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NICOTINE AND METHYLPHENIDATE CHRONIC EXPOSURE ON ADULT CANNABINOID RECEPTOR AGONIST (CP 55,940) PLACE CONDITIONING IN MALE RATS

A Thesis

Presented to the

Faculty of

California State University,

San Bernardino

In Partial Fulfillment

of the Requirements for the Degree

Master of Arts

in

General-Experimental Psychology

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by

Christopher Philip Plant

June 2016

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ABSTRACT

A problematic connection has been reported between those who use nicotine related products alone or in combination with ADHD medications, like methylphenidate (MPH), in late childhood or early adolescence and the increased likelihood of later marijuana abuse in adulthood. Pre-clinical studies have found that the use of nicotine during the early adolescence period produces enduring changes to the endocannabinoid system in the brain. Since CB agonists, like marijuana, exert their effect through the eCB system, it is possible that early nicotine use may alter the rewarding nature of CB agonists in adulthood. In addition, MPH has also been shown to increase nicotine self-administration and abuse related behaviors of nicotine in rats. Thus, the current study consisted of two experiments looking at the effects of early nicotine and methylphenidate exposure on adult CB-agonist place conditioning in rats. In the first experiment, rats were pre-exposed to either saline or nicotine (0.16, 0.32, or 0.64 mg/kg) from PD 31 to PD 40. On PD 60, rats began a 13-day biased CPP procedure with the CB agonist, CP 55,940 (10, 20 or 30 µg/kg), or vehicle. No significant group differences were found, suggesting that early nicotine exposure does not influence the rewarding nature of CB agonists. Additional individual subgroup comparisons were conducted to determine if any subgroups significantly differed from 0 or no mean change in preference from preconditioning to testing. These analyses revealed that rats pre-exposed to the moderate (0.32 mg/kg) dose of nicotine

showed a significant aversion to the high (30 μg/kg) dose of CP 55,940, suggesting that early nicotine exposure may reduce the rewarding nature of CB agonists in adulthood. In the second experiment, rats were pre-exposed to either saline or MPH (0.5, 2, 0r 5 mg/kg) from PD 21 to PD 30. Similar to the first experiment, rats began a 13-day biased CPP procedure on PD 60 with CP 55,940 (10, 20 or 30 μg/kg) or vehicle. Rats conditioned with the moderate (20 μg/kg) dose of CP 55,940 showed a significant preference for the CB agonist as compared to rats conditioned with the high (30 μg/kg) dose of CP 55,940. CP 55,940 exposed rats did not significantly differ from control rats. There was no significant effect of MPH or a MPH x CP 55,940 interaction, suggesting that early MPH exposure does not alter the rewarding nature of CB agonists in adulthood. Together these findings suggest that early nicotine, but not MPH, exposure may influence the rewarding nature of CB agonists in adulthood, suggesting an additional risk factor of early nicotine use. However, future studies should evaluate the effects of persistent nicotine and MPH exposure starting in early adolescence or childhood through adulthood to determine whether the effects of nicotine and MPH are altered if use is continued into adulthood.

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CHAPTER ONE

INTRODUCTION

Adolescence is a transitional period in development between childhood and adulthood. There are both biological and social changes occurring during this period that make it an especially vulnerable period for substance use and abuse. During this period subcortical structures in the brain responsible for the experience of emotions are developed, but cortical structures necessary for higher order cognition, like the prefrontal cortex, are just beginning to develop (Konrad et al., 2013; Casey & Jones, 2010). Thus, adolescents are able to experience “adult” emotions, but they do not have the ability to control and process these emotions in an effective way. This biological immaturity is believed to be a major contributor to the increased impulsivity and risky decision-making often associated with this time period (Konrad et al., 2013).

Adolescence is also a period in development when people begin to separate from their parents and put a greater emphasis on their peer group (Gorrese & Ruggierri, 2012). This transition away from parental control increases the role of peer influence on behavior during adolescence. De Looze et al. (2012) revealed that decreased parental involvement and increased time spent with friends were associated with an increase in risky behaviors, including substance use and sex initiation, in adolescence. The combination of the adolescent desire for independence from parental control, increased peer influence, and impulsivity and risky decision-making all lead to

a greater vulnerability during this period to enter into environments where illicit substances are being used (Casey & Jones, 2010). This is important in understanding the vulnerability during this period because the exposure to and availability of substances are key factors in the initiation of substance use (Merikangas & McClair, 2012).

Adolescence is an especially vulnerable period for the initiation of nicotine, the psychoactive component of tobacco, use and the progression to nicotine dependence (Moyer, 2013). In fact, around 90 percent of chronic smokers begin smoking in adolescence (SAMHSA, 2012). The chronic use of tobacco-related products has been implicated in a wide variety of medical illnesses, and remains one of the leading causes of preventable deaths around the world. Researchers are beginning to discover another dangerous role for these products as potential “gateways” to other drugs of abuse. For example, early onset of nicotine use has been associated with early marijuana and stimulant use (Behrendt et al., 2012; Hayatbakhsh et al., 2009; Weinberger & Sofuoglu, 2009; McQuown et al., 2007). This is particularly concerning because the early use of these substances is associated with a greater risk for the development of substance use disorders (Copeland & Swift, 2009). The exact nature of the relationship between early nicotine use and marijuana use has not been determined, but preclinical studies have shown that nicotine exposure during the adolescent period increases cannabinoid (CB) receptor density in the ventral tagmental area, prefrontal

cortex, dentate gyrus and hippocampus in rats (Werling et al., 2009). This suggests a possible biological mechanism through which the effects of CB receptor agonists, like marijuana, may be altered from early exposure to nicotine.

Troubling connections have also been reported between the use of nicotine and methylphenidate, one of the most common stimulant treatments for attention deficit hyperactivity disorder (ADHD). Methylphenidate use with and without an ADHD diagnosis has been shown to increase cigarette smoking in humans (Vanisckel et al., 2011; Vansickel et al., 2009) and enhance abuse-related behaviors of tobacco in rats (Wooters et al., 2008). Associations have also been reported between those diagnosed with ADHD and the abuse and dependence of marijuana, nicotine and other drugs of abuse (Lee et al., 2011; Aksoy et al., 2012; Lambert, 2005). Interestingly, this relationship has been shown to decrease when individuals with ADHD are treated with methylphenidate, although the association is still stronger than in the general population (Wilens, Biederman & Gunawardene, 2003). However, there is little known about the effects of early methylphenidate exposure on those without symptoms of ADHD or the combined effect of nicotine and methylphenidate. This is largely due to the difficulty in experimentally investigating the effects of early methylphenidate exposure in human children. Thus, animal models become particularly important in elucidating the potential

long term biological effects of substances like nicotine and methylphenidate on adult substance misuse.

In the current study we conducted two independent experiments. The first experiment was designed to investigate the role of adolescent nicotine exposure on the rewarding properties of cannabinoid agonists in young adult rodents. Similarly, the second experiment investigated the effects of late childhood exposure to methylphenidate (Ritalin) on the rewarding properties of cannabinoid agonists in young adult rodents. The following chapters discuss in detail the relevant neurotransmitter systems, nicotine, methylphenidate, marijuana, CP 55,940, and the rational for the current study.

CHAPTER TWO

CATECHOLAMINE NEUROTRANSMITTERS

Catecholamine neurotransmitters are distinguished by a chemical structure called a catechol, and all are derivatives from the amino acid tyrosine (McTavish, Cowen, & Sharp, 1999). This family of neurotransmitters includes dopamine (DA), norepinephrine (NE) and epinephrine. However, only DA and NE will be discussed in depth due to their relevance to the current study.

DA is known to be involved in a variety of functions, including movement, mood, cognition, sexual behaviors, attention, and, most importantly for the purposes of this study, reward mechanisms (Clark et al., 2012; Adachi et al., 2012; McHenry et al., 2012; Brown et al., 2011; Missale et al., 1998). DA is believed to play a major part in neuronal reward mechanisms underlying recreational substance use, and is also implicated in various other disorders, including attention-deficit hyperactivity disorder (ADHD), schizophrenia, Parkinson's disease, various affective disorders, and Tourette's (Volkow et al., 2011; Miller et al., 2012; Bortolato, Chen & Shih, 2008; Missale et al., 1998).

NE is involved in a variety of functions both in the central and peripheral nervous systems. It is known to play a part in attention, arousal, impulse control, emotion, memory, stress, motivation as well as reward and reward learning (Roychowdhury et al., 2012; Goddard et al., 2010; Robinson, 2012; Segal et al., 2012; Thoma et al., 2012; Young & Williams, 2010; Gallagher et

al., 2013). The noradrenergic system is also implicated in many psychiatric disorders, including ADHD, major depressive disorder, bipolar disorder, post-traumatic stress disorder, and anxiety disorders (Park et al., 2012; Machado & Einarson, 2010; Wiste et al., 2008; Blanchard et al., 2012; Goddard et al., 2010).

The process of DA and NE synthesis occurs in the axon terminals of dopaminergic and noradrenergic neurons, respectively. DA and NE synthesis begins with the conversion of tyrosine to L-dyhydroxy-phenylalanine (L-dopa) in the presence of tyrosine hydroxylase, the rate-limiting step in the synthesis of all the catecholamines (Elsworth & Roth, 1997). L-dopa is then converted into DA in the presence of aromatic L-amino acid decarboxylase (Elsworth & Roth, 1997; Smidt, Smits & Burbach, 2003; Sourkes 1979). Once DA is synthesized it is actively transported into vesicles by vesicular monoamine transporters located on the vesicles where they are stored for release in dopaminergic neurons (Elsworth & Roth, 1997). In noradrenergic neurons, NE is synthesized from DA after DA is stored in vesicles in the presence of dopamine beta-hydroxylase (May, Qu & Meredith, 2012).

Catecholamine vesicles are released through calcium dependent exocytosis (Leviel, 2011). Once the vesicles begin to move towards the active zone where docking and fusion occur with the help of SNARE (soluble n-ethylmaleimide-sensitive-factor attachment protein receptor) complexes and complexion and final fusion to the active zone occurs with synaptotagmin

(Ramakrishnan, Drescher, & Drescher, 2012). Once fusion occurs catecholamine neurotransmitters are released into the synaptic cleft where they bind to their respective receptors on the postsynaptic density (Ford et al., 2010; Elsworth & Roth, 1997).

There are two classes of DA receptors, D_1-like and D_2-like, which are further divided into five subtypes, D_1-D_5. All the DA receptors are G-protein coupled and operate through second messenger systems. The D_1-like receptors, D_1 and D_5, are coupled to either G_s or G_{olf}. When G_s and G_{olf} are activated from the binding of DA they stimulate the enzyme adenylyl cyclase, which is responsible for converting ATP to cyclic adenosine monophosphate (cAMP) (Billington & Hall, 2012). As cAMP levels rise due to the stimulation of adenylyl cyclase, the enzyme protein kinase A (PKA) is activated and can lead to a slight depolarization of the postsynaptic neuron (Billington & Hall, 2012; Binder et al., 2001). The D_2-like receptors, D_2-D_4, are coupled to either G_i or G_o, which are inhibitory G-proteins. Their activation decreases adenylyl cyclase, which in turn decreases cAMP formation and PKA activity leading to a slight hyperpolarization of the postsynaptic neuron (Missale et al., 1998).

NE is unique because it doesn't have receptors exclusive for it, but instead it shares the same receptors with epinephrine, the other member of the catecholamine class (Moore & Bloom, 1979). There are two primary groups of receptors used by NE: alpha (α) and beta (β) receptors. The α group contains subtypes of α_1 and α_2 and the β group contains subtypes of β_1, β_2,

and β_3. All the receptors used by norepinephrine are G-protein coupled receptors. The α_1 receptor is G_q coupled and activates the diacylglycerol (DAG)/inositol-1,4,5-trisphosphate (IP3) second messenger system. IP3 increases intracellular calcium and DAG activates protein kinase C, which phosphorylates other proteins producing changes within noradrenergic neurons (Exton, 1985). The α_2 receptor is G_i coupled and decreases cAMP (Yi et al., 2012; Exton, 1985). β receptors 1-3 are all coupled to G_s and increase cAMP levels (Rebois et al., 2012).

After DA and NE bind to their respective receptors, the remaining neurotransmitters in the synapse are removed through active reuptake by either DA transporters (DAT) or NE transporters (NETs) (Elsworth & Roth, 1997; Ford et al., 2010). DATs and NETs are implicated in various disorders, including ADHD (Miller et al., 2012), and they also are the site at which many drugs like methylphenidate, cocaine and amphetamines produce their biochemical effects (Hannestad et al., 2010; Le Foll et al., 2009; Missale et al., 1998). Once DA and NE are taken back into the presynaptic neuron's axon terminal they are either repackaged into vesicles or enzymatically destroyed by monoamine oxidase (Elsworth & Roth, 1997).

The dopaminergic system is a localized system that primarily exerts its effects through three pathways: the mesocortical, nigrostriatal and the mesolimbic (Rieckmann et al., 2011). The mesocortical pathway refers to dopaminergic neurons from the ventral tagmental area (VTA) connecting to

the frontal cortex (Thierry et al., 1976). The nigrostriatal pathway signals from the substantia nigra to the striatum (Thierry et al., 1976). The mesolimbic pathway, also known as the reward/motivation pathway, consists of dopaminergic axonal projections from the VTA to various areas of the limbic system (nucleus accumbens, amygdala and hippocampus) as well as the medial prefrontal cortex (Koob & Kreek, 2007; Wanat et al., 2009). This is the pathway through which many abused drugs are thought to exert their reinforcing effects, and it is believed to be an essential aspect of the biological mechanisms underlying substance addictions (Leroy et al., 2012; Le Foll et al., 2009; Missale et al., 1998).

In contrast to DA, NE pathways in the brain are much more diffuse. The locus coeruleus in the pons contains the vast majority of CNS noradrenergic cell bodies and is primarily responsible for the synthesis of NE (Ishibashi et al., 2009). The locus coeruleus noradrenergic cell bodies project axons throughout the entire CNS, including both cortical and subcortical structures in the brain and the spinal cord (Jodo, Chiang & Aston-Jones, 1998; Lipski, 2013; Bruinstroop et al., 2012). NE can also act as a hormone by being released directly into the blood stream by the adrenal medulla (Schneider et al., 2011).

CHAPTER THREE

ACETYLCHOLINE

Acetylcholine (Ach), like DA and NE, is a small molecular weight neurotransmitter. Much of our knowledge about neurons and chemical transmission was first discovered on cholinergic neurons in the peripheral nervous system (Holmstedt, 1975). The cholinergic system is essential for the functioning of both the autonomic and somatic nervous systems because in its absence essential organs like the heart and lungs would no longer function, and we would not be able to complete even the simplest of motor tasks (Fregoso & Hoover, 2012; Ikeda et al., 2012; Murray et al., 2013). Apart from its essential role at all neuromuscular junctions, ACh is implicated in a variety of psychological phenomena such as motivation, learning, memory, stress, attention, mood, addiction and reward (Serreau et al., 2011; Pepeu & Giovanni, 2010; Mora at al., 2012; Williams & Adinoff, 2008; Picciotto et al., 2008). Dysfunction in the cholinergic system has also been linked to several psychiatric disorders including Alzheimer's, Parkinson's, schizophrenia, bipolar disorder, and substance use disorders (Ni, Marutle & Nordberg, 2013; Aosaki et al., 2010; Luckhaus et al., 2012; Thomsen, Weyn & Mikkelsen, 2011; Chatterjee & Bartlett, 2010).

Like DA, ACh is synthesized within the presynaptic neurons axon terminal, packaged in vesicles through active transport, and released through calcium dependent exocytosis. ACh is synthesized from acetyl coenzyme A

and choline in the presence of choline acetyltransferase (ChaT) (Fujii, Takada-Takatorie, & Kawashima, 2012; Fulton & Nachmansohn, 1943). Cholinergic neurons are often identified by the presence of ChaT (Bellier & Kimura, 2011; Hedrick & Waters, 2010). Choline, which is extracted from the extracellular fluid, is the rate-limiting step in ACh synthesis (Birks, 1985). We have very little excess ACh in our bodies, so any disturbance in choline levels could have dire consequences on the body (Ghoshal & Farber, 1984). Once ACh is synthesized it is packaged into vesicles through the action of vesicular ACh transporters located on the vesicles in preparation to be released (Tayebati, Di Tullio, & Amenta 2008; Siegal, et al., 2004).

Once ACh is released into the synaptic cleft it either binds to receptors (Cooper, Floyd, & Roth, 1991; Israel & Dunant, 1993) or is enzymatically degraded by acetylcholinesterase into acetic acid and choline (Massoullie et al., 1993). Choline is then pumped into the presynaptic terminal through choline transporters and reused for the synthesis of ACh. Interestingly, due to the importance and diffuse nature of the cholinergic system in the human body many toxins, poisons, bacteria, and other natural threats that exert their effects biochemically work on ACh synapses (Utkin et al., 2012; Sudof, 2001).

ACh receptors fall into two classes: nicotinic (nAChRs) and muscarinic (mAChRs) receptors (Kester, Karpa & Vrana, 2011). These receptors gained their distinctive names because nicotine was found to bind exclusively to the nAChRs, whereas the psychoactive component of mushrooms, muscarine,

was found to bind exclusively to the mAChRs (Kester et al., 2011). All the nAChRs are ligand-gated ion channels that when bound by ACh allow the flow of sodium and calcium (Komal, Evans, & Nashmi, 2011). When ACh binds at both of the binding sites on the two alpha subunits of the nAChR simultaneously, a conformational change occurs to the nAChR, which opens the pore so ions can flow through (Kosower, 1987). Once the pore is opened the ionic flow produces changes in the net charge of the cytosol of the postsynaptic neuron (Kosower, 1987). Nicotinic receptors are located at neuromuscular junctions, where fast transmission is essential, and in various places throughout the brain (Williams, et al., 2011; Katzung, 2003).

The mAChRs are much more complex in their functioning than the nAChRs because they operate exclusively through G-proteins and second messenger systems (Ehlert et al., 1995; Caufield, 1993; Wess 1996). There are five types of mAChRs, labeled M_1-M_5 (Caulfield & Birdsall, 1998). The differences between these receptors rest on the second messenger systems they activate. The mAChRs labeled M_1, M_3 and M_5 are grouped together because they work on the diacylglycerol (DAG)/inositol-1,4,5-trisphosphate (IP3) second messenger system (Alberts et al., 2002; Ehlert et al, 1995). These receptors are coupled to the G-protein labeled G_q (Markovic et al., 2012; Burford & Nahorski, 1996). When G_q is activated the substrate phosphatidylinositol-4,5-biphosphate (PIP_2), which is part of the plasma membrane, is broken down in the presence of the effector enzyme

phosopholipase C into the second messengers DAG and IP3. DAG and IP3 work by activating protein kinase C and increasing intracellular calcium signaling (Baylis & Vasquez, 2012; Alberts et al., 2002). The other mAChRs, M_2 and M_4, are grouped together because they work on the cAMP second messenger system. These receptors work through G_i and G_o. When $G_{i/o}$ is activated a decrease in cAMP occurs, there is an outward flow of potassium ions, and an inhibition of calcium channels, which together lead to an inhibitory postsynaptic potential (Guo, Mao & Wang, 2010).

Cholinergic neurons are present throughout the central and peripheral nervous systems, and thus their pathways and effects are much more diffuse than those seen in the dopaminergic system (Lucas-Meunier et al., 2003; Dringenberg et al., 2006; Caulfield, 1993; Wess et al., 1990). The cholinergic system has areas of action throughout the brain, spinal cord, and body and is actually the most diffuse neurotransmitter system in the human body (McCormick, 1989).

CHAPTER FOUR

ENDOCANNABINOID SYSTEM

Compared to other neurotransmitter systems, the endocannabinoid (eCB) system is much less understood. This lack of information stems from two reasons: (1) the known endogenous cannabinoids were only recently discovered in the early 1990s, and (2) the cannabinoid system operates very differently from other neurotransmitter systems. Despite the lack of information regarding the eCB system, studies have shown that it is involved in a variety of psychological phenomena, including mood, pain, appetite, memory and reward (Bambico, 2012; Miller et al., 2012; Fulton, 2010; Abush & Akirav, 2013; Hell et al., 2012). Interestingly, the eCB system has increasingly become a promising target for new drug therapies for many psychiatric disorders, including Parkingson's, Huntington's, various mood disorders, substance use disorders, and Tourette's (Fernandez-Ruiz et al., 2011; Micale et al., 2013; Panlilio, Justinova & Goldberg, 2013; Muller-Vahl, 2013). The eCB system's ability to modulate the signaling of other neurotransmitter systems seems to be the primary reason it is becoming a target for new drug therapies (Piomelli et al., 2000).

One of the most important distinguishing characteristics of the eCB system is that it operates through retrograde signaling. Specifically, eCBs are synthesized for release by the postsynaptic neuron and bind to cannabinoid (CB) receptors on the presynaptic neuron's terminal. This retrograde signaling

mechanism is believed to be important in mediating the release of neurotransmitters at both excitatory and inhibitory synapses, which ultimately has important implications for synaptic plasticity for neurons that contain eCBs and/or their receptors (Chevaleyre, Takahashi & Castillo, 2006; Jian-Yi et al., 2010).

There are two eCBs that have been identified as binding to CB receptors, N-arachidonoyl-ethanolamine (anandamide; AEA) and 2-arachindonoyl-glycerol (2-AG) (Mechoulam & Parker, 2013; Devane & Hanus, 1992; Mechoulam et al., 1995). AEA is synthesized from N-arachidonoyl-phosphatidylethanolamine (NAPE) and 2-AG from "the hydrolytic metabolism of 1,2-diacylglycerol (DAG) mediated by two sn-1-selective DAG lipases, DAGL-alpha and DAGL-beta" (Sidhpura & Parsons, 2011 p. 1071; Ueda et al., 2011; Piomelli, 2003). Both these eCBs are lipids and are able to pass through plasma membranes without protein transporters, which is another unique characteristic of the eCB system.

The synthesis of eCBs is stimulated by elevations in calcium levels in both the intra- and extra- cellular environments of certain postsynaptic neurons (Placzek et al., 2008). eCBs are synthesized from precursors of membrane lipids, and once synthesized they diffuse out of the postsynaptic neuron. Unlike the catecholamines and other neurotransmitters, eCBs are not packaged into vesicles for release; instead their hydrophobic nature allows them to simply pass through the neuron's membrane (Sidhpura & Parsons,

2011). After the eCBs bind to CB receptors, the neurotransmitters are taken up through reuptake mechanisms into both neurons and glial cells (Bisogno et al., 2006). Once reuptake occurs the transmitters are enzymatically degraded by either fatty acid amide hydrolase (FAAH) for AEA or monoacylglycerol lipase (MAGL) for 2-AG (Feledziak et al., 2012; Ueda et al., 2011).

There are two known CB receptors, CB_1 and CB_2 receptors. CB_1 receptors are much more prevalent in the central than the peripheral nervous systems, and the opposite is true of the CB_2 receptors. Both of these receptors are G-protein coupled receptors that operate through G_i and G_o. Activation of the CB receptors reduces adenylyl cyclase, slows the flow of calcium into the presynaptic terminal, and activates potassium channels. This produces an influx of potassium, and suppresses the release of neurotransmitters from the presynaptic terminal to which the CB receptors are attached (Gebremedhin & Lange, 1999; Reis et al., 2011). This suppression effect produces either a slight inhibition or excitation of the postsynaptic neuron depending on the properties of the synapse affected (Basavarajapppa, Ninan & Arancio, 2008; Best & Regehr, 2008; Pistis et al., 2002). Thus, the eCB system produces behavioral effects by working in conjunction with another neurotransmitter system.

The eCB system is thought to modulate many neuronal systems producing a wide range of physiological and behavioral effects (Schlicker & Kathmann, 2001). The eCB system is known to influence nearly every

neurotransmitter system in some way, including glutamate and GABA systems, which are the primary excitatory and inhibitory neurotransmitter systems in the brain (Pistis et al., 2002; Schnlicker & Kathmann, 2001). In many areas of the brain, the majority of CB receptors are found on GABAergic and glutamatergic neurons (Kofalvi et al., 2005).

Many studies have shown that the eCB system plays a part in almost all aspects of drug abuse and addiction, including the rewarding effects, usage, drug seeking behavior, and relapse and cravings (Hell et al., 2012; Gamaleddin et al., 2012; Higuera et al., 2008; Fattore et al., 2011; Solinas, Goldberg & Piomelli, 2008; Rodriguez et al., 2011). Due to the ability of CB antagonists to decrease the administration and reinstatement of many substances in rats, it is not surprising that researchers are optimistic that pharmaceutical treatments for individuals struggling with substance addictions through the antagonism of CB_1 receptors may be possible (Shindler et al., 2010; Shoaib, 2008).

The eCB system's primary role in various aspects of drug abuse and addiction seem to be related to its ability to mediate synaptic plasticity in areas of the brain that are commonly associated with drugs of abuse, like the VTA, nucleus accumbens, certain areas in the limbic system and the prefrontal cortex (Zhiqiang et al., 2010; Luchicchi et al., 2010; French, Dillon, & Wu, 1997; Mato et al., 2004; Chiu et al., 2010). Some investigators believe that the eCB system is so important that they often posit it as being among the most

crucial factors in the neuronal basis of substance addictions (Onaivi, 2008). This is not unexpected as the use of CB_1 receptors agonists increase firing rates of dopaminergic neurons in the mesolimbic “reward/motivation” pathway (Diana, Melis & Gessa, 2003).

CHAPTER FIVE

NICOTINE

Nicotine is a highly addictive substance found in tobacco products, and is used by around 56.8 million people 12 or older in the United States (SAMHSA, 2012). Around 90 percent of chronic smokers start smoking in adolescence (SAMHSA, 2012). Approximately 19.5 percent of high school and over five percent of junior high students are smokers (Centers for Disease Control and Prevention, 2010). This is especially problematic because smoking is the leading cause of preventable deaths, and it also has been among the biggest contributors to lung related illnesses, including cancer, for decades (Center for Disease Control and Prevention, 2003). To make matters worse, smoking is one of the most difficult types of substance addictions to treat (Ray et al., 2008; Balfour, 2004). According to Rosenthal, Weitzman, and Benowitz (2011), approximately 80 percent of all people who try to quit smoking will relapse within a month. With the combination of an increased risk of disease, early mortality and chronic relapse associated with nicotine addiction, the need for research and effective treatments for nicotine abuse is becoming increasingly more of a concern.

In general, nicotine produces positive effects on mood, alertness, and anxiety (Rosenthal et al., 2011). However, the perceived positive effects of nicotine are believed to be at least partially due to reductions in withdrawal symptoms, which include anxiety, difficulty concentrating, irritability, and

restlessness (Benowitz, 2010). Thus, consuming nicotine often becomes a form of negative reinforcement (escape from withdrawal symptoms) to chronic smokers. In addition, nicotine causes an increase in the release of catecholamines from the adrenal medulla into the bloodstream producing increases in heart rate, blood pressure, and respiration (Haas & Kuebler, 1997).

When nicotine is inhaled, it enters the lungs where it is absorbed and carried in the blood stream to the brain (Caldwell, Sumner & Crane, 2012). Nicotine exerts it biochemical effects by binding to cholinergic nicotinic receptors (nAChRs), in both the CNS and PNS, producing a slight depolarization of the postsynaptic neurons through the opening of sodium and potassium channels (Barron, 2010). Thus, nicotine increases the activity of Ach. This altered transmission of cholinergic neurons in the CNS also increases the firing of dopaminergic neurons in the mesolimbic and mesocortical pathways (Besson et al., 2012; Novak, Seeman, & Foll, 2010), and this interaction with the dopaminergic system is how nicotine is believed to produce its reinforcing and abuse-related effects. There is also evidence that the cannabinoid system is also involved in mediating the rewarding and abuse-related effects of nicotine on the dopaminergic system. Gamaleddin et al. (2012) showed that stimulating CB1 receptors increased nicotine self-administration, nicotine seeking behaviors and nicotine cue-induced reinstatement. Also, nicotine use in adolescence, but not adulthood, is known

to increase cannabinoid receptor density in the ventral tagmental area, prefrontal cortex and hippocampus in rats (Werling et al., 2009), suggesting that adolescence is a particularly sensitive period for the effects of nicotine on the CB system. It may be possible that these effects during adolescence could alter the effects of psychoactive substances that work through the CB system, like marijuana. However, no studies have assessed whether these nicotine-induced structural changes to the CB system result in notable behavioral or psychological changes when CB agonists, like marijuana, are used later in life.

CHAPTER SIX

METHYLPHENIDATE

Methylphenidate was first synthesized in 1944, and marketed to the public as Ritalin (Leonard et al., 2004). It is one of the most commonly prescribed stimulants for the treatment of attention-deficit hyperactivity disorder (ADHD) (Leonard et al., 2004). In general, methylphenidate is considered a fairly safe drug with minimal side effects if used appropriately (Leonard et al., 2004). However, many researchers and clinicians are growing increasingly concerned about the potential long-term effects of prescribing stimulant treatments to children and adolescents (Marco et al., 2011). ADHD is often diagnosed in late childhood or early adolescence while the brain is still developing, especially in prefrontal regions (Casey & Jones, 2010). This is concerning because during childhood and adolescence the brain is thought to be more open to substance induced neuronal alterations than a fully developed adult brain (Casey & Jones, 2010). Casey and Jones (2010) hypothesize that the differential development between the early developing subcortical structures compared to the slow developing prefrontal, “cognitive control,” regions seem to make years 13 to 17 especially vulnerable times to the effects of drugs and alcohol. Findings such as this contribute to concerns about the lack of research on the long-term neurobehavioral effects of methylphenidate exposure during the late childhood and adolescent developmental periods.

Methylphenidate improves symptoms for many people diagnosed with ADHD (Leonard et al., 2004). It generally produces a greater ability to stay focused and sustain attention, while reducing restlessness and aiding in problems with impulsivity. Conversely, those without a diagnosis of ADHD tend to report the opposite effects, including high levels of anxiety and restlessness (Leonard et al., 2004). Methylphenidate is also known to increase heart rate and blood pressure, which is a commonality between most stimulant drugs (Leonard et al., 2004).

Methylphenidate is considered a fairly safe drug, but it does come with some unwanted side effects. The common side effects include sleep problems, nervousness, dizziness and changes in appetite and affect (Leonard et al., 2004). Borcherding et al. (1990) also found that methylphenidate use in humans often produces unusual movements and/or compulsive behaviors. Similarly, stereotypy, constant, repetitive movements, is often reported in rats given high doses of methylphenidate (Sheel-Kruger, 1971). More severe side effects, like cardiovascular problems and increased stroke risk, are extremely rare, but still are concerning for those on methylphenidate for extended periods of time (Leonard et al., 2004).

Methylphenidate exerts its effects primarily through the noradrenergic and dopaminergic systems in the brain by increasing extracellular norepinephrine and dopamine (Yano & Steiner, 2007; Pascoli et al., 2005). It has a very limited effect on the serotonergic system, which is one of the

notable distinctions between methylphenidate and other stimulant ADHD medications like amphetamines. Interestingly, in more recent studies it has been shown that methylphenidate also indirectly influences glutamate and GABA systems, which may mediate its wide-ranging effects on the brain (Wanchoo, Swann & Dafny, 2009; Wiguna et al., 2012).

Methylphenidate binds with the highest affinity to norepinephrine transporters (NETs), followed closely by dopamine transporters (DATs) (Yano & Steiner, 2007; Pascoli et al., 2005). Methylphenidate binds to between 70 and 80 percent of NETs in humans (Hannestead et al., 2010). When methylphenidate binds to the transporters, it blocks the reuptake of the respective neurotransmitters from the synaptic cleft ultimately prolonging the effect of the neurotransmitters on the receptors. This is thought to be the primary mechanism through which methylphenidate produces improvements in ADHD related symptoms (Rosler et al., 2010; Volkow et al., 2012). This is important for this study because blocking NETs in frontal and subcortical regions is known to affect both the eCB and dopaminergic systems (Richter et al, 2012; Carboni & Sivagni, 2004; Borgkvist et al., 2012).

The striatum, where methylphenidate exerts some of its effects on noradrenergic and dopaminergic neurons, is an important forebrain structure in reward learning and decision-making processes (Balleine, Delgado & Hikosaka, 2007). Methylphenidate's effect on the striatum is important because the dopaminergic neurons that are part of the mesolimbic,

reward/motivation, pathway project axons to the striatum (Leroy et al., 2012; Le Foll et al., 2009). The use of methylphenidate has also been shown to produce changes in the plasticity and functionality of pathways in the striatum (Adriana et al., 2006). Since the mesolimbic pathway is an important target for most drugs of abuse, methylphenidate-induced changes to this pathway may have implications for the effects of other drugs later in life. This includes marijuana that is known to work on CB receptors that influence dopaminergic neurons in the mesolimbic and mesocortical pathways (Gessa et al., 1998).

CHAPTER SEVEN

MARIJUANA AND THE CANNABINOID AGONIST CP 55,940

Marijuana is the mostly widely used illicit drug in the United States. According to the annual survey conducted by the U.S. Department of Health and Human Services, about 7 percent (approximately 18 million) of people 12 or older are current marijuana users, a significant increase from 5.8 percent in 2007 (SAMHSA, 2012). Approximately 68 percent of new drug users start with marijuana, and most of these new users start when they are under 18 years old (SAMHSA, 2012). Marijuana users have the second highest rates of dependence or abuse, trailing only alcohol users (SAMHSA, 2012), and in recent years marijuana use has been reported more frequently by high school students than nicotine use (NIDA, 2011). This upward trend in marijuana use will likely continue as more states legalize marijuana for medical and recreational purposes.

The psychoactive compound in marijuana, Δ^9- tetrahydrocannabinol (THC), is generally considered a partial agonist at both CB_1 and CB_2 receptors (Paronis, Nikas, Shukla & Makriyanisa, 2012), and is known to produce positive mood states, including euphoria and calmness, at low doses (Nahas, 2001). As dose increases negative mood states are more likely and can include paranoia and high levels of anxiety (Englund et al., 2013; Harte-Hargrove & Dow-Edwards, 2012). THC has many short-term side effects including impaired short-term and working memory, motor functions,

judgment, cognitive performance and considerable increases in heart rate (Ranganathan & D'Souza, 2006; Ramaekers et al., 2006; De Melo et al., 2005; Panlilio et al., 2012; Metrick et al., 2012; Ramaekers et al., 2009; Nahas, 2001). In addition to the more immediate effects of THC there is some evidence for long-term effects as well. Long-term use of marijuana has been found to be associated with poorer education and work related outcomes, diminished life satisfaction, respiratory issues, and permanent cognitive impairment (Senn et al., 2008; Caldeira et al., 2012; Hall, 2009).

Marijuana is approved for medicinal use in 18 states as well as Washington DC and has actually been legalized for recreational use in several states. Marijuana has been shown to be useful for relieving pain, appetite stimulation, and controlling nausea (Walker & Huang, 2002; Nelson et al., 1994; Cotter, 2009). However, its medical uses continually come into question, as many believe its negative side effects outweigh the benefits it may bring patients. The negative side effects most commonly discussed are related to impaired cognitive functions and exposure to carcinogens from smoking marijuana (NIDA, 2011).

In recent years more potent, synthetic CB agonists have been developed. The CB receptor agonist CP 55,940 (CP) is considered to be between 10 to 100 times more potent than THC (Herkenham et al., 1990). However, its behavioral and pharmacological effects are generally thought to be similar to those of THC (Fan et al., 1994; Xie, Melvin & Makriyannis, 1996).

CP binds to both CB1 and CB2 receptors with approximate equal affinity, similarly to THC, but CP has higher affinity at both receptor sites (Wiley et al., 1995; Gatley et al., 1997; Griffin et al., 1998; Thomas et al., 1998). CP increases firing rates of mesolimbic dopaminergic neurons (Gessa et al., 1998), which may be a possible mechanism through which CB agonists produce their rewarding effects. Also, Craft et al. (2012) showed that there are sex differences in how CB agonists affect CB receptors. Specifically, cannabinoid agonists appear to bind with higher affinity to CB1 receptors in females than males. Interestingly, Duan, Liao, Jain, & Nicholson (2008) showed that CP is also able to inhibit the function of voltage-gated sodium channels independent of its influence on CB receptors. This effect, however, only occurs with large doses of CP as binding to CB1 receptors is about 10,000 times more potent than its effect on sodium channels. CP's effect on sodium channels does raise concerns about very high doses, and may also explain why higher doses tend to be aversive.

Experimental findings with CP seem to show that its effects are highly dose-dependent. For example, low doses of CP seem to decrease anxiety-like behaviors, while higher doses appear to increase anxiety as measured by the elevated plus maze task (Marco et al., 2004). Also, like THC, there are conflicting results in place conditioning procedures, which are believed to measure the reward/aversive properties of substances. Some studies report conditioned place preferences to CB agonists, which is expected considering

the wide spread use of substances like marijuana, (Braidi et al., 2001; Valjent & Maldonado, 1990) while others report place aversions to the same agonists (McGregor, Issakidis & Prior, 1996). However, when focusing on the experimental procedures, it seems when care is taken to avoid the dysphoric effects commonly associated with the initial use, long half-life, and dosing of cannabinoid agonists a conditioned place preference is often reported (Braidi et al., 2001; Valjent & Maldonado, 1990). It seems that higher doses of CP are more likely to produce an aversion than lower doses.

CHAPTER EIGHT

CONDITIONED PLACE PREFERENCE

One of the most common methods to investigate the rewarding/aversive properties of a drug in animal models is the conditioned place preference (CPP) paradigm (Bardo & Bevins, 2000). There are notable advantages to the use of the CPP paradigm, including its ability to test both the rewarding/aversive properties of a drug and locomotor activity simultaneously (Bardo & Bevins, 2000). It also can be adjusted in many ways to investigate both short-term and long-term behavioral changes produced by early exposure to drugs, drug-associated learning and drug induced biological alterations (Bardo & Bevins, 2000).

According to Bardo & Bevins (2000), the CPP paradigm is based on classical/Pavlovian conditioning principles. The paradigm is often conducted in three stages. The first stage is preconditioning where the animals are allowed to roam freely in two connected but distinct chambers for a specific amount of time. The basic idea is to get the rats accustomed to the apparatus, and to assess whether there is an unconditioned preference for one of the chambers (Bardo & Bevins, 2000). The second stage is conditioning. During this stage there are a number of drug and vehicle alternating sessions. In half of the sessions the animals are administered the drug of interest and then placed inside only one of the chambers for a specific amount of time. In the biased CPP paradigm the drug is paired with the chamber that the rats did not prefer

in the preconditioning stage. For the rest of the sessions the rats are injected with saline and then put into the other chamber for the same amount of time as the drug-paired session. The purpose of this stage is to condition an association between the chamber (conditioned stimulus-CS) that is paired with the drug of interest and the effects of the drug (unconditioned stimulus-UCS) (Bardo & Bevins, 2000). The last stage is testing. In this stage the animals are not exposed to any drugs. They are placed in the CPP apparatus and allowed to roam freely in the two main chambers for the same amount of time as the preconditioning session. The amount of time spent in each chamber is measured. If animals spend significantly more time in the drug-paired compartment they are described as having a conditioned place preference (CPP), and if the animals spend more time in the non-drug paired compartment they are described as having a conditioned place aversion (CPA). The general idea is that if the drug is rewarding the animal will be much more likely to spend time in the drug-paired compartment that has been associated with the positive effects of the drug. However, if the drug is aversive to the animal, the drug-paired compartment will be associated with negative feelings. This will make the animal less likely to spend time in the drug-paired compartment and more likely to spend time in the non-drug (saline) paired compartment, which should be “neutral.”

Despite its many advantages, there are some criticisms levied against the CPP paradigm. Bardo and Bevins (2000) assert that some question

whether the CPP paradigm actually tests the rewarding/aversive properties of drugs at all. Some believe that the results found in studies using this paradigm could be the result of novelty seeking behavior rather than anything to do with the properties of the drug itself. They believe that the effects of the drug may cloud the ability of the rat to familiarize itself with the drug-paired compartment, and thus result in the rat spending more time in the drug-paired compartment simply because it appears more novel than the neutral (saline-paired) compartment during testing. This would mean that findings showing that the rats preferred (spent more time in) the drug-paired compartment would not be the result of the rewarding properties of the drug, but just the novelty of the drug-paired compartment when the drug is not present. However, this interpretation makes it difficult to account for findings that show that most drugs that are considered pleasurable tend to show CPPs while drugs that are viewed as aversive tend to show CPAs (Tzchentke, 2007). If novelty seeking is what is being assessed the drug-paired compartment should be more novel to the rats whether the drug being assessed is pleasurable or aversive, but CPPs and CPAs are both reported. Thus the evidence tends to support the claim that the CPP paradigm measures the rewarding/aversive properties of drugs rather than just novelty seeking behaviors.

Another criticism often directed at the CPP paradigm relates to its difficulty in producing a full dose-effect curve (Bardo & Bevins, 2000). This

tends to be especially problematic with pharmacological questions that require detailed dose-effects. However, one way to help alleviate this problem is to assign doses to independent groups in a between subjects manner (Bardo & Bevins, 2000). This does not entirely eliminate the problem, but it improves the ability of the CPP paradigm to give more detailed dose-effect information.

The CPP paradigm has been used to evaluate the motivational properties of nearly all of the commonly used and abused drugs. Its ability to assess reward and aversion has been reliably shown with many drugs, including heroin, methamphetamine, cocaine, MDMA and various other drugs of abuse (Tzchentke, 2007; Conrad et al., 2013; Ribeiro et al., 2012). Most of the drugs that are thought to be highly rewarding show CPPs when administered to animals (Tzchentke, T., 2007). However, marijuana (THC) and other CB agonists do not consistently produce CPPs when administered to animals, and in some cases they actually produce CPAs (Bardo & Bevins, 2000). This is problematic because marijuana is the most used illicit drug in the United States and clearly is perceived as highly rewarding by those that use it. Some believe that the inconsistencies seen in the results with cannabinoid agonists are due to dysphoric effects produced by the initial use, high sensitivity to dose changes, and the long half-life of these substances (Murray & Bevins, 2010; Braidi et al., 2001; Bardo & Bevins, 2000; Valjent & Maldonado, 2000). Studies using cannabinoid agonists in a CPP paradigm should take these factors into account. A possible approach could be to

pre-expose the animals to the drug prior to the start of conditioning (i.e to avoid dysphoric effects of initial use), use lower doses of the drug (i.e. avoid aversive effects of higher doses), and have longer waiting periods between conditioning sessions (i.e. avoid possible negative side effects of the long half-life). If these factors are accounted for CPPs seem to be more commonly reported for many cannabinoid agonists.

CHAPTER NINE

PROPOSAL AND HYPOTHESES

This study explores the problematic connection commonly reported between those who use nicotine related products alone or in combination with ADHD medications, like methylphenidate, in late childhood or early adolescence and the increased likelihood of later marijuana abuse in adulthood (Lee et al., 2011; Gray & Upadhyaya, 2009; Jardin, Looby & Earleywine, 2011; Faroane et al., 2007). Despite this association, very few empirical studies have been conducted to elucidate the reasons for this connection. The goal of this study was to first investigate whether nicotine alters the rewarding properties of CB agonists in adulthood. Secondly, we assessed whether pre-exposure to methylphenidate altered the rewarding nature of CB agonists in adulthood. The rewarding nature of the CB agonist (CP 55,940) was assessed using the conditioned place preference (CPP) paradigm. The CPP paradigm is one of the most common methods used to assess the rewarding properties of drugs in animal models (Bardo & Bevins, 2000).

Nicotine influences areas in the brain commonly associated with the experience of reward (Besson et al., 2012; Novak, Seeman, & Foll, 2010), and its effect on nAchRs has been shown to increase dopamine activity in the mesolimbic and mesocortical pathways (Cohen et al., 2012; Brandon et al., 2011; Dani & Harris, 2005). Nolley and Kelley (2007) found that nicotine use in

adolescence can halt the development of reward systems potentially increasing the probability of substance related problems in adulthood. In addition, exposure to nicotine in adolescence has been shown to produce long-term increases in CB receptor activity (Mateos, et al., 2011). Since CB agonists exert their effects through CB receptors, it is reasonable to suspect that early exposure to nicotine may alter the rewarding nature of CB agonists in adulthood. It was hypothesized that exposure to nicotine in early adolescence would increase the rewarding nature of CB agonists in adulthood. If early exposure to nicotine does make CB agonists more rewarding then the nicotine-exposed rats will spend significantly more time in the drug (CP)-paired compartment compared to controls.

As stated previously, there are known relationships between nicotine and methylphenidate use (Vanisckel et al., 2011; Wooster et al., 2008) and nicotine and marijuana use (Gamaleddin et al., 2012; Werling et al., 2009). Researchers estimate that more than twice as many people with ADHD use nicotine related products compared to the general population (Lambert & Hartsough, 1998; Milberger et al., 1997). Since methylphenidate is commonly prescribed in adolescence and is the most common pharmaceutical treatment for ADHD, it is highly likely that nicotine, which is also frequently used in adolescence, is used in combination with methylphenidate (Wheeler et al., 2013). In addition, methylphenidate has been shown to increase cigarette smoking in human studies (Vanisckel et al., 2011), and Wooster et al. (2008)

have shown that methylphenidate may actually increase the abuse related behaviors associated with nicotine use in rats. With all this known, the potential additive effect of early exposure to nicotine and methylphenidate on the rewarding properties of CB agonists is also of interest in this study.

It is hypothesized that the combined effect of early exposure to nicotine and methylphenidate will produce changes to the eCB system in a way that makes CB agonists more rewarding in adulthood than exposure to either one alone. If the combination of nicotine and methylphenidate does produce an additive effect on the rewarding nature of CB agonists then the rats treated with both drugs will spend more time in the drug (CP)-paired compartment then the nicotine and control groups.

However, if nicotine does not alter the rewarding nature of CP the second experiment with nicotine and methylphenidate will not be conducted. Instead, we will assess whether exposure to methylphenidate alone will alter the rewarding nature of CP. There is a common association reported between those who are diagnosed with ADHD and thus likely use stimulant medications, like methylphenidate, and an increased risk for marijuana use and abuse later in life (Lee et al., 2011; Aksoy et al., 2012; Kousha, Shahrivar & Alagnhband-rad, 2012; Galera et al., 2008; Jardin et al., 2011). There are very few, if any, studies directly linking the effects of methylphenidate to the cannabinoid system, although reduced CB1 receptor density is associated with ADHD rat models (spontaneously-hypertensive-rat; SHR) and

cannabinoid agonism has been shown to reduce some ADHD related behaviors in adolescent SHR rats (Adrianni & Laviola, 2004). There is also evidence that shows that the noradrenergic and dopaminergic systems, which are the systems through which methylphenidate exerts its effects, interact with the CB system in a bidirectional manner (Richter et al., 2012; Daigle, Wetsel & Caron, 2011; Giuffrida et al., 1999). Thus, it is possible that the increases in NE and DA activity produced by methylphenidate use may alter the cannabinoid system in a way that could make cannabinoid agonists more rewarding in adulthood.

It was hypothesized that early exposure to methylphenidate will increase the rewarding nature of CB agonists in adulthood. If methylphenidate does increase the rewarding nature of CB agonists then the rats treated with methylphenidate will spend significantly more time in the drug (CP)-paired compartment than the control group.

CHAPTER TEN

MATERIALS AND METHODS

Subjects

Two-hundred and ninety-four male rats of Spague-Dawley descent were used in this study. One hundred and sixty-one rats were used in the nicotine experiment and 133 rats were used in the methylphenidate experiment. Rats were given unlimited access to both food and water throughout the study. Litters were culled to 10 pups and weaned on PD 25. The rats were group housed for the duration of the study. They housed in the California State University, San Bernardino (CSUSB) colony room under a 12-hour light/dark cycle. Both the injections and behavioral testing occurred during the light portion of the cycle. The rats were randomly assigned into groups of approximately equal number. All guidelines for the treatment of animals were followed according to the “Guide for the Care and Use of Laboratory Animals” (Institute for Laboratory Animal Research, 2011). The Institutional Animal Care and Use Committee at CSUSB also approved the experimental procedures before the start of the study.

Apparatus

The CPP apparatus was a T-shaped wooden chamber consisting of two large adjacent compartments measuring 24 x 30 x 45 cm and a smaller side compartment measuring 24 x 10 x 45 cm. The main compartments were

separated by an adjustable partition that either had an opening allowing free movement between the two compartments (preconditioning and testing) or a solid divider that restricted movement to a single compartment (conditioning). Each compartment contained distinct cues that allowed the rats to easily distinguish between the two main compartments. One compartment was painted all white (visual) and had mesh flooring (tactile and visual) and pine bedding (olfactory). The other compartment was painted all black and had straight bar flooring and cedar bedding. The gray side compartment was separated from the main compartments by a partition that was easily opened and closed. The side compartment was used as a neutral starting point between the two main compartments during preconditioning and testing sessions.

Drugs

(-)- Nicotine hyrdrogen tartrate and methylphenidate hydrochloride were obtained from Sigma (St. Louis, MO). Both drugs were dissolved in saline at a volume of 1 ml/kg. Nicotine injections were administered subcutaneously (SC) and methylphenidate was injected intraperitoneally (IP). 2-[(1R,2R,5R)-5-hydroxy-2-(3-hydroxypropyl) cyclohexyl]-5-(2-methyloctan-2-yl)phenol (CP-55,940) was also obtained from Sigma (St. Louis, MO). CP 55,940 was dissolved in 50% DMSO/distilled water and was injected IP at a volume of 1 ml/kg.

Experiment 1: Nicotine Pre-Exposure

In Vivo Drug Treatment

The rats were weighed and then injected with nicotine (0.16, 0.32, or 0.64 mg/kg) or saline for ten consecutive days starting at PD 31. In rats, this injection period (PD 31-40) is developmentally comparable to early adolescence in humans (Anderson, 2003). Once the drug treatment was completed the animals were left undisturbed until PD 55 when handling began for the CPP procedure.

CP-55,940-Induced CPP Procedure

On PD 60 all rats began the CPP procedure. The same conditioning procedures were used for all the experiments. A 13-day biased CPP procedure was used. This included one preconditioning/priming injection day, one rest day, 10 conditioning days, and one testing day. On the preconditioning day, rats received no injection and were placed in the gray side compartment of the apparatus. Once the rats entered either the black or white compartment the partition to the side compartment was closed, and they were allowed to move freely between the main compartments for 15 minutes. The initial compartment preference was determined, and all injections of CP 55,940 (CP) were administered in the non-preferred compartment. Immediately following the preconditioning session, the rats received a priming injection of CP (10, 20, or 30 µg/kg) in their home cages in order to avoid the dysphoric effects commonly associated with the first administration of

cannabinoid agonists (Valjent & Maldonado, 2000; Parker & Gillies 1995; McGregor et al., 1996). The priming doses were the same as those that the rats received during the conditioning stage. Due to the long half-life of CB agonists, the rats had a day break prior to the first day of conditioning.

On conditioning days, the rats were injected with either their respective doses of CP and placed in their non-preferred compartment or saline and placed in their preferred compartment for a 20 min session. There was a 10 min delay between the injection and placement in the CPP apparatus. Initial drug order was counterbalanced within groups. An alternating day schedule continued for 10 days until five CP conditioning days and five saline days were completed. Locomotor activity was assessed on the first and last exposure to the CP drug during the conditioning stage.

The test day was on the 13th day of the CPP procedure. The rats received no injection, and as in the preconditioning stage, the rats started in the gray side compartment and were allowed to move freely between the black and white compartments for 15 minutes. The amount of time spent in each compartment was assessed. The preconditioning, first and last two days of conditioning and testing were videotaped, and automatically scored using the Noldus EthoVision XT 9 software.

Experiment 2: Methylphenidate Pre-Exposure

In Vivo Drug Treatment

Rats were weighed and injected with methylphenidate (0.5, 2 or 5 mg/kg) starting at PD 21 for 10 consecutive days. In rats, the period from PD 21 to PD 30 is developmentally comparable to late childhood in humans (Anderson, 2003). There were two injections of methylphenidate six hours apart per day for the 10-day period. Once injections were completed the rats were left undisturbed in their home cages until PD 55 when handling began for the CPP procedure. An identical CPP procedure was used for this experiment as described in the first experiment.

Data Analysis

Data for all sessions were recorded using *Noldus EthoVision XT 9* video and animal tracking software. Time spent in each compartment was recorded on both the preconditioning and testing days. Change in compartment preference from preconditioning to testing was determined by calculating a difference score between the time spent in the non-preferred compartment at preconditioning and the time spent in the same compartment at testing. Positive scores indicate an increase in the time spent in the drug paired compartment at testing, and negative scores indicate an decrease in the time spent in the drug paired compartment. Rats showing no preference (i.e. preferred compartment <= 455) and rats with extreme preferences (i.e. 75% or more time spent in one compartment at preconditioning) were

excluded from analyses to facilitate data interpretation. This resulted in the removal of 15 cases for the nicotine study and 12 cases for the MPH study. Also, rats with discrepant overall times spent in the CPP boxes at preconditioning and testing due to tracking software errors (preconditioning overall time / testing overall time was <= .95) were also excluded from analyses. This resulted in the removal of three additional cases for the nicotine study and one case for the MPH study. Thus, the data from 143 rats were included for the nicotine study and 120 rats for the MPH study were included in the final analyses.

The first and last two days of drug conditioning were also recorded, and the change in activity from the first to the last exposure of CP 55,940 was assessed. A difference score was calculated between the distance traveled by the rats on the first exposure and the last exposure to CP. Positive scores represented an decrease in activity (behavioral sensitization) and negative scores represented an increase in activity on the last exposure to CP (behavioral habituation).

The data for both experiments was analyzed using separate two-way ANOVAs, and Tukey tests were used for any post hoc comparisons. The alpha level was set at 0.05 for all analyses. In addition to the ANOVAs todetermine group differences, individual t-tests were conducted to determine whether or not a significant preference or aversion occurred. To determine whether a change in preference occurred from preconditioning to testing, the

difference scores for each subgroup were compared to 0 (no difference) using t-tests. The comparisons began with the most extreme difference score and progressively towards the least extreme difference score. The t-tests comparisons were discontinued once a non-significant result was found to limit the number of comparisons. Alpha was corrected using the following formula α/k. Positive values indicated that more time was spent in the CP-paired side after conditioning compared to preconditioning, whereas negative values indicated that a significant aversion for the CP-paired side occurred.

CHAPTER ELEVEN

RESULTS

Experiment One: Nicotine Pre-Exposure

In the first experiment, rats were pre-exposed to nicotine from PD 31 to PD 40, and on PD 60 (early adulthood) began a 13-day biased CP 55,940 – induced CPP procedure. During the conditioning phase, locomotor activity (i.e. distance traveled) on the first or last exposure to CP 55,940 was not affected by either nicotine pretreatment or CP 55,940 treatment. Moreover, there was no interaction of the pretreatment and treatment drugs as activity did not change from the first to the last exposure of CP 55,940 suggesting that early exposure to nicotine did not influence CB agonist-dependent activity.

To determine whether early nicotine exposure altered the rewarding nature of the CB agonist, two-way (Nicotine × CP) ANOVA was conducted. The results indicated that neither nicotine nor CP 55,940 significantly altered compartment preference nor was there any interaction between the two drugs (Nicotine × CP interaction: $F_{9,145} = 0.699$, $p = 0.709$). Thus, contrary to the proposed hypothesis, early nicotine exposure does not seem to affect the rewarding nature of CB agonists (see Figure 1).

Table 1. Mean Distance Traveled (cm) on the First and Last Exposure to CP 55,940. Rats were Pre-Exposed to Nicotine (0, 0.16, 0.32, or 0.64 mg/kg) from PD 31 to 40 and Began the CPP Procedure on PD 60 where they Received Alternating Injections of Saline or CP 55,940 (0, 10, 20, or 30 µg/kg) for Ten Days

	Nicotine Pre-Exposure (PD 31-40)							
	0.0 mg/kg		0.16 mg/kg		0.32 mg/kg		0.64 mg/kg	
CP 55,940	First	Last	First	Last	First	Last	First	Last
Vehicle	*M* = 7853.90 SEM = 574.59	*M* = 6727.49 SEM = 409.62	*M* = 7680.22 SEM = 370.01	*M* = 6309.58 SEM = 273.36	*M* = 8071.20 SEM = 465.15	*M* = 7215.65 SEM = 369.94	*M* = 7934.37 SEM = 420.03	*M* = 6869.46 SEM = 382.95
10 µg/kg	*M* = 8195.95 SEM = 348.59	*M* = 6177.76 SEM = 368.70	*M* = 7780.43 SEM = 491.68	*M* = 6371.50 SEM = 546.85	*M* = 7550.36 SEM = 405.39	*M* = 6141.72 SEM = 344.13	*M* = 8144.42 SEM = 408.09	*M* = 6947.67 SEM = 367.68
20 µg/kg	*M* = 7297.85 SEM = 418.54	*M* = 6631.84 SEM = 532.73	*M* = 8165.44 SEM = 584.50	*M* = 6184.01 SEM = 313.53	*M* = 8206.42 SEM = 365.36	*M* = 6811.42 SEM = 299.08	*M* = 7512.76 SEM = 287.63	*M* = 6981.42 SEM = 486.45
30 µg/kg	*M* = 7795.27 SEM = 450.48	*M* = 6915.79 SEM = 440.97	*M* = 8175.30 SEM = 405.16	*M* = 6918.31 SEM = 544.37	*M* = 8175.84 SEM = 428.58	*M* = 6866.98 SEM = 510.08	*M* = 7965.41 SEM = 467.10	*M* = 6664.38 SEM = 555.26

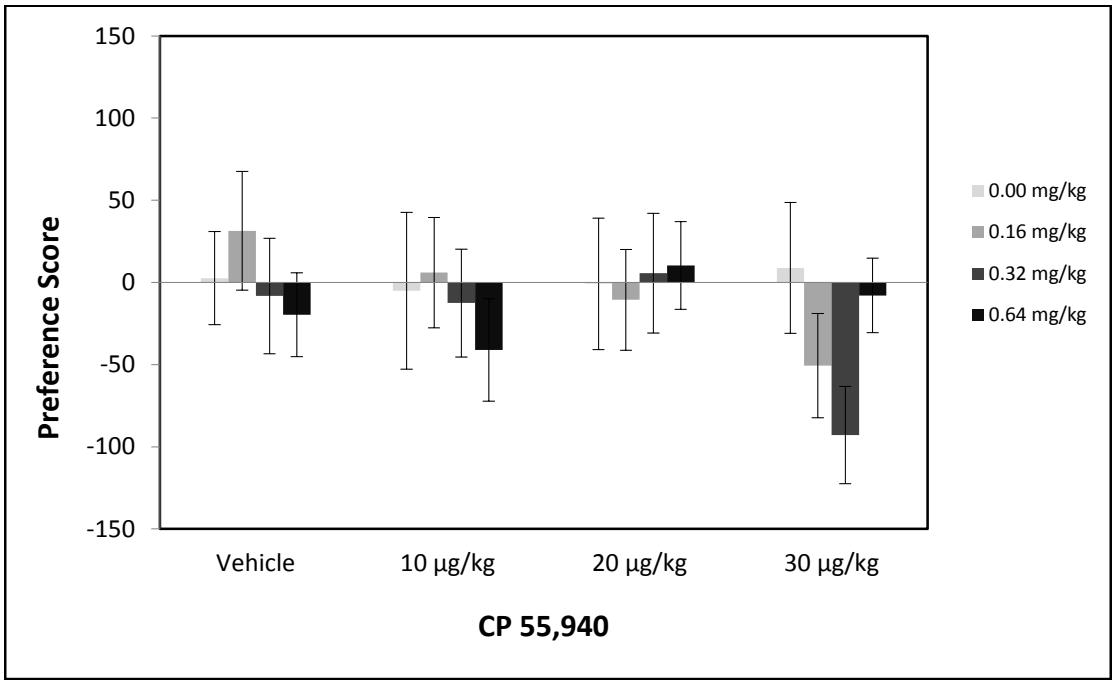

Figure 1. Mean Preference Score (±SEM) on the CPP Test Day. Rats were Pre-Exposed to Nicotine (0, 0.16, 0.32, or 0.64 mg/kg) from PD 31 to 40 and began the CPP Procedure on PD 60 where they Received Alternating Injections of Saline or CP 55,940 (0, 10, 20, or 30 µg/kg) for Ten Days. Positive Scores Indicate an Increase in the Time Spent in the Drug-Paired Compartment and Negative Scores Indicate a Decrease in the Time Spent in the Drug Paired Compartment at Testing.

Individual t-tests were conducted to determine whether each (nicotine x CP 55,940) subgroup was significantly different from 0 (no change in preference). The rats pre-exposed to 0.32 mg/kg nicotine (moderate dose) showed a significant aversion to the 30 µg/kg CP 55,940 (high dose)

[$t(10) = -3.138$, $p \leq 0.01$], suggesting that the moderate dose of nicotine decreased the rewarding properties of CP 55,940. Rats exposed to 0.16mg/kg nicotine (low dose) and conditioned with the high dose of CP 55,940 had the second most extreme mean difference score. However, the mean difference score for this subgroup was not significantly different from 0 [$t(9) = -1.592$, $p = .146$].

Experiment Two: Methylphenidate Pre-Exposure

Rats were pre-exposed to methylphenidate at PD 21 to 30, and on PD 60 they began a CP 55,940 13-day biased CPP procedure. Similar to the first experiment, locomotor activity (i.e. distance traveled) was assessed on the first and last exposure to CP 55,940 (see Table 2). However contrary to nicotine, pretreatment with methylphenidate (0.5 mg/kg) significantly increased activity on the first CP exposure day (MPH main effect, $F_{3, 128} = 3.378$, $p = 0.020$, Tukey Test, $p < 0.05$). This effect on activity however was transient as methylphenidate did not alter activity on the last CP exposure day. Similar to the nicotine pre-exposure experiment, the CP drug had no significant effect on activity either drug exposure day. Again, similar to experiment 1, activity levels for rats exposed to methylphenidate did not change from the first to the last exposure of CP 55,940. Thus, preadolescent methylphenidate pre-exposure did not alter CP 55,940-induced activity in adulthood.

Table 2. Mean Distance Traveled (cm) for the First and Last Exposure to CP 55,940. Rats were Pre-Exposed to MPH (0.0, 0.5, 2.0, or 5.0 mg/kg) from PD 21 to 30 and Began the CPP Procedure on PD 60 where they Received Alternating Injections of Saline or CP 55,940 (0, 10, 20, or 30 µg/kg) for Ten Days

	MPH Pre-Exposure (PD 21-30)							
	0.0 mg/kg		**0.5 mg/kg**		**2.0 mg/kg**		**5.0 mg/kg**	
CP 55,940	**First**	**Last**	**First**	**Last**	**First**	**Last**	**First**	**Last**
Vehicle	*M* = 7499.06 SEM = 307.96	*M* = 6885.41 SEM = 1088.89	*M* = 9583.49 SEM = 365.20	*M* = 7976.02 SEM = 381.04	*M* = 7356.55 SEM = 486.43	*M* = 6438.81 SEM = 599.66	*M* = 7710.03 SEM = 572.37	*M* = 6685.39 SEM = 498.94
10 µg/kg	*M* = 7937.92 SEM = 657.47	*M* = 6066.16 SEM = 588.50	*M* = 8323.90 SEM = 784.76	*M* = 6226.19 SEM = 467.95	*M* = 7360.35 SEM = 464.54	*M* = 6363.68 SEM = 504.38	*M* = 7895.09 SEM = 600.88	*M* = 7023.69 SEM = 405.16
20 µg/kg	*M* = 7579.32 SEM = 309.93	*M* = 6598.96 SEM = 391.64	*M* = 8212.83 SEM = 446.53	*M* = 6558.42 SEM = 488.28	*M* = 7747.41 SEM = 533.89	*M* = 7304.54 SEM = 409.76	*M* = 7711.90 SEM = 714.04	*M* = 6852.74 SEM = 738.93
30 µg/kg	*M* = 8057.83 SEM = 177.87	*M* = 6960.62 SEM = 292.87	*M* = 8504.38 SEM = 243.28	*M* = 6988.73 SEM = 218.86	*M* = 8647.87 SEM = 617.77	*M* = 6998.28 SEM = 636.98	*M* = 7867.78 SEM = 499.85	*M* = 7578.10 SEM = 649.88

A two-way (MPH x CP) ANOVA revealed a non-significant main effect of methylphenidate ($F_{3,117} = 0.230$, $p = .875$). However, there was a significant main effect of CP 55,940 ($F_{3,117} = 3.077$, $p < .05$, $\eta^2 = .073$), and a Tukey HSD post hoc analysis revealed that rats treated with the moderate dose of CP 55,940 (20 µg/kg) showed a significantly greater preference for the drug-paired compartment than rats treated with the high dose (30 µg/kg) (see Figure 2). CP 55,940 exposed rats did not differ significantly from vehicle-treated rats. The hypothesized interaction between methylphenidate and CP 55,940 was not significant ($F_{9,117} = 1.555$, $p = 0.137$), suggesting that methylphenidate did not alter the rewarding nature of the CB agonist (see Figure 3).

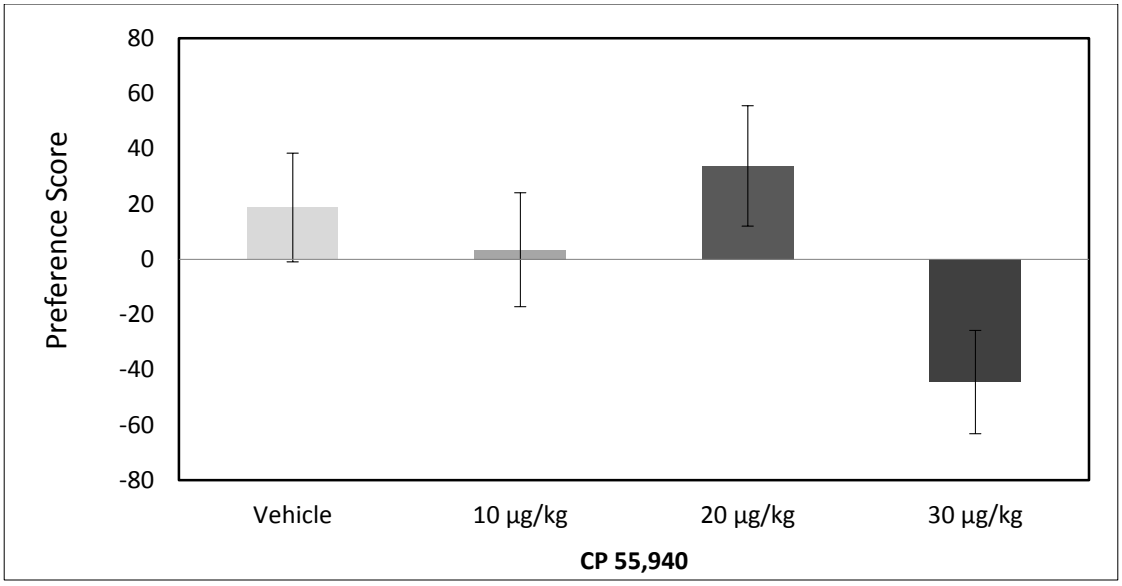

Figure 2. Mean Preference Score (±SEM) on CPP Test Day. Rats were Pre-Exposed to MPH from PD 21 to 30 and began the CPP Procedure on PD 60 where they Received Alternating Injections of Saline or CP 55,940 (0, 10, 20, or 30 µg/kg) for Ten Days. Positive Scores Indicate an Increase in the Time Spent in the Drug-Paired Compartment and Negative Scores Indicate a Decrease in the Time Spent in the Drug-Paired Compartment at Testing. There was a Significant Main Effect of CP 55,940 such that Rats Treated with the Moderate Dose (20 µg/kg) Showed a Significantly Greater Preference for the CP-Paired Compartment than Rats Treated with the High Dose (30 µg/kg)

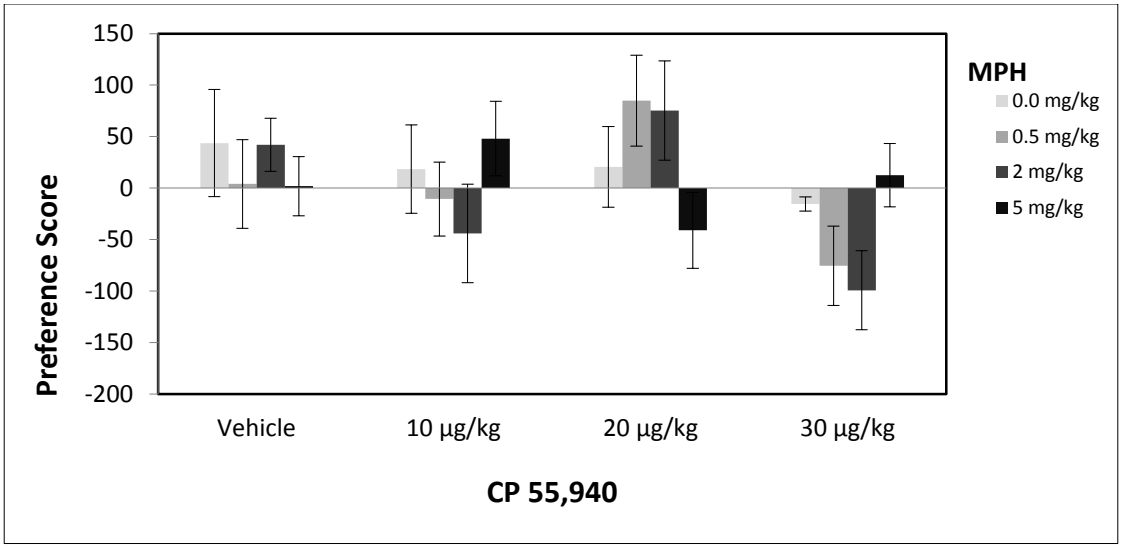

Figure 3. Mean Preference Score (±SEM) on the CPP Test Day. Rats were Pre-Exposed to MPH (0.0, 0.5, 2.0, or 5.0 mg/kg) from PD 21 to 30 and Began the CPP Procedure on PD 60 where they Received Alternating Injections of Saline or CP 55,940 (0, 10, 20, or 30 µg/kg) for Ten Days. Positive Scores Indicate an Increase in the Time Spent in the Drug-Paired Compartment and Negative Scores Indicate a Decrease in the Time Spent in the Drug Paired Compartment at Testing

Similar to the first experiment, individual t-tests were conducted to determine whether each (MPH x CP 55,940) subgroup was significantly different from 0 (no change in preference). The rats pre-exposed to 2 mg/kg MPH (moderate dose) showed a significant aversion to the 30 µg/kg CP 55,940 (high dose) [$t(7) = -2.588$, $p = .036$], suggesting that the moderate dose of MPH decreased the rewarding nature of CP 55,940. However, after correcting alpha for multiple comparisons (.05/2 = .25) the result was no

longer significant. Rats pre-exposed to 0.05 mg/kg MPH (low dose) and conditioned with the 20 μg/kg (moderate) dose of CP 55,940 had the second most extreme mean difference score. The mean difference score for this subgroup was not significantly different from 0 [$t(7) = 1.919$, $p = .096$].

CHAPTER TWELVE

DISCUSSION

Adolescence is a transitional developmental period that is associated with increased impulsivity and risky-decision making (Konrad et al., 2013). This is a vulnerable period for the initiation of substance use, particularly nicotine/tobacco products (SAMHSA, 2012), and effects of psychoactive substance exposure on the brain (Mateos, et al., 2011; Nolley & Kelley, 2007). This is problematic because early nicotine use is associated with the early use of marijuana (Behrendt et al., 2012; Hayatbakhsh et al., 2009) and early marijuana use is considered a risk factor for the development of cannabis use disorders (Copeland & Swift, 2009). Similarly, early adolescence is also a period when individuals are often first exposed to stimulant medications, like methylphenidate, for either prescribed (e.g. ADHD) or recreational purposes (Klein-Schwartz & McGrath, 2003, McCabe et al., 2004), and there is increasing concern as to whether early stimulant exposure influences later substance abuse (Nolley & Kelley, 2007).

The current study was conducted to assess the effect of early exposure to nicotine and methylphenidate on the rewarding nature of cannabinoid (CB) agonists, like marijuana, in adulthood utilizing rats as animal models. The conditioned place preference (CPP) behavioral paradigm was utilized to assess the rewarding nature of the CB agonist CP 55,940. CP 55,940 works similarly to marijuana on both CB1 and CB2 receptors in the central and

peripheral nervous systems, but is substantially more potent at each receptor site than the psychoactive component in marijuana, Δ^9- tetrahydrocannabinol (THC) (Fan et al., 1994; Herkenham et al., 1990; Xie, Melvin & Makriyannis, 1996).

Adolescent Nicotine Exposure and Adult Cannabinoid Preference

Previous pre-clinical studies have shown a link between early exposure to nicotine and enduring changes to the endocannabinoid (eCB) system into adulthood, including increased CB receptor density and activity (Mateos, et al., 2011; Werling et al., 2009). Based on these findings, it was hypothesized that nicotine exposure during the adolescent period would result in behavioral and potentially perceptual changes to the phenomenological experience of CB agonists in adulthood. Specifically, it was hypothesized that early exposure to nicotine would enhance the rewarding nature of CB agonists in early adulthood. This is particularly important because if early exposure to nicotine enhances the rewarding nature of CB agonists this would suggest that the relationship between early nicotine use and the risk for adult cannabis use disorders could be explained through the enduring biological changes nicotine has on the eCB system during adolescence. Thus, identifying another clear risk factor for early nicotine initiation and possibly elucidating a potential pharmacological target to treat or possible prevent marijuana abuse in adulthood.

The group based results indicated that early exposure to nicotine did not enhance the rewarding nature of CP 55,940. However, individual subgroup comparisons revealed that rats pre-exposed to the moderate dose of nicotine showed a significant aversion to the high dose of CP when compared to a mean difference score of 0 or no change in preference from preconditioning to testing. This suggests that the effect early exposure to nicotine has on the eCB system may translate into significant changes to CB agonist-induced reward learning in adult rats. This is an important finding as this may suggest that the enduring changes to the eCB system caused by early nicotine use in adolescence may influence CB agonist use and possibly abuse in adulthood. The study also revealed that locomotor activity was not changed from the first to the last exposure of CP 55,940, suggesting that early exposure to nicotine does not alter the behavioral effects of CB agonists in adulthood.

Although it appears that early exposure to nicotine may alter the rewarding nature of CB agonists, there are some factors to consider when attempting to interpret and/or generalize the results of this study. First, the difficulty in producing conditioned place preferences using CB drugs (Murray & Bevins, 2010) may make it extremely difficult to translate study findings to humans, when marijuana is the most widely used illicit drug and thus, is considered a highly rewarding psychoactive substance for many humans. Second, although the effects of CP 55,940 are reasonably comparable to THC, it may be inappropriate to conclude that early exposure to nicotine may

alter the rewarding nature of THC specifically, as the pharmacodynamics of THC are similar but not identical to those of CP 55,940 (Fan et al., 1994; Xie, Melvin & Makriyannis, 1996). From this study we can conclude that early exposure to nicotine may alter the rewarding nature of CP 55,940 as assessed by the CPP paradigm (substance dependent cue-based learning). Although, the current study does provide evidence to suggest that the biological effects of nicotine exposure on the eCB system in adolescence may translate into notable changes in the rewarding nature of CB agonists in adulthood.

Late Childhood Methylphenidate Exposure and Cannabinoid Preference

Methylphenidate (Ritalin) is frequently prescribed to older children as a psychopharmacological treatment for ADHD (Leonard et al., 2004). Over the last decade researchers have discovered an association between individuals diagnosed with ADHD in late childhood, and thus have likely been exposed to stimulate medications like methylphenidate early in life, and an increased likelihood of marijuana abuse in adulthood (Lee et al., 2011; Aksoy et al., 2012; Kousha, Shahrivar & Alagnhband-rad, 2012; Galera et al., 2008; Jardin et al., 2011). Although no previous studies have directly linked methylphenidate use to biological changes in the eCB system, the aforementioned association and pharmacodynamics of methylphenidate could suggest that early exposure to stimulate medications, like methylphenidate, may alter the eCB or reward/motivation-based systems in such a way to alter

the rewarding nature of CB agonists, like marijuana, later in life. Similar to the first experiment, it was hypothesized that early exposure to methylphenidate would alter the rewarding nature of the CB agonist CP 55,940. If the hypothesis was confirmed it would suggest that early methylphenidate use influences the abuse potential of drugs like marijuana in adulthood. This is particularly concerning because methylphenidate is one of the most frequently prescribed drugs to children with ADHD (Leonard et al., 2004). However, the current study revealed that early exposure to methylphenidate did not alter the rewarding nature of CP 55,940, suggesting that the association reported between ADHD and marijuana abuse is not likely attributable to enduring biochemical effects of methylphenidate on the eCB system in the brain. This finding is consistent with a recent meta-analysis conducted by Humphreys, Eng and Lee (2013) suggesting that stimulant medication does not influence the risk for adult substance abuse.

Similar to Experiment 1 (Nicotine pre-exposure), rats treated with CP 55,940 in Experiment 2 (MPH pre-exposure) did not significantly differ from controls in their preference for the drug-paired compartment. However, rats conditioned with the moderate dose of CP 55,940 (20 µg/kg) showed a greater preference for the drug-paired compartment at testing compared to rats exposed to the high dose (30 µg/kg). This is consistent with previous findings that show that high doses of CB agonists tend to be aversive (Murray & Bevins, 2010; Braidi et al., 2001; Bardo & Bevins, 2000; Valjent & Maldonado,

2000). However, the significant effect of CP 55,940 was somewhat surprising in this study as the CPP procedure was conducted in exactly the same way as in the first experiment, but no significant group based results were found in the first experiment. Considering the only differences between the first and second experiments were the pre-treatment drugs (nicotine vs. methylphenidate) and the developmental timing of the pre-exposure (PD 31-40 vs. PD 21-30), it's possible that early methylphenidate exposure may have had some effect on the rewarding nature of CP 55,940. However, no definitive conclusions can be drawn, as there was no significant interaction between methylphenidate and CP 55,940 (p = .137) in the group based comparisons. As in the first experiment, interpretations and generalizations should be made with caution as CP 55,940 was used in place of THC in this study.

Conclusion

The association commonly reported between individuals who start using nicotine products in early adolescence and the greater likelihood for the abuse of marijuana in adulthood may be related to the biological effects of nicotine on the eCB system in adolescence. However, the group based results did not show significant difference from controls. Considering these findings, it is still important to consider other ways in which nicotine could influence the use of marijuana through potentially non-reward based biological mechanisms. In addition, the reported association between early nicotine use and adult marijuana abuse could also be explained through biological or social

influences distinct from the biological effects of nicotine, including factors related to impulsivity, risk taking and peer group influences. Also, the current study limited nicotine exposure to a 10-day period in early adolescence. Future studies may benefit from maintaining nicotine exposure throughout the course of the study to more closely represent the persistent nature of nicotine use for those who begin smoking during early adolescence. The findings may be altered if nicotine exposure is maintained for an extended period.

Also, the early use of stimulant medications does not seem to directly affect the rewarding nature of marijuana in adulthood. However, future studies should consider assessing whether early methylphenidate exposure has an enduring biological effect on the eCB system, similar to the aforementioned studies conducted on the early effects of nicotine (Mateos, et al., 2011; Werling et al., 2009), in order to clarify whether early methylphenidate exposure has an enduring effect on the cannabinoid system. In addition, it will be important to consider factors specific to ADHD, especially marked impulsivity, as potentially more salient explanatory factors in the association of early ADHD diagnoses and increased marijuana abuse in adulthood. Similar to the first experiment, future studies should also consider the duration of methylphenidate exposure when assessing its effects on the rewarding nature of CB agonists, like marijuana, in adulthood.

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