UNIVERSIDADE FEDERAL DO PARANÁ

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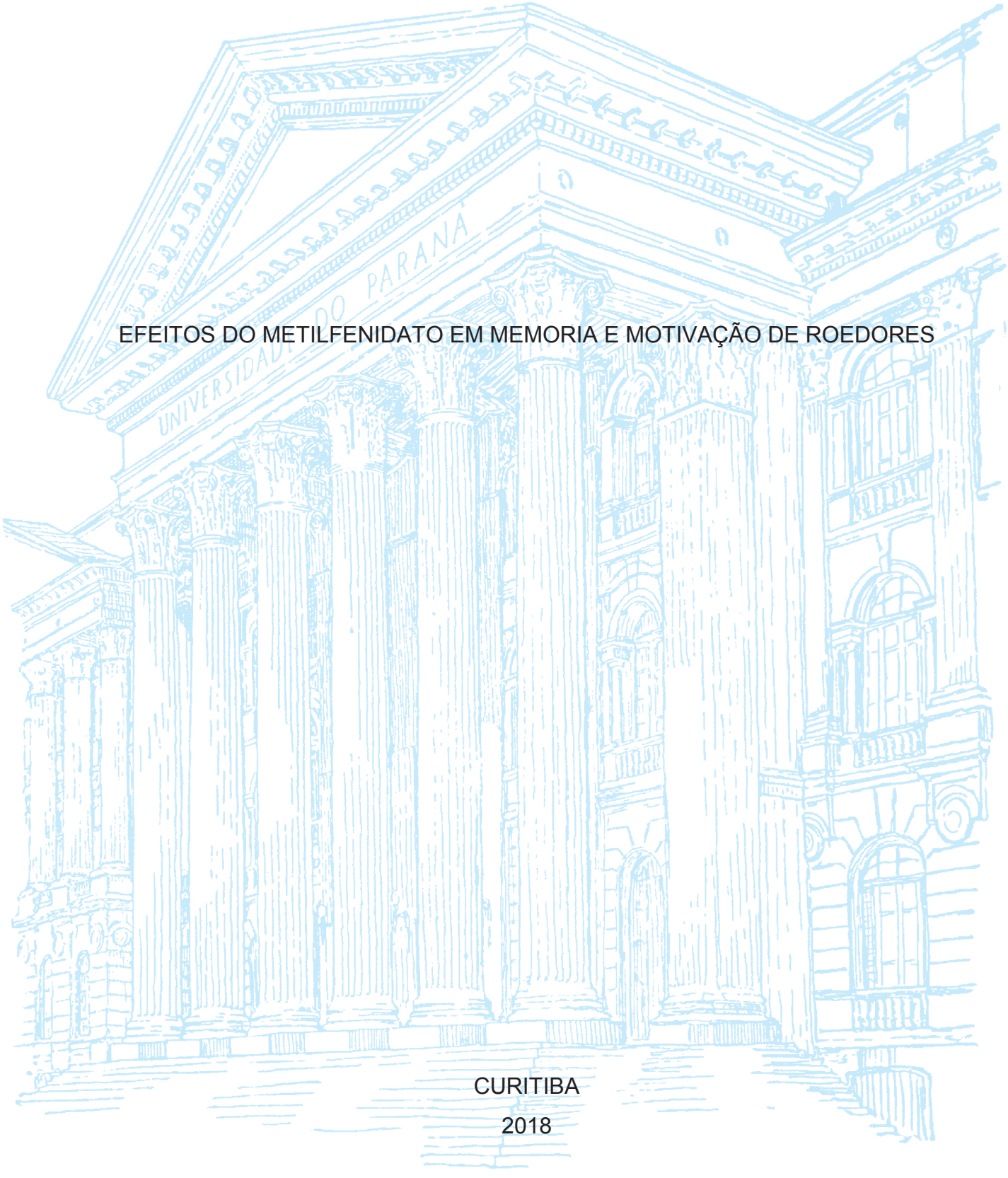

EFEITOS DO METILFENIDATO EM MEMORIA E MOTIVAÇÃO DE ROEDORES

CURITIBA

2018

LAURA MILENA NITOLA PULIDO

EFEITOS DO METILFENIDATO EM MEMORIA E MOTIVAÇÃO DE ROEDORES

Tese apresentada como requisito para obtenção do título de Doutora em Farmacologia, Departamento de Farmacologia, Setor de Ciências Biológicas, Universidade Federal do Paraná

Orientador: Prof. Dr. Claudio Da Cunha
Co-orientador: Prof. Dr. Romulo Antônio Fuentes Flores

CURITIBA

2018

Universidade Federal do Paraná. Sistema de Bibliotecas.
Biblioteca de Ciências Biológicas.
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Pulido, Laura Milena Nitola
Efeitos do metilfenidato em memoria e motivação de roedores. / Laura Milena Nitola Pulido. – Curitiba, 2018.
58 p.: il. ; 30cm.

Orientador: Claudio da Cunha
Co-orientador: Romulo Antônio Fuentes Flores
Tese (Doutorado) - Universidade Federal do Paraná, Setor de Ciências Biológicas. Programa de Pós-Graduação em Farmacologia.

1. Metilfenidato. 2. Memória. 3. Motivação. 4. Estimulantes. I. Título II. Cunha, Claudio da. III. Flores, Romulo Antônio Fuentes. IV. Universidade Federal do Paraná. Setor de Ciências Biológicas. Programa de Pós-Graduação em Farmacologia.

CDD (20. ed.) 615.785

MINISTÉRIO DA EDUCAÇÃO
SETOR CIÊNCIAS BIOLÓGICAS
UNIVERSIDADE FEDERAL DO PARANÁ
PRÓ-REITORIA DE PESQUISA E PÓS-GRADUAÇÃO
PROGRAMA DE PÓS-GRADUAÇÃO FARMACOLOGIA

TERMO DE APROVAÇÃO

Os membros da Banca Examinadora designada pelo Colegiado do Programa de Pós-Graduação em FARMACOLOGIA da Universidade Federal do Paraná foram convocados para realizar a arguição da tese de Doutorado de **LAURA MILENA NITOLA PULIDO** intitulada: **EFEITOS DO METILFENIDATO EM MEMORIA E MOTIVAÇÃO DE ROEDORES**, após terem inquirido a aluna e realizado a avaliação do trabalho, são de parecer pela sua aprovação no rito de defesa.

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Curitiba, 08 de Agosto de 2018.

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Dedicated to Mariana, Lucía, Emma, Elizabeth, Julieta and Anna

ACKNOWLEDGEMENTS

This accomplishment is possible thanks to the help, support and love of many people, to whom I would like to express my gratitude.

To my grandmother Ligia and my godmother Mary, who planted the first seeds to make me the woman that I am today. Thank you for being excellent role models, I hope I can become half the women you are.

To my mother Claudia and my stepfather Joseph, for your unconditional love and support. To my brother Felipe and sister in law Sina, who have been there for me in difficult moments, and who make me feel loved when I need it the most. And of course to my brother Jose and my sister in law Aura, my brother Nicolas, and my little sisters Mariana and Lucía.

To my cousins Daniela and Ana, who are my sisters and best friends, with you I can be myself, and I know I am never alone.

To Alexis Lagos for his support and love.

To Laura Leon for being such an amazing, patient, generous and understanding friend, and the best role model.

To Fernando Cardenas who was my first advisor, who introduced me in this amazing path of neuroscience. His love for science and research was an inspiration. And of course to my former colleagues from Fernando's Lab, Rodrigo and Johanna, who are excellent professionals and very dear friends.

To my colleagues in professor Claudio's Lab, Suelen, Amanda, Julie, Ana, Gabriela, Dani, Gisele, Mayra, Jacke, Alex, David, Adam, Jose, William, João, and Gabriel. For their friendship, their support in the lab and in life. For borrowing their shoulders for me to cry, and their ears for me to speak, for laughing and celebrating with me when things went well. More than friends and colleagues, they were my brothers and sisters in science.

To Jéssica, Lilian, Taysa, Meira and Isabella for their friendship. They are very dear friends and outstanding researchers. It is an honor to have had the opportunity to work by their side, and a great blessing to have them as friends.

To all my colleagues and friends in the pharmacology department, without exception, who always made me felt among my family. Sharing this experience with them made it so much more special.

To all the professor in the pharmacology department, especially professors Cristina, Maria, Janaina, Roberto, Paulo, Alexandra, Joice and Aleksander. For all the support, the help, and everything that I have learned from them.

To all the people who work in the pharmacology department, Gabriela, Ely, Fernando, and Silvia. For all their help, support and kindness.

To my friends in Curitiba outside the pharmacology department, Silvia, Mayara, Alexandre, Rocio, Gerson, Abigail, Ariel, Amanda, Rafael and Leticia. They were my family in Curitiba for six years.

To Romulo Fuentes, for his support, his advice –academic and personal-, and for his friendship. I hope I can keep collaborating and learning from him for many years.

The last and most important person that I want to thank is professor Claudio, who has been much more than an advisor for me. There are so many reasons for being grateful to professor Claudio, he has done so much more for me than he realises. For accepting me in his team and welcoming me into his family. For his kindness, his generosity, his patience, his support. For helping me through the difficult times that I experienced during this process. For all the things that I have learned from him, for being a passionate, ethic and dedicated researcher and an excellent teacher. But most of all, for being such an amazing human being, that combines humbleness with greatness. I approached him expecting to learn how to be a scientist, and he taught me how to be a human.

And thank you to this beautiful land, that made me feel like home: Brazil.

Out of the night that covers me,
Black as the pit from pole to pole,
I thank whatever gods may be
For my unconquerable soul.

In the fell clutch of circumstance
I have not winced nor cried aloud.
Under the bludgeonings of chance
My head is bloody, but unbowed.

Beyond this place of wrath and tears
Looms but the Horror of the shade,
And yet the menace of the years
Finds and shall find me unafraid.

It matters not how strait the gate,
How charged with punishments the scroll,
I am the master of my fate,
I am the captain of my soul.

By William Hernest Henley

RESUMO

O metilfenidato (Ritalina®) é um fármaco estimulante utilizado no tratamento do transtorno de déficit de atenção e hiperatividade. Recentemente, houve um incremento consumo de metilfenidato entre adultos jovens, principalmente estudantes de universidade, que usam o fármaco com o intuito de aprimorar as suas funções cognitivas e melhorar o seu desempenho académico. Contudo, ainda não é claro se o uso do metilfenidato traz benefícios para usuários saudáveis (sem o diagnóstico de transtorno de déficit de atenção). Além disso, não há claridade sobre os efeitos do metilfenidato na motivação e como eles interagem com os efeitos em cognição para influenciar no desempenho. O objetivo desse estudo é avaliar os efeitos da administração aguda ou crónica de metilfenidato (0.3, 1 ou 3 mg/kg i.p.) na memoria e na motivação em ratos. Para isso ratos foram treinados na tarefa de labirinto radial de oito braços, com três braços reforçados com 1, 3 ou 6 pellets de sacarose, que se mantiveram estáveis ao longo do experimento. Nós avaliamos o desempenho no labirinto quantificando o número de entradas nos braços não reforçados e o número de reentradas nos braços reforçados. A administração aguda de metilfenidato foi avaliada no último dia do treino e não mostrou nenhum efeito no desempenho, sugerindo que o metilfenidato não tem nenhum efeito sobre memorias já estabelecidas. A administração crónica de 3mg/kg metilfenidato incrementou o número de reentradas aos braços com 3 e 6 pellets a partir do día 11 do treino. Este aumento nas reentradas não foi observado para o braço com 1 pellet, e nem em animais treinados com a mesma quantidade de pellets em todos os braços. Nós sugerimos que este aumento nas reentradas não reflete uma piora na memoria, mas pode refletir um aumento na motivação a procurar recompensas altas. Nós concluímos que o metilfenidato não possui efeitos na memoria, porem, pode piorar o desempenho indiretamente, afetando outros processos como motivação.

ABSTRACT

Methylphenidate (Ritalin ®) is a stimulant used in the treatment of attentional deficit and hyperactivity disorder. The use of methylphenidate by healthy young adults has increased recently, mainly among college students who use it with the intention to improve their cognitive functions and have a better academic performance. However, it is not clear if the use of methylphenidate has benefits for healthy subjects (people without the diagnose for attention deficit and hyperactivity disorder). Furthermore, it is not clear what are the effects of methylphenidate on motivation and how they interact with the effects in cognition, influencing performance. This study aims to evaluate the effects of acute or chronic methylphenidate administration (0.3, 1 or 3 mg/kg i.p.) in memory and motivation, in rodents. To this end, rats were trained in the radial arm maze task, with three reinforced arms that remained stable across the experiment, and that contained 1, 3 or 6 sucrose pellets. We evaluated performance quantifying the entries in the non-reinforced arms and the re-entries in the reinforced arms. Acute administration of methylphenidate was evaluated on the last day of training, and it did not show any effect on performance, suggesting that methylphenidate does not have any effect on memory that is well established. From the 11^{th} day of training, chronic administration of 3mg/kg of methylphenidate increased the number of re-entries in the arms that contained 3 or 6 sucrose pellets. This increase in the re-entries did not occur for the arm baited with 1 pellet, nor for animals trained with the same amount of reward in the three arms. We propose that this increase in re-entries does not reflect impairment in working memory, but it may reflect an increase in motivation to search for high rewards. We conclude that methylphenidate does not have effects on memory, but it can affect performance indirectly, by effects in other domains such as motivation.

ABBREVIATIONS

ADHD – Attention Deficit and Hyperactivity Disorder

ANVISA – National Health Surveillance Agency (in Portuguese *Agencia Nacional de Vigilancia Sanitária*)

DAT – Dopamine Transporter

SNc – Substantia Nigra pars compacta

VTA – Ventral Tegmental Area

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PRESENTATION

This thesis presents original results divided in seven sections: introduction, methods, results, discussion, conclusion and references. At the end of the document four figures are found as appendixes.

1. INTRODUCTION

Methylphenidate is a stimulant that was synthesised for the first time in 1944 in Switzerland, and now is the active compound of Ritalin ® and Concerta ®. It is used primarily to treat attention deficit and hyperactivity disorder (ADHD), and it can be used to treat narcolepsy and depression as well (Challman e Lipsky, 2000).

Although methylphenidate has therapeutic uses, it also has some problematic side effects. In the Controlled Substances Act, a federal U.S. drug policy, methylphenidate is classified as a schedule II drug, which means methylphenidate is considered a substance with a high potential for abuse, and yet it has therapeutic uses. In Brazil, the National Health Surveillance Agency (ANVISA) also recognises methylphenidate as a substance with potential for abuse and classifies it under the black strip category, which is reserved for the highest controlled drugs.

The abuse potential of methylphenidate arises from the fact that it blocks dopamine transporters and increases dopaminergic transmission (Ritz et al. 1987; Ritz and Kuhar 1988; Volkow 1998). This mechanism of action is similar to cocaine and amphetamine, and virtually, all drugs with the potential of abuse increase dopaminergic transmission either directly or indirectly (Volkow e Morales, 2015).

1.1. EFFECTS OF METHYLPHENIDATE ON DOPAMINERGIC TRANSMISSION.

Dopamine is a catecholamine neurotransmitter (Carlsson *et al.*, 1957; Carlsson e Waldeck, 1958; Carlsson, 1959) produced in the mammalian brain in ten different regions identified as A8-A17 (Figure 1) by Dahlstrom and Fuxe (1964). The most studied nuclei are A9 and A10, located in the midbrain, also known as substantia nigra pars compacta (SNc) and ventral tegmental area (VTA). These two nuclei innervate structures in the forebrain, forming three different pathways: one that reaches the dorsal and ventral striatum, one that reaches limbic structures (for example the amygdala and the septum), and one that reaches the cortex (Lindvall *et al.*, 1974; Lindvall *et al.*, 1977; Fallon, Koziell*, et al.*, 1978; Fallon e Moore, 1978a; b; Fallon, Riley*, et al.*, 1978).

These dopaminergic pathways are of particular interest because of their involvement in motivation and learning (Bromberg-Martin *et al.*, 2010; Da Cunha *et*

al., 2012). Regarding motivation, evidence suggests that dopamine is involved in reward approach or reward-seeking behaviours (Salamone e Correa, 2012). An example of this notion is an experiment that shows that animals with chronically elevated dopamine show higher motivation to work for food reward (measured as the breakpoint in a progressive ratio) (Cagniard *et al.*, 2006). This example illustrates that higher dopamine levels correlate with states of higher motivation to search for rewards. Likewise, dopamine release decreases if the animal has a low motivation for food reward (for example if the animal is satiated), and if the cost of the reward increases (Ostlund *et al.*, 2011). This example illustrates that lower dopamine levels correlate with states of lower motivation to search for a reward. Considering this, authors have proposed that dopamine signals incentive salience or 'wanting' for a reward (Robinson *et al.*, 2005; Berridge, 2007).

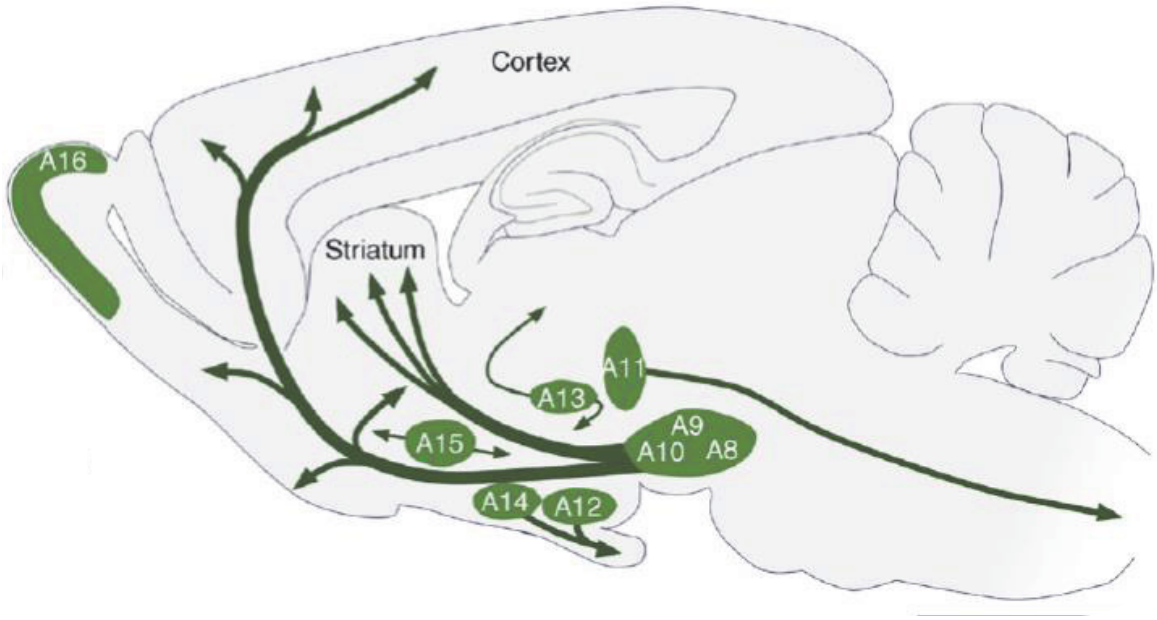

Figure 1. Sagittal view of dopaminergic nuclei in the rat brain. Source: Bjorklund and Dunnet (2007).

In addition, extensive evidence demonstrates that dopamine conveys a signal for reward prediction error: unexpected rewards trigger a rapid increase in the firing of dopaminergic neurons and dopamine release (Schultz, 1997; Schultz *et al.*, 1997; Bromberg-Martin e Hikosaka, 2009; Hart *et al.*, 2014). A similar rapid increase happens with cues that predict the apparition of reward (Bromberg-Martin e Hikosaka, 2009) and with rewards that are higher than expected (Bayer e Glimcher, 2005). On the other hand, if an expected reward fails to appear there is a pause in

dopaminergic neurons firing (Schultz *et al.*, 1997). These reward prediction error signals are proposed to mediate associative learning (Steinberg *et al.*, 2013).

The Dopamine transporter (DAT) is one of the mechanisms that regulate extracellular dopamine levels. It takes dopamine from the synaptic cleft and brings it back to the presynaptic cell. Acute administration of therapeutic doses of methylphenidate blocks between 50% - 75% of DAT (Figure 2) in the human brain (Volkow *et al.*, 1998), which increases extracellular levels of dopamine in the striatum (Volkow *et al.*, 2001). Chronic administration of methylphenidate produces some adaptations in the system: it reversibly increases the expression of DAT in the striatum (Robison *et al.*, 2017); and it also increases the rates of dopamine uptake and increased dopamine release (Calipari *et al.*, 2014).

At the behavioural level, studies show that chronic methylphenidate alters the circadian rhythms of male and female rats (Algahim *et al.*, 2009; Lee *et al.*, 2011) and increases self-administration of cocaine, nicotine and amphetamine in rats (Thanos *et al.*, 2007; Wooters *et al.*, 2008; Crawford *et al.*, 2011; Calipari *et al.*, 2013). Both effects are likely related to alterations in dopamine release described earlier. Thus, chronic methylphenidate induces a sensitisation dopaminergic transmission reflected at the molecular, physiological and behavioural levels.

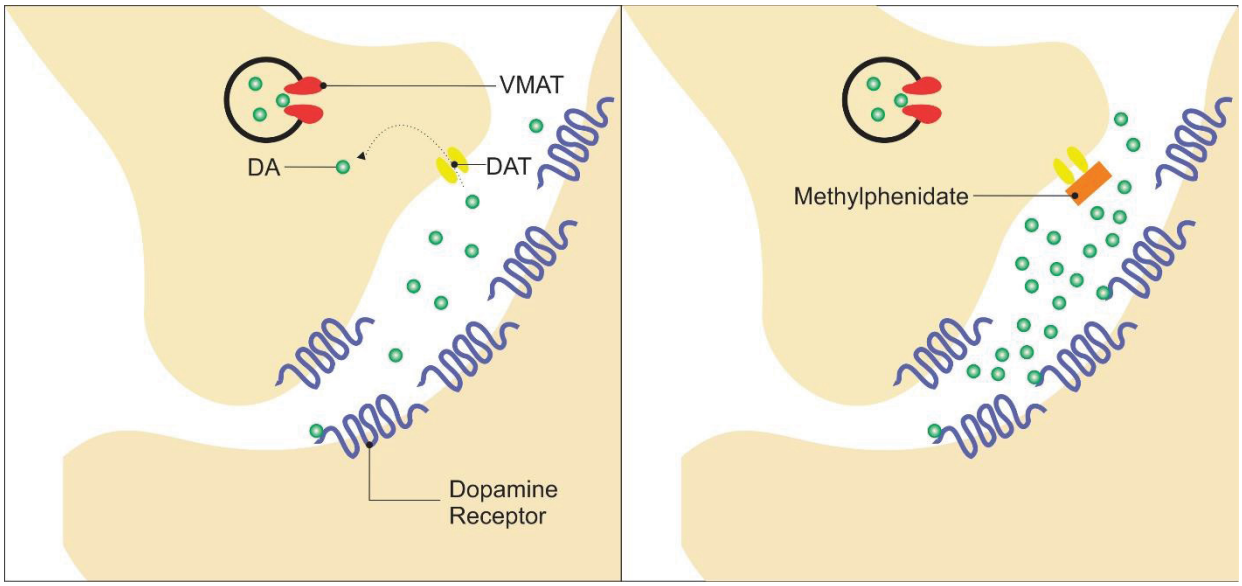

Figure 2. Mechanism of action of methylphenidate. This figure was prepared by MSc. William Sanchez Luna.

1.2. OFF-LABEL METHYLPHENIDATE USE IN BRAZIL

Methylphenidate is available in Brazil since 1998, and its consumption shows a trend toward increase. Data from ANVISA indicate that consumption went from 156.62 kg in 2009 to 413.38 kg in 2011 (Anvisa, 2012). Off-label use may partially explain this phenomenon (off-label refers to the use of prescription drugs by healthy people who do not have a medical prescription).

Off-label methylphenidate use among young adults is a marked phenomenon in countries such as the United States, where it went from 8% in 2003 to 12% in 2013 (Mccabe *et al.*, 2014 & Boyd, 2014). In Brazil, this phenomenon seems to have momentum as well. Multiple studies show that the prevalence of off-label use of methylphenidate among college students goes from 5.30% to 60% (see Table 1). However, to the best of our knowledge, no studies have assessed this phenomenon in the general population of young adults or the whole country. Thus, these percentages are likely lower if we consider the general population, as college students are at higher risk to engage in off-label methylphenidate use (Herman-Stahl *et al.*, 2007). In addition, we cannot estimate how this trend of off-label methylphenidate use has changed over time in Brazil.

The main reason reported by students to engage in off-label methylphenidate use is to improve their academic performance (Lage *et al.*, 2015). However, only two studies assessed if methylphenidate consumption correlates with better performance. Both studies were carried out in the United States, and neither could find a positive correlation between off-label methylphenidate use and academic performance (Arria e Wish, 2006; Munro *et al.*, 2017). We could not find studies addressing the relation of academic performance and off-label methylphenidate use in Brazil. Thus, the relationship between off-label use of methylphenidate and academic performance is not clear yet.

Table1. Studies addressing the prevalence of off-label use of methylphenidate in college students in Brazil

Author, Year	**City/State**	**Population**	**Sample**	**% off-label methylphenidate use**
Carneiro *et al.,* 2012	City: Volta Redonda / State: Rio de Janeiro	Students enrolled in the undergraduate program of Medicine	160 participants 45% women and 55% men	23.72%
Pasquini, 2013	City: several cities / State: São Paulo	Students enrolled in 30 universities of the state	5128 participants	44.1%
Mota and Pessanha, 2014	City: Campos dos Goytacazes / State: Río de Janeiro	Students enrolled in undergraduate programs of medicine and pharmacy	150 participants 65% women and 35% men Between 18 - 30 years old	60%
Coli *et al*, 2016	City: not specified / State: Minas Gerais	Students enrolled in the undergraduate program of Medicine	120 participants 58.33% women and 41.67% men Between 16-30 years old	25%
Cordeiro and Pinto, 2017	City: Ponta Grossa / State: Paraná	Students from universities in the city, pursuing academic degrees in health sciences: physical education, nursery, phonoaudiology, pharmacy, physiotherapy, nutrition, odontology and radiology	793 participants 71% women and 29% men Between 18-25 years old	5.3%

1.3. EFFECTS OF METHYLPHENIDATE ON COGNITION

Most of the studies that evaluate the effects of methylphenidate focus on laboratory tests that measure a specific cognitive domain. Here we report studies in two domains that are of particular interest for this study: episodic and working memory.

1.3.1. EFFECTS OF METHYLPHENIDATE ON EPISODIC MEMORY

Episodic memory is the ability to acquire, consolidate, store and retrieve information related to episodes (Tulving, 2002; Moscovitch *et al.*, 2016). It contains specific information about the time and place of the acquisition and can be understood as the mental replay of the situation. Typical laboratory tests that measure episodic memory are testing memory for narratives, and word recognition and recall such as the Rey's Auditory Verbal Learning Test (Moradi *et al.*, 2017).

Methylphenidate seems to enhance episodic memory. A meta-analysis that assessed 42 single administration studies found a positive effect of methylphenidate (Repantis *et al.*, 2010). Likewise, a review concluded that single administration methylphenidate and amphetamine benefit memory and the longer the delay between the training and the test, the stronger the benefit (Smith e Farah, 2011). More recently, another meta-analysis found a small but significant positive effect in short- and long- term episodic memory, although the authors suggest a possibility of publication bias (Ilieva *et al.*, 2015)

1.3.2. EFFECTS OF METHYLPHENIDATE ON WORKING MEMORY

Working memory is the capability to temporarily store and manipulate information to use in the current task (Baddeley, 2003; 2010; 2012). Several tests measure working memory; the oldest and most common is the Sternberg test (Sternberg, 1966). In this task, participants are exposed to a list called memory set. After a few seconds, the participant is exposed to an item and needs to identify if the item belongs to the memory set or not. To resolve this task, the participant needs to maintain the information of the set in the working memory.

Methylphenidate has controversial effects in working memory. A review found mixed results, with some studies that found improvement and some did not find effects (Smith e Farah, 2011). The authors argue that this may be due to individual differences: for example, amphetamine administration does not have any effect on people with an allele that decreases the enzyme that metabolizes dopamine and norepinephrine, while it has a beneficial effect in people with most active form of the enzyme (Mattay *et al.*, 2003). Likewise, a most recent review found an improvement in working memory with doses between 10 and 20 mg of methylphenidate, but not

with doses above or below this range (Linssen *et al.*, 2014). Thus, methylphenidate may improve working memory depending on the dose and individual characteristics of the person taking it.

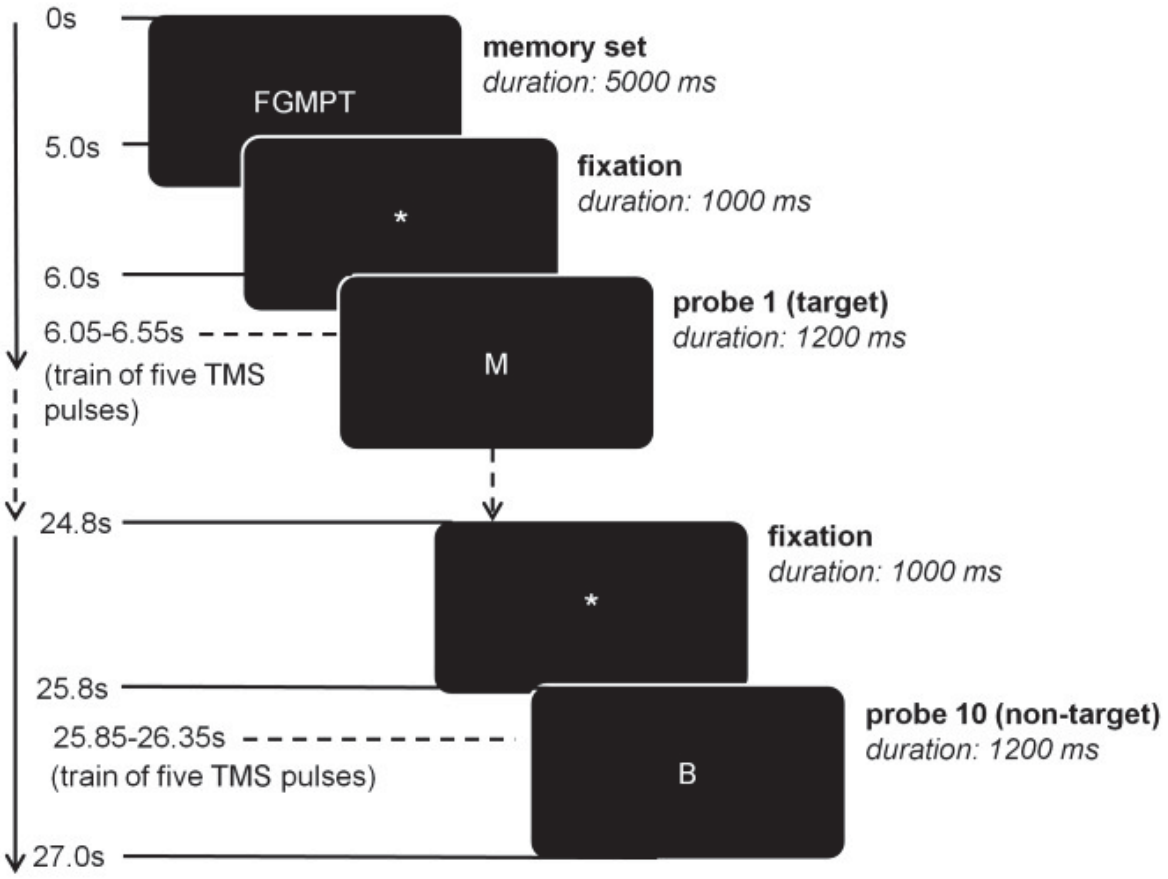

Figure 3. Example of temporal sequence of the Sternberg Task. Source: Jansma, (2013)

1.3.3. USE OF RODENTS IN COGNITIVE AND BEHAVIORAL STUDIES

Studies in laboratory settings in humans are useful assessing the effects of drugs on cognition and behavior, but they have limitations such as a high heterogeneity among participants, and lack of control of several variables that may interfere in the results, and a difficulty to make chronic treatments. These limitations can be surpassed with the use of animal models in the behavioral and pharmacological studies. In particular, mice and rats are suitable subjects, commonly used in the study of behavior and cognition; and one of the cognitive functions most studied in rodents is spatial memory.

Spatial memory is the ability to acquire, consolidate, store and retrieve information regarding locations and routes; and to use this information to navigate successfully in the environment (Vorhees e Williams, 2014). It is widely studied in rodents, because is one of high cognitive functions that they display, and because it shares neural mechanisms with episodic memory in humans. There are several behavioural tests used to assess spatial memory in rodents. In this study, we used the eight-arm radial arm maze. This test was developed by Olton in 1976 and measures spatial memory (Olton e Samuelson, 1976). The radial arm maze consists of a central octagon from which eight runaways or arms irradiate (Figure 4), at the distal end of each arm there is a receptacle where the animal can find a food reward. The maze is surrounded by distal visual cues that remain stable through the experiment, and that the animal can use to navigate. Other common test used to evaluate spatial memory is the water maze, which is a circular pool with a hidden platform; animals must learn the location of the platform based on visual cues that surround the maze (Vorhees e Williams, 2014).

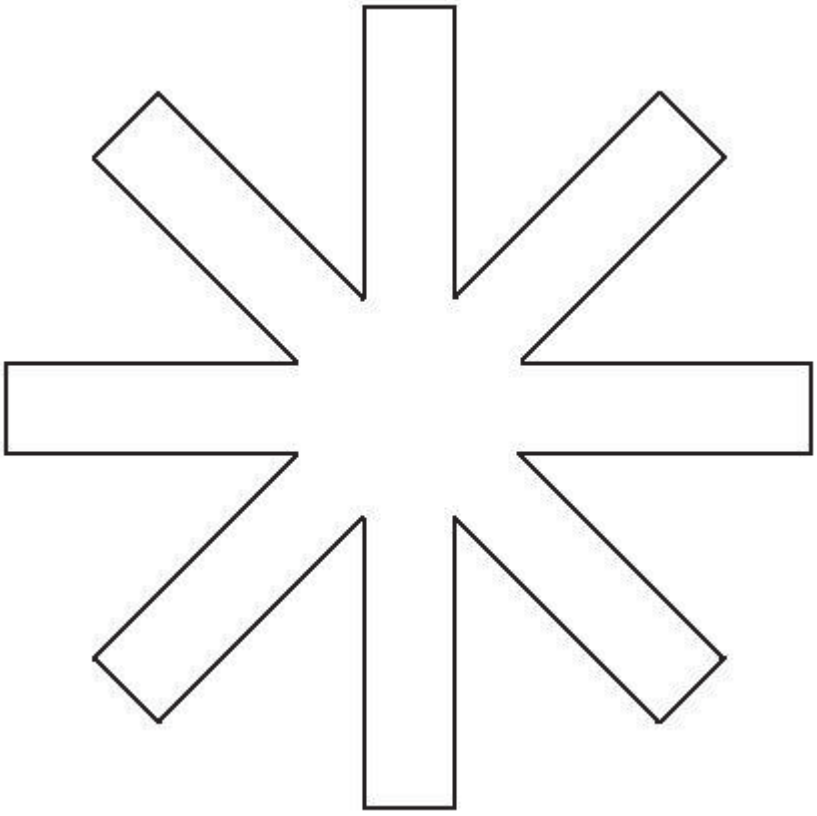

Figure 4. Schematic representation of an eight-arm radial arm maze.

1.3.4. EFFECTS OF METHYLPHENIDATE ON RODENTS SPATIAL MEMORY

Few studies have assessed the effects of methylphenidate on spatial memory and have yielded controversial results. Veetil (2011) found an improvement in the radial arm maze with 3mg/kg of methylphenidate administered i.p. every day before training, for four days. Sontag (2011) did not have any effect of 2.5 mg/kg of methylphenidate on working and reference memory, administered for five days, every day before behavioural testing, to animals that were well trained. In the same study, Sontag found that 10 mg/kg impaired memory. Haleem (2015) found that methylphenidate improved memory consolidation at doses from 0.25-1 mg/kg administered every day after water maze training during one week; after a gap of a week without training, the animals that received the methylphenidate remembered the task better than the controls. Sloan (2016) found no effects of 0.75 and 1.5 mg/kg of methylphenidate in the performance of the radial arm maze. The animals were well trained on the task, and the drug was administered 15 minutes before testing. Hence, it seems that methylphenidate will yield different effects on memory depending on the dose, the moment of the administration and whether the animals are well trained or not.

One issue that has not been explored is how the effects of methylphenidate on motivation influence performance in cognitive tasks; to answer this question is the aim of this study. To this aim, we treated separate groups of rats with four different doses of methylphenidate (0, 0.3, 1 or 3 mg/kg) and trained them in an eight-arm radial arm maze that had three reinforced arms that remained stable across the training. Each reinforced arm had a different amount of reward, one arm contained 1 sucrose pellet, other arm contained 3, and the last arm contained 6 sucrose pellets. We trained a separate group of animals in the same task, but with the same amount of reward in the three arms, to isolate the effects on memory from the effects on motivation.

We found that animals that received chronic treatment with 3 mg/kg of methylphenidate displayed a higher number of re-entries in the high rewarded arms (the ones that contained 3 or 6 sucrose pellets). This increase did not occur in animals trained with the same amount of reward in all arms; hence, we propose that this effect is the result of increased motivation to search for high rewards, rather than impairment in working memory. In addition, we assessed both the acute and chronic effects of methylphenidate on anxiety and locomotion, to rule out that the observed

effects were mediated by emotional or motor processes. This assessment did not reveal any effect of methylphenidate in anxiety or locomotion. We conclude that the effects of methylphenidate are complex, and presented in several domains such as cognition and motivation.

2. AIM

To investigate what are the effects of methylphenidate on memory and motivation, and how each influences performance in cognitive tasks.

2.1. OBJECTIVES

To evaluate the effects of acute methylphenidate (0, 0.3, 1 and 3 mg/kg; i.p.) in the scores for working and reference spatial memory in the radial arm maze, in well-trained rats.

To evaluate the effects of chronic methylphenidate (0, 0.3, 1 and 3 mg/kg; i.p.) in the scores for working and reference spatial memory in the radial arm maze, in rats.

To evaluate the effects of chronic and acute methylphenidate in the scores for anxiety in the elevated plus maze, in rats.

To evaluate the effects of chronic and acute methylphenidate in the scores for locomotor behaviour and anxiety in the open field, in rats.

To evaluate the effects of acute methylphenidate in sucrose consumption.

3. METHODS

3.1. ANIMALS

Two hundred and eight adult male Wistar rats from our breeding stock were used in these experiments. The animals were maintained in standard conditions, in a room with controlled temperature (22±2ºC), on a 12h/12h light/dark cycle, with the light period beginning at 7:00 am. The rats used in the radial arm maze experiments were kept under food restriction to maintain 90% of their free-feeding weight (300–350 g). The rats used in the open field and elevated plus-maze experiments had food ad libitum. Water was available ad libitum for all rats. The food, the water intake, and the weight of the animals were monitored daily. All experimental procedures were approved by the Federal University of Parana Ethical Committee for Animal Care (protocol 932) and complied with Brazilian (11.794/8 October 2008) and International Laws (EC Council Directive, 24 November 1986; 86/609/EEC). Efforts were made to minimise the rats' discomfort.

3.2. DRUGS

Methylphenidate (Ritalin, Novartis Pharma) was dissolved in saline (0.9% NaCl). The doses of methylphenidate (0.3 mg/kg, 1.0 mg/kg and 3.0 mg/kg) were chosen based on the allometric calculation (Nevill, 1994) for therapeutic doses for humans corresponding to 10 – 60 mg for adults of 70 kg (0.14-0.85 mg/kg). Each rat received an intraperitoneal (i.p.) administration of one of the three doses, or saline, 30 min before the behavioural tests. In the acute experiment the drug was injected only on the testing day; in the chronic experiments, the drug was injected once a day during the whole duration of the experiments (20 days).

3.3. BEHAVIOURAL PROCEDURES

The radial maze task. The radial maze consisted of 8 62x14cm arms with 2cm barriers, and a central octagon arena (36 cm diameter) elevated 66 cm above the floor, all painted in black. A circular receptacle (1 cm diameter, 0.1 cm depth) was located at the distal end of each arm. The maze remained in the same location in all sessions. The walls of the room were covered with a white curtain. Several coloured geometric figures were attached in the wall to serve as distal cues. The behaviour of the rats in the maze was videotaped by an overhead camera (Stingray) operating at

60 fps and digitised by the CinePlex® behavioural research system (Plexon Inc., Texas). Before training, rats underwent behavioural shaping. On Day 1 they were allowed to explore the whole maze for 5 minutes. On Day 2, 5 mg sucrose pellets were distributed along each arm of the maze (total of 40 pellets), and the rats were allowed to explore the maze for 10 minutes. This procedure was repeated daily until each rat had consumed at least 36 pellets in a session. In the next two days the procedure was repeated with three pellets per arm and, and in the next day the exploration time was reduced to 5 min and each arm was baited with only one pellet located in the receptacle. Only the animals that did not consume all pellets were further used and underwent daily training sessions with four trials per session. On each trial, three arms were baited, and this arms remained stable across the experiment. The baited arms were counterbalanced among rats. For each rat, the baited arms were the same, and there was at least one non-baited arm between two baited arms. In each trial the rat was released in one of the non-baited arms, alternated among trials in a counterbalanced manner, and allowed to explore the maze until all pellets were consumed or until 5 minutes had elapsed. The rats remained in a holding cage in the 1-min interval among trials.

The elevated plus maze test. Independent groups of rats were evaluated in this test as a control to evaluate whether the effect of methylphenidate on the radial maze was a consequence of the effect of this drug on anxiety. The elevated plus-maze consisted of two open arms, (50x10cm) and two closed arms (50x10cm and 50 cm height walls) elevated 50 cm above the floor. The closed arms were perpendicular to the open arms, and the all arms give access to a 10x10cm maze. The rats were allowed to explore the maze freely for 5 minutes. Rats were videotaped by an overhead camera operating at 60 fps, and their trajectories of the animals were digitalised by the ANY-maze® Behavioural tracking system (Stoelting Co, IL). The number of entries and time spent in the open and closed arms were scored. Anxiety index was calculated as 1 – [(time in open arms/total time[300 seconds])+(entries in open arms/total entries)]/2; this index had values that vary from 0 to 1; higher values indicate higher anxiety (Huynh, Krigbaum, Hanna, & Conrad).

The open field test. The open field apparatus consisted of a circular arena of 100 cm diameter surrounded by a 45 cm wall. The floor and the walls were painted in white. Rats were videotaped from above for 5 minutes. The same video-camera and

tracking system described above were used. The travelled distance was used as a score of locomotor behaviour. The time spent in the periphery vs the time in the central zone (20 cm far from the walls) were used as scores of anxiety (Prut & Belzung, 2003).

3.4. EXPERIMENTAL SCHEDULES

Experiment 1. Acute effects of methylphenidate on the radial maze scores. During the training and test sessions, three arms of the radial maze were baited with 1, 3, or 6 sucrose pellets. All the animals were trained for 30 days or until they entered three times or less in non-baited arms during the entire session (75% of correct choices) for three consecutive days. In the last day, thirty minutes before the last session, the rats received vehicle or 0.3, 1.0, or 3 mg/kg methylphenidate (i.p.).

Experiment 2. Chronic effects of methylphenidate on the radial maze scores. The number of pellets in the baited arms and drug treatment was the same as in Experiment 1. However, i.p. injections were given 30 min before each training for 20 days. The trajectory of the animals within the mazes was videotaped by an overhead camera operating at 60 fps and digitalised by the ANY-maze® Behavioural tracking system (Stoelting Co, IL). From the video recordings of the test, it was extracted: the number of visits and revisits to the baited and non-baited arms, and the time spent in the central part of the maze, and in the distal part of each baited arm (20x14cm around the reward receptacle).

Experiment 3. Chronic effects of methylphenidate on the radial maze scores. Three arms were baited with one sucrose pellet each. Rats were given 20 daily sessions of training. Thirty minutes before each training session rats received saline or 3 mg/Kg of methylphenidate i.p.

Experiment 4. Acute and chronic effects of methylphenidate on the open field test. Independent groups of rats received the same drug treatment of Experiment 2 and were tested in the open field on days 1 and 20. Drugs were given 30 min before each test.

Experiment 5. Acute and chronic effects of methylphenidate on the EPM test. Another independent group of rats received the same drug treatment of

Experiment 2 and were tested in the elevated plus-maze on days 1 and 20. Drugs were given 30 min before each test.

Experiment 6. Acute effects of methylphenidate on sucrose intake. Food-deprived rats were habituated to sucrose pellets, a week before the behavioural testing. On the test day, rats received i.p. injections of vehicle or 3 mg/kg of methylphenidate. Thirty minutes after administration they were put in a cage identical to their home cage to have access to 20 g of 5 mg sucrose pellets. The animals were allowed to stay in this cage for 15 minutes, and then they returned to the home cage. The amount of food eaten during these 15 minutes was measured. This procedure repeated for two days.

3.5. STATISTICAL ANALYSIS

Experiment 1. Four parameters were analyzed from the data collected in experiment 1: a) the entries in non-reinforced arms from the first to the penultimate day (day 1 – day 34), to determine if all the groups learned the task and if all of them had a similar performance; b) the entries in non-reinforced arms on the last day, to evaluate the effects of acute methylphenidate on the expression of reference memory; c) the reentries in the reinforced arm, to evaluate the effects of acute methylphenidate on working memory; and d) the reentries on each separate reinforced arm, to assess the effects of methylphenidate on motivation.

The first parameter, entries in the non-reinforced arms from the first ot the penultimate day, was analyzed using a two-way repeated measures ANOVA, with time and treatment as the two factors. The second and the third parameters, the entries in the non-reinforced arms and the reentries in the reinforced arms, were analyzed with a one-way ANOVA, with treatment as a factor. Finally, the fourth parameter, the reentries on each separate arm, was analyzed with a two-way ANOVA, with treatment and reward amount as factors.

Experiment 2. From the data collected in experiment 2 were analyzed the entries in the non-reinforced arms, the reentries to all reinforced arms and to each arm separate, the time spent on each region of the maze (on each reward zone and on the center), the total entries, and the distance travelled. All of them were analyzed for the entire experiment (20 days), and all of them were analyzed with two-way

repeated measures ANOVA, with the time and treatment as factors; except the reentries on each separate reinforced arm, which was analyzed with a three-way repeated measures ANOVA, with time, treatment and reward amount as factors. In addition, the time spent on the center of the maze was correlated with the entries in the non-reinforced arms and with the reentries to all reinforced arms. These correlations were made for each treatment separately.

Experiment 3. For the experiment were also analyzed the entries in the non-reinforced arms and the reentries in all reinforced arms, during the entire experiment. Both parameters were analyzed with a two-way repeated measures ANOVA, using time and treatment as factors.

Experiments 4 and 5. Three parameters were analyzed in experiment 4: the time in the closed arms, the percentage of entries in the open arms, and the index anxiety as explained above. Regarding experiment 5, the parameters analyzed were the distance travelled and the percentage of time spent in the center of the maze. Both analysis were performed for day 1 and day 20 of the treatment, to assess both the acute and chronic effects of methylphenidate. In all cases the data was analyzed using one-way ANOVA, with treatment as factor.

Experiment 6. One parameter was assessed in experiment 6, the amount of sucrose pellets eaten. This parameter was measured both days that lasted the experiment and the data was analyzed with a two-way repeated measures ANOVA, with day and treatment as factors.

4. RESULTS

Experiments 1, 2, and 3, assessed the effects of methylphenidate on a radial arm maze paradigm. The aims were to assess: a) reference memory by comparing the number of entries to non-rewarded arms among groups, and b) working memory by comparing the number of re-entries to rewarded arms. Experiment 2 also assessed the effects of methylphenidate in motivation, comparing the preference of the animal for different amounts of reward.

Experiments 4, 5 and 6 assessed the effects of methylphenidate on the open field and the elevated plus maze, and on sucrose consumption. These experiments were performed to rule out any effect on anxiety, locomotion and appetite that could blunt the effects on memory.

4.1. EFFECTS OF ACUTE ADMINISTRATION OF METHYLPHENIDATE ON RADIAL ARM MAZE PERFORMANCE.

Experiment 1. In this experiment assessed the effects of acute methylphenidate on memory. As expected, all groups decreased the entries to the non-reinforced arm in a significant and progressive manner, and their performance showed no significant differences (Figure 5A; Table 3). Thirty minutes before the last session, rats received an injection of vehicle or one of the three doses of methylphenidate. The number of entries to non-reinforced arms in the last day did not differ among groups (Figure 5B; Table 2). The number of re-entries in all reinforced arms in the last day also did not differ among groups (Figure 5C; Table 2); however, when we separated the re-entries per arm, we found that animals from the vehicle group entered more in the high-rewarded arm than in the low reward arm (Figure 5D; Table 3). Thus, acute methylphenidate did not affect working memory or the evocation of reference spatial memory.

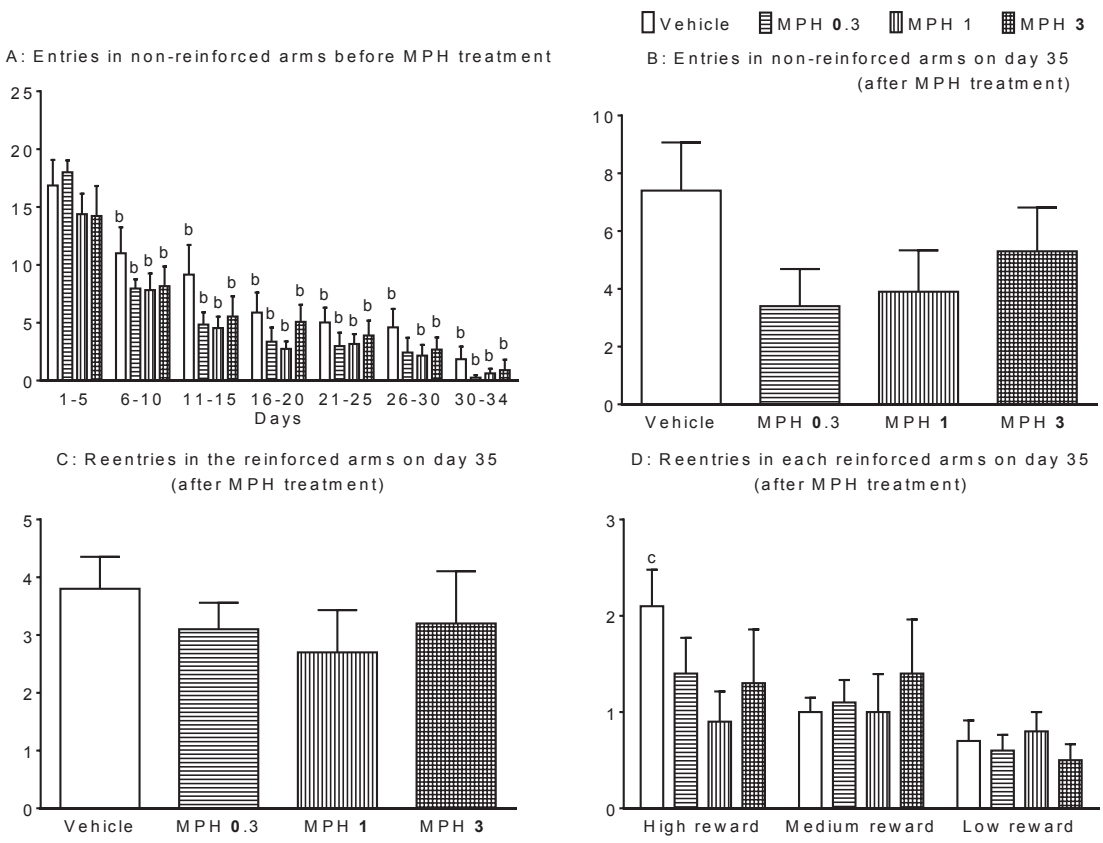

Figure 5. The acute treatment with methylphenidate did not altered the performance in the radial arm maze. Three out of the eight arms were baited with 1, 3, or 6 pellets. Bars express mean ± SEM. A: Entries in non-reinforced arms in 5-days bins. B: Entries in non-rewarded arms the next day after the last day in A . bin. C: Reentries in all rewarded arms. D: Reentries in each separated rewarded arm. MPH 0.3, 1 and 3: methylphenidate 0.3, 1 and 3 mg/kg, respectively. Each group N = 10. Data are expressed as mean ± SEM. b: $p < 0.05$ compared to the 1st bin of the same group, Tukey post hoc test after ANOVA.

Table 2. Effects of methylphenidate on reference and working memory, and on anxiety and locomotor measures analyzed with one-way ANOVA statistics

	One-way ANOVA	
	F	p
Entries in non reinforced arms last day (Exp. 1)	1.46	0.24
Reentries in all reinforced arm last day (Exp. 1)	0.44	0.72
Distance travelled in the OF (acute)	0.09 (3, 37)	0.96
Distance travelled in the OF (chronic)	1.54 (3, 37)	0.21
% of time in the center of the OF (acute)	0.92 (3, 37)	0.43
% of time in the center of the OF (chronic)	0.36 (3, 37)	0.78
% of time in the closed arms of the EPM (acute)	3.71 (3, 42)	**0.01**
% of time in the closed arms of the EPM (chronic)	1.22 (3, 42)	0.31
% of entries in the closed arms of the EPM (acute)	0.03 (3, 42)	0.98
% of entries in the closed arms of the EPM (chronic)	0,43 (3, 42)	0.72
Anxiety index (acute)	1.27 (3, 42)	0.29
Anxiety index (chronic)	1.82 (3, 42)	0.15
Total number of entries in arms EPM (acute)	0.07 (3, 42)	0.97
Total number of entries in arms EPM (chronic)	1.16 (3, 42)	0.2

MPH 0.3, 1 or 3 methylphenidate 0.3, 1, 3 mg/kg. For open field measures: Vehicle N=10, MPH 0.3 N=10, MPH 1 N=10, MPH 3 N=11. For elevated plus maze measures: Vehicle N=11, MPH 0.3 N=11, MPH 1 N=12, MPH 3 N=12.

Table 3. Effects of methylphenidate on reference and working memory, and sucrose intake analyzed with two-way ANOVA statistics

	Factor 1		Factor 2		Interaction	
	F	p	F	p	F	p
Reentries in each arm in the last day (Exp. 1) Factor 1=Treatment, Factor 2=Reward	5.31 (2,108)	**<0.01**	0.59(3,108)	0.61	1.01 (6,108)	0.42
Entries in non reinforced arms (Exp. 2) Factor 1=Treatment, Factor 2=Time	0.59 (3,36)	0.63	96.14(3,108)	**<0.0002**	3.53 (9,108)	**<0.002**
Reentries in reinforced arms (Exp. 2)Factor 1=Treatment, Factor 2=Time	3.19 (3,36)	**<0.05**	17.22 (3,108)	**<0.0001**	4.48 (9,108)	**<0.0001**
Time spent in the central compartment (Exp. 2) Factor 1=Treatment, Factor 2=Time	8.10 (3,36)	**<0.05**	21.30 (3,108)	**<0.0001**	1.90 (9,108)	0.059
Distance travelled in the RAM (Exp. 2)Factor 1=Treatment, Factor 2=Time	3.28 (3, 36)	0.04	13.36 (3, 108)	<0.0002	6.51 (9, 108)	<0.0002
Entries in non reinforced arms (Exp. 3) Factor 1=Treatment, Factor 2=Time	1.72 (1,19)	0.20	66.76 (3,57)	**<0.0001**	3.08 (3,57)	**<0.05**
Reentries in reinforced arms (Exp. 3) Factor 1=Treatment, Factor 2=Time	0.19 (1,19)	0.65	15.61 (3,57)	**<0.0001**	1.09 (3,57)	0.36
Sucrose intake test (Exp. 6)Factor 1=Treatment, Factor 2=Time	26.99 (1, 18)	<0.0001	29.25 (1, 18)	<0.0001	7.75 (1, 18)	0.01

MPH 0.3, 1 or 3 methylphenidate 0.3, 1, 3 mg/kg.

4.2. EFFECTS OF CHRONIC ADMINISTRATION OF METHYLPHENIDATE ON RADIAL ARM MAZE PERFORMANCE

Experiment 2. In this experiment, three arms of the radial arm maze were rewarded, and each arm contained a different amount of reward; this arrangement allowed us to evaluate reference and spatial memory, and at the same time to evaluate motivation by comparing the number of visits the animals did to each rewarded arm.

All groups decreased the entries to the non-reinforced arm in a significant and progressive manner (Figure 6A). In the first five days, rats treated with 1 and 3 mg/kg of methylphenidate entered less in the non-reinforced arms than rats treated with

vehicle (Figure 6A; Table 3). Chronic methylphenidate did not have any further effect in reference spatial memory.

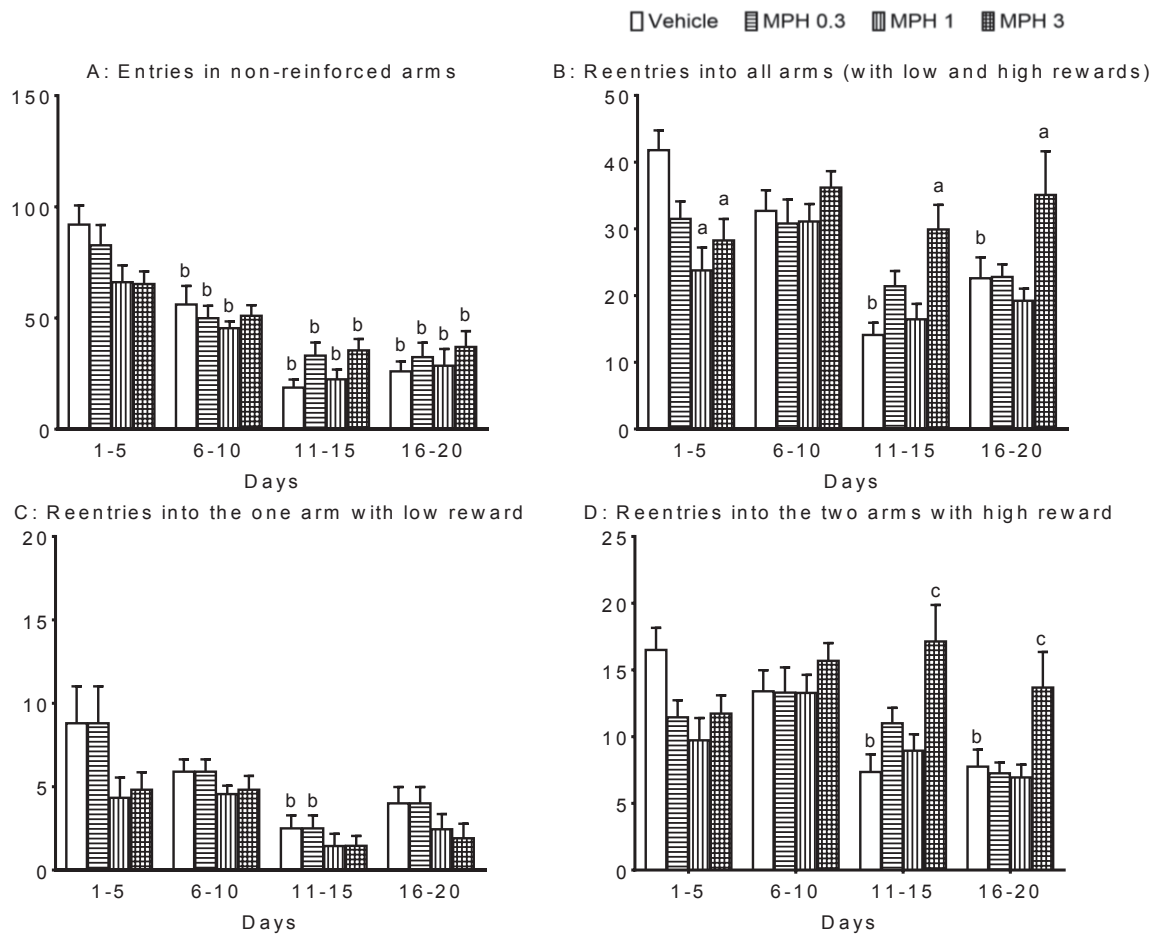

Figure 6. Effects of the chronic treatment with methylphenidate on the rats performance of the radial arm maze. Three out of the eight arms were baited with 1, 3, or 6 pellets. Bars express mean ± SEM. A: Entries in non-reinforced arms in 5-days bins. B: Reentries in all reinforced arms. C: Reentries in arm baited with 1 sucrose pellet. D: Reentries in arms baited with 3 and 6 sucrose pellets. MPH 0.3, 1 and 3: methylphenidate 0.3, 1 and 3 mg/kg, respectively. Vehicle N = 10, MPH 0.3 N = 10, MPH 1 N = 9, and MPH 3 N = 11 Data are expressed as mean ± SEM. b: $p < 0.05$ compared to the 1st bin of the same group, Tukey post hoc test after ANOVA.

Next, we evaluated the effects of the chronic administration of methylphenidate over working memory. Animals treated with vehicle decreased the re-entries in the reinforced arm in a significant a progressive manner; this decrease did not occur for animals treated with any dose of methylphenidate (Figure 6B; Table

3). In the first five days, animals treated with 1 and 3 mg/kg of methylphenidate had a lower number of re-entries in the reinforced arms than animals treated with vehicle. From day 11 to 20, animals treated with 3 mg/kg had a higher number of re-entries in the reinforced arms than animals treated with vehicle.

To know if the increased re-entries to the reinforced arms correspond to both reinforced arms or just to the high-rewarded ones, we compared the number of re-entries to the 6-pellets arms with 3-pellets arms and found no differences between them (Figure 7; Table 4). Hence, for further comparisons, we decided to merge the data from the 6 and 3 pellets arms by averaging the entries to each and consider it a single category. Here upon we will refer to the merged data of the 6 and 3 pellets arms as high-reward arm and as low-reward, the arm baited with 1 pellet. From day 11 to 20, animals treated with 3 mg/kg of methylphenidate showed more re-entries in high-reward arms than in low-reward arms; none of the animals treated with the other doses of methylphenidate or with vehicle showed this difference (Figure 6C and 6D; Table 4).

Table 4. Effects of methylphenidate on the reentries to arms with high vs low reward analyzed with three-way ANOVA statistics

	Reentries in reinforced arms (Experiment 2)	
	F(DFn,DFd)	p
Treatment (Vehicle, MPH 0.3, MPH 1 or MPH 3)	1.84 (3,288)	0.314
Reward Amount (high/medium, low)	94.45 (1,288)	**<0.000**
Interaction Treatment * Reward	3.46 (3,288)	**<0.02**
Time	25.21 (3,288)	**<0.000**
Interaction Time * Treatment	4.55 (9,288)	**<0.000**
Interaction Time * Reward	5.73 (3,288)	**0.001**
Interaction Time * Treatment * Reward	2,36 (9,288)	**0.014**

MPH 0.3, 1 or 3 methylphenidate 0.3, 1, 3 mg/kg.

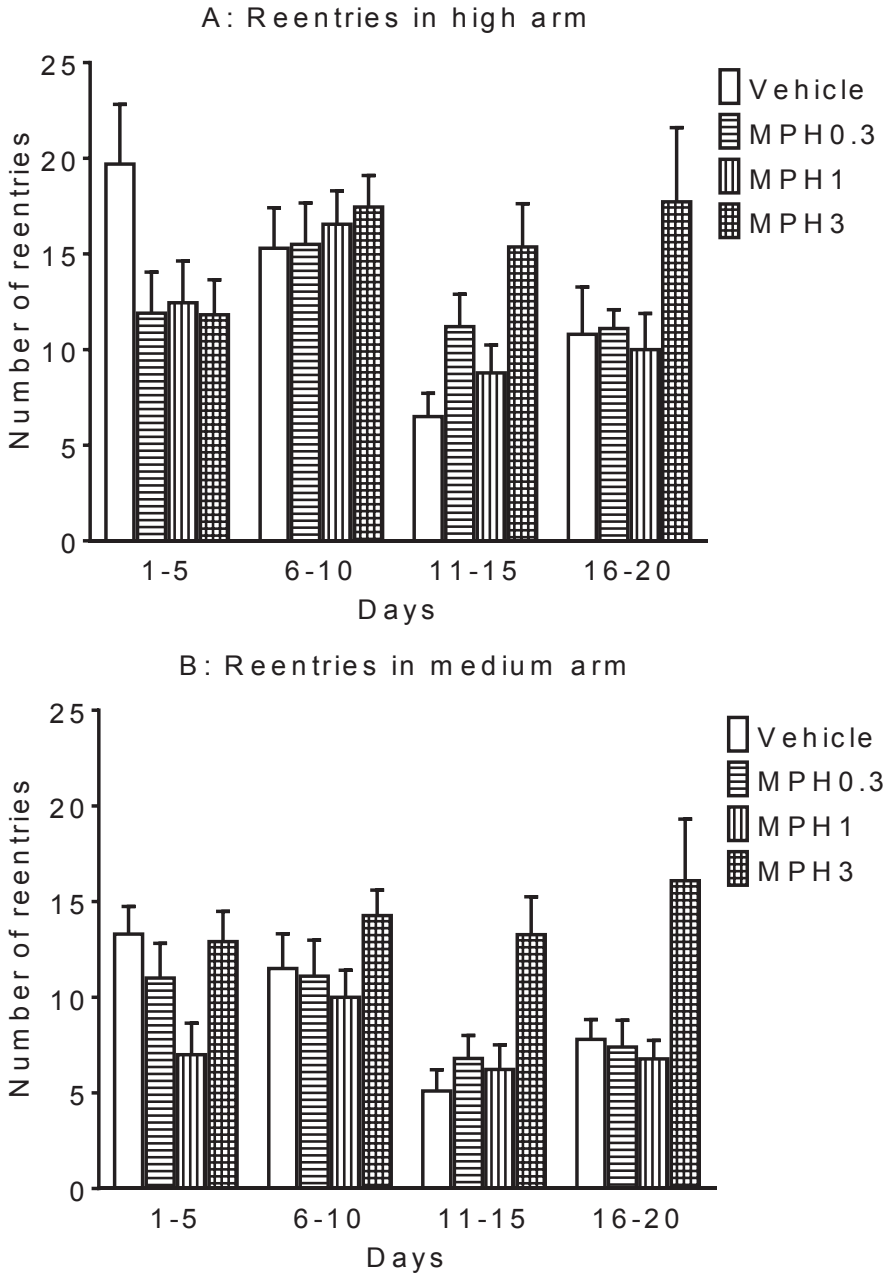

Figure 7. Effects of chronic treatment with methylphenidate in reentries to each separate arm. No differences found between reentries in high and medium arm for any group. All data are expressed as mean ± SEM.

The analyses above show a reduction in entries to the non-reinforced arms and the number of re-entries to the reinforced arms for animals treated with 1 and 3 mg/kg. Since it happened for both parameters, we decided to investigate if this reduction was related to a decreased exploratory activity rather than an enhanced

memory. To answer this, we analysed the number of total entries, the total distance travelled, and the time the animals spent on each zone of the maze. Regarding the total number of entries, during the first five days, animals treated with 1 and 3 mg/kg of methylphenidate had a lower number of total entries compared with animals treated with vehicle (Figure 8). In addition, from days 11 to 15, animals treated with 3 mg/kg had a higher number of total entries compared with animals treated with vehicle. Regarding the distance travelled, animals treated with any of the three doses of methylphenidate travelled a shorter distance during the first five days, compared with animals treated with vehicle; and animals treated with 3 mg/kg travelled a longer distance than animals treated with the vehicle from days 11 to 15 (Figure 9; Table 3).

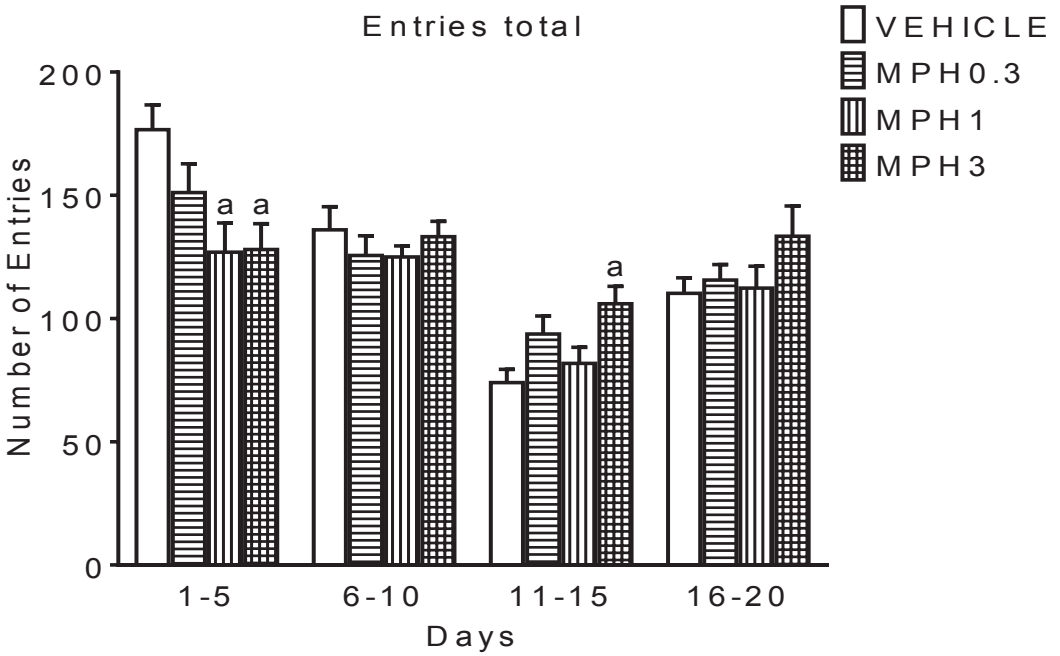

Figure 8. Effects of chronic treatment with methylphenidate in exploratory behaviour in the radial arm maze. Animals treated with 1 and 3 mg/kg of methylphenidate entered fewer times, considering all arms, during the 1st block, compared to animals treated with vehicle. Animals treated with 3 mg/kg entered more times in all of the arms in the 3rd block, compared to vehicle. All data are expressed as mean ± SEM. a: $p < 0.05$ compared to vehicle, Tukey post hoc test after two-way ANOVA.

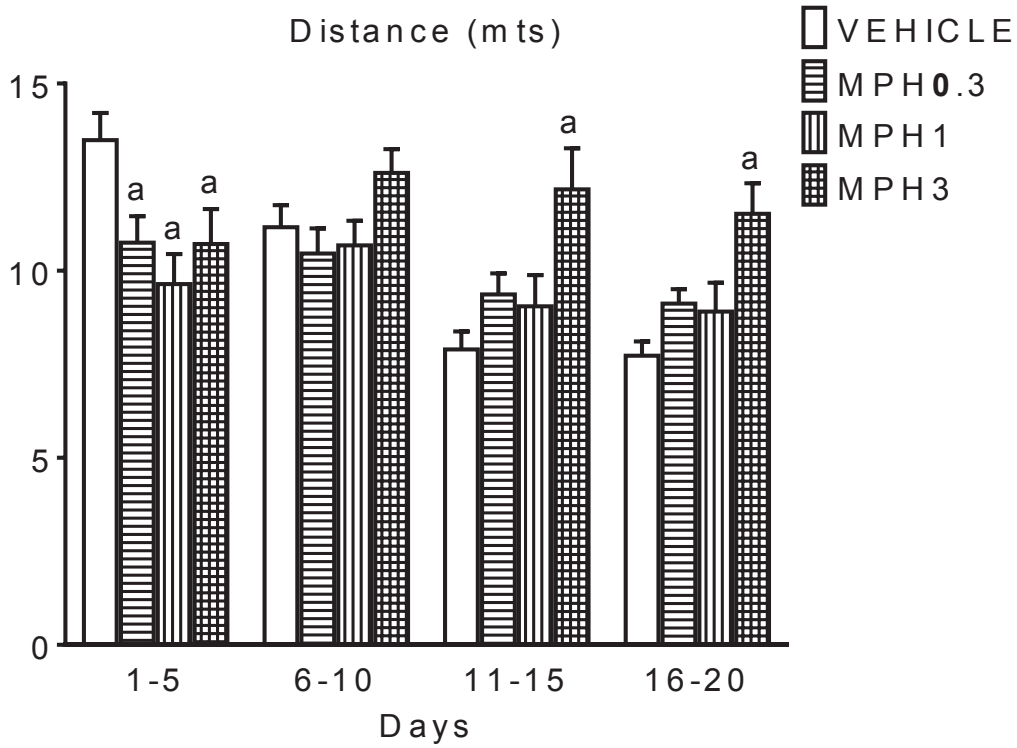

Figure 9. Distance travelled in the radial arm maze in the experiment with different amount of reward on each arm. MPH 0.3, 1 and 3: methylphenidate 0.3, 1 and 3 mg/kg, respectively. Data are expressed as mean ± SEM. Bars represent mean ± SEM of the total distance travelled. a: $p < 0.05$ compared to the vehicle group, Tukey post hoc test after two-way ANOVA.

Next, we evaluated the exploratory activity of the rats by analysing the time spent on each zone of the maze. Animals treated with 0.3 and 1 mg/kg of methylphenidate spent less time in the high reward area (the distal end of the arm in the area near the reward receptacle) in the 3rd and 4th bins compared with the 1st bin; in addition, animals treated with 0.3 mg/kg of methylphenidate spent more time in the high reward arm compared with low reward (Figure 10).

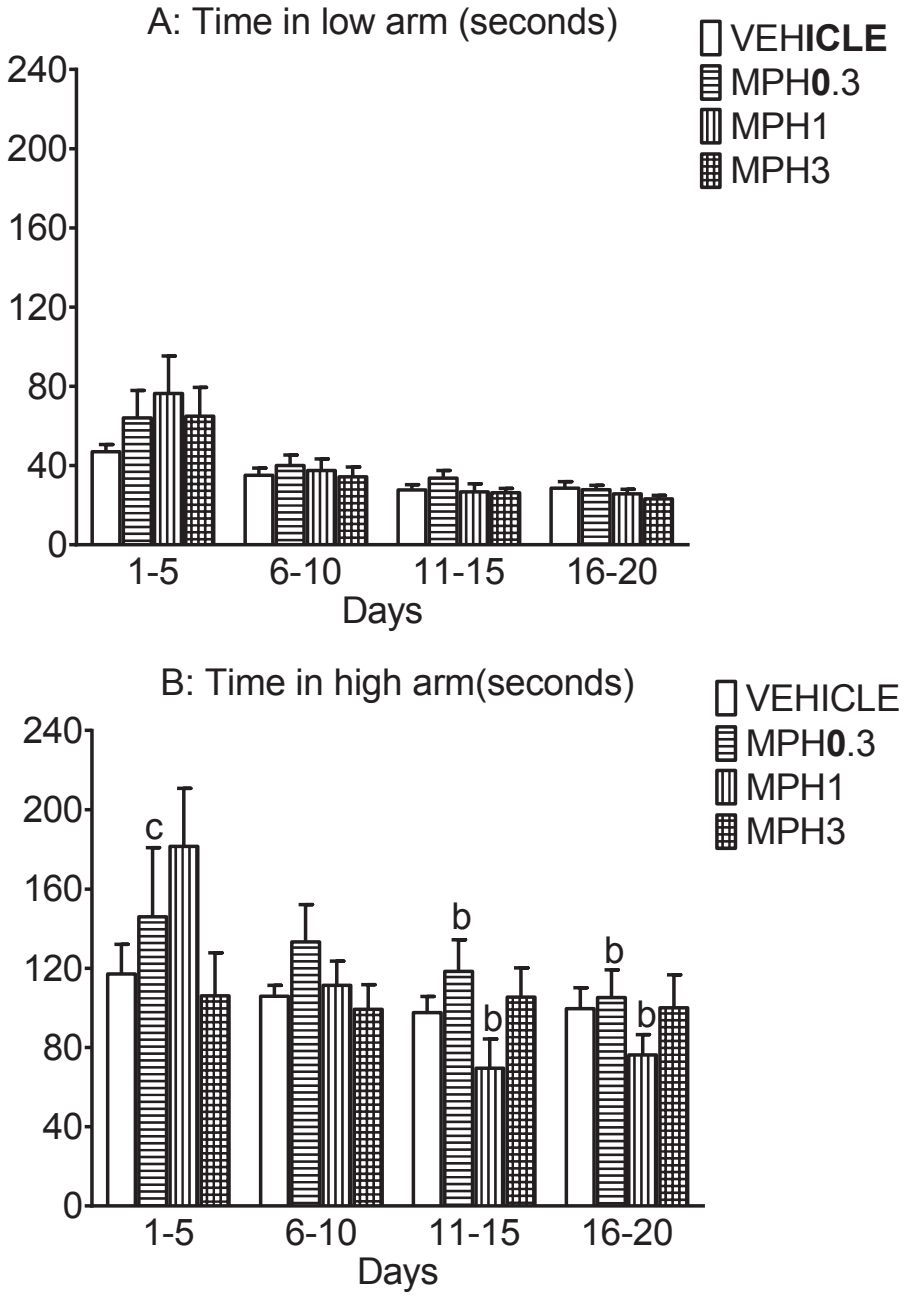

Figure 10. Effects of chronic methylphenidate in the time the animals spent on each reward area. A: No differences were found between groups or across time in the time the animals spent in the low reward arm. B: No differences found between groups in the time the animals spent in the high reward arm. Animals treated with 0.3 and 1 mg/kg of methylphenidate spent less time in the high reward arms during blocks 3 and 4 than in block 1. Finally, animals treated with 0.3 mg/kg spent more time in the high reward arm than in the low reward, during block 1. All data are expressed as mean ± SEM. b: $p < 0.05$ compared to 1st block, Tukey post hoc test after two-way ANOVA. c: $p < 0.05$ compared to low arm, Tukey post hoc test after two-way ANOVA

Likewise, the time the animals spent in the central compartment of the maze was also analysed (Figure 11). Rats treated with 1 and 3 mg/kg of methylphenidate

spent more time in the central compartment of the radial maze than those treated with the vehicle, during the 1st bin (Figure 11A; Table 2). Overall, rats treated with vehicle spent 11 ± 9 s (mean ± SD) in the central compartment. Thus, we separated the trials within each group according to their duration: more than 20 seconds spent in the central area or 20 seconds or less. A Chi-square test revealed that there was a significant increase in the number of trials on which animals spent more than 20 s in the central compartment in the groups treated with 1 and 3 mg/kg of methylphenidate (Figure 11B).

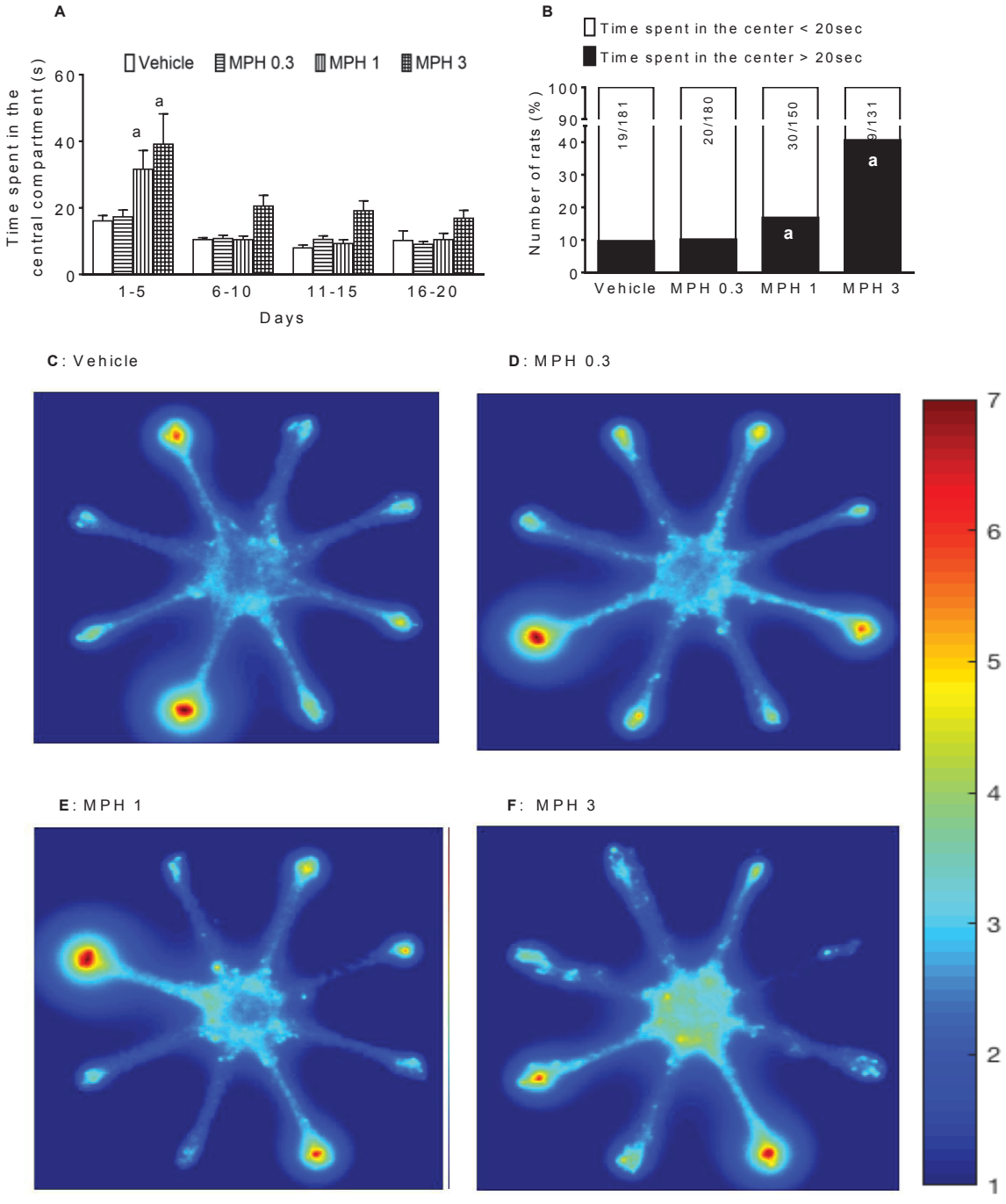

Figure 11. Effect of methylphenidate in the time rats spent in the central compartment of the radial maze. The arms were baited with 1, 3 or 6 sucrose pellets. A: time spent in the central compartment of the maze, arms were baited with 6 pellets (high reward), 3 or 1 pellets (low reward). Data are expressed as mean ± SEM. $p < 0.05$ compared to the vehicle group, Tukey post hoc test after two-way ANOVA. B: black represents the percentage of trials in which the time spent more than 20 s in the central zone; white represents the percentage of trials in which the time spent in the central zone was equal or lower than 20 s. The mean ± SD time vehicle rats spent in the central area was 11 ± 9 s; a: $p < 0.05$ compared to the vehicle group, after Chi-square analysis. C-F: Representative heat maps of exploratory behaviour in

the radial arm maze. L, arms baited with low reward (1 pellet); H, arms baited with high reward (3 or 6 pellets). A warmer colour indicates a higher time spent in the area.

In addition, we represented the exploratory activity of every animal during the whole experiment in colour maps. This representation is a qualitative assessment of the exploratory activity, which helped us visualise on which regions of the maze the animals spent more time. A warmer colour indicates a higher level of exploratory activity in a determined zone of the maze. Illustrative examples of animals treated with vehicle, 0.3, 1 and 3 mg/kg of methylphenidate are in figures 11C, D, E and F. In this examples we can confirm that the animals treated with methylphenidate spent more time in the central compartment of the maze than the animals treated with vehicle. Colour maps of the entire group of animals treated with vehicle, 0.3, 1 and 3 mg/kg of methylphenidate are in appendixes A, B, C and D.

Next, we investigated how the amount of time the animal spent in the central compartment was related to their performance on the task. For each group, we made a correlation between the time spent at the central compartment of the maze and the number of entries in the non-reinforced arms. We made two separate correlations, one considering the trials with 20 seconds or less on the central compartment of the maze, and another one considering the trials with more than 20 seconds con the central compartment of the maze. Using the Pearson test, we confirmed that there is a positive correlation between the time spent in the central compartment of the maze and the number of re-entries in reinforced arms; this is true for all the trials on which the animals spent 20 seconds or less in the central compartment of the maze. When the trials on which the animals spent more than 20 s are considered, this correlation disappears for animals treated with vehicle and 0.3 mg/kg of methylphenidate and is the opposite for animals treated with 1 and 3 mg/kg. The Pearson coefficients are stated in Figure 12 A-H.

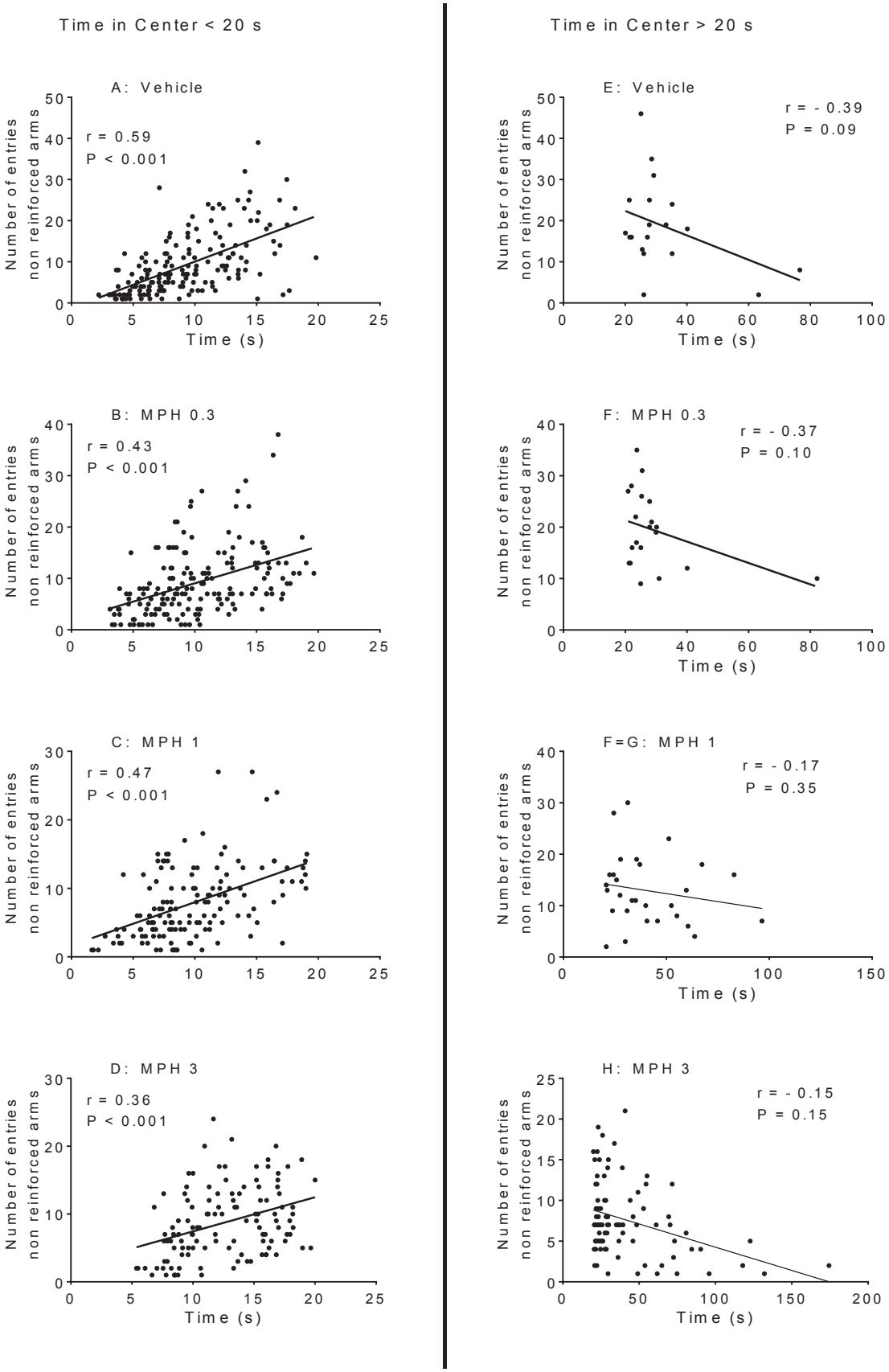

Figure 12. Correlation between the time spent in the central compartment before entering a non-reinforced arm and the number of entries in the non-reinforced arms. A-E: Only data of the trials in which rats spent less than 20 s in the central

compartment were included. F-G: Only data of the trials in which rats spent more than 20 s in the central compartment were included. * P < 0.05 Pearson's test.

The same correlation between the time spent in the central platform and the re-entries in the reinforced arms was done for the entries in the non-reinforced arms. As in the previous analysis, this correlation was done for each treatment, and separating the trials on which the animals spent 20 seconds or less from the trials that the animals spent more than 20 seconds. Pearson correlation also found a positive correlation between the time the animals spent in the central platform and the number of entries in the non-reinforced arms for all the treatments considering the trials on which the animals spent 20 seconds or less. No correlation was found between the time the animals spent in the central platform and the number of entries in the non-reinforced arms when the animals spent more than 20 seconds. The Pearson coefficients are stated in Figure 13 A-H.

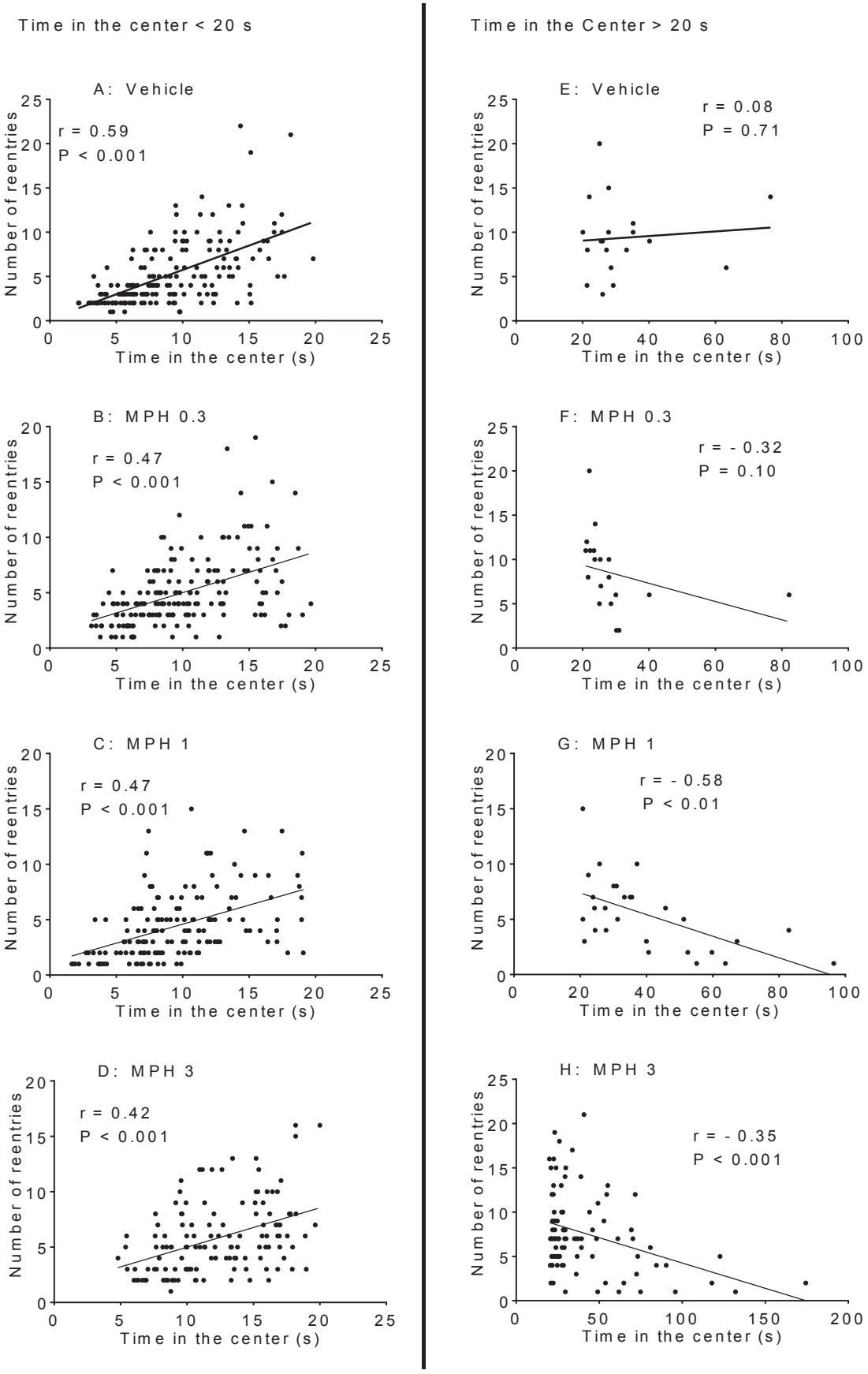

Figure 13. Correlation between the time spent in the central compartment before reentering a reinforced arm and the number of reentries in the reinforced arms. A-E:

Only data of the trials in which rats spent less than 20 s in the central compartment were included. F-G: Only data of the trials in which rats spent more than 20 s in the central compartment were included. * P < 0.05 Pearson's test.

Experiment 3. The increase in re-entries to rewarded arms had two possible explanations: a) it could be the result of an impairment in working memory or b) it could be the result of an increase in motivation to search for high rewards. To resolve this point we performed the experiment 3. This experiment was similar to experiment 2, but the three reinforced arms had the same amount of reward. Rats of both groups decreased the entries into the non-reinforced arm in a significant and progressive manner(Figure 14A). In addition, during the first five days, rats treated with 3 mg/kg of methylphenidate entered fewer times in the non-reinforced arms than the rats treated with saline. No further effects of methylphenidate were found.

Regarding re-entries in all reinforced arms, both groups decreased the re-entries in a progressive and significative manner. Rats treated with 3 mg/kg of methylphenidate did not have any difference from animals treated with vehicle (Figure 14B; Table 2).

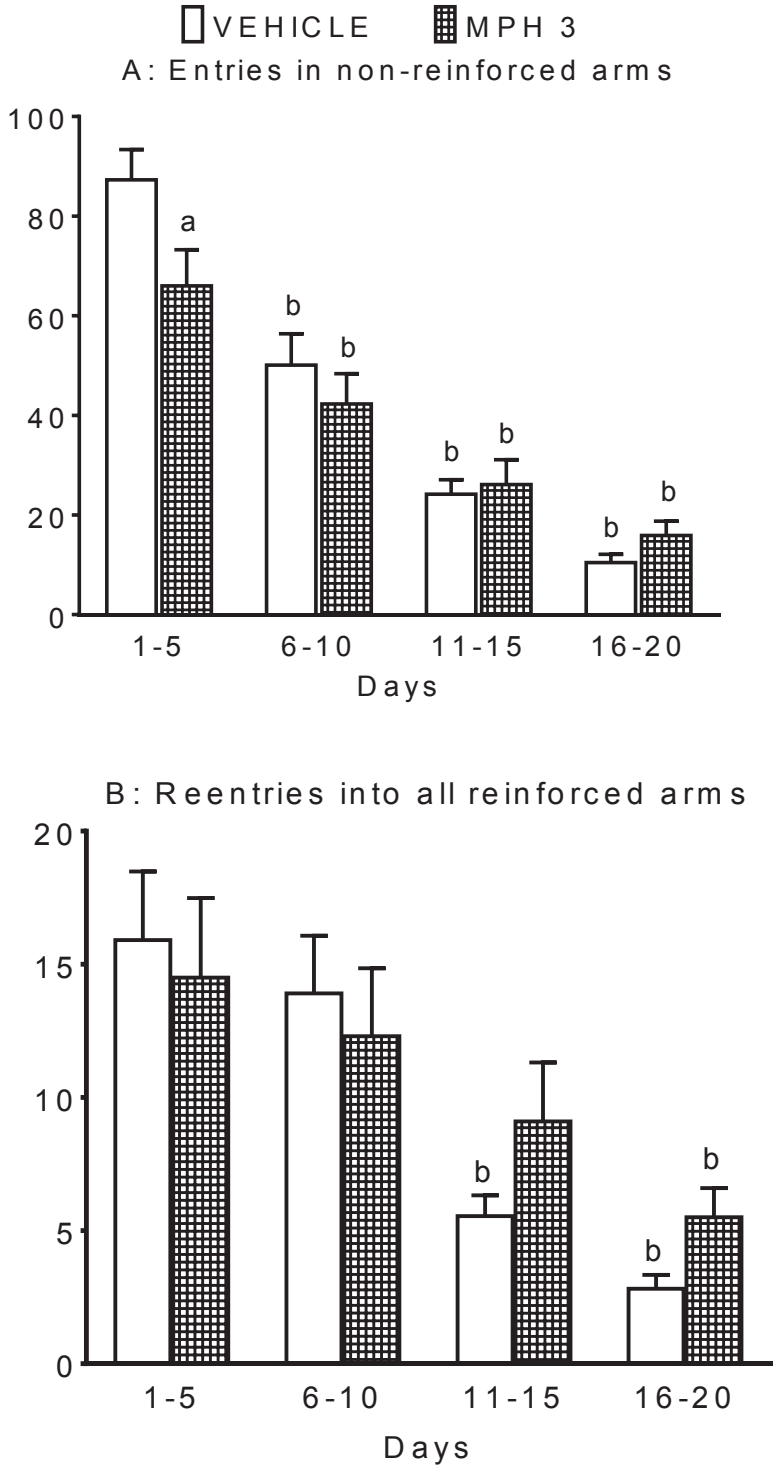

Figure 14. Effects of the chronic treatment with methylphenidate on the rats performance of the radial arm maze. Three out of the eight arms were baited with 1pellet, each. Bars express mean ± SEM. A: Entries in non-reinforced arms in 5-days bins. B: Reentries in all reinforced arms. MPH 3: methylphenidate 3 mg/kg. Vehicle N = 11, MPH 3 N = 10. Data are expressed as mean ± SEM. b: $p < 0.05$ compared to the 1st bin of the same group, Tukey post hoc test after ANOVA.

4.3. EFFECTS OF METHYLPHENIDATE IN THE ELEVATED PLUS MAZE AND THE OPEN FIELD

Finally, we studied if methylphenidate, in the acute or chronic doses, had effects in locomotion or anxiety measures. For open field measures methylphenidate did not have any effect on the locomotion measured as travelled distance or anxiety, using the percentage of time spent in the central compartment of the open field as an inverse measure of anxiety (Table 2 and 5). The same was true for the chronic treatment with methylphenidate for both locomotion and anxiety (Table 2 and 5).

In the elevated plus maze, animals treated with methylphenidate did not show any difference in the percentage of entries in the closed arms, nor in the anxiety index, compared with animals treated with vehicle (Table 2 and 5). However, a difference in the percentage of time spent in the open arms was revealed between the animals treated with 0.3 and 1 mg/kg (Table 2 and 5). Finally, chronic methylphenidate did not have any effect in any of the scores of the elevated plus maze.

Table 5. Effects of methylphenidate in open field and elevated plus maze scores

	Vehicle	MPH 0.3	MPH 1	MPH 3
	Mean ± SEM	Mean ± SEM	Mean ± SEM	Mean ± SEM
Distance travelled in the OF (acute)	27,34 ± 1,76	27,1 ± 2,59	25,77 ± 2,29	26,62 ± 2,34
Distance travelled in the OF (chronic)	17,1 ± 2,98	18,37 ± 2,90	23,31 ± 3,80	25,75 ± 2,71
% of time in the center of the OF (acute)	5,10 ± 0,83	5,89 ± 1,06	6,78 ± 1,63	7,63 ± 0,93
% of time in the center of the OF (chronic)	7,32 ± 3,42	7,86 ± 3,01	7,46 ± 1,80	11,5 ± 4,46
% of time in the closed arms of the EPM (acute)	58,07 ± 3,29	59,92 ± 2,36	46,9 ± 2,92	53,79 ± 3,29
% of time in the closed arms of the EPM (chronic)	72,95 ± 6,86	66,21 ± 2,71	59,65 ± 5,87	58,52 ± 7,23
% of entries in the closed arms of the EPM (acute)	48.54 ± 2.27	47.9 ± 3.16	47.3 ± 2.62	48.28 ± 2.91
% of entries in the closed arms of the EPM (chronic)	57,13 ± 4,26	56,26 ± 4,24	46,9 ± 3,08	52,06 ± 4,95
Anxiety index (acute)	0,59 ± 0,02	0,60 ± 0,02	0,54 ± 0,02	0,58 ± 0,02
Anxiety index (chronic)	0,72 ± 0,03	0,69 ± 0,03	0,62 ± 0,03	0,62 ± 0,04
Total number of entries in arms EPM (acute)	29,73 ± 1,82	28,55 ± 1,32	28,83 ± 2,02	29,58 ± 2,72
Total number of entries in arms EPM (chronic)	22,55 ± 4,90	35,45 ± 3,13	34,5 ± 4,12	32 ± 5,56

Data are shown as mean ± SEM. Bonferroni's test after two-way ANOVA. *MPH 0.3, 1 or 3* methylphenidate 0.3, 1, 3 mg/kg.

4.4. EFFECTS OF METHYLPHENIDATE ON SUCROSE PELLETS INTAKE

Methylphenidate reduced the consumption of sucrose pellets. Animals treated with 3 mg/kg of methylphenidate consumed less sucrose on both days compared with the vehicle group; in addition, animals in the vehicle group eat more sucrose in day 2 compared with day 1, while animals treated with 3 mg/kg of methylphenidate did not increase the amount of sucrose eaten (Figure 15; Table 3).

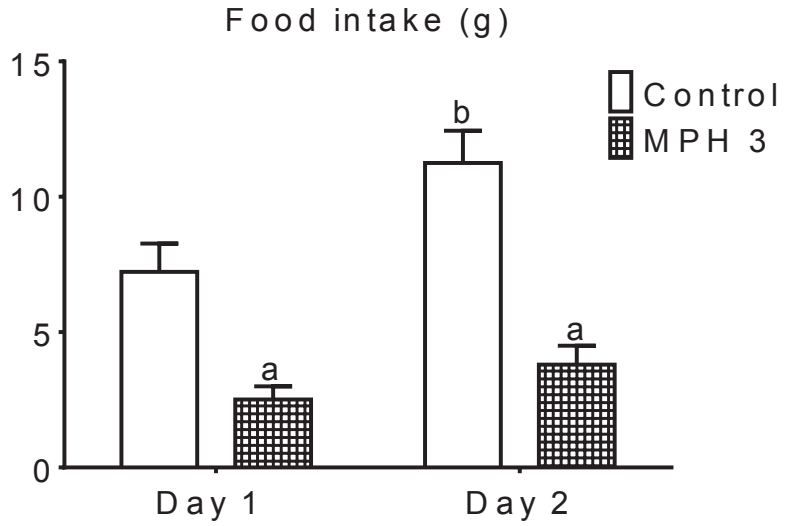

Figure 15. Effect of methylphenidate on food intake. Animals were exposed to 20 grams of sucrose pellets and allowed to eat for 15 minutes. MPH 3: methylphenidate 3 mg/Kg. Both groups had a N = 10. All data are expressed as mean ± SEM. a: $p < 0.05$ compared to the vehicle group. b: $p < 0.05$ compared to the same group on Day 1. Tukey post-hoc test after two-way ANOVA

5. DISCUSSION

This study aimed to evaluate the cognitive and motivational effects of acute and chronic treatments with methylphenidate and whether the effect on one domain impacts the effect of the other domain.

The acute methylphenidate treatment did not affect the number of reference memory errors (entries in the non-reinforced arms) and the number of working memory errors (re-entries into a reinforced where the sucrose pellet has been previously eaten).

Chronic methylphenidate, early on training (days 1-5) reduced the number of entries in non-reinforced arms and the number of re-entries in the reinforced arms. Such effects would be taken as suggestive of spatial reference and working memory effects. However, the reduction in the total number of entries and the decrease in the travelled distance suggest that these results are more likely a consequence of a decrease in locomotor activity - which reduces the number of entries into both the reinforced and non-reinforced arms. The decrease in the number of visited arm per session may also be caused by a long time to choose in which arm to enter since methylphenidate increased the time spent in the central arena.

Later on training (days 11-20), chronic methylphenidate increased the number of re-entries into the reinforced arms. Although this finding could be interpreted as an impairment in working memory induced by methylphenidate, this effect was observed only for the arms baited with a higher number of pellets. Also, this effect did not appear in the group of rats that were trained with the same amount of reward in all the arms. Therefore, the increased re-entries to the arms with high rewards are more likely a consequence of an effect of methylphenidate on motivation than on spatial working memory *per se.*

5.1. EFFECTS OF METHYLPHENIDATE ON MEMORY

Several studies in humans assessed the effects of acute methylphenidate in declarative memory (Smith & Farah, 2011) and found a positive effect, although the size of this effect varies with the task and dose (Smith & Farah, 2011). The authors argue that this difference in the size of the effect may be due to individual differences

so that the ideal dose would be different for each person (Smith & Farah, 2011). In rodents, the one study of acute effects of methylphenidate showed improvement in fear conditioning (Carmack, Howeell, et al., 2014). While all the mentioned human studies and the rodent one tested the effects of methylphenidate on memory acquisition or consolidation, we tested the effects of acute methylphenidate in the expression of an already established memory. Sloan et al. (Sloan *et al.*) found similar results: intraperitoneal administration of 0.75 and 1.5 mg/kg of methylphenidate did not have any effect on the performance on the radial arm maze, in a memory well established. Therefore, the human and rodent studies suggest that methylphenidate improves memory acquisition and consolidation, but not the expression of well-established memories.

Regarding the chronic effects of methylphenidate on memory, previous findings are controversial. Sontag et al. (2011) reported that administration of 10, but not 2.5 mg/ kg methylphenidate for five days before training impaired learning of a spatial reference memory task. On the other hand, Haleem et al. (2015) reported that oral 0.25-1 mg/kg methylphenidate administered immediately after each training session for one week of training improved memory consolidation, and after a gap of one week without any treatment or training, methylphenidate administered during 4 weeks of retraining improved retention and memory persistence.

Contrary to the absence of effects reported by Sontag et al. (2011), Veetil and Kurian (2011) reported an improvement in memory acquisition and retention in the radial arm maze task triggered by 3 mg/kg methylphenidate administered i.p. to rats 30 minutes every day before training, for four days. One possible explanation for this discrepancy is the difference in the task designs used. In the tasks used by Sontag and in the current study, animals received methylphenidate before the test, and all the behavioural training was carried out with the animals under the effect of the drug. In the task used by Veetil and Kurian, animals received the drug before the first trial, and the five remaining trials happened with an interval of one hour. As the half-life of the drug in rodents is 1 hour, only one of the six trials was performed under the peak concentration of the drug. Thus the increased improvement found by Veetil and Kurian occurred mostly during the residual effects of methylphenidate. It is likely that molecular events related to memory consolidation were triggered by the first trial and

methylphenidate peak concentration coincided with a critical time window of these processes, enhancing the outcome.

The interpretation above is compatible with the observation made by Haleem et al. (2015), which suggest that a small dose of methylphenidate does not have an enhancing effect on memory when learning occurs under the effect of the drug; however, it may have a beneficial effect when taken after learning, probably strengthening memory consolidation. On the other hand, high doses of methylphenidate seem to have detrimental effects on memory.

Which mechanism could explain this apparent enhancement of consolidation? The answer is the effects of dopamine in long term potentiation.

After the acquisition of a new memory, there is a process that strengthens this memory known as consolidation (Dudai *et al.*, 2015). According to a current and well-accepted hypothesis, at the cellular level, the mechanism of memory consolidation is long-term potentiation (Takeuchi *et al.*, 2014; Dudai *et al.*, 2015). The process of long-term potentiation (LTP) was described first by Bliss and Lomo in 1973 (Bliss e Lomo, 1973). In their experiment, Bliss and Lomo demonstrate how the efficiency of synaptic transmission -the ability of a presynaptic neuron to activate a postsynaptic neuron- increased after repetitive electrical stimulation (Bliss e Lomo, 1973).

Extensive evidence indicates a role for dopamine in LTP and memory consolidation. Dopamine antagonists -particularly D1 receptor antagonists- (Frey *et al.*, 1990; O'carroll e Morris, 2004; Saddoris *et al.*, 2015) and dopamine depletion (Yang *et al.*, 2002) prevent the formation of LTP. Likewise, the administration of Dopamine D1 receptor antagonist, just before learning, impairs memory formation (O'carroll *et al.*, 2006; Bethus *et al.*, 2010). Thus, dopamine D1 receptors likely mediate the improvement in memory consolidation reported after methylphenidate use. Future studies should address the question whether dopamine d1 antagonists prevent the beneficial effect of methylphenidate.

5.2. EFFECTS OF METHYLPHENIDATE ON EXPLORATORY ACTIVITY

Animals that received the two higher doses of methylphenidate reduced their exploratory activity, and this effect was likely not a consequence of increased anxiety or reduced locomotion (we did not observe any of those in the elevated plus maze or in the open field, both tasks designed to assess anxiety and locomotion). Two factors may be contributing to this decrease in exploratory activity: an effect on motivation for eating the sucrose pellets, and an effect on the decision-making time.

Animals treated with 3 mg/kg of methylphenidate ate fewer pellets when exposed to 20 g sucrose pellets for a limited interval of time. This observation complies with previous studies (Alam & Najam, 2015; Elfers & Roth, 2011; Thanos et al., 2015), which also showed a reduction in food intake after chronic methylphenidate treatment. This evidence is compatible with our observation that the reduction in exploratory activity only happened during the first five days. Thus, it is possible that the reduction in locomotor activity observed in the first five days of treatment reflects a reduction in appetite.

The other factor that may explain the reduction in exploratory activity is an effect in decision making: rats treated with the higher doses of methylphenidate might take longer to decide which arm to enter. Methylphenidate increased the number of trials on which animals spend more than 20 seconds in the central arena of the radial maze (this breaking point was selected because the mean + SD of the time the control group spent in the central arena was 11 + 9 seconds). In these trials, we found a negative correlation between the time spent in the central compartment and the number of re-entries in the reinforced arms, only for the animals treated with 3 mg/kg. Thus, rats treated with 3mg/kg of methylphenidate made a better choice (fewer re-entries) but took longer to decide which arm to enter next. The time rats spent deciding might reduce the time they spent exploring, which may explain the reduced exploratory activity.

This finding is in agreement with human studies by Franke et al. (2017) that tested the effects of methylphenidate on the performance of highly experienced chess players. They found that participants under the effect of methylphenidate took longer to complete chess moves, but they also made better moves. The authors argue that players took a long time to decide their actions, which led to an enhanced quality of their game. Moreover, another study in humans assessing the effects of

modafinil –a stimulant with a similar clinical profile to methylphenidate- showed that participants under the effect of modafinil had a better performance than controls in a decision-making task and a spatial planning task, but also had a slowed latency to respond(Turner *et al.*, 2003). Thus, both methylphenidate and modafinil seem to affect decision-making by increasing accuracy but decreasing speed. These effects may be explained by actions in prefrontal cortex areas (Goldstein e Volkow, 2011; Moeller *et al.*, 2014)

Methylphenidate seems to alter decision-making, by increasing the latency to make a decision, but enhancing accuracy. We propose this interpretation based on the negative correlation between the time in the central platform and the number of re-entries in the reinforced arms in the animals treated with 3 mg/kg of methylphenidate. Previous studies in humans showed similar results with methylphenidate and modafinil: people under the effect of methylphenidate and modafinil make more accurate decisions, but take longer to decide (Turner *et al.*, 2003; Franke *et al.*, 2017). In the most recent of these studies, Franke et al. (2017) suggest that this improvement may be attributed to an action of methylphenidate in prefrontal cortex.

The prefrontal cortex is the region located in the most anterior part of the brain and it is linked to cognitive functions such as planning, problem-solving, and decision-making –grouped under the umbrella term “executive functions”- (Yuan e Raz, 2014). There is extensive evidence showing the involvement of dopaminergic modulation in the prefrontal cortex during cognitive tasks (Wass *et al.*, 2013; Puig, Antzoulatos*, et al.*, 2014; Puig, Rose*, et al.*, 2014). At the cellular level, dopamine increases the signal-to-noise ratio in the prefrontal (Kroener *et al.*, 2009); during a cognitive task, this increase in signal-to-noise ratio could mean an enhancement of the neuronal activity devoted to the task, and a decrease of the background activity (Kroener *et al.*).

Thus, Franke et al. (2017) proposed that methylphenidate improved the accuracy in decision-making by an increase in dopamine levels in the prefrontal. Indeed, previous studied demonstrated that methylphenidate normalized prefrontal activity in cocaine users (Goldstein *et al.*, 2010) and optimized prefrontal activity during a cognitive task (Moeller *et al.*, 2014). However, Franke et al. (2017) do not

provide an explanation for the increase in the latency to make a decision; further studies should clarify this question.

Another issue to consider regarding the potential benefits of methylphenidate in decision-making is that the actions of dopamine in cognitive control follow a U inverted shape (Cools e D'esposito, 2011; Htun *et al.*, 2014). This means that there is an optimum level of dopamine in the prefrontal that would be beneficial for cognitive performance, while levels above or below would have detrimental effects. Furthermore, some features like the task demands and the baseline performance modulate the relationship between dopamine levels and performance. For example, a dopaminergic agonist improved working memory in participants with poor baseline performance, and the same drug impaired working memory in participants with high baseline performance (Kimberg *et al.*, 1997). Thus, even though the results here suggest a beneficial effect of methylphenidate in decision-making, this may change for each individual depending on the task that they are executing and on their baseline performance.

5.3. EFFECT ON METHYLPHENIDATE IN MOTIVATION TO SEARCH FOR HIGH REWARDS

One possible explanation for the increase in re-entries is that methylphenidate increased the motivation to search for high rewards. Methylphenidate increases extracellular levels of dopamine in the striatum (Volkow et al., 2002) and higher levels of extracellular dopamine in this system are known to increase motivation to work for rewards (Cagniard, Balsam, Brunner, & Zhuang, 2006). Hence, the increase in the re-entries to high rewarded arms may reflect an increase in motivation to search for high rewards, likely mediated by dopamine.

Animals treated with the highest dose of methylphenidate showed an increase in the re-entries to the high rewarded arms. We propose that this is not an effect in working memory –which would be the traditional interpretation- because this increase in the number of re-entries did not occur in the low reinforced arms, or in a separate group of animals that was trained with the same amount of reward. We propose that this increase in the re-entries in the high reward arm may reflect that the animals treated with methylphenidate had a higher motivation to search for high rewards and

that their memory for the locations for high rewards was stronger than for low rewards. Both explanations –the higher motivation and the stronger memory for high rewards- require dopamine release in the nucleus accumbens (Saddoris *et al.*, 2015)

These results can also be explained by a model that was recently proposed by our group. According to the model, there are neurons in the nucleus accumbens that represent all the locations in the environment called place-to-go cells; when a neuron that represent a specific location is activated, the animal will approach toward that location (Da Cunha *et al.*, 2009). Thus, the dopamine release evoked by the high reward would strengthen the long term potentiation in the neuron that represents the location for the high reward, and as a consequence this neuron would be more easily activated the next time the animal is exploring the maze; the activation of this neuron would result in the approach of the animal toward the location of the high reward.

Experiments currently carried out by our group have made possible to observe some neurons in the nucleus accumbens that behave as place-to-go (unpublished data). Future studies could assess the effect of methylphenidate administration in these neurons to determine if the explanation proposed above is correct.

6. CONCLUSIONS

In summary, our results demonstrate that methylphenidate does not affect only cognitive processes such as memory, but also has an effect on other processes such as motivation. Effects on both domains may interact and improve or impair performance, depending on the task. The results presented in this study, and the literature reviewed, reveal that methylphenidate has complex effects in different cognitive and behavioral processes. Although methylphenidate has beneficial effects for people with ADHD, it is not clear if its use may benefit healthy subjects given the complex effects that it has.

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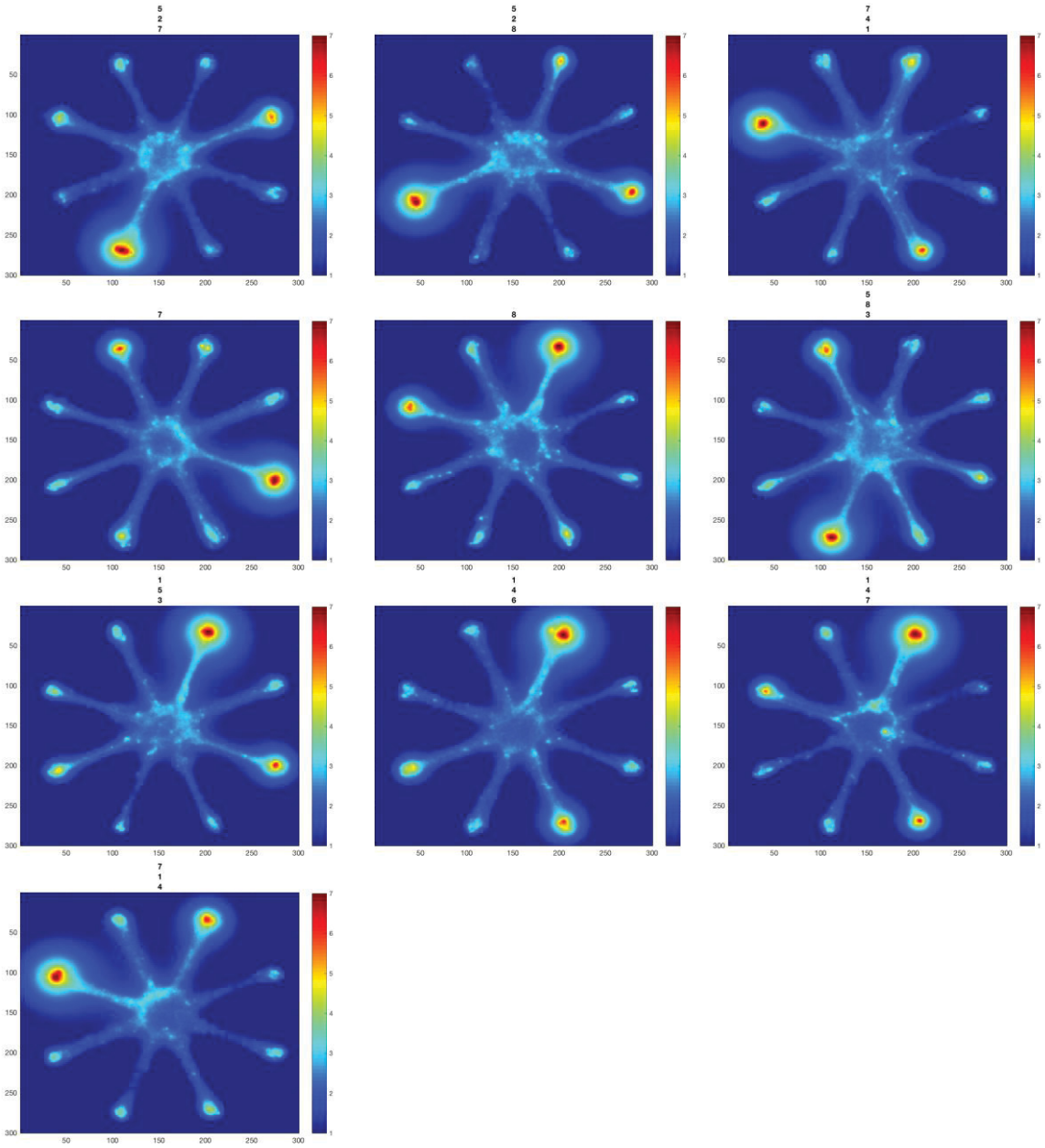
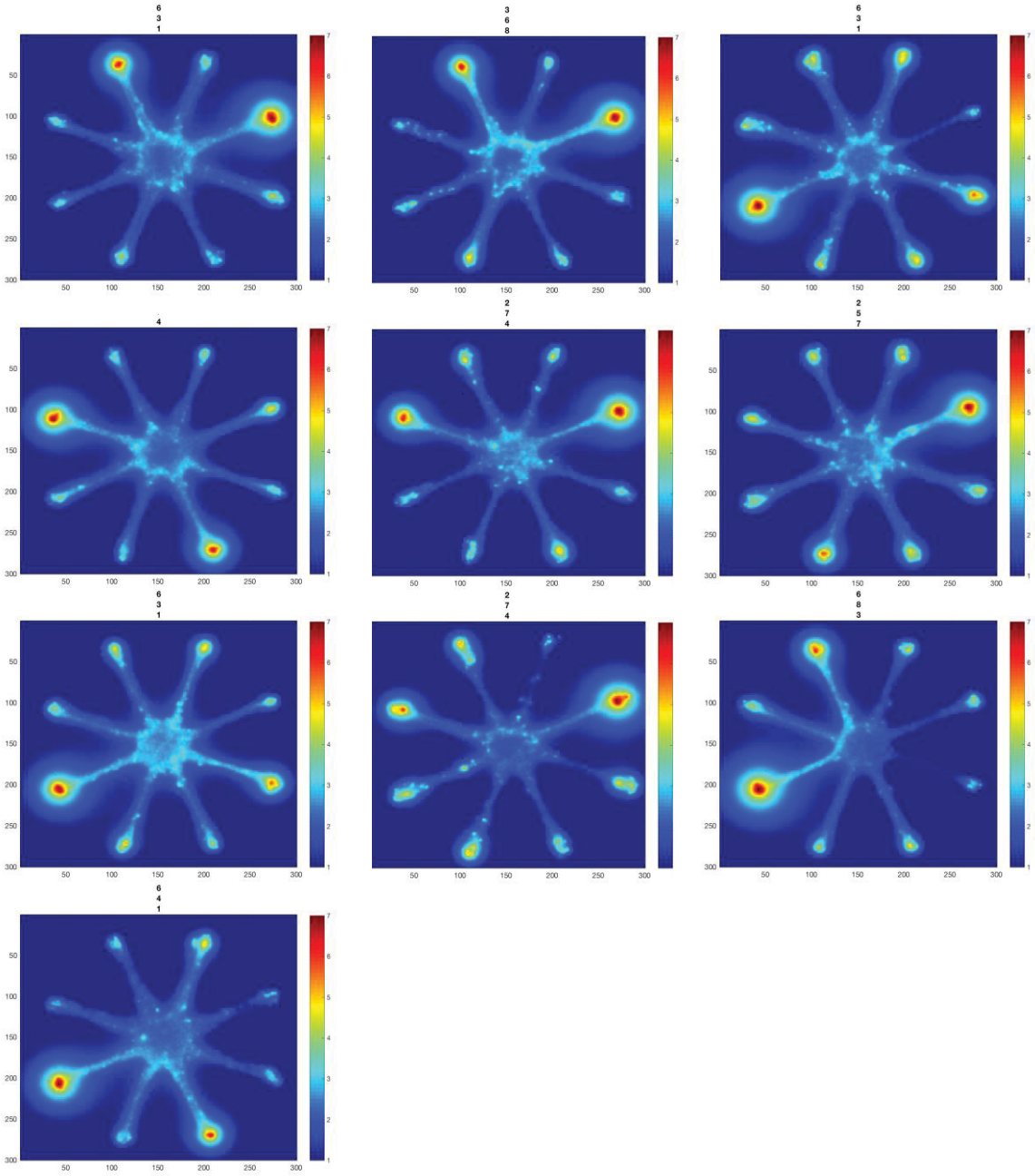
APPENDIX A



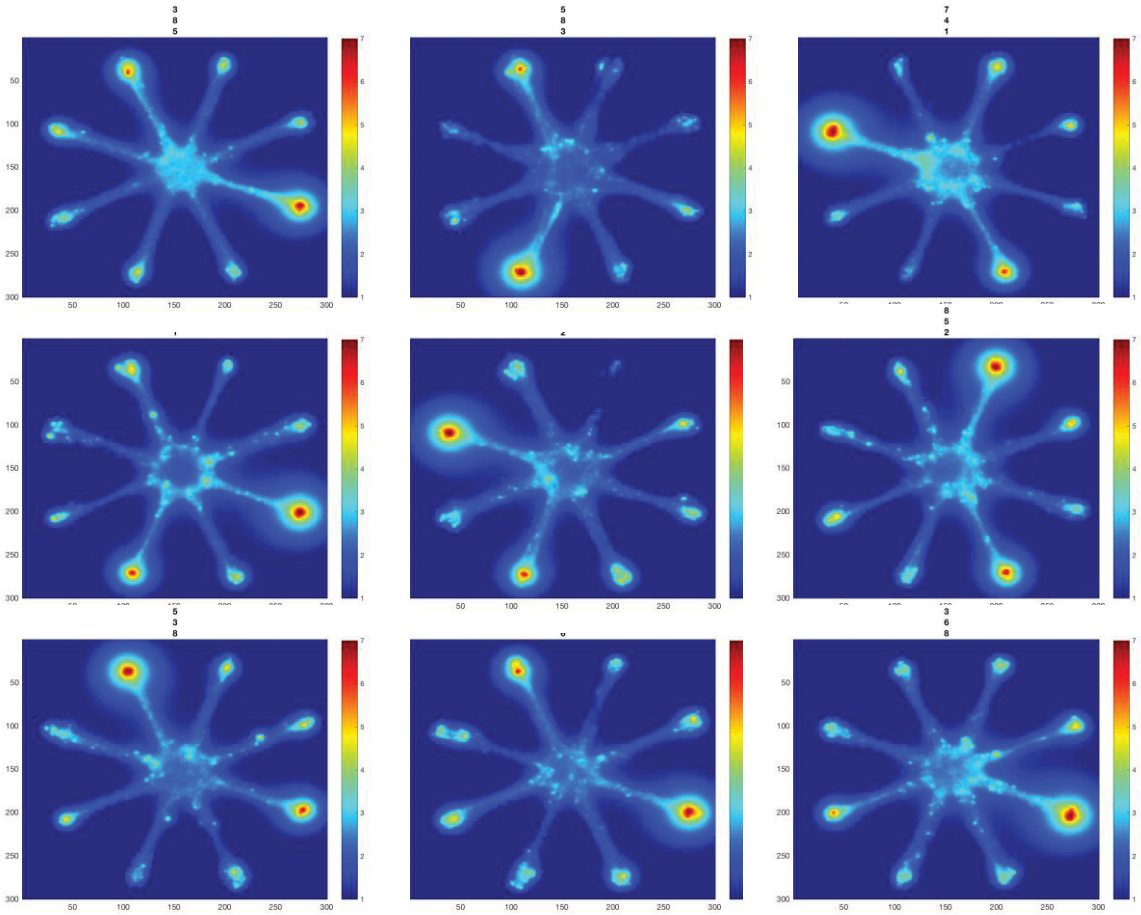
Appendix A. Colour map is representing the exploratory behaviour of the animals from the vehicle group in the radial arm maze. Warm colours indicate higher exploration than cold colours. The image represents the mean from the entire experiment.

APPENIX B



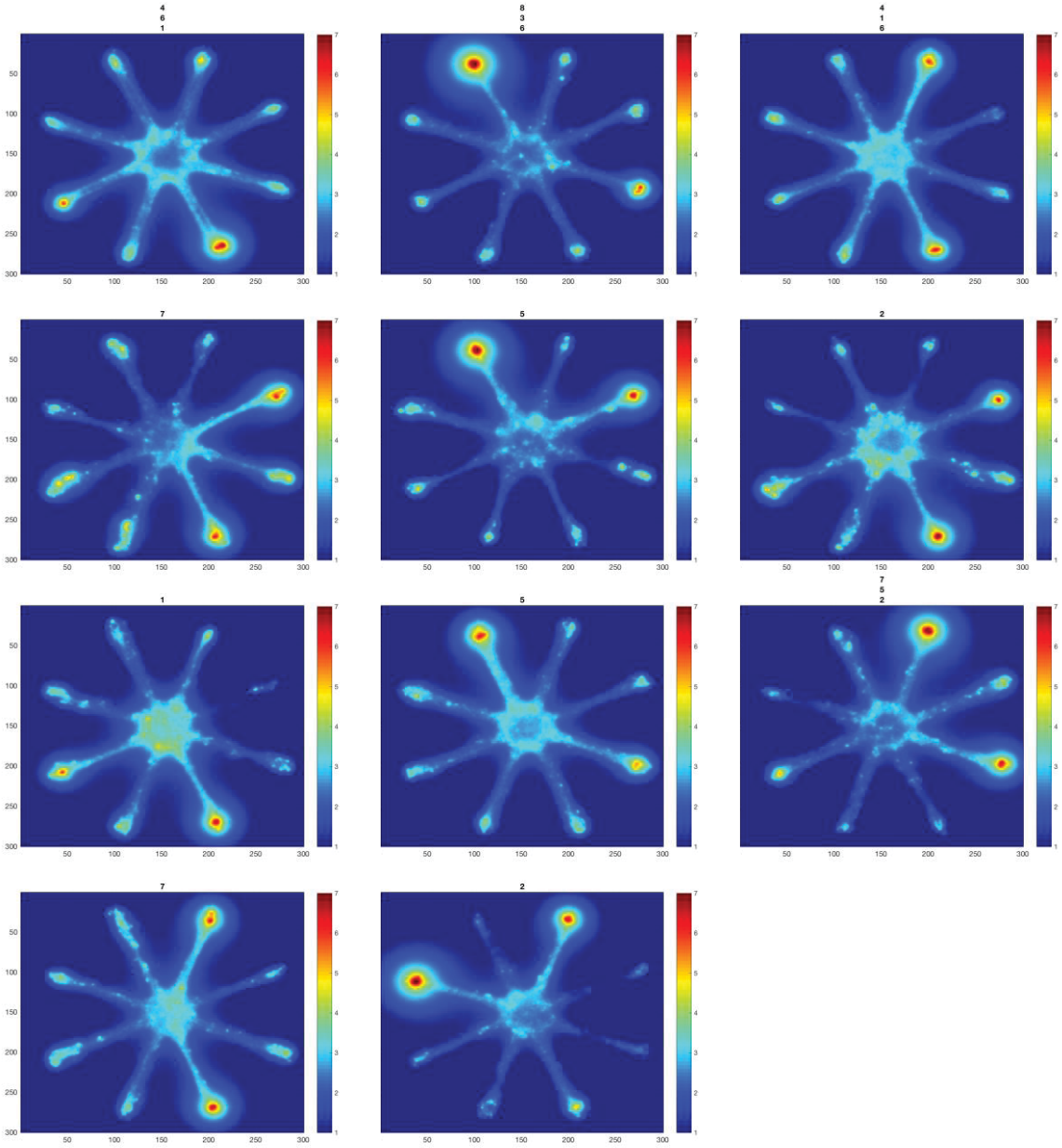
Appendix B. Colour map representing the exploratory behaviour of the animals treated with 0.3 mg/kg of methylphenidate in the radial arm maze. Warm colours indicate higher exploration than cold colours. The image represents the mean from the entire experiment.

APPENDIX C



Appendix C. Colour map representing the exploratory behaviour of the animals treated with 1 mg/kg of methylphenidate in the radial arm maze. Warm colours indicate higher exploration than cold colours. The image represents the mean from the entire experiment.

APPENDIX D



Appendix D. Colour map representing the exploratory behaviour of the animals treated with 3 mg/kg of methylphenidate in the radial arm maze. Warm colours indicate higher exploration than cold colours. The image represents the mean from the entire experiment.