=66) were exposed to odor and fluid as described above. Animals did not receive conditioned training on the day of the testing session. Thirty five minutes after receiving haloperidol (0 or 0.05 mg/kg) mice were introduced in the OF with no odor, and 15 minutes later one of the odors (CS+ or CS-) was introduced in the upper part of the OF attached to the wall, and centered in one of the quadrants. Separate analyses were done for the quadrant where the CS was located and for the rest of quadrants. A between-groups design was used.

Statistical analyses

Different statistical analyses were used depending on the experiment. In experiments 1, and 3A, data were analyzed using repeated measures ANOVA. Experiments 2 and 3B were analyzed using a one-way simple ANOVA. In experiment 4 a two-way between-groups factorial ANOVA was used. 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). The relationship between sucrose consumption and locomotor behavior was also examined using correlation tests with a 95% confidence interval. STATISTICA 7 software was used. All data were expressed as mean ±SEM, and significance was set at $p<0.05$.

RESULTS

Experiment 1: *Effect of haloperidol on 10% sucrose and water preference and intake.* Previous to the haloperidol dose/response test phase, preference for 10% sucrose or water consumption was assessed by comparing the average volume consumed during the last four weeks before testing started. A Students t-test analyses comparing water vs sucrose, showed a significant effect (t (6)=77.66, $p<0.01$) among the animals with home cage restricted access, and also among the group of animals with *ad libitum* water access (t (6)=593.59, $p<0.01$). Animals in both groups consumed a significantly higher volume of sucrose compared to water (0.29 ± 0.04 ml of water, 2.62 ± 0.26 ml of sucrose in the deprived animals, and 0.15 ± 0.03 ml of water, 2.52 ± 0.09 ml of sucrose in the *ad libitum* group).

For the drug testing phase (0.0, 0.025, 0.05 and 0.1 mg/kg haloperidol), the one-way ANOVA did not yield a statistically significant effect for either sucrose (F(3,39)=0.38, n.s) or water intake (F(3,39)=0.14, n.s) among non-restricted animals. For the restricted group the one-way ANOVA for sucrose (F(3,42)=0.34, n.s) or water intake (F(3,42)=1.17, n.s) also did not yield a

statistically significant effect. Thus, haloperidol at this range of doses had no effect on intake under any condition tested. These data are shown in table 1.

	Haloperidol Dose (mg/kg)	10% Sucrose Intake (ml)	Water Intake (ml)
Water ad libitum	0.0	1.57 ± 1.08	0.18±0.28
	0.025	1.60 ± 0.70	0.19±0.19
	0.05	1.73 ± 1.06	0.16±0.19
	0.1	1.40 ± 0.86	0.16±0.12
Restricted water	0.0	1.69 ± 0.84	0.23±0.23
	0.025	1.48 ± 0.86	0.17±0.20
	0.05	1.47 ± 1.04	0.13±0.09
	0.1	1.52 ± 0.77	0.15±0.15

Table 1. Effect of haloperidol (0.0, 0.025, 0.05 and 0.1 mg/kg) on sucrose and water intake in non-deprived and water restricted animals. Mean (±SEM) ml consumed in 60 min.

Experiment 2: *Effect of different doses of haloperidol on locomotion in the OF*. The one-way ANOVA for the haloperidol factor (0.0, 0.025, 0.05 or 0.1 mg/kg) showed a significant effect of dose on horizontal locomotion (F (3,41)=85.63, p<0.01; Fig. 1A). Planned comparisons yielded significant differences between vehicle and the highest dose of haloperidol (p<0.01). For the vertical locomotion data (Fig. 1B) the ANOVA also showed a significant effect of haloperidol (F (3,41)=6.49, p<0.01). Planned comparisons revealed significant differences between vehicle and the lowest (p<0.05) and highest (p<0.05) doses of haloperidol. Thus, 0.1 mg/kg haloperidol consistently reduced spontaneous horizontal and vertical locomotion in a novel OF, while 0.05 mg/kg did not significantly affect any of the locomotion measurements.

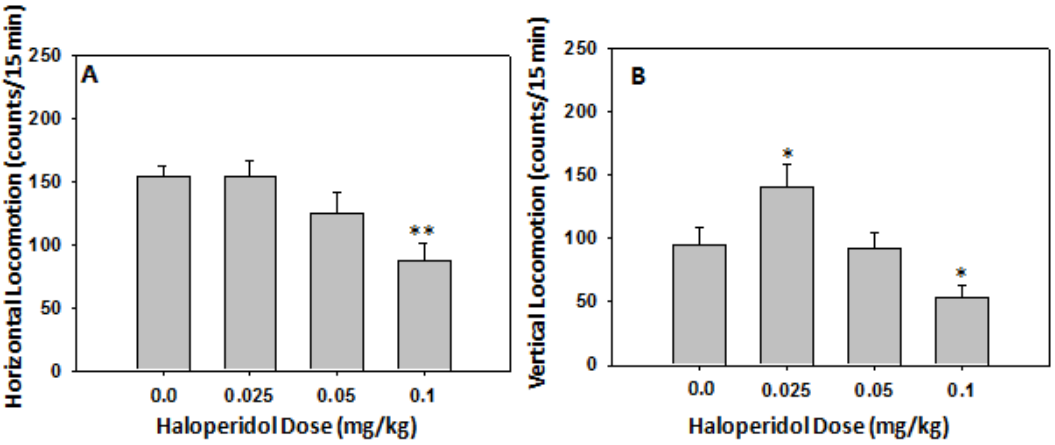

Fig. 1. Effect of haloperidol (0.0, 0.025, 0.05 or 0.1 mg/kg) on A) horizontal locomotion and B) vertical locomotion. Mean (±SEM) number of counts in the OF during 15 minutes. *p<0.05 **p<0.01 significantly different from vehicle.

Experiment 3: *Effect of olfactory CS associated to sucrose or water on meassures of spontaneous locomotion.* Figure 2 shows sucrose and water intake during the last conditioning sessions before locomotion tests started.

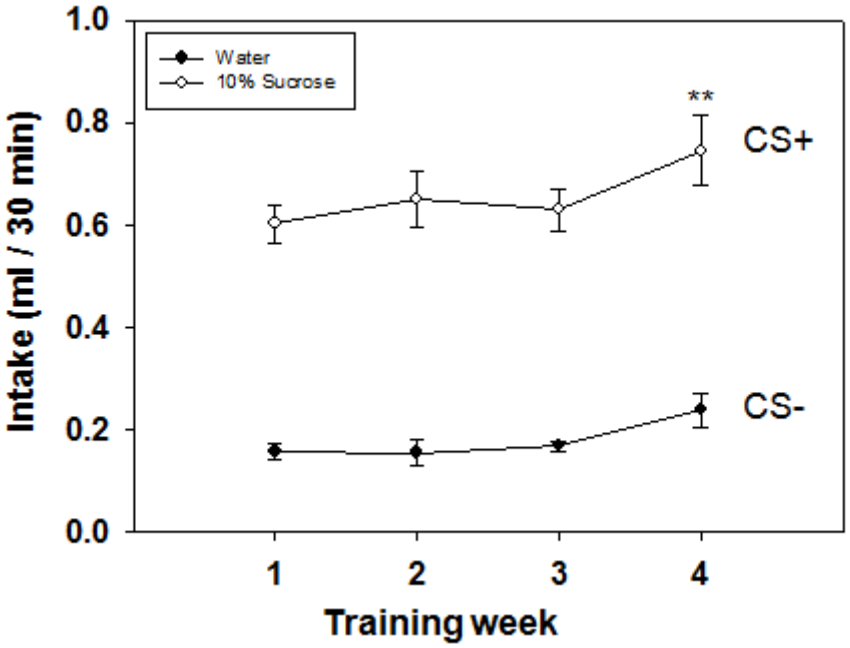

Fig 2. Water and sucrose intake during olfactory conditioning sessions across the 4 weeks before locomotion tests started. Mean (±SEM) ml of water and sucrose consumed in 30 min. **p<0.01 significantly different from week 1.

The repeated measures ANOVA showed no significant changes in water intake ($F(3,204)=2.12$, n.s) over four successive weeks of training. However, ANOVA yielded a significant effect on sucrose intake (F (3,204)=3.15, $p<0.05$) over weeks. Planned comparisons revealed significant differences on sucrose intake between first and last week ($p<0.01$). Students t-test analyses comparing the average volume consumed during the last four weeks water vs. sucrose yield a significant effect (t (6)=319.13, $p<0.01$). Mice preferred 10% sucrose over tap water.

3.A. Effect of CS on locomotion in the RW. Repeated measures ANOVA yielded a significant effect of the CS ($F(1,8)=5.79$, $p<0.05$) on RW locomotion. Mean number (+SEM) of RW turns in 15 minutes when the CS- was present was 780.3 ± 78.7, and when the CS+ was present; 840.2 ± 88.9 (see Fig. 3). Thus, mice habituated to running in the wheel showed an increase in wheel running when the CS+ was presented relative to wheel running when the CS- was present in the RW cage.

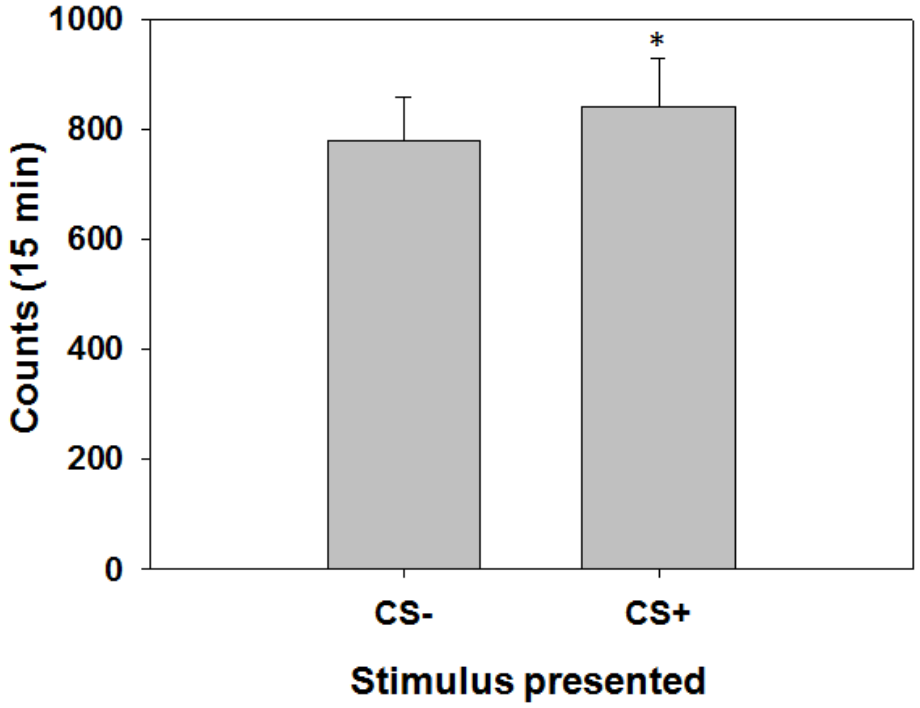

Fig. 3. Effect of CS+ or CS- on locomotion in the RW. Mean (±SEM) number of counts during 15 minutes. *p<0.05 significantly different from CS- condition.

3.B. Effect of CS on horizontal and vertical locomotion in the OF. The Student t test comparing the two independent groups of mice presented with CS- or with CS+ demonstrated that there was a significant effect (t=5.90, df=33, p<0.05) of CS+ presentation relative to CS- presentation on both horizontal (Fig. 4A) and vertical locomotion (Fig. 4B; t=9.03, df=33, p<0.01). As in the RW experiment, the presence of the CS+ enhanced locomotion in both measures.

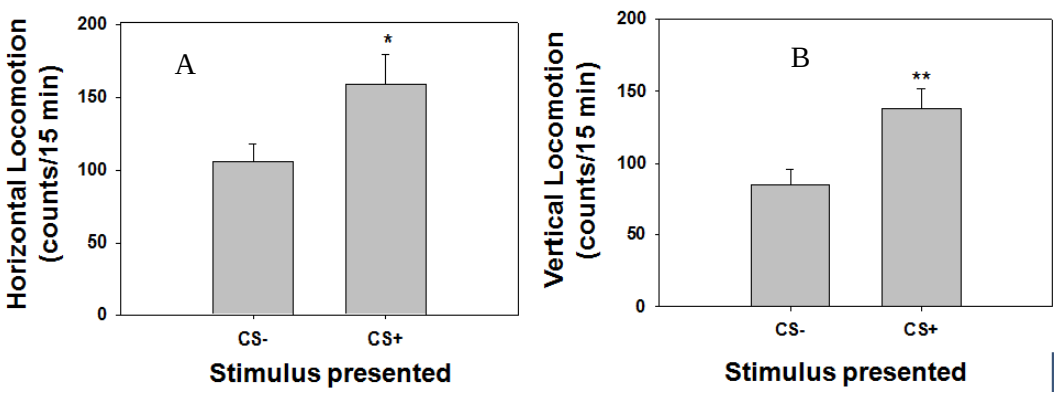

Fig. 4. Effect of CS+ or CS- on A) horizontal locomotion and B) vertical locomotion. Mean (±SEM) number of counts in the OF during 15 minutes. *p<0.05 **p<0.01 significantly different from CS-.

Experiment 4: *Effect of haloperidol on horizontal and vertical locomotion in the OF in the presence of a CS*. A two-way factorial ANOVA of the conditioned stimulus factor (CS+ or CS-) and the haloperidol dose factor (0.0 or 0.05 mg/kg) for horizontal locomotion in the CS quadrant (i.e., the quadrant in which the CS was located; Fig. 5A), showed no significant effect of stimulus condition (F(1,62)=8.22, *n.s*), a statistically significant effect of haloperidol (F(1,62)=8.25, p<0.01), and a significant stimulus x haloperidol interaction (F(1,62)=4.08, p<0.05). Planned comparisons yielded significant differences between CS+ and CS- groups in the vehicle condition (p<0.01), but not in the haloperidol condition. Moreover, haloperidol (0.05 mg/kg) significantly reduced horizontal locomotion in the CS+ group (p<0.01) but not in CS- group. Analysis of the vertical locomotion data for the CS quadrant (Fig. 5B), showed a significant effect of stimulus condition (F(1,62)=8.22, p<0.01), a statistically significant effect of haloperidol (F(1,62)=13.42, p<0.01), and a significant stimulus x haloperidol interaction (F(1,62)=13.05, p<0.01). Planned comparisons yielded significant differences between CS+ and CS- in the vehicle condition (p<0.01), but not the haloperidol condition. As with horizontal locomotion, haloperidol (0.05 mg/kg) also significantly reduced horizontal locomotion in the CS+ group (p<0.01) but not in CS- group.

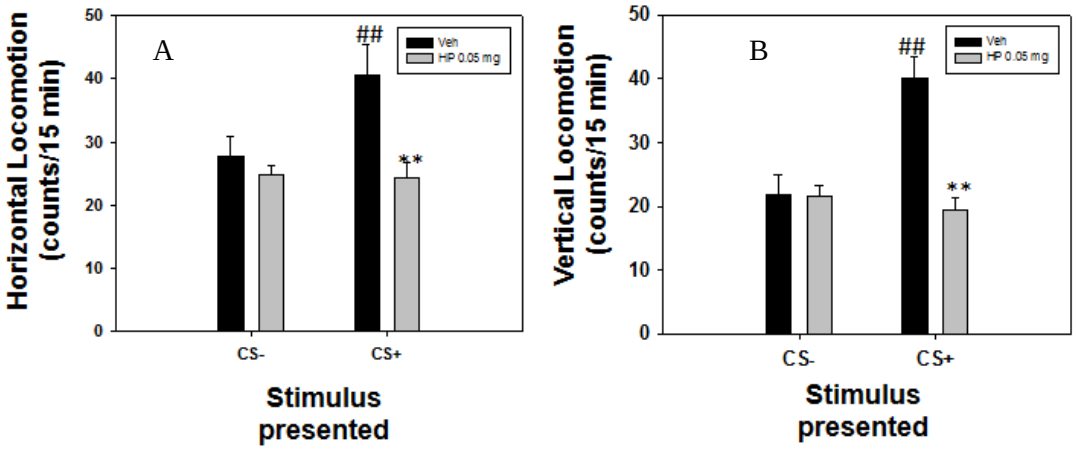

Fig. 5. Effect of haloperidol (0.0 or 0.05 mg/kg) on locomotor effects induced by CS+ or CS- presentation on A) horizontal locomotion and B) vertical locomotion, in the quadrant where the CS was located. Mean (±SEM) number of counts in the OF during 15 minutes. ***p*<0.01 significantly different between doses in the same group; ##*p*<0.01 significant difference between CS+ and CS- at the same dose.

Figures 6A and 6B show horizontal and vertical locomotion in the quadrants with no CS. A two-way factorial ANOVA with the two main factors (stimuli: CS+ or CS-) and haloperidol dose (0.0 or 0.05 mg/kg) for horizontal locomotion in the non-CS quadrants showed a significant effect of stimulus condition ($F(1,62)=4.89$, $p<0.05$), and of haloperidol treatment ($F(1,62)=7.54$, $p<0.01$), and also showed a significant stimulus x haloperidol interaction ($F(1,62)=4.18$, $p<0.05$). Planned comparisons yielded significant differences between CS+ and CS- in the vehicle condition ($p<0.01$), but not the haloperidol condition. Haloperidol (0.05 mg/kg) significantly reduced horizontal locomotion only in the CS+ group ($p<0.01$), and not in CS- group. Finally, the two-way factorial ANOVA of the vertical locomotion data in non-CS quadrants showed a significant effect of the stimulus factor ($F(1,62)=6.80$, $p<0.05$), but neither the

haloperidol factor ($F(1,62)=3.97$, *n.s*), nor the interaction ($F(1,62)=2.46$, *n.s*) were significant. These data indicate that the CS+ increased horizontal locomotion even in the areas of the chamber in which there was no CS, but in the case of vertical locomotion the results were less robust. There was a slight increase in vertical locomotion in animals exposed to the CS+, and the overall effect of haloperidol approached statistical significance ($p<0.0507$), but these actions were not enough to drive a significant interaction.

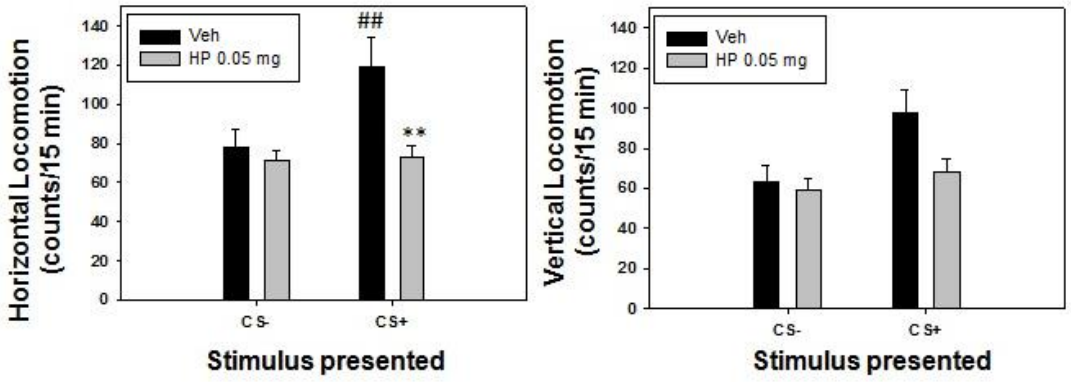

Fig. 6. Effect of haloperidol (0.0 or 0.05 mg/kg) on locomotor effects induced by CS+ or CS- presentation on A) horizontal locomotion and B) vertical locomotion, in the rest of quadrants where no CS was present. Mean (±SEM) number of counts in the OF during 15 minutes. $^{**}p<0.01$ significantly different between doses in the same group; $\#\#p<0.01$ significant difference between CS+ and CS- at the same dose.

Additional analyses were performed to better understand the relation between consummatory behavior and activational effects of the CS. Thus we plotted the amount of sucrose consumed during the last 2 weeks previous to the OF test against locomotor measures in the OF. There were no significant

correlations between these two types of variables in any of the groups (see tables).

Group: CS-/vehicle

Sucrose intake (ml)- Locomotion (counts)	Correlation coefficient (r)	*P* value
Vertical in CS quadrant	0.09	0.70
Vertical other quadrants	-0.02	0.94
Horizontal in CS quadrant	0.02	0.95
Horizontal other quadrants	0.05	0.84

Group: CS+/vehicle

Sucrose intake (ml) – Locomotion (counts)	Correlation coefficient (r)	*P* value
Vertical in CS quadrant	0.17	0.50
Vertical other quadrants	-0.10	0.71
Horizontal in CS quadrant	-0.09	0.72
Horizontal other quadrants	-0.29	0.25

Group: CS-/haloperidol 0.05 mg/kg

Sucrose intake (ml) – Locomotion (counts)	Correlation coefficient (r)	*P* value
Vertical in CS quadrant	-0.31	0.46
Vertical other quadrants	-0.34	0.42
Horizontal in CS quadrant	0.14	0.74
Horizontal other quadrants	-0.15	0.72

Group: CS-/haloperidol 0.05 mg/kg

Sucrose intake (ml) – Locomotion (counts)	Correlation coefficient (r)	*P* value
Vertical in CS quadrant	0.34	0.45
Vertical other quadrants	0.30	0.51
Horizontal in CS quadrant	0.33	0.47
Horizontal other quadrants	0.35	0.44

Correlational analyses also were performed for sucrose intake averaged from the

two last weeks of training and locomotion measures obtained in the OF for the four experimental groups. The dependent variables are depicted in the far left column followed by the correlation coefficient (r) and the level of significance (P value).

DISCUSSION

The present results show that although DA D_2 receptor antagonism was able to dose dependently was reduce measures of spontaneous horizontal and vertical activity in a novel OF in mice, it did not reduce total intake of sucrose and water, and did not alter sucrose preference. Our data are in agreement with previous results showing that Nacb DA has been clearly implicated in the regulation of spontaneous, novelty-induced, food-induced, and drug-induced locomotion (Kelley and Iversen, 1976; Koob et al., 1978; Ahlenius et al., 1987; McCullough and Salamone, 1992; Correa et al., 2002, 2004). However, consummatory behaviors remain intact after Nacb DA depletions or low doses of DA antagonists (Salamone et al., 2010). Moreover, directional aspects of motivation and emotional reactions to tastants are spared after DAergic antagonism, while activational aspects of motivation seem to be very susceptible to disruption by interference with DA transmission. Moderate doses of the D_2 receptor antagonist haloperidol do not modify the hedonic or aversive reactions to sucrose or quinine in rats (Treit and Berridge, 1990). Mice with DA deficiency show intact discrimination between saccharin, sucrose and water solutions, showing a clear preference for sucrose over the other two, and for saccharine over water (Cannon and Palmiter, 2003). Also, rats with DA depletions in the shell of the Nacb showed no alterations in preference for sucrose (Martinez-Hernandez et al., 2012). D_2 or D_1 receptor antagonists,

(raclopride and SCH23390) did not diminish the maximal lick rate of 10% sucrose achieved by rats (Cannon and Bseikri, 2004). The non-selective DA antagonist flupentixol injected in Nacb reduced speed to approach sucrose, but had no effect on final sucrose intake in rats (Ikemoto and Panksepp, 1996).

Our results also indicate that an odor associated with sucrose presentation and consumption (Pavlovian conditioned stimulus, CS) was able to acquire properties that result in an increase of behavioral activation when the CS+ is presented. This potentiated activity is manifested in more running in a RW, as well as in increases of exploration in an OF a measured by increased horizontal locomotion and rearing. Horizontal locomotion was increased in all the areas of the OF, near the location of the CS+ but also in more distal areas, indicating a general increase in exploration. However, although vertical locomotion or rearing was substantially increased in proximity to the CS+, the stimulation of vertical activity by the CS+ in the other 3 quadrants was less robust. This pattern suggests that vertical activity was more focused in the direction of the location of the CS+. Moreover, these increases in exploration are DA-dependent since a dose of haloperidol (0.05 mg/kg) that did not affect locomotion on its own (see fig 1 and also the CS- group in figures 6 and 7) was able to selectively decrease the effects of the CS+ in both measures of locomotion in the OF. Thus, the increases in both general and specific aspects of behavioral activation seem to be DA mediated. This conclusion seems to be supported by data showing that CSs evoke DA responses within the Nacb (Day et al., 2007; Roitman et al., 2004; McCutheon et al., 2012), and more specifically by results showing D_1 and D_2 receptor adaptations underlying approach behaviors directed towards signals associated to rewards (Flagel et al., 2007). Some of the motivational functions of

mesolimbic DA represent areas of overlap between aspects of motivation and features of motor control, which is consistent with the well known involvement of Nacb in locomotion and related processes (Salamone et al., 1991, 1997, 2002, 2003, 2005, 2007; Yurgelun-Todd et al., 2007; Barbano and Cador 2007; Niv et al., 2007; Phillips et al., 2007; Robbins and Everitt 2007). NAcb DA seems to be required for forward locomotion in response to novel stimuli and to stimuli associated with reward or punishment (Ikemoto and Panksepp, 1999). However, pavlovian associations do not specify fixed actions (Nicola, 2010). NAcb DA is required for CS to promote locomotor approach to the CS itself and to the reward (Di Ciano et al., 2001; Corbit et al., 2007; Lex and Hauber, 2008; Nicola, 2010). Both stimuli (i.e., the unconditioned stimulus as well as CS) elicit activation, and DA manipulations can directly affect this activity, while the directional aspect of motivation is influenced by manipulations of other neural systems such as GABA systems in the brainstem, ventral pallidal systems where lesions produce aversion, or opioid systems in the nucleus accumbens shell (Berridge, 1996; Berridge and Peciña, 1995; Cromwell and Berridge, 1994; Peciña and Berridge, 1996a,b). In the present studies, the fact that the amount of sucrose consumption was not affect by DA antagonism and was not related to the amount of exploration, argues for a separation between the unconditioned reinforcing properties of sucrose and its predictive and invigorating properties.

The CS does not elicit the same pattern of behavior in all animals. Thus, two different patterns of behavior have been described in an operant task in which a CS is presented before the food. Sign-trackers are animals that responded to the CS by approaching the CS and directly interacting with it (i.e. almost attempting to “consume” it). Goal-trackers are animals that responded to

the CS by approaching the location where the US (e.g. food or sucrose) would be delivered (Hearst and Jenkins, 1974; Boakes, 1977; Flagel et al., 2007, 2008, 2009; Robinson and Flagel, 2009; Yager and Robinson, 2010; Morrow et al., 2011). The present paradigm does not allow for the differentiation between animals directed towards the goal or towards the sign, since the goal was never present in the OF and the animals can not reach and grasp the sign. However, the present results demonstrate that the CS+ acquires activational properties, thus increasing active exploration in a novel area in which the unconditioned stimuli could be located, but also instigating more focused exploration towards the CS+, effects that were both blunted after DA antagonism.

All these results suggest that DA activates and invigorates reward-seeking behavior by allowing Pavlovian CSs to evoke general exploration of the environment and approach to potentially significant stimuli. It is important to characterize the neural correlates involved in behavioral activation and effort-related processes in animals, since there is a striking similarity between the brain mechanisms involved in behavioral activation and effort-related processes in animals and energy-related disorders such as anergia, fatigue and psychomotor slowing seen in depressed humans (Salamone et al. 2007).

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GENERAL CONCLUSIONS

Along with previous data in the literature, a pattern of results that emerges across all the present studies is that DA mediates the willingness to exert effort for natural reinforcers (food or sucrose), and also mediates activation involved in exploratory behaviors. This conclusion is in agreement with the hypothesis that DA is involved in the activational component of motivation, rather than in the directional component. DA receptor antagonism or depletion does not change the preference for appetitive reinforcers, animals were still directed towards them, but they changed strategies in order to minimize the effort demands to obtain these reinforcers. When no effort was required, no change in behavior was observed.

Work output in an instrumental task and locomotor exploration are components of naturalistic foraging behaviors to obtain food. Nacb DA does provide the neural substrate for this essential set of behaviors. This nucleus then acts as the interface between motor and motivated behavior. Nacb is part of a circuit that involves other striatal structures, that participate in the more pure motoric components, as well as in the stablisment of habitual responses, and also is modulated by other frontocortical and limbic structures. Moreover, neurochemical interactions between the DArgic system and the neuromodulator system adenosine are very important for the regulation of the energizing component of motivated behaviors. Thus, co-localization of adenosine and DA receptors, and the intracellular cascades that they initiate, regulates neural activity in the Nacb in a very precise way. The specificity of the receptor type is also key in this regulation, being D_2- A_{2A} interaction central for the types of behaviors studied in the present group of experiments.

CONCLUSIONS FROM THE EXPERIMENTAL CHAPTERS

Chapter 1: Selection of sucrose concentration after DA depletion and selective DA antagonists depends on the effort required by the instrumental response: studies using tetrabenazine and D_1, D_2 and D_3 antagonists.

Control animals press the lever under a FR7 schedule to obtain the preferred 5% sucrose concentration while consuming little 0.3% sucrose volume.

- DA depletion by tetrabenazine as well as D_1 and D_2 antagonism produced a shift on the behavior; lever pressing was decreased and a significant increase in intake of free available sucrose was seen, effect that did not resemble those obtained after pre-exposing the animals to both sucrose solutions.
- D_3 antagonism does not modify this behavior.
- DA antagonism does not affect sucrose consumption when no effort was required.
- Non-selective adenosine antagonists do not reverse the effects caused by D_1 antagonism. However, theophylline attenuated the choice effects produced by D_2 antagonism effects.

Chapter 2: Dopaminergic modulation of effort-related choice behavior as assessed by a progressive ratio chow feeding choice task: Pharmacological studies and the role of individual differences.

- In each operant session, the PROG schedule represents a continuous challenge to work more and more to obtain preferred palatable food. This challenge is affected by the presence of freely available chow. Thus, more that with a FR schedule, PROG ratio forces animals to reach a break point after which they stop lever pressing and consume high quantities of the chow. This

characteristic of the task brings out individual differences in the amount of work output that the animals are willing to pay for the preferred food.

- The DA D_2 antagonist haloperidol decreased number or lever presses, maximum ratio achieved, and active lever time. Haloperidol does not decrease chow intake, which indicates that primary food motivation is intact in haloperidol-treated rats.

- The A_{2A} antagonist, MSX-3 increased number of lever presses and maximum ratio achieved, and also increased the amount of time that animals kept the lever active during the session. MSX-3 was observed to decrease chow consumption at the highest dose.

- Both DA and adenosine manipulations do not resemble the effects of pre-feeding the animals or the effects of appetite suppressants. Pre-feeding animals, to reduce food motivation and thereby devalue the food reinforcement, decreased number of lever presses and highest ratio achieved, and also substantially reduced chow consumption. CB1 receptor antagonist/inverse agonist AM251 produced similar effects to those resulting from pre-feeding. AM251 decreased number of lever presses, maximum ratio achieved, and chow consumption.

- The PROG/choice procedure is characterized by substantial individual variability. High responders did show greater DARPP-32 expression in Nacb core than low responders reflecting greater DA transmission in the animals working harder on the lever pressing component of the task.

Chapter 3: Effect of subtype-selective adenosine receptor antagonists on basal or haloperidol-regulated striatal function: studies of c-Fos expression and motor activities in outbred and A_{2A}R KO mice.

- D_2 antagonism decreased behavioral activation in an OF.
- Non-selective adenosine antagonism was able to reverse the effects of D_2 antagonism and this effect seems to be mediated by A_{2A} receptors rather than A_1 receptors. MSX-3, an A_{2A} receptor antagonist completely reversed haloperidol effects while CPT, an A_1 antagonist was not able to attenuate D_2 antagonism.
- KO mice for the A_{2A} receptor showed resistance to haloperidol effects.
- Parallel results were obtained in the expression of the cellular marker, c-Fos, on Nacb and dorsal striatum.

Chapter 4: Adenosine A_{2A} receptor antagonism and genetic deletion attenuate the effects of dopamine D_2 antagonism on effort-based decision making in mice: Studies using a T-maze with barrier.

- In a T maze with barrier in the arm that has a high amount of food and free access on the other arm with a smaller quantity of the same type of food, control animals select to climb a barrier to obtain more food.
- D_2 antagonism decreased HD arm selection redirecting the behavior towards the less effortful option. This effect does not resemble the results obtained after prefeeding the animals. Pre-fed animals do not shift behavior, they increase omissions.

- Theophylline as well as the selective A_{2A} antagonist MSX-3, attenuated haloperidol effects, reaching control levels with the A_{2A} antagonist. The A_1 antagonist, CPT failed to reverse D_2 antagonism.

- KO animals for the A_{2A} receptor showed protection against D_2 antagonism on this task.

- Parallel results were obtained in the expression of the cellular marker c-Fos on Nacb and dorsal striatum.

- These data validate this effort based choice paradigm for mice used previously in rats.

Chapter 5: DA D_2 receptor antagonism modulates the preference for primary reinforcers based on their effort requirements: studies using running wheels and sucrose consumption in mice.

- Under control condition, mice showed a clear preference towards the RW and spend less time in contact with sucrose.

- D_2 antagonism by haloperidol redirects the behavior towards the less effort demanding option, effect that did not resemble devaluation by pre-exposition to both reinforcers.

- This new task allows the study of preference based on the activational costs of every reinforcer.

Chapter 6: Impact of DA D_2 receptor antagonism on the activational effects produced by olfactory conditioned stimuli associated to voluntary sucrose consumption.

- D_2 antagonism at doses that reduce vertical and horizontal exploration in a novel OF, do not alter 10% sucrose or water intake and preference.

- An olfactory stimulus previously paired with sucrose presentation, increased locomotion in a RW and in the OF.

- Enhanced locomotion on a novel environment due to the presence of the CS+ was blocked after haloperidol at a low dose that did not alter by itself locomotion.

FUTURE DIRECTIONS

In summary, the present results are consistent with the hypothesis that DA is involved in effort-related processes, and support the concept that adenosine A_{2A} receptors interact with DA in modulating these functions. Future research should investigate the effects of additional genetic manipulations on effort-related choice behavior, including DA receptor knockouts as well as regionally-specific deletion of A_{2A} receptors. Moreover, the search for individual differences in neural markers of Nacb activity in response to effort demanding tasks can be a new approach to understand this basic process.

The present work also has clinical relevance. DA has been implicated in aspects of depression, including such fundamental symptoms as psychomotor slowing, anergia, feelings of listlessness, decreased energy levels and fatigue. It has been suggested that psychomotor slowing in depression may be functionally similar to the impairment in activational aspects of motivation that results from a reduction of DA activity in the brain. Thus, research on DA/adenosine interactions involved in effort-related processes may yield insights into the brain mechanisms involved in motivational symptoms of depression and other disorders. Future studies involving effort-based functions in genetically altered mice could prove to be a critical aspect of this research.

APPENDIX

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.

Abstract

Tremulous jaw movements are defined as a rapid vertical deflection of the lower jaw that resembles chewing but is not directed at any particular stimulus. In rats, tremulous jaw movements can be induced by a number of neurochemical conditions that parallel those seen in human parkinsonism, including dopamine depletion, dopamine antagonism, and cholinomimetic administration. Moreover, tremulous jaw movements in rats can be attenuated using antiparkinsonian agents such as L-DOPA, dopamine agonists, muscarinic antagonists, and adenosine A_{2A} antagonists. In the present studies, a mouse model of tremulous jaw movements was established. The focus of these studies was to investigate the effects of adenosine A_{2A} antagonism, and a conditional neuronal knockout of adenosine A_{2A} receptors, on cholinomimetic-induced tremulous jaw movements in mice. The muscarinic agonist pilocarpine significantly induced tremulous jaw movements in a dose dependent manner (0.25-1.0 mg/kg IP). These movements occurred largely in the 3-7.5 Hz local frequency range. Administration of the adenosine A_{2A} antagonist MSX-3

(2.5-10.0 mg/kg IP) significantly attenuated pilocarpine-induced tremulous jaw movements. Furthermore, adenosine A_{2A} receptor knockout mice showed a significant reduction in pilocarpine-induced tremulous jaw movements compared to litter-mate controls. These results demonstrate the feasibility of using the tremulous jaw movement model in mice, and indicate that adenosine A_{2A} receptor antagonism and deletion are capable of reducing cholinomimetic-induced tremulous jaw movements in mice. Future studies should investigate the effects of additional genetic manipulations using the mouse tremulous jaw movement model.

INTRODUCTION

Resting tremor is a cardinal symptom of Parkinson's disease (PD), presenting in more than 70% of patients with idiopathic PD (Deuschl et al., 2000). Moreover, tremor and other parkinsonian symptoms can be induced by various drugs, including dopamine (DA) antagonists (Marsden, 1984) and cholinomimetics (Ott and Lannon, 1992; Song et al., 2008). In recent years, adenosine A_{2A} antagonists have emerged as a potential treatment of parkinsonian motor impairments, including tremor (Schwarzschild et al., 2006; Ferré et al., 2008; LeWitt et al., 2008). Adenosine A_{2A} receptors are highly expressed in neostriatum, and A2A antagonists exert motor effects in rodents and primates that are consistent with antiparkinsonian actions (Ferre et al., 1997, 2004; Chen et al., 2001; Morelli and Pinna, 2001; Wardas et al., 2003; Morrelli et al., 2007; Salamone et al., 2008; Collins et al., 2010). Human clinical reports have indicated that the adenosine A_{2A} antagonists significantly improve motor deficits, reduce OFF time, and increase ON time in parkinsonian patients (LeWitt et al., 2008; Hauser et al., 2008; Factor et al., 2010). Given the potential utility of adenosine A_{2A} antagonists for the treatment of parkinsonism, further investigations into their behavioral effects using animal models are critical.

One model that has proven to be useful for assessing the role of adenosine A_{2A} receptors in motor function is the tremulous jaw movement (TJM) model, an extensively validated rodent model of parkinsonian resting tremor (Simola et al., 2004; Miwa et al., 2009; Collins et al., 2010a, 2011; for reviews, see Salamone et al., 1998; Collins-Praino et al., 2011). TJMs are defined as rapid vertical deflections of the lower jaw that are not directed at any stimulus (Salamone et al., 1998), and occur in phasic bursts of repetitive jaw

movement activity, with multiple movements within each burst. TJMs have many of the neurochemical, anatomical, and pharmacological characteristics of parkinsonism, and thus meet a reasonable set of validation criteria for use as an animal model of parkinsonian tremor (Salamone et al., 1998; Collins-Praino et al., 2011). These movements can be induced by many conditions that are associated with parkinsonism, including neurotoxic or pharmacological depletion of striatal dopamine (DA; Jicha et al., 1991; Salamone et al., 2008a,b), and acute or subchronic administration of DA antagonists (Ishiwari et al., 2005; Betz et al., 2007; Salamone et al., 2008). TJMs also are induced by cholinomimetic drugs, including muscarinic agonists such as pilocarpine, arecoline and oxotremorine (Salamone et al., 1986, 1998; Collins et al., 2010), and the anticholinesterases physostigmine, tacrine, and galantamine (Salamone et al. 1998; Simola et al., 2004, 2006; Collins et al. 2011). As shown by studies using analyses of video recordings or electromyographic methods, TJMs occur largely within the 3-7 Hz frequency range that is characteristic of parkinsonian resting tremor (Finn et al., 1997; Ishiwari et al., 2005; Collins et al., 2010). TJMs also can be attenuated by several classes of antiparkinsonian drugs, including DA agonists and anticholinergics (Cousins et al., 1997; Salamone et al., 1998, 2005; Betz et al. 2007). In recent years, several adenosine A_{2A} antagonists have been shown to significantly reverse the TJMs induced by DA depletion, DA antagonism and cholinomimetic administration (Correa et al., 2004; Simola et al., 2004; Tronci et al., 2007; Salamone et al., 2008a; Betz et al., 2009; Collins et al., 2010; Pinna et al., 2010; Collins et al., 2011).

With the rising importance of genetic manipulations in mice (i.e. transgenic, knockout, knockin, etc.) to the field of neuroscience, it is necessary

to investigate whether it is possible to extend well-validated behavioral paradigms currently being used in rats to mouse models. The TJM model is no exception. Although one previous study showed that mice with a knockout of muscarinic M4 receptors showed significantly fewer cholinomimetic-induced TMMs than wild type mice (Salamone et al., 2001), every other study of TJM activity has employed rats. Given the putative antiparkinsonian properties of adenosine A_{2A} receptor antagonists, it is of great interest to determine if mice with a deletion of the adenosine A_{2A} receptor show reduced levels of TJM activity compared to type mice.

The present experiments sought to determine whether mice with a knockout of the adenosine A_{2A} receptor would be resistant to the development of pilocarpine-induced TJMs compared to their wild type littermates. In order to investigate this research question, several preliminary experiments were necessary. The first experiment studied the ability of the muscarinic agonist pilocarpine (0.25 mg/kg – 1.0 mg/kg) to induce TJMs in the specific strain of mice being used for the knockout study (C57/BL6). In the second experiment, the local frequency range of the TJM "bursts" induced by pilocarpine was characterized using freezeframe video analysis. A third study investigated the ability of the adenosine A_{2A} antagonist MSX-3 to attenuate pilocarpine-induced TJMs. Finally, in the fourth study, both wild-type and adenosine A_{2A} receptor knockout mice were assessed for pilocarpine-induced TJMs. Based upon previous findings, it was hypothesized that A_{2A} knockout mice would show fewer tremulous jaw movements than their wild type littermates.

MATERIALS AND METHODS

Animals

Male C57BL/6 mice (25; Harlan Laboratories, Indianapolis, IN) with no prior drug experience were used for the first 3 studies. For the final study, a total of 24 neuronal A_{2A} receptor conditional knockout mice and their littermate controls (12 CaMKIIα-cre, A_{2A} flox/flox and 12 non-transgenic [no cre] A_{2A} flox/flox mice) congenic for the C57BL/6 background and with no prior drug experience were obtained from Massachusetts General Hospital (Boston, MA; see Bastia et al. (2006) and Xie et al., (2006) for details on the generation of these mice). Mice weighed 15-40 g throughout the course of the experiment and had *ad libitum* access to lab chow and water. The mice were group-housed in a colony that was maintained at approximately 23□ C and had a 12-h light/dark cycle (lights on at 0700 h). These studies were conducted according to University of Connecticut and NIH guidelines for animal care and use.

Pharmacological agents

The muscarnic agonist pilocarpine was purchased from Sigma Aldrich Chemical (St. Louis, MO) and dissolved in 0.9% saline. The adenosine A_{2A} antagonist MSX-3 ((E)-phosphoric acid mono-[3-[8-[2-(3-methoxyphenyl)vinyl]-7-methyl-2,6-dioxo-1-prop-2-ynyl-1,2,6,7-etrahydropurin- 3-yl]propyl] ester) was synthesized at the Pharmazeutisches Institut (Universität Bonn; Bonn, Germany; Hockemeyer et al., 2004), and was dissolved in 0.9% saline. MSX-3 is a pro-drug of the active adenosine A_{2A} antagonist, MSX-2. In order to ensure that a similar dose was also appropriate for use in the mouse strain used for these experiments, extensive pilot work was

performed; the dose of 1.0 mg/kg pilocarpine used in experiments 2-4 was based upon the

results of the first experiment.

Apparatus and testing procedures

Observations of mice took place in a 11.5 × 9.5 × 7.5 cm clear glass chamber with a plastic mesh floor, which was elevated 26 cm from the table top. This allowed for the viewing of the mouse from several angles, including underneath. TJMs were defined as rapid vertical deflections of the lower jaw that resembled chewing but were not directed at any particular stimulus (Salamone et al., 1998). Each individual deflection of the jaw was recorded using a mechanical hand counter by a trained observer, who was blind to the experimental condition of the mouse being observed. Separate studies with two observers demonstrated an inter-rater reliability of $r = 0.98$ ($p<0.001$) using these methods.

Experiments

Experiment 1: *Ability of pilocarpine to induce tremulous jaw movements*. A group of 11 male C57BL/6 mice was used to assess the effect of pilocarpine (0.25 mg/kg- 1.0 mg/kg) on TJMs. All mice received IP injections of either 1.0 ml/kg saline or 0.25 mg/kg, 0.5 mg/kg, 0.75 mg/kg, or 1.0 mg/kg pilocarpine in a within-groups design, with all mice receiving all drug treatments in a randomly varied order (one treatment per week; no treatment sequences were repeated). Five min after IP injection, mice were placed in the observation chamber and allowed 5 min to habituate. Following this habituation period, TJMs were counted for 10 min.

Experiment 2: *F reeze-frame video analysis of local frequency of the tremulous jaw movements induced by pilocarpine.* Three male C57BL/6 mice received an IP injection of 1.0 mg/kg pilocarpine. Five min later, mice were placed in a flat bottom mouse restrainer (myNeuroLab.com, Richmond, IL) so that a consistent view of the orofacial area could be achieved. After habituating for 5 min, each mouse was videotaped for 15 min using a FlipVideo UltraHD (Cisco Systems, Farmington, CT). The sections of these video files that allowed for clear observation of the orofacial area were then subjected to a freeze-frame analysis (1 frame = 1/30 s), in which the observer went frame-byframe through each burst of jaw movements (i.e., each group of at least two jaw movements that were within 1.0 s of each other). The observer recorded the inter-movement interval for each pair of jaw movements within these bursts, which was defined as the number of frames between each point at which the jaw was fully closed during successive jaw movements. This information was then used to determine the local frequency within bursts of jaw movements.

Experiment 3: *Ability of the adenosine A2A antagonist MSX-3 to attenuate the tremulous jaw movements induced by pilocarpine.* A group of 11 male C57BL/6 mice was used to assess the effects of the adenosine A_{2A} antagonist MSX-3 (2.5-10.0 mg/kg) on the tremulous jaw movements induced by administration of 1.0 mg/kg pilocarpine. A within-groups design was utilized for this study, with all mice receiving all drug treatments in a randomly varied order (one treatment per week; no treatment sequences were repeated). On test day each week, each mouse was given an IP injection of either 1.0 ml/kg saline or 2.5 mg/kg, 5.0 mg/kg, or 10.0 mg/kg MSX-3. Ten min later, all mice received an IP injection of 1.0 mg/kg pilocarpine to yield the following combined

treatment conditions: 1.0 mg/kg pilocarpine + saline vehicle, 1.0 mg/kg pilocarpine + 2.5 mg/kg MSX-3, 1.0 mg/kg pilocarpine + 5.0 mg/kg MSX-3, and 1.0 mg/kg pilocarpine + 10.0 mg/kg MSX-3. Five min after injections, mice were placed in the observation chamber and allowed 5 min to habituate, after which TJMs were counted for 10 min.

Experiment 4: *Ability of pilocarpine to induce tremulous jaw movements in mice with a knockout of the adenosine A2A receptor.* A total of 24 male C57BL/6 mice (n = 12 postnatal neuronal A_{2A} receptor conditional KO mice (A_{2A} -/-); n = 12 litter mate controls (A_{2A} +/+)) were used to assess the effect of the knockout of the adenosine A_{2A} receptor on the induction of TJMs by 1.0 mg/kg pilocarpine. For this experiment, only homozygous A_{2A} KO mice and littermate controls were used. All mice received an IP injection of 0.1 mg/kg pilocarpine in a between-groups design. Five min after IP injection, mice were placed in a glass observation chamber and allowed 5 min to habituate. Following this habituation period, TJMs were counted for 10 min by an observer blind to the condition of the mouse (i.e. littermate control vs. A_{2A} KO).

Statistical analysis

The behavioral data for the first two experiments were analyzed using a repeated measures analysis of variance (ANOVA). Average TJMs over the two five-min observation periods were calculated and then used in the ANOVA calculations. A computerized statistical program (SPSS 12.0 for Windows) was used to perform these analyses. When there was a significant ANOVA, planned comparisons using the overall error term were used to assess the differences between each dose and the control condition; the total number of comparisons

was restricted to the number of treatments minus one (Keppel, 1991). The behavioral data from the knockout experiment (Experiment 4) was analyzed using a Student's independent samples t-test to contrast the knockout group with littermate controls. A computerized statistical program (SPSS 12.0 for Windows) was used to perform these analyses.

RESULTS

3.1 Experiments 1 and 2: *Ability of pilocarpine to induce tremulous jaw movements*. Figure 1A shows the effects of injections of pilocarpine (0.25 mg/kg- 1.0 mg/kg) on the induction of TJM activity. Repeated measures ANOVA revealed that there was a significant overall effect of drug treatment on TJM activity ($F(4, 40) = 24.46$; $p < 0.001$). Planned comparisons showed that all doses of pilocarpine were capable of significantly inducing tremulous jaw movements ($p < 0.001$) compared to mice treated with saline vehicle. Figure 1B displays the results of the freeze-frame analyses of videotaped samples of pilocarpine-induced jaw movement activity in three wild-type C57BL/6 mice. A total of 509 jaw movements were analyzed. 83.69% of these jaw movements took place within "bursts," defined as a group of at least two jaw movements that were within 1.0 s of each other. Data are shown as the number of inter-movement intervals (i.e., the number of 1/30 sec frames that elapsed from jaw closing through jaw opening to the next jaw closing during each movement) from jaw movements in bursts, assigned to four different frequency bins. To interpret these data in terms of frequencies (i.e. jaw movements per second), the reciprocal of the inter-movement interval was calculated (e.g. 10/30 frames per

second corresponds to 3 Hz; 4/30 frames per second to 7.5 Hz, etc.) The majority (77.60%) of the jaw movement activity took place in the 3.0-7.5 Hz frequency range. There were no jaw movements in the 1-3 Hz or > 10 Hz bins.

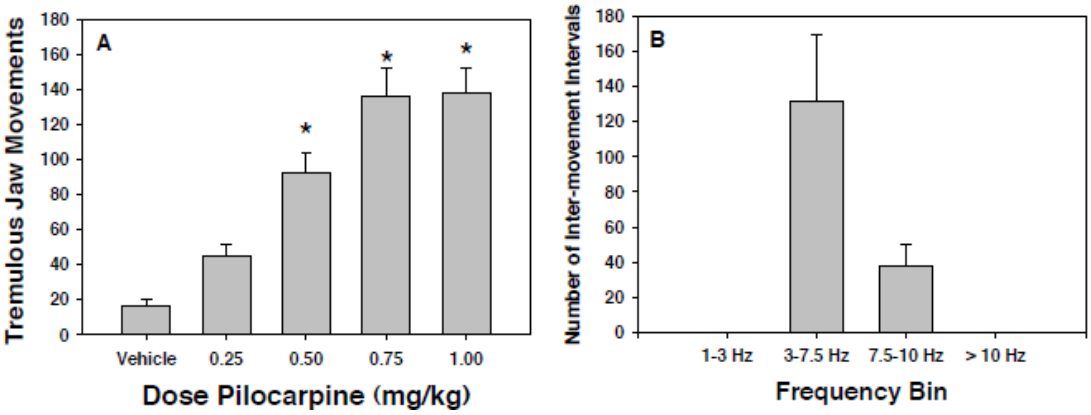

Fig 1A, B. (A): Effects of different doses of pilocarpine (IP) on tremulous jaw movements. Mean (+SEM) number of jaw movements in mice (n = 11) treated with either saline vehicle or pilocarpine. **significant difference from vehicle control ($p < 0.05$). (B): This figure shows the results of the freeze-frame analysis of inter-movement intervals using the video analysis methods described above. Inter-movement times were determined by freeze-frame analysis of video obtained from 3 mice treated with 1.0 mg/kg pilocarpine, and were assigned to one of four local frequency bins. Distribution of the mean (+ SEM) number of inter-movement intervals within each frequency bin is shown.

3.2 Experiments 3 and 4: *Ability of the adenosine A_{2A} receptor antagonism and knockout attenuate the tremulous jaw movements induced by pilocarpine.* Co-administration of the adenosine A_{2A} antagonist MSX-3 attenuated the TJMs induced by a dose of 1.0 mg/kg pilocarpine (Figure 2A). Repeated measures ANOVA revealed that there was a significant overall effect of MSX-3 treatment on the induction of TJMs by 1.0 mg/kg pilocarpine ($F(3,30) = 35.88$; $p < 0.001$). Planned comparisons showed that the 2.5, 5.0 and 10.0

mg/kg doses of MSX-3 were capable of significantly reducing the TJMs induced by 1.0 mg/kg pilocarpine (i.e., compared to pilocarpine plus saline; $p < 0.05$). Figure 2B shows the effects of injection of 1.0 mg/kg pilocarpine on the induction of TJM activity in mice homozygous for neuronal knockout of the adenosine A_{2A} receptor (A_{2A} -/-) and littermate controls (A_{2A} +/+). An independent samples t-test revealed that adenosine A_{2A} neuronal knockout mice showed significantly fewer TJMs than the littermate controls ($t(22) = 2.45$; $p < 0.05$).

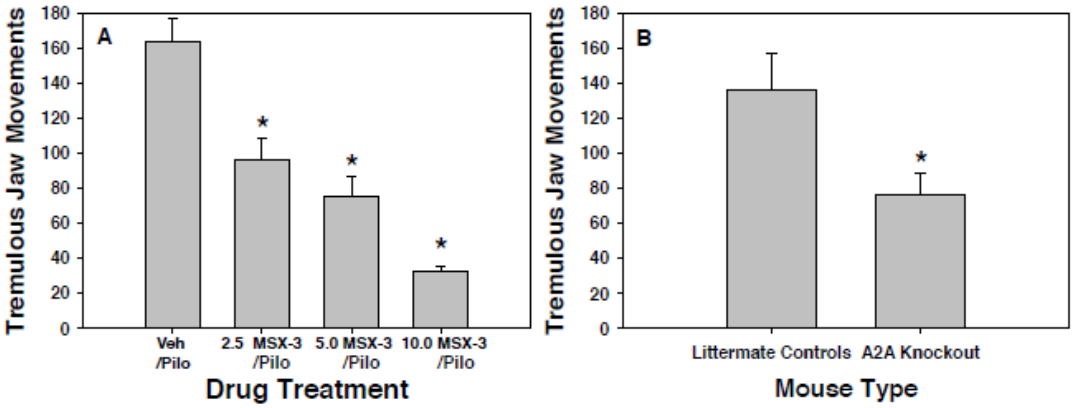

Fig 2A,B. (A): Effect of the adenosine A_{2A} antagonist MSX-3 on the tremulous jaw movements induced by 1.0 mg/kg pilocarpine. Mean (+ SEM) number of jaw movements in mice (n = 11) treated with pilocarpine plus vehicle (Veh/Pilo), and pilocarpine (Pilo) plus various doses (2.5, 5.0 and10.0 mg/kg IP) of MSX-3. *significant difference from pilocarpine plus vehicle control ($p< 0.05$). (B): Effect of neuronal adenosine A_{2A} receptor knockout on the tremulous jaw movements induced by 1.0 mg/kg pilocarpine. Mean (+ SEM) number of jaw movements in knockout mice (n = 12) and littermate controls (n = 12) treated with pilocarpine. *significant difference from littermate controls ($p < 0.05$)

DISCUSSION

The current studies describe the development of a mouse model of TJM activity. The first experiment investigated the ability of the muscarinic agonist pilocarpine to induce TJMs in C57BL/6 mice. Acute administration of pilocarpine has been well documented to induce TJMs in rats (Salamone et al., 1986, 1998; Finn et al., 1997; Betz et al., 2007; Collins et al., 2010). Pilocarpine administration was able to induce TJM activity in C57BL/6 mice at all doses tested (i.e. 0.25-1.0 mg/kg). This is consistent with findings from a previous study indicating that administration of pilocarpine induced tremulous jaw movements in 129SvEv (50%) × CF1 (50%) mice (Salamone et al., 2001). Local frequency analysis of the pilocarpine-induced jaw movements in mice using freeze frame video analysis indicated that the TJMs induced by pilocarpine occurred largely in the 3-7.5 Hz frequency range, which is consistent with the findings from previous studies of the local frequency of TJMs induced by DA depletion, D_2 antagonism, and administration of cholinomimetic drugs in rats (Finn et al., 1997; Ishiwari et al., 2005; Collins et al., 2010). Moreover, this 3-7.5 Hz frequency range is similar to that reported during resting tremor in parkinsonian patients (Deuschl et al., 2000, 2001). These findings are consistent with the hypothesis that the oral motor movements induced by acute pilocarpine administration are potentially a useful mouse model of parkinsonian resting tremor.

Taken together with previous studies, the finding that pilocarpine administration is capable of significantly inducing tremulous jaw movements in mice highlights the role that Ach plays in striatal motor functions related to parkinsonism. Cholinomimetic drugs, such as muscarinic agonists and

anticholinesterases used for the treatment of Alzheimer's disease, have been shown to induce or exacerbate parkinsonian symptoms, including tremor, in humans (Ott and Lannon, 1992; Song et al., 2008; Collins-Praino et al., 2011). In addition, muscarinic receptor antagonists have been used as treatments for the motor symptoms of parkinsonism (Bezchlibnyk-Butler and Remington, 1994). Furthermore, several studies have implicated neostriatal muscarinic receptors in the regulation of TJM activity (Salamone et al., 1998; Betz et al., 2007, 2009).

Tremor responds relatively poorly to most currently available antiparkinsonian medications, including L-DOPA (Bain, 2002). In recent years, adenosine A_{2A} antagonists have emerged as a potential treatment of parkinsonian motor impairments. One clinical report suggested that tremor was particularly sensitive to the effects of adenosine A_{2A} antagonism (Barra-Jimenez et al., 2003). Adenosine A_{2A} receptors are highly expressed in neostriatum, and A_{2A} antagonists exert motor effects in rodents and primates that are consistent with antiparkinsonian actions (Ferre et al., 1997, 2004; Chen et al., 2001; Morelli and Pinna, 2001; Hauser and Schwarzschild, 2005; Morrelli et al., 2007; Salamone et al., 2008; Collins et al., 2010). For that reason, the final two experiments sought to investigate the ability of adenosine A_{2A} receptor antagonism or genetic deletion to attenuate the TJMs induced by 1.0 mg/kg pilocarpine. In experiment 3, the adenosine A_{2A} antagonist MSX-3 significantly attenuated pilocarpineinduced TJMs, which is consistent with previous findings in rats (Correa et al., 2004; Simola et al., 2004; Tronci et al., 2007; Salamone et al., 2008; Pinna et al., 2010; Collins et al., 2010, 2011). Furthermore, deletion of the adenosine A_{2A} receptor also resulted in significantly lower levels of pilocarpine-induced TJMs compared to wild-type mice. This is consistent with previous

research, which has shown that knockout of the adenosine A_{2A} receptor is capable of reversing the catalepsy induced by the DA D_2 antagonist haloperidol (Chen et al., 2001; El Yacoubi et al., 2001), the DA D_1 antagonist SCH 23390 (El Yacoubi et al., 2001), and the muscarinic agonist pilocarpine (El Yacoubi et al., 2001). Moreover, genetic deletion of the adenosine A_{2A} receptor in mice has been shown to alter the locomotor response to adenosine antagonists (Yu et al., 2008), and to affect amphetamine sensitization (Chen et al., 2003), drug self-administration of cocaine and MDMA (Ruiz-Medina et al., 2011), aspects of cognition (Wei et al., 2011), and effort-related choice behavior (Pardo et al., 2012). Furthermore, striatal adenosine A_{2A} receptors appear to be required for the motor stimulating effects of adenosine A_{2A} antagonists, because mice lacking these receptors showed an absence of motor stimulation in response to adenosine A_{2A} antagonists (Yu et al., 2008; Wei et al., 2011).

Taken together, these results demonstrate the feasibility of using the tremulous jaw movement model in mice, and indicate that adenosine A_{2A} receptor antagonism and deletion are capable of reducing cholinomimetic-induced TJMs in mice. The results of these experiments add to the growing body of evidence demonstrating that adenosine A_{2A} function is involved in regulating motor functions in animals that are potentially related to parkinsonism. Additional studies will seek to more completely characterize the effect of adenosine A2A receptor deletion on motor function, and future research should investigate the effects of additional genetic manipulations using the mouse TJM model, including regionally-specific knockout of A_{2A} receptors (e.g. Lazarus et al., 2011).

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