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EFFECTS OF CANNABIDIOL ON MORPHINE AND METHYLPHENIDATE-INDUCED CONDITIONED PLACE PREFERENCE DURING EXTINCTION/ REINSTATEMENT IN MONKEYS AND RATS

Adel Kashefi

Data da defesa

30 January 2020

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Thesis presented as part of the requirements for obtaining a PhD degree in Animal Biology (area: Neuroscience and Behavior) by the Post-Graduate Program in Animal Biology of the University of Brasília.

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Elaborada por: Adel Kashefi

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You make my heart melt, and fill it with love... .

“With a wife like you, I feel so blessed... .

“I will love you more and more each day with every beat of my heart, until the day I die and my heart stops beating.

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The only way to do great work is to love what you do!!!

Steve Jobs

RESUMO

Justificativa e objetivos: O metilfenidato (MPH) é um estimulante leve do SNC que tem sido usado em crianças hiperativas, pacientes com neurodegenerativa e principais transtornos depressivos. Exposição a pistas associadas ao MPH aumenta o desejo e a excitação fisiológica em usuários de drogas. Por outro lado, o canabidiol (CBD) é um importante composto não psicoativo e os pesquisadores sugeriram que ele pode ser útil no tratamento da dependência de drogas. O objetivo do nosso estudo foi avaliar os efeitos da administração de CBD no MPH durante a extinção e restabelecimento de preferência-por-lugar condicionado (CPP) em macacos e ratos.

Métodos: Noventa e quatro ratos Wistar adultos machos pesando 200–250 g e cinco machos adultos de sagui-de-tufos-pretos (Callithrix penicillata) pesando 352 ± 5 g foram utilizados neste projeto de pesquisa. No estudo 1 usando macacos como sujeitos, para indução de CPP, a injeção IP de MPH (5 mg / kg) foi usada diariamente durante uma fase de condicionamento de cinco dias. Sob condições de extinção, os saguis recebiam injeção diária de IP do veículo ou do CBD. A primeira sessão de teste de reintegração ocorreu 24 horas após a última sessão de extinção e os animais receberam uma dose inicial de MPH (1mg / kg) e foram submetidos a um julgamento de reteste de 15 minutos. No Estudo 2, ratos machos adultos receberam MPH (1, 2,5 ou 5 mg / kg, I.P.) ou morfina (5 ou 10 mg / kg, s.c.) durante a fase de condicionamento da CPP. Após o estabelecimento da CPP, durante a fase de extinção, 60 minutos antes da sessão da CPP, os animais receberam diariamente CBD de ICV (10, 50 µg / 5 µl), veículo sozinho (DMSO) ou eram ingênuos. No dia do restabelecimento,

os animais receberam a dose inicial de MPH, 0,5 mg / kg, e foram colocados na caixa de CPP para avaliar o escore de CPP durante 10 minutos.

Resultados: Nossos achados indicaram que 5 e 10 mg / kg de morfina ou 1 e 2,5 mg / kg de MPH em ratos e 5 mg / kg de MPH em sagüis induziram CPP. Além disso, a administração de CBD não pôde inibir o restabelecimento da resposta de CPP induzida por MPH em macacos saguis. No entanto, em ratos, a administração ICV de ambas as doses de CBD (10 e 50 µg / 5 µl) suprimiu a reintegração de MPH e exibiu significativamente latências de extinção mais curtas nos grupos tratados em comparação aos grupos ingênuos e / ou DMSO.

Conclusão: Nossos resultados indicam que as injeções de morfina e MPH induzem o condicionamento da CPP. O CBD evita efetivamente a reposição do MPH em ratos, mas não em saguis. É possível que o CBD possa ser considerado como um tratamento que reduz o risco de recaída; no entanto, isso requer mais investigação.

Palavras-chave: Metilfenidato, Morfina, Canabidiol, preferência-por-lugar condicionado, Extinção, restabelecimento, Rato, Macaco.

ABSTRACT

Background and aims: Methylphenidate (MPH) is a mild CNS stimulant that has been used in hyperactive children, patients with neurodegenerative and major depressive disorders. Exposure to MPH-associated cues enhance craving and physiological arousal in drug users. On the other hand, cannabidiol (CBD) is a major non-psychoactive compound and researchers have suggested that it might be useful in the treatment of drug addiction. The aim of our study was to evaluate the effects of administration of CBD on MPH during extinction and reinstatement of the conditioned preference place (CPP) in monkeys and rats.

Methods: Ninety four male adult Wistar rats weighing 200–250 g and five male adult black-tufted-ear marmosets (*Callithrix penicillata*) weighing 352±5 g were used in this research project. In **Study 1** using monkeys as subjects, for induction of CPP, IP injection of MPH (5 mg/kg) was used daily during a five-day conditioning phase. Under extinction conditions, marmosets were given daily IP injection of either vehicle or CBD. The first reinstatement test session occurred 24 h after the last extinction session and animals received a priming dose of MPH (1mg/kg) and were submitted to a 15 min retest trial. In **Study 2**, Adult male rats received MPH (1, 2.5 or 5 mg/kg, I.P.) or morphine (5 or 10 mg/kg, s.c.) during the CPP conditioning phase. Following the establishment of CPP, during the extinction phase, 60 min before CPP session, animals were given daily ICV CBD (10, 50 µg/5µl), vehicle alone (DMSO) or were naïve. On the reinstatement day animals received the priming dose of MPH, 0.5 mg/kg, and were placed into the CPP box to evaluate the CPP score during 10-min.

Results: Our findings indicated that 5 and 10 mg/kg of morphine or 1 and 2.5 mg/kg of MPH in rats and 5 mg/kg of MPH in marmosets induced CPP. In addition, CBD administration could not inhibit the reinstatement of the MPH-induced CPP response in marmoset monkeys. However, in rats, the ICV administration of both doses of CBD (10 and 50 µg/5µl) suppressed the reinstatement of MPH and significantly displayed shorter extinction latencies in treated groups compared to both naïve and/or DMSO groups.

Conclusion: Our results indicate that Morphine and MPH injections induce CPP conditioning. CBD effectively prevents reinstatement of MPH in rats but not in marmosets monkeys. It is possible that CBD can be considered as a treatment that reduces the risk of relapse; however, this requires more investigation.

Key words: Methylphenidate, Morphine, Cannabidiol, Conditioned Place Preference, Extinction, Reinstatement, Rat, Monkey.

LIST OF FIGURES

LIST OF ABBREVIATIONS

ADHD – Attention Deficit Hyperactivity Disorder.

AMPH – Amphetamine.

BNST – Bed Nucleus of the Stria terminalis.

CBD – Cannabidiol.

CC – Conditioned Compartment.

CPP - Conditioned Place Preference.

DMSO – Dimethyl Sulfoxide.

DA - Dopamine.

DAT – Dopamine Transporter.

DLPFC – Dorsolateral Prefrontal Cortex.

GLU – Glutamate.

ICV - Intracerebroventricular.

IP - Intraperitoneally.

IV- Intravenous.

METH - Methamphetamine.

MPH – Methylphenidate.

NHP – Nonhuman Primates.

NE – Norepinephrine (NE).

NAc – Nucleus Accumbens.

OFC – Orbitofrontal Cortex.

5-HT – Serotonin.

SPECT – Single Photon Emission Computed Tomography.

VTA - Ventral Tegmental Area.

CONTENTS

1. INTRODUCTION

1.1. Brief overview of the status of drug abuse in Iran and Brazil

Drug abuse has been raised as a national problem in Iran and Brazil for a long time (Miraglia, 2015; Amin-Esmaeili *et al.*, 2017). Iran has a lengthy history of opiates being the most common drug of abuse (Assari *et al.*, 2014). In the early 2000s, methamphetamine (METH) entered Iran's market. Nowadays, METH use is becoming one of the most serious social problem in Iran (Ekici and Ozbay, 2013). It has led to significant health problems, affected the use of methadone for addiction treatment, and has also become a serious menace to HIV/AIDS prevention programs among drug users nationwide (Shariatirad, Maarefvand and Ekhtiari, 2013). Brazil has recently become a main destination country for cocaine and crack. Indeed, the use of cocaine and crack has skyrocketed in the past decade among young people (Abdalla *et al.*, 2014). Although cocaine use in North America decreased significantly between 2006 and 2012, the annual prevalence of cocaine use among Brazil's college students has remained at 3 percent (Miraglia, 2015). The estimated prevalence of cocaine use among the general population is estimated at 1.75 percent; this is also consistent with the increasing trend of cocaine consumption in Brazil. The use of cocaine has more than doubled since 2005— when about 0.7 percent of the population had used cocaine— and is four times higher than the average worldwide (0.37 percent) (Federal, 2011; Miraglia, 2015). The growth of Brazil's urban population and increases in affluence and disposable income appear to be the principal causes of

expanding substance use (Miraglia, 2015).

1.2. Methylphenidate

Methylphenidate (MPH) is an amphetamine-like central nervous system stimulant that has been used in the clinical treatment of major depression, neurodegenerative disorders, cognitive improvement in patients with brain tumors, AIDS disease, fatigue and as a treatment for delirium and sedation related with opioid use (Prommer, 2012). On the other hand, this medicine is most frequently prescribed for the management of Attention Deficit and Hyperactivity Disorder (ADHD) in children and teenagers (Goldman et al., 1998; Pliszka, 2007). Actually, MPH is the first option of prescribed treatment for children and adolescents who have been diagnosed with ADHD (Klein-Schwartz and McGRATH, 2003; DuPont et al., 2008; Prommer, 2012). Moreover, within the population of children, ADHD is one of the most common neurodevelopmental disorders of childhood, with approximately 3% to 10% of school aged children in the United States (Buitelaar, 2002). Worldwide, it is estimates about 5% for children and adolescents diagnosed with ADHD (Polanczyk et al., 2007). Diagnosis is dependent on parent and teacher accounts as there is no existing laboratory test able to verify diagnosis (Rowland, Lesesne and Abramowitz, 2002). Thus, the incidence of ADHD can be a complex process because of the subjective nature of parent and teachers account of a child's behavior. Furthermore, this disorder has a high rate of comorbid diagnosis such as learning disability, behavior disorder and anxiety disorder (Rowland, Lesesne and Abramowitz, 2002). The behavioral symptoms of these disorders often simulate the similar behavioral symptoms of ADHD that can

cause symptoms and behaviors that may be mistaken for ADHD. New diagnosed cases of ADHD have been increasing considerably and concomitantly the prescription of stimulants for treatment (Safer, Zito and Fine, 1996; Greenhill, Findling and Swanson, 2002; Cox et al., 2003; Olfson et al., 2003; Rowe, Robinson and Gordon, 2005). It is important to note that a significant population of children who are diagnosed may not have ADHD (Mayes, Bagwell and Erkulwater, 2008). However, prescription rates of MPH tripled during the early 1990s (Safer, Zito and Fine, 1996; Zito et al., 2000). The rise in prescription rates of MPH appeared to coincide with news that varying dose amounts of MPH elicited tolerance, sensitization, and withdrawal (Yang, Swann and Dafny, 2006), suggesting that MPH can elicit dependence and display a potential for abuse.

1.2.1. MPH Addiction

The potential for abuse and dependence of MPH is alarming considering the rise in the amount of children misdiagnosed and then prescribed a stimulant medication for treatment (Klein-Schwartz and McGRATH, 2003; DuPont *et al.*, 2008; Prommer, 2012). Evidence suggests that MPH abuse has substantially increased over the past several years (Looby and Earleywine, 2011; Brookshire and Jones, 2012). In the USA, of those persons age 12 and older, 4.2 million have used MPH recreationally at least once in their life (Substance Abuse and Mental Health Services Administration, NSDUH, 2005). Additionally, in 2005 there were 3,212 MPH drug-related hospital emergencies and by 2010 that number had risen to 4,089 (Substance Abuse and Mental Health Services Administration, DAWN, 2010). There are some factors that may contribute to the illegal use

of MPH, and route of administration is one that appears to play an important role (DuPont *et al.*, 2008; Volkow and Swanson, 2008). Oral administration is the most common method of administration (McGough *et al.*, 2006). For instance, (DuPont *et al.*, 2008) confirmed that 86% of those college students who used MPH recreationally did so by oral and intranasal routes of administration. Previous studies have reported higher incidences of MPH abuse through inhalation, with 75% of abusers self-administering MPH through this route (Morton and Stockton, 2000; Bright, 2008). Additionally, studies have also suggested that oral administration of MPH may result in reinforcing effects and this effect appears to be dose-dependent (Jasinski, 2000; Rush and Baker, 2001), while other studies using self-report data have suggested that recreational use is very common (Teter *et al.*, 2006; DuPont *et al.*, 2008). Lastly, past studies have verified that the use of extended-release MPH formulations have helped to eliminate the abuse liability (Kollins *et al.*, 1998; Berridge *et al.*, 2006; Parasrampuria *et al.*, 2007). However, given the route of administration (oral and intranasal) of typical recreational abuse, extended-release formulations of MPH may not negate the abuse responsibility of MPH. The most popular route of administration for recreational use of MPH is through intranasal administration (Bright, 2008) after several pills have been crushed. Intranasal administration avoids the first-pass metabolism in liver and is quickly absorbed into the bloodstream through the soft tissues in the mucous membrane. This allows for faster onset of bioavailability of the drug. Hence, MPH administered via this route negates differences in formulations, and rapid onset of the effects of the drug has been reached. If the drug is

taken through other routes of administration, it can reach the brain more rapidly and produce more important effects on the reward system (Volkow and Swanson, 2003; DuPont *et al.*, 2008). Studies have shown that MPH bioavailability in the brain is increased in rats using the intraperitoneal (ip) route (Berridge *et al.*, 2006).

1.2.2. Mechanism of MPH

The primary mechanism of MPH is to bind and block the dopamine transporter (DAT) and to a lesser extent, the norepinephrine (NE) transporter (Schweri et al., 1985; Solanto, 1998). The blockade of both the DAT and NE transporter diminishes synaptic clearance of these neurotransmitters, leaving behind high levels of monoamines in the synaptic cleft. This mechanism is similar that of cocaine and should be a matter of concern considering cocaine is a drug of abuse that has been shown to have reinforcing effects and is commonly abused (Swanson and Volkow, 2003). In fact, cocaine is considered one of the most commonly abused drugs, and like amphetamine (AMPH) as well as MPH, causes increases of extracellular dopamine in the brain (Volkow and Swanson, 2003). Studies have shown that MPH produces an increase of dopamine within the nucleus accumbens, which is believed to underlie the rewarding effects of drugs of abuse (Di Chiara and Imperato, 1988). MPH-induced increases of dopamine presumably underlie the reinforcing aspects of this drug, although its rewarding effects are dose-dependent (Nora D Volkow *et al.*, 1999).

1.2.3. MPH Effect on DAT

DAT is the main mechanism responsible for regulating of extracellular dopamine and blockade of the DAT is the neurobiological mechanism for MPH effect. DAT mediates the majority of DA uptake into neurons that is the primary mechanism through which DA is cleared from the synapse. MPH has been shown to produce a considerable decrease of DAT protein in younger animals (Moll et al., 2001) and in adults (Izenwasser et al., 1999) and reverse the enhancement in striatal DAT in an animal model of ADHD (Roessner et al., 2010). Further, in adult male rats, in vivo quantification of the DAT using small animal Single Photon Emission Computed Tomography (SPECT) discovered a dose-dependent decrease of striatal DAT after Intravenous (IV) administration of MPH (3 and 10 mg/kg) 2 h post drug treatment, but nucleus accumbens was not analyzed (Nikolaus et al., 2010). Finally, one study which analyzed 0.75 and 1.5 mg/kg MPH given for 7 days reported no changes in the DAT of several brain areas, including the nucleus accumbens shell and core (Bello and Hajnal, 2006). Hence, it appears that individuals with elevated DAT levels, such as ADHD sufferers, may be more susceptible to the addictive effects of amphetamine-like drugs.

1.2.4. Effect of MPH on Reward

Previous studies have established that MPH is able to induce conditioned place preference in rodents (Martin-Iverson, Ortmann and Fibiger, 1985; Mithani *et al.*, 1986). Most relevant to our work, (Meririnne, Kankaanpää and Seppälä, 2001) reported that doses of MPH ranging from 1.25 to 20 mg/kg produced conditioned place preference (CPP). Interestingly, a dose of 0.62 mg/kg showed only a trend to preference, and a dose of 0.31 mg/kg did not

produce any preference. These two lower doses, when given IP, produce brain concentrations that are similar to therapeutic doses of MPH (Berridge et al., 2006; Devilbiss and Berridge, 2006). It has also been verified that rats will self-administer MPH dose dependently on a fixed ratio (FR1) and progressive ratio (PR) schedules (Botly *et al.*, 2008). Interestingly, the effects of the dopamine D1 receptor antagonist SCH 23390 and dopamine D2 receptor antagonist eticlopride at a dose of 0.01 and 0.03 mg/kg increased the number of MPH infusions on FR1 schedule and decreased breaking points on PR schedule. These above results demonstrate the rewarding aspects of MPH and other psychostimulants regulated by both D1 and D2 receptors and contribute to reinforcement behavior (Figure 1).

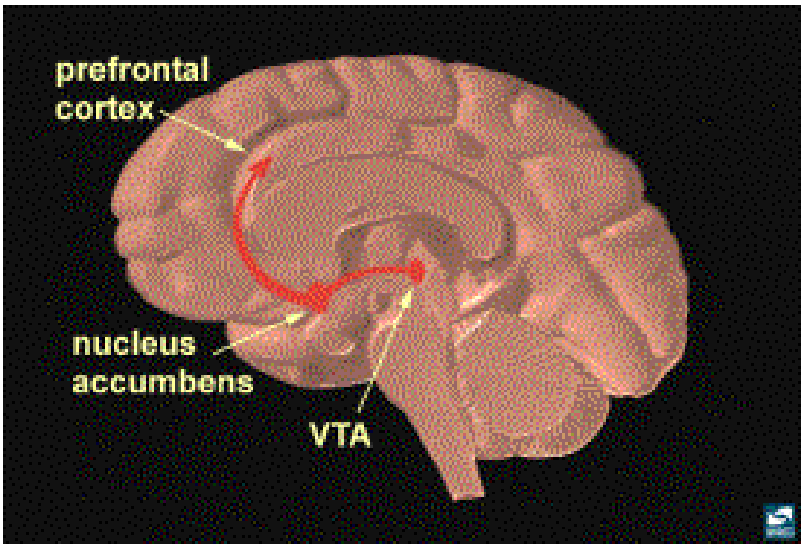

Figure 1. Illustrative scheme of reward circuit and positive reinforcing effects of drug addiction. The neurons of the VTA contain dopamine which is released into the nucleus accumbens and prefrontal cortex in response to artificial reward stimuli. stimulation of the reward pathway produces highly pleasurable sensations, providing positive reinforcement whith promotes futher drug use. (Figure taken from the :https://www.slideshare.net/dawnvtomy/physiology-of-drug-addiction).

1.3. The Dopamine System

The mesolimbic dopaminergic system is organized by dopaminergic cell bodies within the ventral tegmental area (VTA). The VTA sends a major axonal projection to both the nucleus accumbens and prefrontal cortex, and this system forms the brain's reward system. These brain areas also send reciprocal projections back to the VTA. This pathway is also identified as the medial forebrain bundle. Dopamine (DA) has been shown to be essential in the rewarding properties of psychostimulants (George, Le Moal and Koob, 2012). All addictive substances have been shown to activate the mesolimbic DA pathway, including cocaine, AMPH, and METH. Research has shown that all drugs of abuse enhance DA release within this pathway of the brain's reward system (Volkow *et al.*, 2009). Dopamine binds to two families of receptors: the D1 and D2. The D1 receptor has two receptor subtypes: the D1 and D5. The D2 receptor family has three receptor subtypes: the D2, D3, and D4. Both of these receptor families are metabotropic G-protein coupled dopamine receptors that negotiate the physiological functions of DA. Behaviorally, DA plays a main role in voluntary movement, reward, hormonal regulation as well as hypertension (Beaulieu and Gainetdinov, 2011). Therefore, many other drugs that target dopaminergic neurotransmission have been clinically prescribed for the management of several neurodegenerative and behavioral disorders including Parkinson's disease, schizophrenia, bipolar disorder, Huntington's disease, Tourette's syndrome as well as ADHD. Previous studies have demonstrated that dopamine D1 receptors are involved in the development of sensitization to the rewarding properties of psychostimulants (Meririnne, Kankaanpää and Seppälä, 2001).

For example, it has been shown that the D1 antagonist SCH 23390 prevents self-administration of AMPH prior to treatment (Pierre and Vezina, 1998) and also cocaine induced conditioned place preference (Shippenberg, Heidbreder and Lefevour, 1996). In contrast to D1-antagonism, the D2-antagonist raclopride (RAC) was ineffective in blocking conditioned place preference to cocaine. On the other hand, it should be noted that D2-antagonists have prevented development of sensitization to locomotor-stimulating effects of AMPH and METH (KURIBARA and UCHIHASHI, 1993; Meng, Feldpaush and Merchant, 1998) Accordingly, there is reasonable evidence to suggest that D2 receptors may be involved in the rewarding properties of particular psychostimulants.

1.3.1. Dopamine dysregulation and ADHD

Research has confirmed that DA dysregulation is involved in those individuals diagnosed with ADHD (Volkow and Swanson, 2008). DA is implicated in the brain as a mediator of reinforcement signals (Carmona *et al.*, 2009) and if ADHD consists of alterations in reward processing, then altered dopamine functioning can cause symptoms of ADHD (Tripp and Wickens, 2009). Tripp and Wickens (2009) have proposed a theory (dopamine transfer deficit) that suggests some symptoms of ADHD are a direct result of the breakdown of the transfer of the DA cell response to a cue that predicts reinforcement. Critically, prior imaging work has shown that children diagnosed with ADHD demonstrate a lower DA response in the ventral striatum to stimuli that involved anticipation of reward (Luman, Tripp and Scheres, 2010). Thus, it is hypothesized that there is a decreased phasic DA neuronal response in those diagnosed with ADHD and MPH

works to normalize this lack of response (Tripp and Wickens, 2008). Therefore, it makes sense that the pharmacological properties of psychostimulants clinically prescribed to treat these disorders act on the mesolimbic DA pathway. However, a variety of addictive substances such as cocaine, AMPH, act directly on this pathway as well. Therefore, delayed reinforcement at the cellular level occurs by a decline in phasic DA cell response to a cue that predicts reinforcement, hence rendering it ineffective. This would only happen after the positive reinforcer is delivered and would explain the unusual response to delay of reinforcement in children with ADHD (Tripp and Wickens, 2009). Children who do not have ADHD experience no delay in anticipatory dopamine signaling.

1.4. Other neurotransmitters

Previous studies indicate MPH has a high binding affinity for NE transporters (Gatley *et al.*, 1996; Kuczenski and Segal, 1997). Kuczenski and Segal (1997) demonstrated that hippocampal levels of NE were elevated following MPH administration. Using glucose metabolism as a measure of MPH's activity within different brain regions, Volkow and colleagues (1998a) found that glucose metabolism in the cerebellum was enhanced following MPH treatment. Typically, MPH's effect on glucose metabolism is attributed to activation of D2-R's. Although, the cerebellum does not contain D2-R's it is postulated that the cerebellar increases in glucose metabolism are due to activity on NE (Volkow *et al.*, 1997; Leonard *et al.*, 2004). MPH has been shown to indirectly increase levels of ACh in the prefrontal cortex via stimulation of the D 1 -R's (Acquas and Fibiger, 1996; Leonard *et al.*, 2004). Generally, ACh levels are increased by Dl-like

receptor activation, whereas D2-R activation reduces ACh release (Berlanga, Simpson and Alcantara, 2005). Cholinergic interneurons in the striatum express both D5 and D2 receptors. These interneurons are essential in associative learning as well as planning and executing movement. It has also been shown that the D 1-like and D2-like receptors can have a synergistic effect that is linked to synaptic plasticity and learning (Kashihara *et al.*, 1999; Silkis, 2001).

1.5. Reward, extinction and reinstatement

Effects of drug abuse on the brain are much more dramatic than natural rewards, such as food and social interactions. Drug abuse is a chronic and enduring phenomenon, which is major public health concern. Relapse, the resumption of drug abuse following abstinence or extinction, remains the major problem for the treatment of addiction. Relapse occurs in response to different precipitating events, including stress and drug priming dose (Gerber and Stretch, 1975; Perry *et al.*, 2014). One of the main aspects in substance abuse is extinction, a form of learning in which associations between cues and the events they predict are weakened by exposure to the appetitive cues in the absence of those events. Evidence from animal models suggests that conditioned responses to drug cues can be extinguished. Investigations into the neurobiological substrates of extinction of conditioned drug craving and withdrawal may facilitate the successful use of drug cue extinction within clinical contexts and treatment programs (Myers and Carlezon Jr, 2010).

In the place conditioning paradigm, extinction training can occur in either of two ways: animals can be given free access to the place conditioning

apparatus in repeated test sessions, or animals can be restricted in the formerly drug- or withdrawal-paired context in the absence of drug administration or precipitated withdrawal (i.e., following an injection of saline) and afterward given free access tests to assess extinction. When extinction has happened, there no longer is a preference for or aversion to the previously drug- or withdrawal-paired context; that is, animals spend approximately equal amounts of time in each of the 2 compartments (Myers and Carlezon Jr, 2010).

Neurophysiologic mechanisms underlie the uncontrolled, compulsive behaviors defining the addicted state. These “hard-wired” alterations in the brain are considered critical for the transition from casual to addictive drug use.

1.5.1. Brain areas involved in reinstatement of drug and reward seeking

An understanding of the role of the addictive process therefore also requires the inclusion of brain regions neuronally linked to the ventral tegmental area (VTA) and nucleus Accumbens (NAc). VTA supplies dopaminergic innervation not only to the NAc, but also to the amygdala and bed nucleus of the stria terminalis (BNST). In addition, the VTA projects DA from a third dopaminergic tract, the mesocortical pathway, which innervates prefrontal cortical regions that include the orbitofrontal cortex (OFC) and anterior cingulate. Coupled with glutaminergic and other reciprocal neurotransmitter connections, the NAc is integrated with the OFC, anterior cingulate, insular cortex, and hippocampus (Adinoff, 2004).

1.5.1.1. Amygdala

Activity of the amygdala has been associated to memory consolidation for emotionally arousing events (Tyng *et al.*, 2017). The amygdala is involved in incentive motivational value of rewards to stimuli and in the conditioning of fear to novel stimuli. For example, animals favoring a specific cage that is identified with drug administration will lose this conditioned stimulus if the amygdala is ablated (Adinoff, 2004).

1.5.1.2. Anterior cingulated

Implicated in human disorders of emotion and attention, the anterior cingulate is involced in emotional self-control, focused problem-solving, error detection, performance monitoring, and adaptive response to changing conditions (Allman et al., 2001). It plays a role in the conflict detection processing, particularly when low-frequency responses are performed (Braver et al., 2001), but is influenced by both motivation and affective state.

1.5.1.3. Bed nucleus of the stria terminalis (BNST)

BNST has been implicated in autonomic, hormonal and behavioral reactions to fearful stimuli, including the stress response (Choi *et al.*, 2007). The BNST is considered part of the extended amygdala and shares with the nucleus accumbens a sensitivity to dopamine stimulation. In rodents, the BNST is involved in the reinstatement of cocaine seeking after foot shock (Erb and Stewart, 1999).

1.5.1.4. Dorsolateral prefrontal cortex (DLPFC)

The DLPFC appears to be specialized for holding/maintaining several pieces of information “on line” or in short-term storage of information (i.e.,

“working memory”) (Petrides, 2000). The DLPFC is essential for the control and regulation of cognitive activities, including the sequencing of events, planning, and the selection of goals (Dixon, 2015).

1.5.1.5. Hippocampus

Crucial for fast acquisition of new factual information and the formation of new memories about personally experienced events (i.e., episodic memory), the hippocampus has been involved in the memory loss in Alzheimer’s disease (Maruszak and Thuret, 2014). Damage to the hippocampus causes anterograde amnesia with a lesser degree of retrograde amnesia (Broadbent, Squire and Clark, 2007).

1.5.1.6. Insular Cortex

Insula is part of the cerebral cortex and plays a role in a wide range of functions including processing of visceral and somatosensory inputs, olfaction, craving, addiction and emotions such as pain (Ghaziri *et al.*, 2018). It possibly plays an important role in relating interceptive signals and often in acute anxiety studies (Shin and Liberzon, 2010).

1.5.1.7. Orbitofrontal cortex (OFC)

As a part of prefrontal cortex implicated in disorders of impulsivity and decision making, the OFC is involved in situations that are unpredictable or uncertain, and modulates the reinforcement value of stimuli in the context of recent experience (Tsuchida, Doll and Fellows, 2010). It determines and decodes the likely value or behavioral relevance of available choices of action and is therefore activated when there is lack of information available to determine an appropriate course of action (Adinoff, 2004). It has been

suggested that the medial OFC (ventromedial cortex), with connections to the hippocampus and cingulate, is implicated in assessing the familiarity or “rightness” of a situation and in integrating outcome expectancies (Adinoff, 2004). The lateral OFC, with connections to the amygdala and insula, is connected with the suppression of previously rewarded responses and is required to change behavior (i.e., to provide “stop” signals) (Elliott, Dolan and Frith, 2000).

1.6. Cannabidiol activity

The plant Cannabis sativa has been used for many centuries. It is known to have therapeutically relevant properties and has about 400 different identifiable chemical constituents; more than 60 of them are cannabinoids (Schillack, 2018). Cannabinoids exert their effects by interaction with specific endogenous cannabinoid receptors such as cannabinoid receptor type 1 (CB1). This receptor is expressed predominantly in central nervous system, in areas that can mediate most of the effects on cognitive function, pain and short-term memory (hippocampus and cerebral cortex), motor control and coordination (basal ganglia and cerebellum), hypothermia and hyperphagia (hypothalamus), and expression of the CB2 receptor is restricted to immune cells, T-cells, B-cells, spleen, tonsils and activated microglial cells and suggested that play a relevant role in the rewarding, reinforcing, and motivational effects (Herkenham et al., 1991; Tsou et al., 1998).

Cannabidiol (CBD), one of the main constituents from the cannabis plant, was previously proposed as a cannabinoid devoid of psychopharmacological activity (Formukong, Evans and Evans, 1988). CBD is a drug with multiple mechanisms of action (Zuardi and Karniol, 1983), including anti-

inflammatory effects (Walter *et al.*, 2003; Costa *et al.*, 2004) antioxidative, a potent inhibitor of cancer cell growth, and neuroprotective effects (Valvassori *et al.*, 2011). In addition, CBD is known by the action on ischaemia, antiepileptic and antipsychotic actions and anxiolytic effects, these effects were observed in animal models, as well as in humans (Fleury-Teixeira *et al.*, 2019). Moreover, it has been suggested also that the endocannabinoid system may be involved in the pathophysiology of depression and that CBD may have agonist properties at 5-HT-1A receptors, which have been related to the therapeutic effect of antidepressant drugs (Shirayama *et al.*, 2002; de Souza Crippa *et al.*, 2004). Also, (Harrison and Markou, 2001) confirmed a modulatory role of 5-HT-1A receptors in brain stimulation reward. However, the mechanisms underlying those effects are not fully understood. One possibility is that activation of 5-HT-1A receptors by CBD could inhibit extracellular concentrations of serotonin and/or attenuates mesolimbic activity. Recently, it reported the CBD attenuates cue-induced reinstatement of heroin seeking (Katsidoni, Anagnostou and Panagis, 2013). A study has shown that the CBD lacks hedonic properties and blocks the reward-facilitating effect of morphine (Parker *et al.*, 2004). Another study showed that the administration of CBD can reverse and/or prevent in rats the behavioral and oxidative stress effects induced by chronic use of D-amphetamine in an animal model of mania. It has been found that CBD may increase activation of 5-HT1A receptors and the subsequent hippocampal expression of brain derived neurotrophic factor (BDNF) (Saarelainen *et al.*, 2003; Duman and Monteggia, 2006).

1.6.1. Mechanisms of CBD in the Brain

CBD may also interact with the endocannabinoid system through indirect mechanisms such as improved action of the endogenous cannabinoid ligand anandamide. This results from blockade of anandamide reuptake and the inhibition of its enzymatic degradation (Mechoulam and Hanuš, 2002; Jiang et al., 2011; Bih et al., 2015). CBD has been shown to modulate several non-endocannabinoid signaling systems. It is not clear which, if any, of these mechanisms are responsible for any of CBD's potential clinical or other effects. Some of these mechanisms include: Inhibition of adenosine uptake, possibly resulting in indirect agonist activity at adenosine receptors, enhanced activity at the 5-HT1a receptor, enhanced activity at glycine receptor subtypes and blockade of the orphan G-protein-coupled receptor GPR55 (Bih et al., 2015).

CBD has also the ability to enhance adenosine signaling through inhibition of its uptake and also has low affinity as a CB1 receptor antagonist (Thomas et al., 2007), leading researchers to search for alternative sites of action to explain its effects. CBD can act as a partial agonist to the 5HT-1A serotonin receptor (Pertwee, 2004; Russo et al., 2005), a weak partial agonist for D2 receptors (Seeman, 2016), a weak negative allosteric modulator to Mu opioid receptors (Kathmann et al., 2006) and activates the GPR55 receptor (Ryberg et al., 2007). Recently, researchers have focused on CBD's involvement with the 5HT-1A receptor to regulate cell-to-cell communication. In general, activation of the 5HT-1A autoreceptors located on post synaptic somato-dendritic sites causes inhibition of that cell's firing output (Tada et al., 2004; Polter and Li, 2010). Following this cell firing

inhibition, CBD administration could potentially inhibit post-synaptic transmission and therefore affect the activity of projected areas. For instance, intracranial infusion of CBD in the shell of the nucleus accumbens (NASh) can attenuate ventral tegmental area (VTA) dopamine neuron firing (Norris et al., 2016; Renard et al., 2017).

1.7. *Callithrix penicillata* monkey as an experimental model

Most preclinical studies use rodents as an animal model to investigate the process of dependence (Puhl *et al.*, 2011; Corwin and Babbs, 2012). Undoubtedly, experiments with rodents are of paramount importance and have given numerous contributions to the current understanding of the neural mechanisms that support the process of dependence. However, studies with nonhuman primates have been employed more frequently in neuropharmacological and behavioral approaches (Valentinuzzi *et al.*, 2008; Arce *et al.*, 2010; Chabrawi and Barros, 2011; Melamed *et al.*, 2013). The use of non-human primates favors the generalization of results for humans, since the phylogenetic aspects, the morpho-functional organization and the behavioral and neurochemical aspects are closer to humans (Hacia *et al.*, 1998; Piggott *et al.*, 1999; Weerts, Fantegrossi and Goodwin, 2007).The genetic homology between non-human and human primates is around 95%, depending on the species studied (Hacia *et al.*, 1998). In addition, the brains of nonhuman primates exhibit all subdivisions of the prefrontal cortex seen in the human brain (Carmichael and Price, 1994; Preuss, 1995). Primates, in general, present a great diversity of social systems (Isbell and Young, 2002) and thus are more vulnerable to psychosocial stress, also exhibiting similar responses to humans (Norcross and Newman, 1999; Pryce *et al.*, 2005).

On the other hand, using primates as experimental subjects requires a lot of care in order to ensure reliable and reproducible results in biomedical research. In order to avoid behavioral and/or physiological abnormalities, it is necessary to observe the specific needs of the species in terms of diet, housing type, physical space, and opportunities for reproduction and social interactions. Other aspects also need to be considered, such as the stress caused by management procedures, hygiene, consanguinity and periodic health examinations. The use of small New World primates, such as marmosets has several advantages that are reflected in their low captive maintenance cost and good reproduction rate, requiring small maintenance spaces and a lower cost when compared to other species of primates (Orsi *et al.*, 2011).

The individuals of the species *Callithrix penicillate* (Hershkovitz, 1977): Order: Primates, Family: Callitrichidae, Callithrix-mico-estrela; Figure 2) have been established as experimental subjects in biomedical, behavioral and neuropsychopharmacological investigations of animal studies (see Barros and Tomaz, 2002).They are small primates with large black tufts behind its ears. The head is black or brown mixed with gray and white patch on the forehead and the body is gray/brown, brush-shaped. They exhibit a characteristic white spot on the forehead in star format, deriving its popular name (de Vivo, 1991; Auricchio, 1995). They are diurnal animals and predominate in the Cerrado and Caatinga biomes of Brazil (Rylands, 2000). With regard to their eating habits, they are omnivorous animals, exhibiting a generalist strategy in obtaining resources (Rylands, 1993).They feed on a wide variety of plant matter (exudates/gums, seeds, flowers, fruits, nectar)

and animals (arthropods, molluscs, small birds and mammals, amphibians and small reptiles) (Ferrari and Ferrari, 1989; Vilela and Faria, 2002)

Studies in our own research group have demonstrated that repeated cocaine administration in marmoset monkeys induces hypervigilance-related behaviors (Cagni *et al.*, 2012). It is also reported that Neurokinin3 receptor modulation of the behavioral and neurochemical effects of cocaine in *Callithrix penicillata* (Souza Silva *et al.*, 2008). In this context, several studies have successfully confirmed the use of *Callithrix penicillata* in studies that investigate the physiological and behavioral responses to drugs of abuse. Therefore, it seems this primate is an unique experimental model for behavioral and psychopharmacology studies and substance use disorder (Barros *et al.*, 2003, 2007; Mello *et al.*, 2005; Silva *et al.*, 2006; Lima *et al.*, 2008; Souza Silva *et al.*, 2008; Melamed *et al.*, 2013).

Figure 2. Callithrix penicillata (mico-star) kept in the Primatology Center of the University of Brasilia (Photo: Aline Borges).

2. JUSTIFICATION

Animal research suggests that, as with the psychostimulants, opiates appear to mediate their reinforcing effects by modulating the activity of the mesolimbic pathway, although not directly (Shippenberg and Elmer, 1998). The opiates enhance NAc dopamine release by increasing the activity of VTA dopamine neurons. It is postulated that this is achieved via activation of mu-opioid receptors located on GABA neurons within the VTA, which play an important role in regulating the activity of VTA dopamine neurons. Opiates also have dopamine-independent effects within the NAc, which play an important role in opiate reward (Koob and Bloom, 1988).

In addition, MPH blocks the DAT, the key mechanism responsible for the removal of extracellular dopamine (DA), thereby elevating extracellular DA levels in various limbic, striatal, cortical, cerebellar terminal fields and increasing DA signaling and duration of DA response (Solanto, 1998). Neuroimaging studies showed that therapeutic doses of MPH increased DA levels in the striatum and NAc and suitable manipulation of this system can be effective in MPH-induced reward, extinction and reinstatement. Although CBD has been shown to affect emotional responses, few researchers have evaluated its effects in relation to brain reward and addiction in monkeys (Ren *et al.*, 2009). In fact, no studies have been done about the effects of CBD on the extinction and reinstatement of morphine and MPH-Induced conditioned place preference in animal models. Therefore, in this research project, we investigated the effects of CBD on brain stimulation reward and reward-facilitating effects of morphine and MPH-induced conditioning in both monkeys and rats.

3. OBJECTIVES

3.1. General objective

The main objective of this study was to investigate the effects of Cannabidiol (CBD) on MPH-induced conditioned place preference during extinction and reinstatement in the non-human primate species *Callithrix penicillate* as well as in rats.

3.2. Specific objectives

1) Investigate the effect of daily injection of the CBD on conditioning and extinction of MPH-induced CPP in *Callithrix penicillate*.

2) Investigate the effect of single injection of the CBD on reinstatement of MPH seeking behavior in MPH-extinguished *Callithrix penicillate*.

3) Investigate the effect of Morphine injection on conditioning and extinction of CPP task in rats.

4) Invetsigate the effect of Morphine on reinstatement and seeking behavior in rats.

5) Investigate the effect of daily injection of the CBD on extinction of MPH-induced CPP in rats.

6) Investigate the effect of single injection of the CBD on reinstatement of MPH seeking behavior in MPH-extinguished rats.

4. MATERIAL AND METHODS

4.1. Ethical aspects

The experiments with monkeys were approved by the Animal Ethics Committee (CEUA) of the Institute of Biological Sciences of the University of Brasília (Annex 1). All the ethical precepts stipulated by COBEA (Brazilian College of Animal Experimentation) have been observed. The study was carried out with animals kept in captivity on the Primate Center / CP-UnB, which is accredited by the Brazilian Institute of Environment and Renewable Natural Resources (IBAMA) as a primates breeding place for scientific purposes (IBAMA Register, 1/53/1999 / 000006-2). The experiments with rats were conducted in Iran and were performed in accordance with the guide for the care and use of laboratory animals (National Institutes of Health Publication No.80-23, revised 1996) and were accepted by the Research and Ethics Committee of Shahid Beheshti University of Medical Sciences (Annex 2).

4.2. Animal Subjects and general housing conditions

In all experimental stages of this study adult (> 18 months) male *Callithrix penicillate*. Were used pair-housed in different home-cages (2 × 1 × 2 m each) of a same colony room at the Primate Center of the University of Brasilia. This room consisted of a semi-outdoor/indoor housing system with two parallel rows of 12 cages each, separated by a common wire-mesh enclosed central corridor. The animals were thus exposed to natural light, temperature and humidity conditions. Fresh food was provided daily at 07:00 h, consisting of a mixture of pieces of fruits and vegetables, and unconsumed items were removed by 17:00 h. Boiled eggs, nuts and/or

cooked chicken breast were given several times a week, also at 07:00 h. Water and chow were available *ad libitum*. Animals were tested in random order on each day and all sessions held between 08:00 and 13:00 h. Housing conditions complied with the regulations of the Brazilian Institute of Environment and Renewable Natural Resources (IBAMA).

The experiments conducted in Iran were performed on male adult Wistar rats (Pasteur Institute, Tehran, Iran) weighing between 220 g and 250 g on the first conditioning day. Rats were housed in groups of four per cage, with *ad libitum* access to food and water, in a temperature-controlled room with a 12/12 h light/dark cycle (lights on at 7:00 PM). Each experimental group consisted of 5–7 animals and were used only once for each test. The tests were performed between 9:00 a.m. and 2:00 p.m.

4.3. Experimental procedure

Our research was divided into two parts: The first study consisted of pharmacological and behavioral experiments on Marmosets; In the second study, behavioral experiments with the CPP paradigm was used to investigate the effects of different doses of Morphine or MPH and also examined effects of intravenous administration of CBD on MPH-induced conditioned place preference, during extinction/ reinstatement in rats.

The methodology, results and discussion of these studies will be presented for each study individually.

4.4. Drugs

4.4.1. Preparation of MPH and Morphine

Methylphenidate hydrochloride (synthesized by Laboratory of Medicinal Chemistry, Novartis Pharma Services, Tehran, Iran) was freshly diluted in saline and injected intraperitoneally (IP) and morphine sulfate (Temad, Iran) was dissolved in physiological saline (0.9% NaCl) and administered by subcutaneous (s.c.) route at the dose of 5 and 10 mg/kg in conditioning phase in rats.

For marmosets, pills of Methylphenidate hydrochloride (MPH; 5.0mg/kg; Ritalin®, Novartis, Brazil) were macerated and dissolved in phosphate-buffered saline.

4.4.2. Preparation of Cannabidiol

For the experiments with rats CBD (Tocris Bioscience, St. Louis, Missouri, USA), was dissolved in a mixture of dimethyl sulfoxide (DMSO) and 10%and 90% phosphate-buffered saline solution. Intracerebroventricular (ICV) microinjections were performed using a 5-µL Hamilton syringe into the lateral cerebral ventricle (10 µg/5 µL) of the rat.

For monkeys, Cannabidiol (CBD; STI Pharm, UK) was dissolved in a 1:19 solution of Tween 80 (Sigma-Aldrich, Brazil) and phosphate-buffered saline and the injections were ip.

5. STUDY 1: EFFECTS OF CANNABIDIOL ON THE EXTINCTION AND REINSTATEMENT OF MPH-INDUCED CPP IN MARMOSETS

5.1. Experimental subjects

Five male adult black-tufted-ear marmosets (*Callithrix penicillata*) were used, weighing 352±5 (range: 340-365g) at the beginning of the study.

5.2. Behavioral section

5.2.1. Conditioning place preference apparatus

CPP paradigm was used to evaluate the effects of CBD on MPH-induced conditioned place preference during extinction and reinstatement.

5.2.1.1. Apparatus

Testing was conducted in a two-compartment CPP box, suspended 1m from the floor. Each compartment (60cm x 60cm x 35cm) had three walls and the floor made of aluminum, whereas the fourth wall and the top were made of glass (Figure 3) (Duarte *et al.*, 2015). Each compartment had different visual and tactile cues. One had a smooth surface and white color, whereas the other had a rough surface and was painted with black and white diagonal stripes. The aluminum wall dividing the CPP box into two compartments consisted of a horizontally-sliding door. If retracted, it gave access to both sides of the apparatus. Each compartment had an independent entry/exit door located on the aluminum side directly opposite the glass wall. Attached to the apparatus, was an aluminum antechamber that encompassed both access doors. The subjects could only access the compartment's sliding doors and enter the respective compartment via this common antechamber, which had a guillotine-type door as its access point.

The CPP box was set-up in a test-room 50m away from the colony facility and subjects were transported between their home-cages and the test-room via a transport-cage (35cm x 20cm x 23cm). This aluminum box prevented them from seeing their surroundings and was attached directly to the guillotine-type door of the CPP box. The apparatus was monitored via a closed-circuit system using two cameras (model C920, Logitech, Brazil): one mounted 1.5m above the arena and the other placed 1.5m in front of its glass wall. Both cameras were connected to the laptop located in an observation-room adjacent to the test-room.

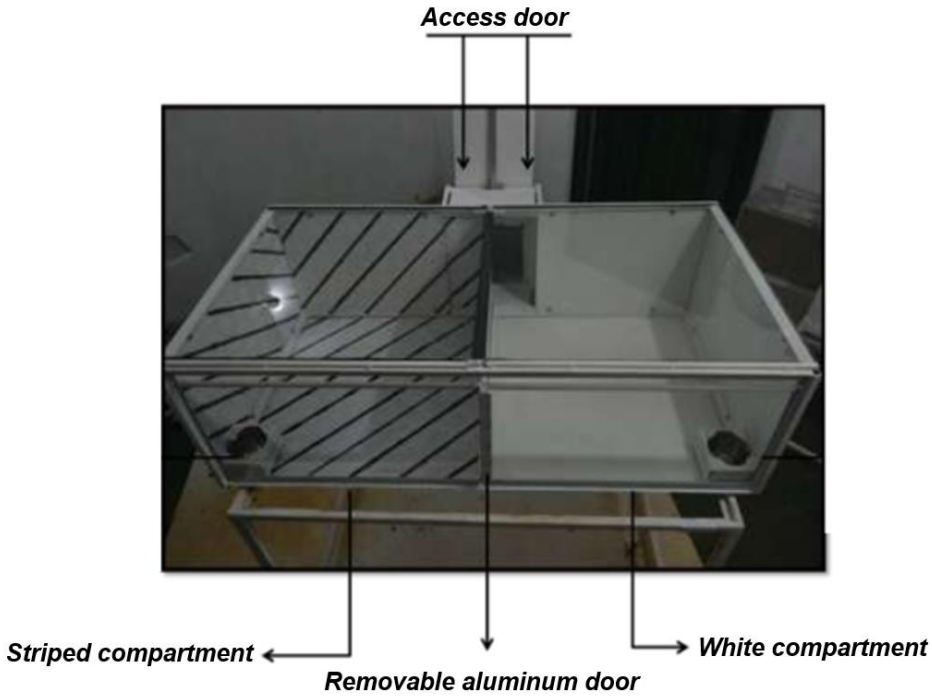

Figure 3. CPP procedure with two different compartments separated by a aluminum wall for evaluation of the reinforcing stimuli effects of MPH stimuli, including rewarding (Photo: Aline Borges).

5.3. Experimental procedure

The procedure consisted of three phases: pre-conditioning, conditioning and post-conditioning phases, as shown in the diagram below (Figure 4).

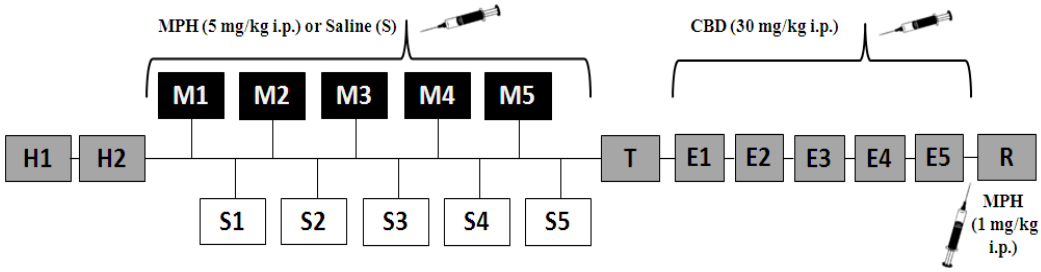

Figure 4. Schematic representation including the two initial habituation trials (H1 and H2) that marmosets had free access to the entire CPP box, and then followed by the methylphenidate (MPH, 5 mg/kg; I.P.; M1–M5) and saline-conditioning trials (S1–S5) held on alternative days with access to one of the compartments. Test trial (T) was held after a sequence of ten MPH/saline-conditionings and the extinction period (E1-E5) was held 24 hours after the test phase with a daily injection of cannabidiol (CBD; 30 mg/kg; I.P.) for five consecutive days. During the test and extinction trials, marmosets had free access to both compartments, and no injections were given prior to the habituation and test trials. One day after the last extinction trial, the reinstatement trial (R) was made, and similar to the test trial, animals had free access to all compartments and received a prime dose of MPH (1 mg/kg; I.P.) before testing session.

5.3.1. Pre-conditioning phase:

The animals were transported from the animal housing room to the testing room at least 30 min before the experiment began, for habituation. Marmosets were submitted to a CPP protocol similar to that used in previous studies from our group (Duarte *et al.*, 2015). Each marmoset initially was submitted to a 15 min habituation trial in the CPP box (Figure 5) on two consecutive days and no drug was available in either compartment and the aluminum sliding-wall was kept partially retracted, providing a 30 cm passage between compartments. The marmosets then were submitted to a daily 15 min conditioning trial in the CPP box during 10 consecutive days. On these trials, the common sliding-wall remained shut.

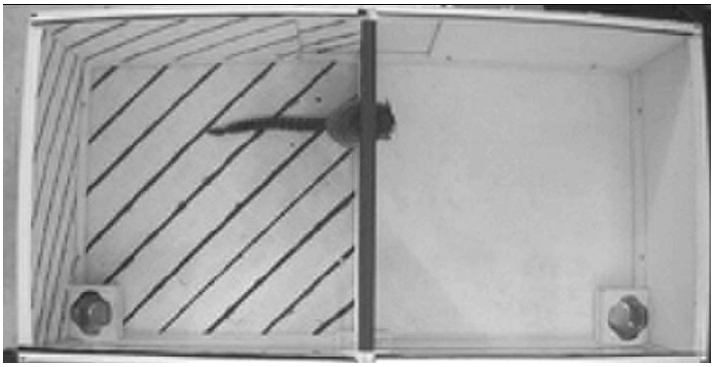

Figure 5. Top view of the CPP with free access to the two side compartments, made possible by the opening of retractable door (Photo: Aline Borges).

5.3.2. Conditioning phase:

The conditioning phase started one day after pre-conditioning test and consisted of a 10-days schedule. On alternate days, each marmoset was given access to either the white or the striped compartment. Subjects received MPH on odd-numbered trials (i.e., 1,3,5,7 and 9) on the conditioned compartment (CC). On even-numbered trials (i.e., 2,4,6,8 and 10) animals received saline. Animals were arbitrarily conditioned in the white or striped context.

Figure 5. Top view of the CPP with free access to the two side compartments, made possible by the opening of retractable door (Photo: Aline Borges).

5.3.3. Post conditioning phase:

Place preference response was determined in a 15 min test trial in the CPP box, 24h after the last conditioning trial. During this trial, each marmoset could access both compartments and no drug was provided, similarly to the habituation trials.

The locomotor activity of each animal was recorded using the locomotion tracking apparatus by a video tracking system. In these experiments, the total distance traveled (in centimeters) for each animal was measured in pre- and post-tests.

5.4. Extinction

After the test trial, subjects received daily I.P. injections of CBD 30 min prior to entrance into the CPP box for 15 min extinction sessions. These trials were made until the extinction of the place preference response. This

extinction was determined when subjects' place preference response was statistically different from the test trial, for two consecutive days.

5.5. Reinstatement

One day after the last extinction trial, one reinstatement trial was made, similarly to the conditions of the test, except that a prime dose (1.0 mg/kg of MPH) was given 10 min before the behavioral test, to evaluate the sensitization and reinstatement.

For all trials, each subject was captured in its home-cage, injected with its treatment and placed in a waiting-cage similar to its home-cage. It was then recaptured, placed in the transport-cage and taken to the test room where it was released into the antechamber of the CPP box. After the end of each trial, the CPP box was cleaned with 70% alcohol. Animals were tested randomly and sessions were held between 07:30-11:30h.

5.6. CPP score and animal movement

The CPP score represents the time spent in the drug paired place minus the time spent in saline paired place. Total distance traveled for each animal was also recorded in order to evaluate the locomotor activity in all control and experimental groups.

5.7. Behavior analysis

For all trials, the *any maze* software (Soelting Co., USA) automatically tracked via the top-view camera the marmosets' total distance and average speed traveled within the CPP box, as well as the time spent in each compartment. In addition, an experienced observer with a 95% intra-rater reliability, manually scored on the same program the following behaviors:

Vigilance (i.e. the duration of continuous sweeping upward or downward movements of the head while stationary); Locomotion (i.e. the duration of continuous movement through the CPP box) (Garland, 1998; Heal, Cheetham and Smith, 2009).

5.8. Statistical analysis

Statistical analysis was completed with the SPSS software (Windows Version 23.0; IBM Corporation, NY, USA). Data were analyzed using the paired t-test for differences in the locomotor and vigilance behaviors; the time in the MPH-paired and SAL-paired on pre and post-CPP (Garland, 1998; Heal, Cheetham and Smith, 2009). A repeated measures one-way analysis of variance (RM ANOVA) was used to analyze the time in the MPH-paired compartment through all the trials. Subsequent multiple pair-wise comparisons were held with Tukey's test whenever applicable. Significance level for all tests was set at $P< 0.05$.

5.9. Results

We found that marmosets did habituate to the CPP box, as we found a significant reduction on the locomotion (t4 =2.92, P = 0.043; Table 1), and no increase in vigilance through the habituation trials (t4 = 2.99, P = 0.40; Table. 1). In addition, subjects did not have an initial preference for either side of the apparatus (t4 = 0.59, P = 0.5; Fig. 6). After 5 alternate days of MPH, the marmosets spent significantly more time in the MPH-paired compartment in comparison to the SAL-paired at post-CPP (t4 = -9.96, P = 0.001; Fig. 6) and to the MPH-paired at pre-CPP session (t4 = -4.826, P = 0.008; Fig. 6). As for the use of CBD on extinction we found a significant difference between the trials (F8.32 = 4.886, P = 0.031; Fig. 7). According to

the pair-wise comparisons, we found a significant difference in time in the MPH-paired zone between pre-CPP x post-CPP (P = 0.008), post-CPP x Extinction 4 (P = 0.016) and Extinction 5 (P = 0.033) and Retest x pre-CPP (P = 0.004).

Parameter	Trial			
	Habituation1	Habituation2	Conditioning1	Conditioning5
Locomotion (s)	69±16	55±16*****	43±16	40±6
Vigilance (s)	718±43	747±43	730±69	695±95

* P <0.05 Habituation 1 vs. Habituation 2

Figure 6. Time marmosets spent (mean±SEM) in locomotion and vigilance on both habituation trials and first and last conditioning trials. * P < 0.05 Habituation 1 vs. Habituation 2

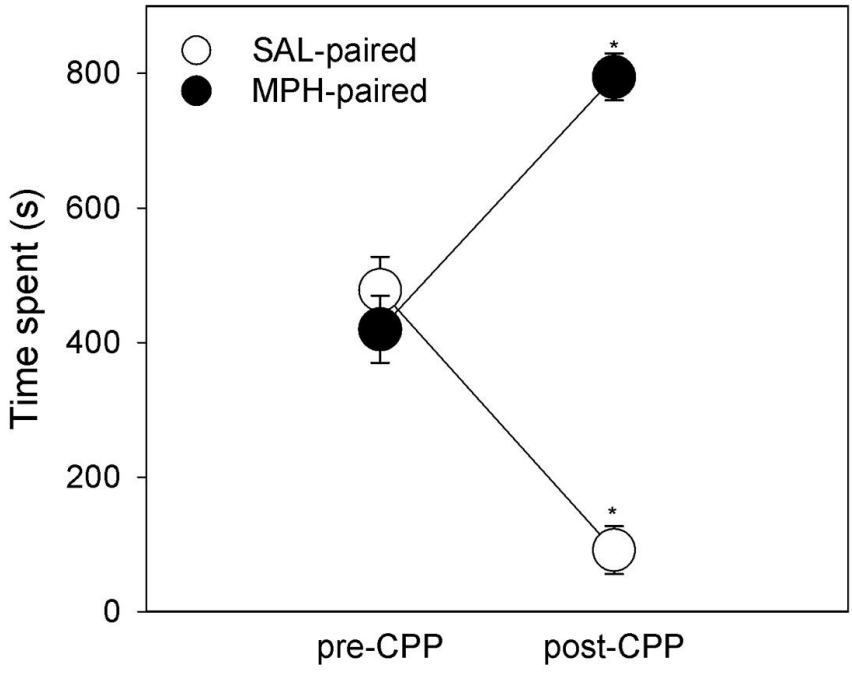

Figure 7. Time marmosets (n=5) spent (mean±SEM; in seconds) in the methylphenidate (MPH) paired compartment and the saline (SAL) paired compartment of the CPP box before (pre-CPP; last habituation trial) and after (post-CPP; test trial) the conditioning trials. *P

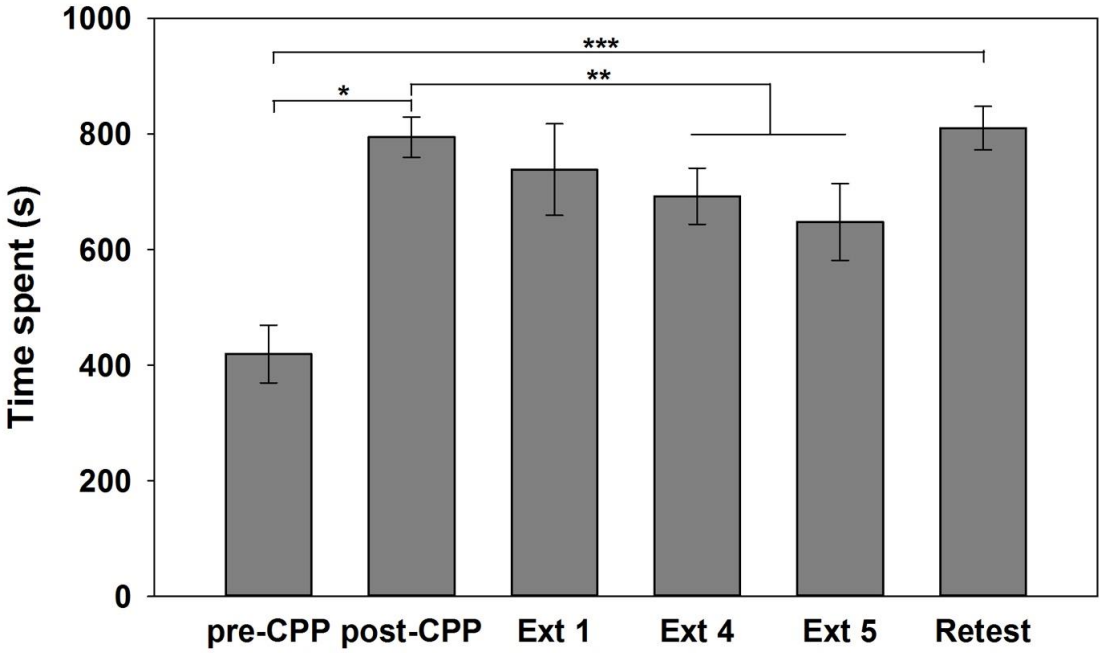

Figure 8. Time marmosets (n=5) spent (mean ± SEM; in seconds) in the methylphenidate (MPH) paired compartment of the CPP box before (pre-CPP; last habituation trial) and after (post-CPP; test trial) the conditioning, on the first, fourth and fifth extinction trial.

5.10. Discussion

To the best of our knowledge, this work is the first study focused on the rewarding properties of MPH in nonhuman primates (NHP) using the CPP behavioral paradigm. Results from the present study suggest that MPH has rewarding effect as indicated by the reinforcing effect of MPH-induced CPP in NHP. Our results are in parallel with previous evidence in male rats (Wooters, Walton and Bardo, 2011). In our study, the marmosets spent significantly more time in the MPH-paired compartment in comparison to the SAL-paired at post-CPP.

MPH acts as a DA and NE transporter inhibitor, leaving behind high levels of monoamines in the synaptic cleft, which will ultimately increase the level of extracellular dopamine in the brain (Volkow and Swanson, 2003). It is generally accepted that DA action in the Nucleus accumbens mediates

the rewarding effects of MPH (Di Chiara and Imperato, 1988). For example, MPH and cocaine have similar actions at the DAT and produce comparable increases in synaptic dopamine levels in baboons (N D Volkow et al., 1999).

However, neurobiological mechanisms underlying the pharmacological effects of MPH in young NHP, particularly marmoset monkey are not known. One possibility is that the key role of MPH effects involves dopaminergic D1 receptors, mediating the rewarding and reinforcing that produces long-lasting conditioning effects and reinstatement.

Vulnerability to relapse is a chronic condition in drug use disorders (Association, 2013). Results from our study showed that the CBD administration could affect extinction phase of MPH-induced CPP while did not decrease reinstatement. First, we argued that CBD is able to broadly block reward mechanisms as well as affect brain centers that lead to relapse. Animal studies have discovered many beneficial effects of CBD relevant for several relapse-promoting conditions including sensitivity to drug-related contexts and stress, anxiety, and impaired impulse control (Gonzalez-Cuevas et al., 2018). One study found that daily injections of CBD after conditioning trials but not during preference trials diminished preference-seeking behavior in the face of drug-related cues and potentiated the extinction of both AMPH-induced and cocaine-induced CPP learning. Thus, CBD facilitates the extinction of amphetamine and cocaine addiction and prevents cue-induced relapse (Parker et al., 2004).

Our findings are in line with previous work showing that CBD (10 and 20 mg/kg, I.P.) did not affect lever pressing induced by heroin during extinction training (Ren et al., 2009). In another study, CBD does not exhibit an impact on the alcohol addiction intoxication phase in humans, and again, no data were found on the other phases of this addiction (Prud'homme, Cata and Jutras-Aswad, 2015).

CBD probably has interaction with dopamine receptors, which play a crucial role in regulating many aspects of behavior and cognition, especially reward-seeking. Apart from dopamine, other neurotransmitter systems may be involved in drug reinforcement initiation including serotonin (5-HT), NE, glutamate (GLU), GABA, opioid peptides and endocannabinoids(Lee et al., 2003). CBD help modulate the endocannabinoid system, it can influence the release of neurotransmitters as well as play a role in the modulation of extracellular levels of DA in the brain (Murillo-Rodríguez et al., 2011). In our study it appears that CBD given alone has little effect on CPP. For instance, rats treated with 10 mg/kg CBD indicated neither CPP nor CPA (Vann et al., 2008). It is also important to note that in this initial study, we used only a dose of CBD that is effective during the conditioning and extinction sessions. Therefore, it is possible that lower or higher doses may have differential effects on factors that facilitate or inhibit the reward systems in NHP.

Our results demonstrate that MPH is a reinforcer and that its reinforcing efficacy may be associated with brain's reward circuitry following increased dopamine activity. Daily injection of MPH may have dramatic and longer-term impact on brain and tend to lead to reinstatement. Also, these results

show that the CBD affect extinction period but could not decrease reinstatement to MPH.

Finally, it is believed that further studies are needed to clarify the real impact of the use of psychostimulants, especially MPH, on the development of behavioral sensitization and neural mechanisms of relapse. It should be noted that effects of MPH on reinstatement depend on several factors, such as the animal model, drug dose administered, type of experimental parameters and maybe role of genetic condition as well as sex.

6. STUDY 2: DOSE-RESPONSE EFFECTS OF MORPHINE AND MPH AND THE EFFECT OF CBD ON MPH-INDUCED CONDITIONED PLACE PREFERENCE, DURING EXTINCTION/ REINSTATEMENT

The second study aimed to evaluate the effective dose of Morphine and MPH in the CPP task.

6.1. Experimental subjects

Ninety four male adult Wistar rats weighing 200–250 g were used in this study.

6.2. Experimental groups

6.2.1. Experiment 1: Dose-response effects of morphine and MPH on the CPP

In this set of experiments, animals were assigned to 5 groups and received different doses of morphine (5, 10, mg/kg, s.c.) or MPH (1, 2.5 and 5 mg/kg; I.P.) during conditioning period, and the control group

animals received saline (Fig.8-A and -B). Conditioning score was calculated for each rat.

6.2.2. Experiment 2: Dose response of MPH on the reinstatement of MPH

In this experiment, animals were divided two groups. We selected two doses of MPH (1, 2.5 mg/kg; I.P.) based on dose response experiment. Animals received 1 or 2.5 mg/kg of MPH during conditioning period. The rats after passed the post-conditioning and extinction phases were treated with an ineffective priming dose of MPH (0.25 or 0.5 mg/kg; I.P.) just before the reinstatement test (Fig.8-C).

6.2.3. Experiment 3: Effect of ICV administration of cannabidiol on the MPH-extinction in CPP

The rats were randomly assigned to four groups, including: Naïve, DMSO, CBD-10 and CBD-50 groups. Animals after been exposed to the effective dose of MPH (2.5 mg/kg, I.P.) during conditioning phase, received - 60 min prior to the MPH-extinction sessions - ICV infusion of CBD (10, 50 µg/5µl), or vehicle (DMSO), or no injection at all (Naïve group; that is, animals were conditioned but did not receive any cannabidiol/vehicle during extinction period (Fig.8D). CPP scores were calculated.

6.2.4. Experiment 4: The effect of ICV administration of cannabidiol on the reinstatement of MPH

The animals were divided to four groups including: Naïve, DMSO, CBD-10 and CBD-50 groups. All animals received the MPH (2.5 mg/kg;

I.P.) during conditioning days and MPH (0.5 mg/kg; I.P.) on the reinstatement day. The Naïve group did not received CBD on the reinstatement day while the CBD-10 and CBD-50 groups received 10 and 50 (µg/5 µl DMSO; icv) of CBD on reinstatement day. One hour after ICV microinjection, animals were placed in the start box with access to the entire apparatus for 10 min and time spent for each chamber was measured on the reinstatement day (Fig. 8E).

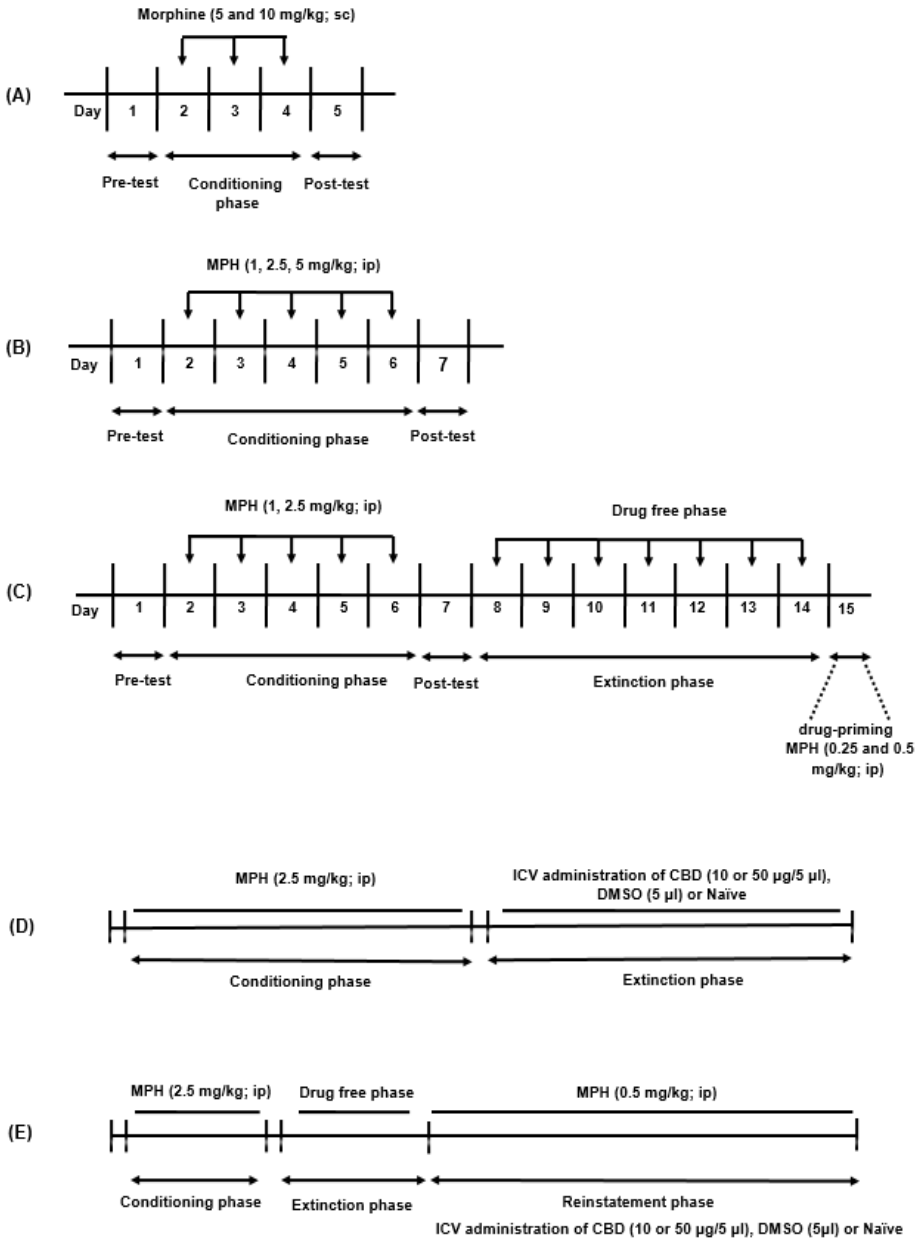

Figure 9. Schematic diagram A, to show different doses of morphine (5, 10 mg/kg, s.c.) and B, MPH (1, 2.5 and 5 mg/kg, I.P.) on the conditioned place preference (CPP) during 3 and 5 days respectively. C, Determine the priming doses of MPH in the reinstatement of MPH-induced CPP, the animals received MPH (0.25 or 0.5 mg/kg; I.P.) on the reinstatement day and then placed into CPP box. D, to investigate the effect of CBD on extinction phase of MPH-induced CPP, after exposed to the effective dose of MPH (2.5 mg/kg, I.P.) the animals were naive or received CBD (10, 50 µg/5µl) or vehicle (DMSO 10%) 60 min before to the extinction sessions. E, to find out whether CBD can inhibit reinstatement of MPH-induced CPP in rats, animals received CBD or vehicle 60 min before the start of the reinstatement session and then injected by MPH (0.5 mg/kg; I.P.).

6.3. Behavioral section

6.3.1. Conditioning place preference apparatus for rats

CPP paradigm was used to evaluate the effects of intracerebroventricular of CBD on MPH-induced conditioned place preference during extinction and reinstatement.

6.3.1.1. Apparatus

METH-induced CPP was conducted in rectangular wooden chambers that had three compartments; two compartments were identical in size (30 cm × 30cm × 40 cm) but differed in shading and texture. The third chamber (null compartment) was just a protruded tunnel (30 × 15 × 40 cm3) connecting two main chambers. In this apparatus, animals demonstrated no consistent preference for either compartment, a study that supports our unbiased CPP paradigm. Time spent in each compartment and motor activity was monitored via recorded by a 3CCD camera (Panasonic Inc., Japan) located 2 m above the apparatus (Figure 9).

All experiments were done in a quiet and isolated room under a constant light and sound situation. The room was equipped with a light centered above the compartment, turned on every session (Arezoomandan et al., 2016; Ebrahimian et al., 2016; Karimi et al., 2014).

Figure 10. CPP procedure for evaluate the reinforcing effects of natural and pharmacological stimuli, including rewarding, (Photo: Reza Arezoomandan,IRAN).

6.4. Experimental procedure

The CPP paradigm consisted of three testing phases occurring on consecutive days including pre-conditioning (1 day), conditioning (5 days) and post-conditioning (1 day).

6.4.1. Pre-conditioning phase:

The male rats were transported from the animal housing room to the testing room at least 30 min before the experiment begins, for habituation. During this phase (day 1), each animal was placed separately in the start box with the removable door removed and the rats were allowed to move freely in all three chambers for 10 min. The distance traveled and time spent in each compartment was recorded using a 3CCD camera (Panasonic Inc., Japan) and locomotion tracking was measured by Ethovision software (Version 7), a video tracking sys-tem for automation of behavioral experiments (Noldus Information Technology, the Netherlands). In unbiased paradigm setup used in this study, the animals should not show any preference for either of two compartments. Animals that spent ≥70% of the total test time in one chamber compared to another were considered to have initial bias and were excluded from the study.

6.4.2. Conditioning phase:

Conditioning phase started one day after pre-conditioning test and consisted of a 5-day schedule. During the conditioning phase, the rats were injected with MPH intraperitoneal (IP) (2.5 mg/kg) in the morning and immediately confined to the drug-paired compartment for 30 min sessions; about 6 h later, the rats were injected with saline as a vehicle and immediately placed in the saline-paired compartment for 30 min. On the next

day, the rats were injected with saline in the morning and MPH in the afternoon. The injection schedule of the fifth day of conditioning was the same as the first day. During sessions, the animals were confined to one compartment by closing the removable wall. This procedure was repeated until day six. Control animals received only saline instead of MPH.

6.4.3. Post conditioning phase:

The post-conditioning test was one day after the last conditioning session on the seventh day of study. For testing, the removable wall was removed and rat was allowed to access the entire apparatus for 10 min and the time spent in each chamber was recorded and analyzed using the Ethovision software. The time spent in the MPH-paired compartment minus time spent in the saline-paired compartment was considered as conditioning scores (CPP scores). The total distance traveled by each animal was also recorded in control and experimental.

The locomotor activity of each animal was recorded using the locomotion tracking apparatus by a video tracking system (Ethovision software). In these experiments, the total distance traveled (in centimeters) for each animal was measured in pre- and post-tests for the control and experimental groups.

6.5. Extinction

Following establishment of CPP, the rats were given daily ICV injection of either vehicle or CBD into the ventricle (10 or 50 µg/5 µl) in their home cages. During this phase, 60 min after injection, rats were placed in the CPP box and tested for CPP. This procedure was repeated for each rat in the control and experimental groups until the measured CPP score in two

consecutive days in extinction period became similar to those in the pre-conditioning day (the CPP score achieved its level on the pre-conditioning day for two consecutive days) (Attarzadeh-Yazdi, Arezoomandan and Haghparast, 2014).

6.6. Reinstatement

Reinstatement can be dependably induced by exposure to cues previously associated with drug reinforcement following extinction (Hiranita et al., 2006). In our study, one day after the last extinction day, the rats received CBD vehicle into the ventricle and 60 min after microinjection; they received a priming injection of MPH (0.25 or 0.5 mg/kg, IP). Then the animals were immediately placed in the start box with access to the entire apparatus and time spent, distance traveled in each compartment and finally conditioning score was measured for 10 min. (Attarzadeh-Yazdi, Arezoomandan and Haghparast, 2014; Arezoomandan et al., 2016).

6.7. Locomotion tracking apparatus

The locomotor activity of each rat was recorded by the locomotion tracking apparatus using a video tracking system (Ethovision software). In these experiments, the total traveled distance (in centimeters) for each animal was calculated in pre- and post-tests for the control and experimental groups.

6.8. CPP score and animal movement

The CPP score represents the time spent in the drug paired place minus the time spent in saline paired place. Total distance traveled for each animal

was also recorded in order to evaluating the locomotor activity in all control and experimental groups.

6.9. Surgery and microinjection procedures

The rats were anesthetized with intraperitoneal injection of a mixture containing ketamine (100 mg/kg) and xylazine (10 mg/kg) and were placed in a stereotaxic frame (Stoelting, USA). The stereotaxic (Figure 11) coordinates for the lateral cerebral ventricle were as follows: 1.6 mm lateral and 0.5 mm posterior to bregma, 4.2 mm deep from dura. The guide cannula was fixed in place using a stainless steel screw in the skull and dental acrylic cement. The rats were maintained and allowed to recover from surgery for 5 to 7 days. The injection unit was a polyethylene tube (PE20) connected to a 5μL Hamilton syringe and a 30 gauge needle with 11mm length in the tip. The syringe was filled with the appropriate drug volume, and then the injection needle was inserted through the guide cannula (10 mm) (Figure 12).

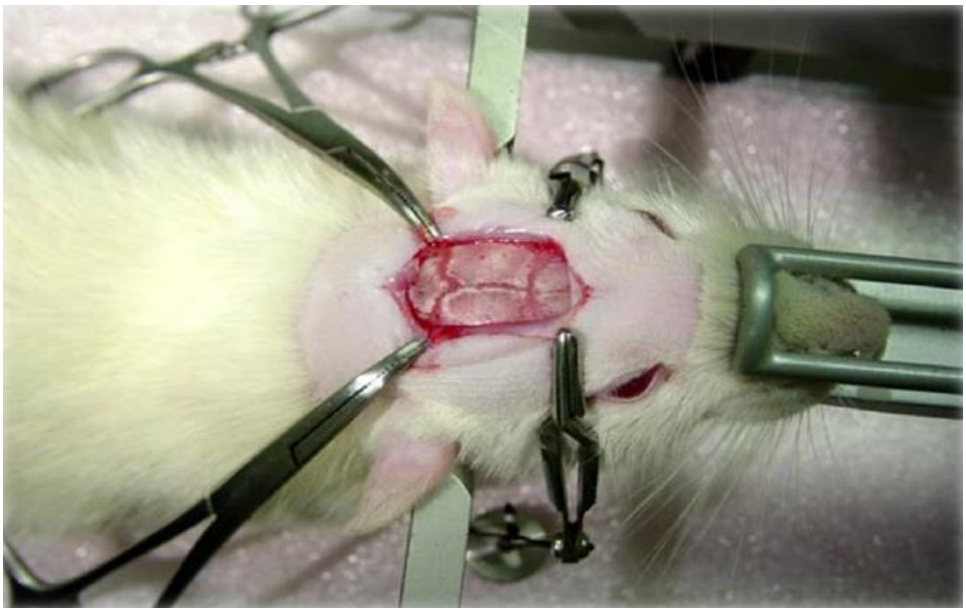

Figure 11. Stereotaxic method in rat (Photo: Adel Kashefi, Iran).

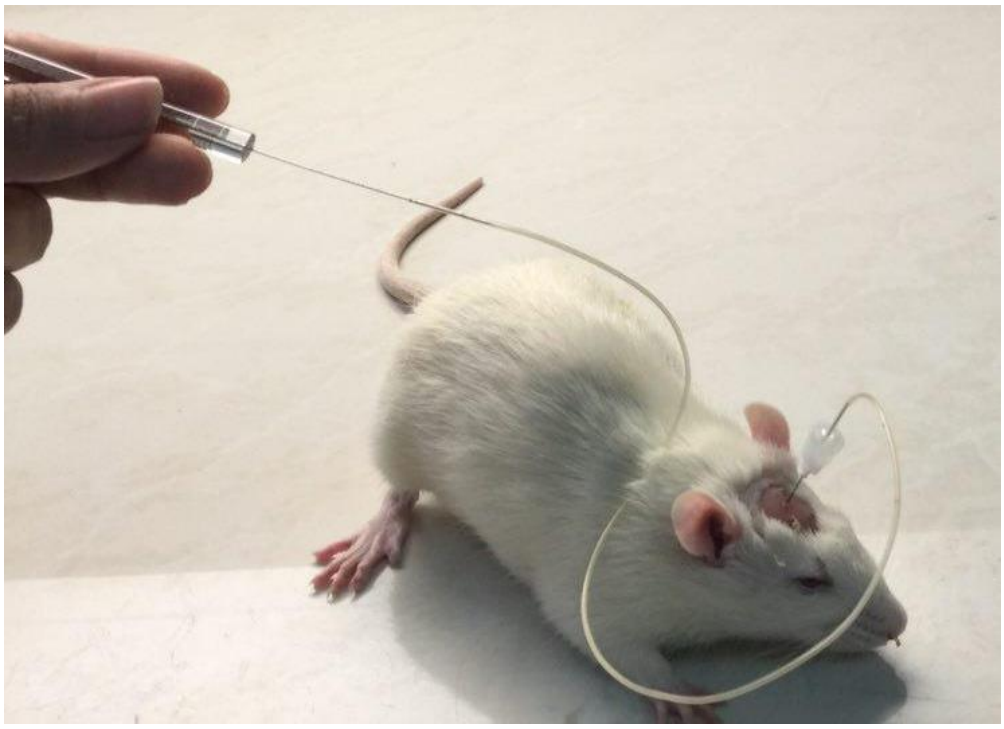

Figure 12. Intracerebroventricular (ICV) injection (Photo: Fatemeh Sadeghzadeh, Iran).

6.9.1. Histology

After completion of behavioral testing, including extinction and reinstatement experiments, the animals were deeply anesthetized with ketamine and xylazine. Then, they were transcardially perfused with 0.9% saline and 10% formalin solution. The brains were removed, fixed, and cut coronally in 50 µm sections through the cannula placement. The neuroanatomical location of cannula tip placement was confirmed using Paxinos and Watson rat brain atlas (Paxinos & Watson, 2007). Only the animals with correct cannulae placements were included in the data analysis (Figure 13).

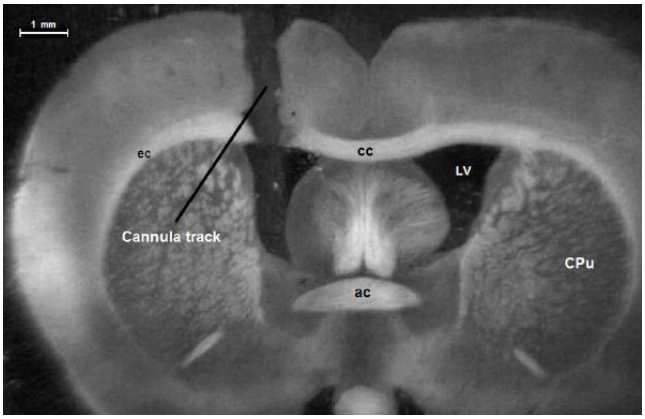

Figure 13. Coronal photomicrograph of representative cannula placement and unilateral microinjection site (CBD or vehicle [DMSO]) in the lateral ventricle of the rat brain (Photo: Adel Kashefi, Iran).

6.10. Statistical analysis

Data were processed by the software GraphPad Prism®5.0. In order to compare the CPP scores and distance traveled obtained in all groups (vehicle and experimental groups). A statistical analysis for place conditioning study was performed using one-way ANOVA followed by post-hoc analysis (Dunnett's or Newman-Keuls test) or Student' t-test (for two-paired comparison). P-values less than 0.05 ($P < 0.05$) were considered to be statistically significant.

6.11. Result

6.11.1. The effect of different doses of morphine and MPH on conditioned place preference

One-way ANOVA followed by Newman Keuls multiple comparison test [$F(3, 37) = 14.79$, $P < 0.001$; Fig. 14] was used to compared the CPP score of saline, morphine and MPH groups. The results showed that there is a significant difference between the conditioning scores of morphine groups (5 and 10 mg/kg; s.c.) and two higher doses of MPH (1 and 2.5 mg/kg; I.P.) with saline group and vehicle ($P < 0.001$). Whereas there is no significant difference between the CPP score of morphine induced-CPP and MPH-induced CPP. Also, 5 mg/kg of MPH, could not induced CPP compare to saline group.

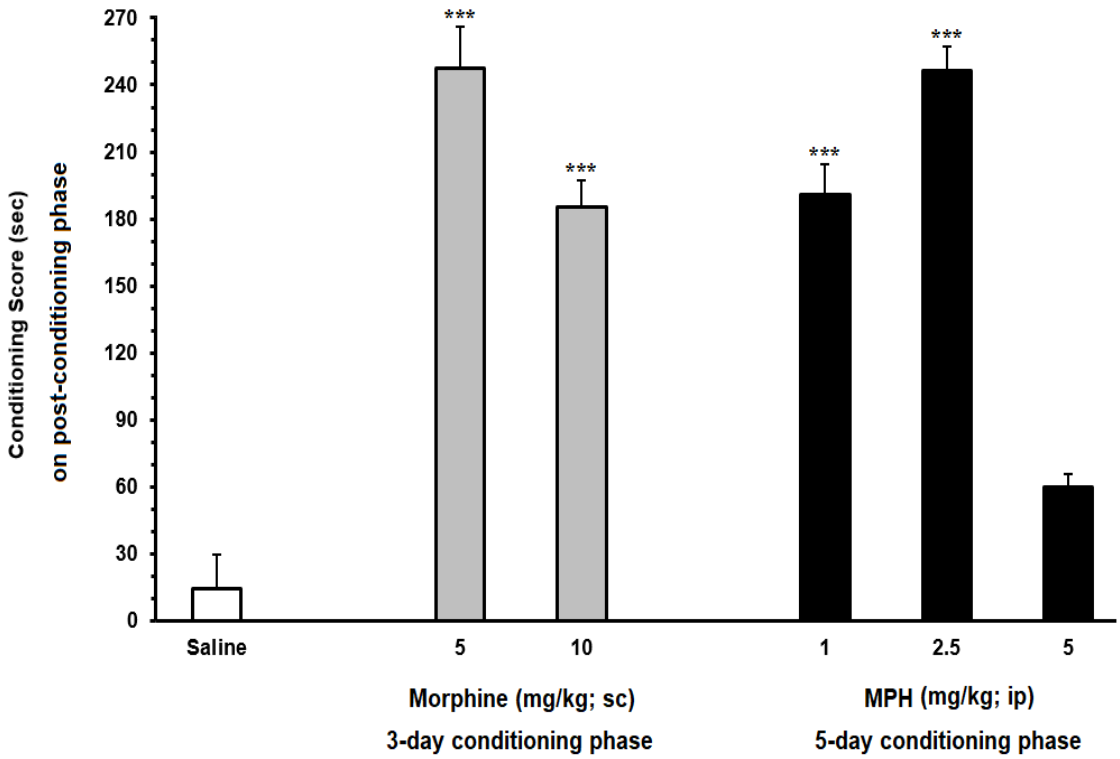

Figure 14. The effects of different doses of morphine and MPH in the CPP paradigm. Morphine induced CPP in 5 and 10 mg/kg and MPH 1 and 2.5 mg/kg doses. Intraperitoneally (IP) administration of 5 mg/kg MPH during conditioning season couldn't induce preference.

6.11.2. The effect of priming dose of MPH on the reinstatement of MPH

After the establishment of MPH-induced CPP, during the extinction period, without any injection, the CPP score was calculated every day. As shown in (Fig. 16A and B), one way repeated measures ANOVA followed by Newman Keuls multiple comparison test or the Tukey's [F (9, 79) = 6.996, P < 0.0001], accepted that the CPP induction by MPH (1 mg/kg) gradually diminished over days and animals in these groups had extinguished their preference for the MPH-paired compartment on the seventh day of extinction. There was no significant difference between the CPP score of

pre-conditioning, the sixth and seventh extinction days. After the last extinction day, the animals were tested for reinstatement. The statistical analysis of acquired data from the paired samples t-test indicated that IP injection of MPH-priming dose (0.25 mg/kg) could induce reinstatement as the CPP score on reinstatement day significantly increased compared to last day of extinction [t (7) = 3.51, $P < 0.05$].

In another part of this experiment (Fig.18), one way repeated measures ANOVA followed by Newman Keuls multiple comparison test or the Tukey's post-test [F (9, 79) = 13.57, $P < 0.0001$] showed that MPH treatment during the conditioning phase (2.5 mg/kg; I.P.) induced the place preference. There was no significant difference between the CPP score of pre-conditioning and the sixth and seventh extinction days. The conditioning score between these groups (1 and 2.5 mg/kg) were not statistically different, but CPP induced by 2.5 mg/kg was slightly greater than 1 mg/kg; therefore we selected (2.5 mg/kg) dose of MPH for the rest of experiments. The statistical analysis of acquired data from the paired samples t-test indicated that injection of MPH-priming dose (0.5 mg/kg) could induce reinstatement and CPP score on reinstatement day significantly increased compared to last day of extinction [t (7) = 8.199, $P < 0.001$].

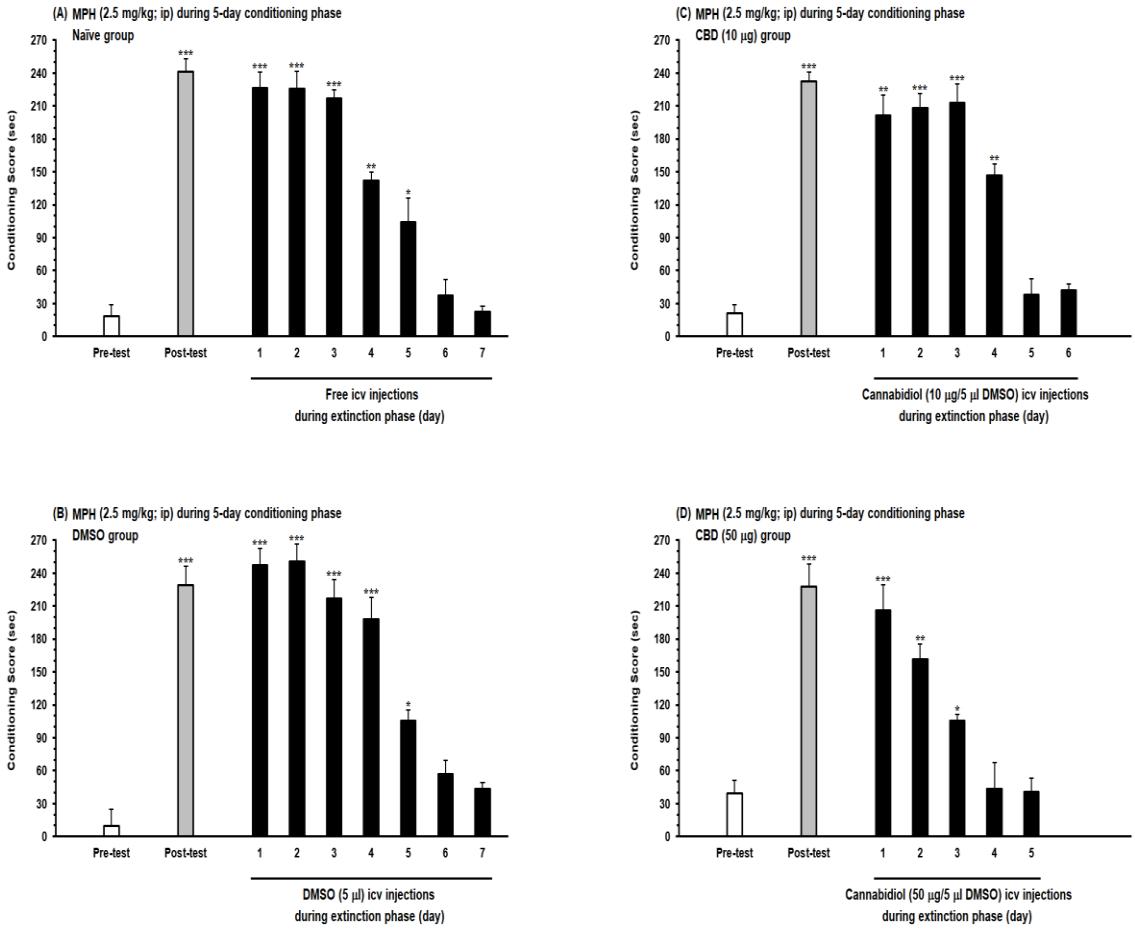

Figure15. The effects of microinjection of (A) Naïve or (B) DMSO compared to different doses of CBD (C) 10 µg/5 µl and (D) 50 µg/5 µl on the extinction phase (days) of MPH-induced conditioned place preference in rats. Each column represents the mean ± SEM of 5–8 rats.

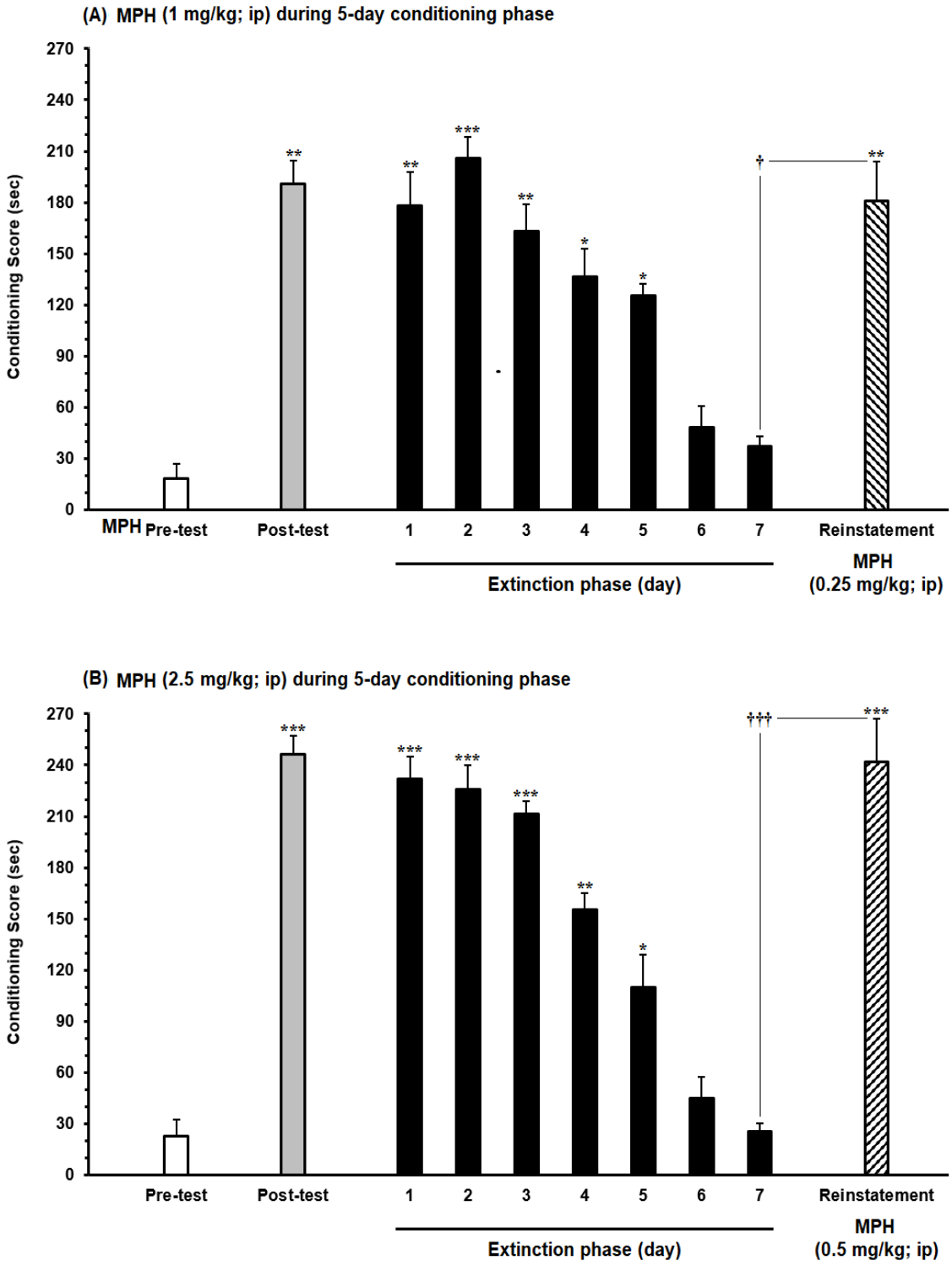

Figure 16. The effects of MPH-administration (0.25 or 0.5 mg/kg; I.P.), as priming dose, on the reinstatement of extinguished MPH-CPP in the rats. The established drug-induced CPP, during the extinction, without any injection (A) MPH 1mg/kg and induced reinstatement by priming dose 0.25 mg/kg (B) MPH 2.5 mg/kg and induced reinstatement by priming dose 0.5 mg/kg. Each column represents the mean ± SEM of 5–8 rats.

*P < 0.05, **P < 0.01, and ***P < 0.001 different from the pretest day.

†P < 0.05, ††P < 0.01 and †††P < 0.001 different from post-test day.

†P<0.05, ††P<0.01, and †††P<0.001 different from the last extinction day.

6.11.3. The effect of ICV administration of CBD during the extinction phase, on the extinction latency of MPH-induced CPP

In this set of experiments, we evaluated the effects of CBD injection in extinction period. The one way repeated-measures ANOVA followed by Newman Keuls multiple comparison test or the Tukey's post-test was used to compare the CPP score among pre-test, post-test and extinction-days. Figure 15-A and -B showed that the MPH-induced CPP was extinguished after seventh day of extinction phase in the both Naïve group and DMSO group [F (8, 62) = 11.89, P < 0.001]. Figure 15-C indicate that the MPH-induced CPP of animals that were microinjected by CBD into ICV during extinction phase (10 µg/5 µl), was extinguished after sixth day [F (7, 63) = 18.28, P < 0.001]. The ICV injection of CBD significantly displayed shorter extinction period in these groups compared to control groups (Naïve and/or DMSO). In addition, duration of extinction phase of the animals which received dose 50 µg/5 µl CBD was shorter compared to those that microinjected by 10 µg/5 µl CBD, 5 and 6 days, respectively (Fig. 15C and D). Therefore, the CBD 50-group displayed shorter days of extinction compared with other groups [F (7, 62) = 23.28, P < 0.001; Fig. 15].

Figure 17 shows the role of ICV injection of CBD (10 and 50 µg/5 µl) in the extinction latency of MPH-induced CPP during extinction phase in four experimental groups including Naïve, DMSO, CBD 10 and CBD 50. The extinction latency was defined as a 50% decrease in CPP score compared to post-conditioning phase. One-way ANOVA followed by Newman-Keuls multiple comparison tests [F (3, 30) =10.31; P = 0.0002] showed that extinction latency changes in treatment groups compared to the Naïve

and/or DMSO group. The CBD-10 and CBD-50 groups that microinjected by the doses 10 and 50 µg/5 µl of CBD significantly displayed shorter extinction latencies compared to both Naïve and/or DMSO groups (P < 0.05 and P < 0.001, respectively) and also shorter extinction latency in CBD-50 compared to CBD-10 (P < 0.05) was observed. Furthermore, no difference was found on extinction latency between the Naïve and DMSO groups (Fig. 17).

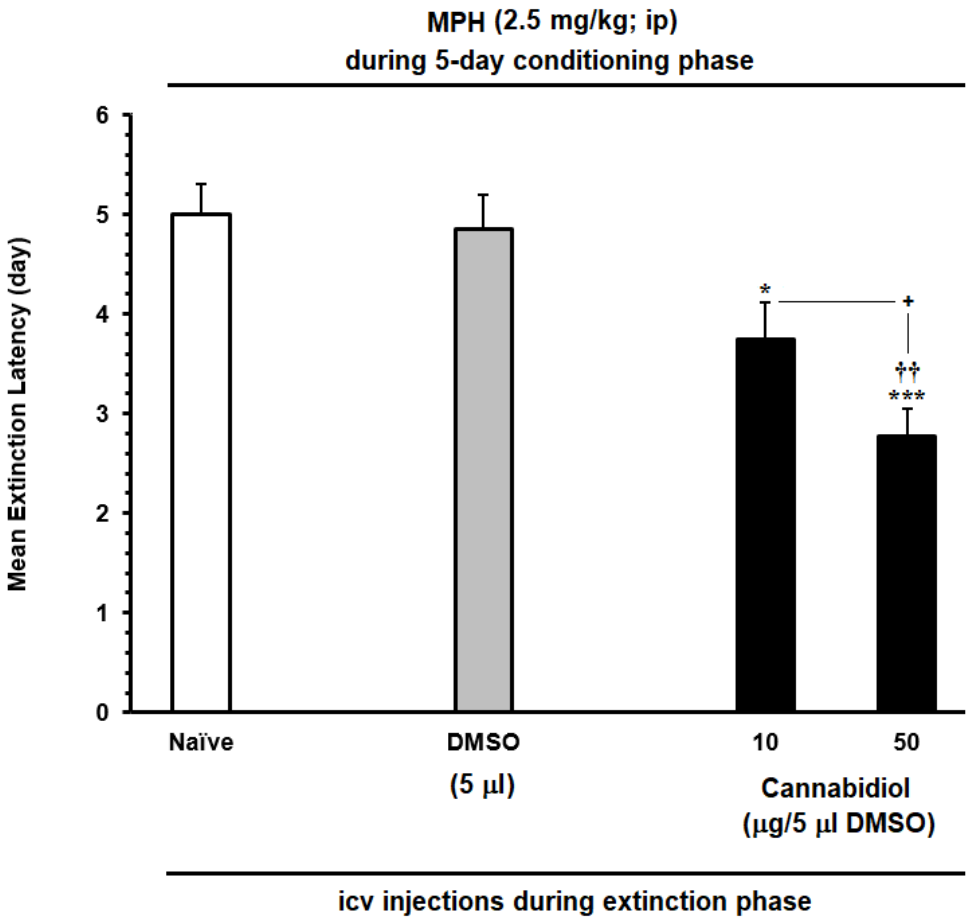

Figure 17. Effects of ICV daily injection of CBD (10 and 50 µg/5 µl) during extinction phase on the extinction latency of MPH-induced conditioned place preference compared to Naïve and/or DMSO in rats. Each bar is represented by the mean ± SEM for 5–8 rats.

*P < 0.05, **P < 0.01, and ***P< 0.001 as compared with the Naïve group.

††P < 0.01 as compared with the DMSO control group.

6.11.4. The effect of ICV administration of CBD on the reinstatement of MPH-induced CPP

Experimental groups were treated MPH (2.5 mg/kg) for five days. On the reinstatement day, the animals were administered with CBD (10 or 50 µg/5 µl) or vehicle alone (DMSO) with ICV injection and then received a single dose of MPH (0.5 mg/kg, I.P.), as priming dose, and were tested for reinstatement to MPH-induced CPP for 10-min. The Naïve group did not receive ICV injection. The one-way ANOVA followed by Tukey's test revealed a significant difference in CPP score between Naïve or DMSO groups compared to animals that received different doses of CBD on reinstatement day [$F(3, 29) = 17.67$, $P < 0.001$; Fig. 18]. In addition, results showed a significant difference between the CPP score of animals microinjected by CBD-10 and CBD-50 ($P < 0.1$). These results demonstrated that ICV-microinjection of CBD during extinction phase could depress the reinstatement to MPH-induced CPP in a dose dependent manner.

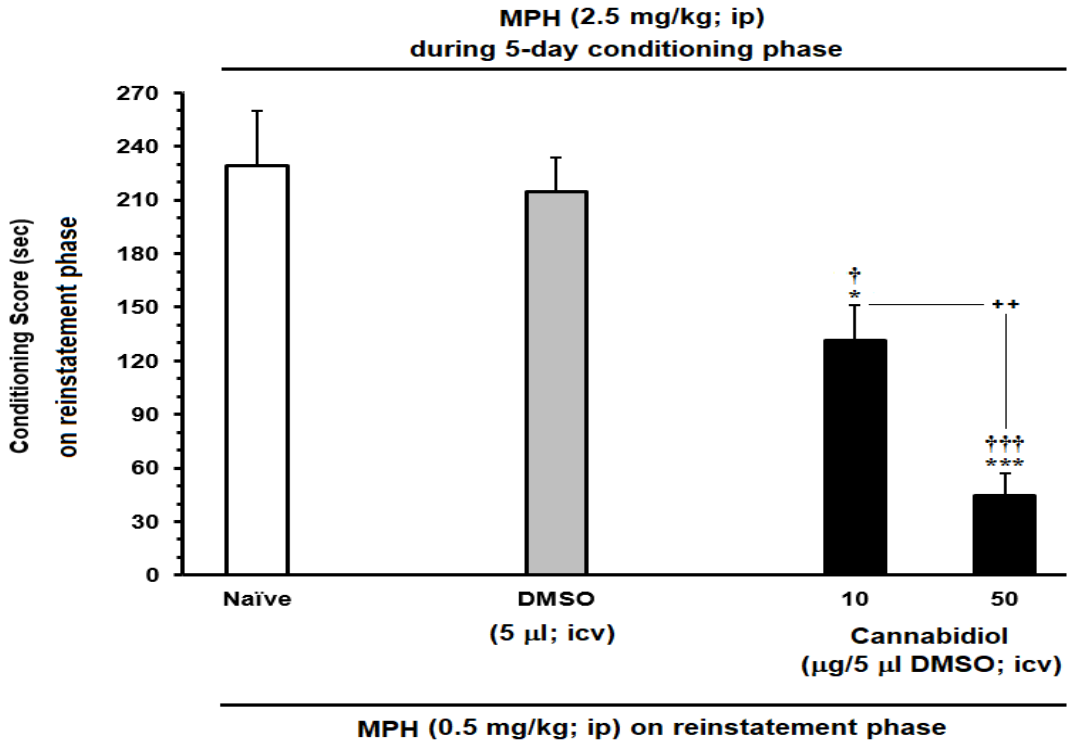

Figure 18. Effect of ICV administration of CBD (10 and 50 µg/5 µl, icv) on the preventing the reinstatement induced by priming dose of MPH (0.5mg/kg; I.P.), compared to Naïve and/or DMSO control group. Animals received CBD or vehicle during on reinstatement day before injecting the priming dose of MPH (0.5 mg/kg, I.P.). Each bar is represented by the mean ± SEM for 5–8 rats.

*P < 0.05, **P < 0.01, and ***P < 0.001 as compared with the Naïve group.

†P < 0.05, ††P < 0.01 and †††P < 0.001 as compared with the DMSO control group.

++P < 0.01 as compared with other dose of CBD.

6.12. Discussion

The results of the current study report several important findings relative to the rewarding associative effects of MPH and are consistent with our previous study in monkeys (Kashefi et al., 2019).

It has been postulated that sensitizing properties of drugs of abuse plays a crucial role in drug-seeking behavior that persists long after withdrawal period (Robinson and Berridge, 1993). Drug-primed reinstatement of CPP is

thought to activate appetitive motivational mechanisms that are involved in the reinitiating drug seeking behavior (Powell, Bradley and Gray, 1992). Thus, by this view, drug craving and addictive behavior are due exclusively to sensitization of incentive salience (Robinson and Berridge, 1993).

Our experiments show that (i) morphine and MPH produced a CPP in adult male rats. Also, this study demonstrated that (ii) the CBD injection produced shorter extinction latency in treated groups compared to control groups, (iii) The priming dose of MPH (0.25 or 0.5 mg/kg; I.P.) could induce reinstatement of MPH, (iv) the CBD able to prevent the reinstatement of MPH-induced CPP. The key point of drug addiction research is the development of treatments that diminish craving and, consequently, reduce the vulnerability to drug-use relapse in psychostimulant abusers (Blanco-Gandía et al., 2018). The results of this study showed that morphine (5 and 10 mg/kg; s.c.) and MPH (1 and 2.5 mg/kg; I.P.) but not 5 mg/kg produced significant CPP. High dose of MPH (5mg/kg), produced a profound suppression of evoked responses and finally could lead to differences in the behavioral response that was probably associated with locomotor activation and stereotypy (see Devilbiss and Berridge, 2008) and this may also be related to the decrease of the dopamine transporter in both the nucleus accumbens and striatum brain areas that mediate reward (Freeman, 2013). Subsequently, priming dose of MPH (0.25 or 0.5 mg/kg; I.P.) could reinstate the CPP induced in rats after extinction phase that is consistent with our earlier study (Kashefi et al., 2019).

Several studies have shown that dopamine (Kim et al., 2016; Sadeghzadeh, Babapour and Haghparast, 2017; Guerrero-Bautista et al.,

2019; Yazdani et al., 2019) and other neurotransmitters such as acetylcholine (Zannone et al., 2018), (Daza-Losada et al., 2007; Vidal-Infer et al., 2012), glutamate (Leão, Cruz and Planeta, 2010; Chesworth et al., 2013; Siahposht-Khachaki et al., 2017; Zhang et al., 2019) and orexin (Qi et al., 2013; Tung et al., 2016; Edalat et al., 2018) plays an essential role in reinstatement. The mesolimbic dopaminergic system appears to be the major neuroanatomical substrate of behavioral sensitization and release of DA from neurons caused reinforcing behavior (De Vries et al., 1998). In the current study, morphine-induced CPP seems to be a result of the rewarding properties of abused drugs involving the mesolimbic dopamine system (Kim et al., 2016). MPH also exerts dopaminergic effects through the mesolimbic pathway in the ventral tegmental area and NAc (Volkow and Morales, 2015). Other studies have shown similar associations of dopamine D1 and D2 receptors in the development of sensitization to the rewarding properties of drugs such as MPH (Robinson and Berridge, 1993). For instance, it has been shown that the D1-receptor antagonist, SCH23390, but not the D2-receptor antagonist, raclopride, blocked high dose (7.5 mg/kg) MPH induced CPP. These data together with our present findings suggest a mechanistic link between dopamine and CPP and that the reported activation of dopamine D1 and D2 receptors can be essential for modulating reward-seeking behavior in several brain regions (Zhu et al., 2011). On the other hand, other common neurotransmitters including serotonin and norepinephrine receptors play an important role in MPH reward behaviors. Some studies show that the administration of MPH causes an upregulation of 5-HT7Rs (Leo et al., 2009; Adriani et al., 2012) and inhibit the reuptake

norepinephrine and increasing the availability of these neurotransmitter in synaptic clefts and thus producing stimulatory effects (Freese et al., 2012).

We found that injection of both doses of CBD (10 or 50 µg/5µl; icv) significantly facilitated extinction of MPH-induced compared to vehicle group in rats.

Our findings are consistent with those of previous studies and showed that CBD had an inhibitory effect on reward-facilitating effect (Katsidoni, Anagnostou and Panagis, 2013; de Carvalho and Takahashi, 2017) and potentiated the extinction of cocaine- and AMPH-induced CPP (Parker et al., 2004; de Carvalho and Takahashi, 2017; Hay et al., 2018). For instance, microinfusion of CBD into the NAc region caused widespread reductions AMPH-induced sensitization and VTA DA neuron activity (Renard et al., 2016).

It has been established that CBD significantly enhanced serotonin and glutamate levels by the 5-HT1A receptor (Linge et al., 2016) and also Norris et al. reported that intra-NAc CBD by blocking the dopaminergic neurons in the VTA region prevented the formation of fear-related memories (Norris et al., 2016) while the expression of c-Fos increases by CBD in NAc (Guimarães et al., 2004) and it's expression can be considered as a marker for neuronal activation (Kovács, 1998). The results of our studies showed that both treatment groups (10 and 50 µg/5 µl) significantly displayed shorter days and latency during of extinction compared to both control groups. It seems that CBD is able to produce changes in neurotransmitters, intracellular signaling and ultimately facilitate MPH extinction.

CBD appeared to be effective in reducing the reinstatement of drug-use. Notably, systemic administration of CBD blocked the reward facilitating effect of morphine (Katsidoni, Anagnostou and Panagis, 2013). It has been made clear that CBD specifically disrupted the reconsolidation of drug-related memories associated with different classes of substance of abuse independent of its emotional nature (hedonic or aversive) in Wistar rats (de Carvalho and Takahashi, 2017). A recent study demonstrated that CBD impaired the reconsolidation of cocaine-CPP and prevented priming-induced reinstatement of METH CPP (Calpe-López, García-Pardo and Aguilar, 2019). Potential therapeutic benefits of CBD have been established in several relevant domains for heroin (Ren et al., 2009), crack and cocaine (Zuardi, Rodrigues and Cunha, 1991; Zuardi, 2008; Leweke et al., 2012; Morgan et al., 2012; Schubart et al., 2014; Iseger and Bossong, 2015) in which use related symptoms and problems. In general, our results on the effects of CBD on MPH-induced CPP are in agreement with the view that CBD may attenuate the rewarding effects of drugs of abuse (Ren et al., 2009; Raineki et al., 2011; Katsidoni, Anagnostou and Panagis, 2013). However, the exact mechanisms underlying the CBD effect are not fully understood. It has been shown that the block of the rewarding effects of cocaine in the CPP by CBD was equal to the increase in the expression of CB1 receptors in the hippocampus (Luján et al., 2018) and an another study demonstrated that CBD administration reduced the gene expression of the CB1 receptor in the NAc (Viudez-Martínez et al., 2018). Another alternative possibility is that CBD mediate activation of 5-HT1A receptors that finally lead to inhibit extracellular concentrations of serotonin and/or attenuate

mesolimbic activity. As mentioned above CBD is probably able to reverse the increase in the activity of the mesolimbic DA reward system.

The other part of the present study showed that the ICV administration of CBD (50 or 10 µg/5 µl) could prevent the MPH-induced reinstatement and in accordance with our study recently reported that CBD (10 µg/5µl, icv) suppressed priming-induced reinstatement of METH CPP in animal models (Calpe-López, García-Pardo and Aguilar, 2019) and also demonstrated that CBD impaired the reconsolidation of cocaine-CPP and prevented priming-induced reinstatement of METH CPP (Calpe-López, García-Pardo and Aguilar, 2019).

Interestingly, the data obtained in this part of our study indicated that CBD had similar results to the extinction period and only a single dose of it could suppress the MPH-induced reinstatement.

It has been reported that D2 partial agonists, such as aripiprazole or terguride, reduced the self-administration of METH (Wee et al., 2007), cocaine (Feltenstein, Altar and See, 2007; Sørensen et al., 2008; Feltenstein, Do and See, 2009) and AMPH (Bäckström, Etelälahti and Hyytiä, 2011). Therefore, CBD may have an anti-reward effect as a partial agonist of DA D2 receptors and inhibit reinstatement of psychostimulant drug seeking behavior (Seeman, 2016). Psychostimulant drugs like METH increase serotonin and demonstrated that selective 5HT1A agonists reduced the hyperactivity and the psychomotor sensitization induced by METH (Ago et al., 2006; Picard et al., 2010). CBD may acts as a modulator of 5-HT receptors (Izzo et al., 2009) and can hypothesis that CBD could block the

rewarding effects of psychostimulant drugs through an agonistic action on post-synaptic 5-HT1A receptors (Müller et al., 2007).

CBD can act clinically as a partial agonist at the dopamine D2 receptors (Seeman, 2016). It has been reported that D2 partial agonist probably had inhibitory effects on reinstatement of cocaine-seeking behavior (Feltenstein, Altar and See, 2007). In addition, CBD was shown to attenuate AMPH induced sensitization of the mesolimbic system, which involves the reward pathway in the brain.

Besides, CBD acts via the enhancement of both serotonergic and glutamate cortical signaling through a 5-HT1A receptor (Katsidoni, Anagnostou and Panagis, 2013). As previously mentioned, these neurotransmitters play a significant role in drug reinstatement. So, it is expected that CBD can inhibit the reinstatement of MPH by manipulating the different types of neurotransmitters.

Although many studies have suggested a diverse set of brain regions in reward-related behaviors, it is still unclear which of these regions contain information that shows the direct effect of CBD on reinstatement of MPH-induced CPP. For example, intra-accumbal administration of CBD dose-dependently inhibits the formation of associative fear memories and prevents the activity of dopaminergic neurons in the ventral tegmental area (Norris et al., 2016). Other study showed that CBD can induce a strong c-Fos immunoreactivity in the NAc, which is involved in the modulation of the reward system (Guimarães et al., 2004).

In conclusion, the observations of this study suggest the potential use of CBD as a treatment strategy given its specificity to attenuate cue-induced

reinstatement of MPH seeking behavior after extinction, possibly preferential impact on mesolimbic neuronal populations, and enduring neural actions. It has known that CBD modulates the endocannabinoid receptors, however, these interaction, are not completely understood. Thus, despite the unknown mechanisms that mediate the CBD actions, it can be seen with potentials for the treatment of addiction. The research shows that CBD may provide a novel therapeutic intervention for drug addiction. However, more studies are necessary to test this hypothesis.

7. FUTURE PERSPECTIVES

The results of our studies open several lines of investigations in the near future as:

1. Investigation of various brain regions of the reward circuitry on the inhibitory effects of CBD on MPH-induced reinstatement.
2. The effect of dopamine and serotonin receptors on the inhibitory effect of CBD on MPH-induced reinstatement.
3. The effect of CBD on the activity of different neurons in hippocampal CA1 region and PRL during MPH reinstatement using single unit recording technique in alert animals.
4. The role of CBD on D1 and D2 dopamine receptors in cortical and central regions of the nucleus accumbens in reinstatement of MPH CPP.

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9. ANNEX 1 - The approval certificate of Animal Ethics Committee (BRAZIL)

UNIVERSIDADE DE BRASÍLIA – UnB
INSTITUTO DE CIÊNCIAS BIOLÓGICA - IBD
COMITÊ DE ÉTICA NO USO ANIMAL - CEUA

11 de junho de 2002.

A QUEM POSSA INTERESSAR

Declaramos que o projeto intitulado ***"Possíveis interações entre os sistemas serotonérgico e dopaminérgico no controle dos efeitos comportamentais e neuroquímicos induzidos pela administração de cocaína em primatas não-humanos (Callithriz penicillata)."***, foi avaliado e aprovado pelo Comitê de Ética no Uso Animal (CEUA) do Instituto de ciências Biológicas da Universidade de Brasília.

Cesar Koppe Grisolia
Comitê de Ética do Uso Animal
Presidente

10. ANNEX 2 - The approval certificate of Animal Ethics Committee (IRAN)

SHAHID BEHESHTI UNIVERSITY OF MEDICAL SCIENCES AND HEALTH SERVICES

Ethical Statement

July 10, 2018

A project entitled "EFFECTS OF CANNABIDIOL ON MORPHINE AND METHYLPHENIDATE-INDUCED CONDITIONED PLACE PREFERENCE DURING EXTINCTION/ REINSTATEMENT IN RATS" has been evaluated and approved by the Ethics Committee of Shahid Beheshti University of Medical Sciences, Tehran, Iran (IR.SBMU.PHNS.REC.1397.108).Committee in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publication No. 80-23, revised 1996).

Sincerely yours,

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