OPPOSING PERSISTENT BEHAVIORAL EFFECTS OF PHYTO- AND ENDOCANNABINOIDS FOLLOWING CHRONIC DEVELOPMENTAL, BUT NOT ADULT, TREATMENTS

by

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April, 2019

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The rate of adolescent Cannabis abuse is increasing for recreational purposes and it is thought to be linked to a range of developmental and social problems. Many studies have demonstrated effects of early Cannabis exposure to produce behavioral effects that persist through adulthood. However, the physiological changes in the CNS that must be responsible for this altered behavior remain poorly understood. Delta-9-tetrahydrocannabinol (THC) is a psychoactive cannabinoid isolated from Cannabis that exerts its effect by partially-activating cannabinoid receptors. Adolescence is a critical period for brain development and maturation. Zebra finches are songbirds that learn vocal patterns during a sensitive period of development that approximates adolescence. Exposure of these animals to a cannabinoid agonist during their period of sensorimotor vocal learning alters song patterns produced in adulthood. Thus, songbirds have unique value in studying developmental effects of drug exposure on a naturally learned behavior. We have adapted place preference methods to study cocaine reinforcement of behavior. Moreover, we pharmacologically manipulated 2- arachidonoyl glycerol (2-AG) levels using JLZ-184 (a selective inhibitor of MAG lipase, the enzyme responsible for

degradation of 2-AG) to determine if augmentation of the endogenous cannabinoid signaling produced THC-like increased cocaine sensitivity. We have found that cocaine dose-dependently reinforces both place preference and aversion at potencies consistent with those observed in mammalian species. THC persistently increases sensitivity to cocaine through adulthood. However, developmental exposure to JZL-184 induced aversion. These effects were not observed following treatment of adults. Moreover, the expression of c-Fos (a marker of neuronal activity) was increased in Area X of striatum in THC-treated animals and nucleus taeniae of amygdala in JZL-184-treated animals. Also, elevated dopamine (DA) and 3,4-Dihydroxyphenylacetic acid (DOPAC) levels were observed in Area X and VTA only in animals that developmentally treated with THC. On the other hand, we have found that developmental chronic THC and JZL-184 exposure resulted in alteration of the song phonology that persist through adulthood.

This indicates that normal endocannabinoid signaling is important to vocal learning, and agonism or antagonism of these processes disrupts this learning, indicating that a “normal tone” of cannabinoid signaling is required.

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A Dissertation

Presented to the Faculty of the Department of Pharmacology and Toxicology

Brody School of Medicine at East Carolina University

In Partial Fulfillment of the Requirements for the Degree

Doctor of Philosophy in Pharmacology and Toxicology

by

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DEDICATION

To whom I love, my parents, wife, sons, brothers, sisters, nephews, nieces, and friends.

ACKNOWLEDGMENT

I would like to thank my mentor, Dr. Ken Soderstrom and my dissertation advisory committee members, Dr. Brian A. McMillen, Dr. Jamie C. DeWitt, Dr. Jacques Robidoux, and Dr. Christopher N. Balakrishnan for their inputs, support, and advice.

Special thanks to my great wife, Maram, for her love, prayers, patience, and support since the first day of my study till the last day. She was always there with me in each moment and without her I could not accomplish my degree. I would like to thank her for taking care of our sons, Alwaleed and Fahad, during the toughest time of my study.

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LIST OF SYMBOLS OR ABBREVIATIONS

2-AG: 2-Arachidonoylglycerol

ACTH: Adrenocorticotropic hormone

AEA: Anandamide

AFN: Anterior forebrain pathway

Area X: The basal ganglia

CB1: Cannabinoid receptor 1

CB2: Cannabinoid receptor 2

CBD: Cannabidiol

CBN: Cannabinol

CMM: Caudomedial mesopallium

CNS: Central nervous system

COMT: Catechol-*o*-methyltransferase

CRF: Corticotropin-releasing factor

CRHR1: Corticotropin Releasing Hormone Receptor 1

CPP: Conditioned Place Preference

CPu: Caudate-putamen

DA: Dopamine

DLM: The Thalamic region

DOPAC: 3,4-Dihydroxyphenylacetic acid

eCB: Endocannabinoid system

FAAH: Fatty acid amide hydrolase

GABA: Gamma-Aminobutyric Acid

GAD: Glutamic acid decarboxylase

GAT-1: GABA transporter 1

HVC: Used as a proper name

HPLC: High-performance liquid chromatography

IHC: Immunohistochemistry

JZL-184: MAG lipase inhibitor

lMAN: lateral magnocellular nucleus of the anterior nidopallium

L-DOPA: Levodopa

MAGL: Monoacylglycerol lipase

mPFC: Medial prefrontal cortex

NAc: Nucleus accumbens

NCM: Neostriatum

PET: Positron emission tomography

PFC: prefrontal cortex

RA: Robust nucleus of the arcopallium

SR: SR141716A

TH: Tyrosine hydroxylase

THC: delta-9-tetrahydrocannabinol

VTA: Ventral tegmental area

CHAPTER 1: INTRODUCTION

1.1 Cannabis Abuse in Adolescence

Use or abuse of illicit drugs mostly starts at early age during the teen and young adult years (Samsha, 2012). Statistics show that approximately 50,000 of American school students in 8th, 10th, and 12th grades had used illicit drugs such as alcohol, cocaine, nicotine, LSD, heroin, amphetamine, and marijuana in their lifetime (Johnson, O'Malley, Bachman, & Schulenberg, 2013). The drinking of alcohol comes in the first place in terms of substances abused among 13 and 14 years old, followed by marijuana (Chadi, Bagley, & Hadland, 2018). Marijuana is used widely for recreational purposes due to its availability to adolescents despite laws making possession a serious crime in many countries, including the United States. Although annual use of marijuana among teenagers decreased in prevalence from 50% in 1970s to 36% in one recent survey, it is still considered a threat for the young adult that causes negative consequences including social and health problems (Chadi et al., 2018). As the teenager's brain continues to develop, any use of illicit drugs during this critical period of life causes many social and health problems that persist through adulthood. Social problems including violence, car accidents, and sexual assaults are higher in adolescents who use illicit drugs compared to those who do not (Decker et al., 2014; Senn, Carey, & Vanable, 2010). In addition, illicit drugs result in altered neurodevelopment and associated cognitive and behavioral functioning (Chadi et al., 2018). Neuroimaging studies have shown a decrease in the volume of some brain structures such as hippocampus and prefrontal cortex (PFC) in alcohol drinkers compared to the non-drinking control which may lead to deficits in

memory (Chadi et al., 2018). Moreover, positron emission tomography (PET) in human studies revealed that repeated and regular cannabis use downregulated the cortical CB1R (Hirvonen et al., 2012). Also, functional imaging studies showed that cannabis abuse reduces activity in brain regions that are involved in memory and attention (Block et al., 2002).

1.2 Adolescence is a Critical Period of Brain Development and Learning

1.2.1 White and Gray Matter Changes

Adolescence is a critical period for brain development and maturation. During the brain maturational processes many changes occur in the context of physiological and social transitions. These changes have an important influence on cognitive function, behavior and emotional processing. Adolescent brain size continues to increase through adulthood (Pfefferbaum et al., 2012). Increases in the white and decreases in the gray matter volume and density in brain regions that are important for memory, attention, and cognitive ability occur during childhood and adolescence. Moreover, these changes include maturation of the neuronal circuity and white matter connections and increase the synaptic density in the cortical regions and limbic system (Barnea-Goraly et al., 2005; Paus et al., 1999).

1.2.2 Development Neurotransmitter Systems

Neurotransmitter systems also appear to develop during adolescence including the dopaminergic, GABAergic, and glutamate NMDA receptor systems (reviewed by He & Hodge, 2007). The development of these neurotransmitter systems during adolescence

is important for PFC function and that of the limbic system which includes the hippocampus, amygdala, nucleus accumbens (NAc), and VTA (Arnsten, Cai, Murphy, & Goldman-Rakic, 1994; Constantinidis, Williams, & Goldman-Rakic, 2002; Sawaguchi & Goldman-Rakic, 1991; Vijayraghavan, Wang, Birnbaum, Williams, & Arnsten, 2007).

1.2.2.1 Dopaminergic System

Dopamine (DA) is a neurotransmitter that belongs to the catecholamine family that plays a role in a variety of physiological functions (i.e. motivation, attention, movement, and hormone regulation) and pathophysiological conditions (i.e. Parkinson's disease), Berridge, Robinson, & Aldridge, 2009). L-DOPA is the primary precursor for DA synthesis by the enzyme aromatic L-amino acid decarboxylase (DOPA decarboxylase) in the presence of a cofactor pyridoxal phosphate. L-DOPA itself is synthesized from L-tyrosine by tyrosine hydroxylase (TH, the rate limiting step) with tetrahydrobiopterin, O_2, and iron (Fe^{++}) as cofactors (reviewed by Björklund and Dunnett 2007). Synthesis of DA takes place in cell bodies in the brain (neurons) and kidneys (adrenal glands) (reviewed by Björklund and Dunnett 2007). After synthesis, DA will be stored in synaptic vesicles and released into the synaptic clefts in response to action potentials. DA then binds to DA receptors (either post or presynaptic receptors) in the synapse. After that, DA can be taken up back by dopamine transporters (DAT) into the pre-synaptic cells or broken down by enzymes. There are a set of enzymes that are responsible of DA degradation including monoamine oxidase (MAO), catechol-O-methyl transferase (COMT), and aldehyde dehydrogenase (ALDH) and the main end products are dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA) (Eisenhofer, Kopin, & Goldstein, 2004). Dopaminergic neurons are located in different parts of the brain including the substantia

nigra and ventral tegmental area (VTA). The dopaminergic neurons in substantia nigra pars compacta are projecting to dorsal striatum (known as nigrostriatal pathway) and play an important role in the control of motor functions (reviewed by Björklund and Dunnett 2007). Damage to these neurons can lead to Parkinson's disease. Whereas the dopaminergic neurons in the VTA are projecting to the prefrontal cortex (PFC, known as the mesocortical pathway) and the Nucleus accumbens (NAc, mesolimbic pathway) and together both of these pathways are known as the mesocorticolimbic pathway. In addition, the VTA is also projecting to other parts of the brain including amygdala and hippocampus. The mesocorticolimbic pathway plays a significant role in reward and motivation (reviewed by Björklund and Dunnett 2007). DA release normally occurs in response to a stimulus (i.e. food, exercise) that creates an action potential in the DA neuron (Berridge et al., 2009). However, in the case of the drugs of abuse such as cocaine and methamphetamine, the levels of DA in the synapses will be higher than normal because of the DAT blocking and facilitating its release from the nerve endings (reviewed by Ikemoto, Yang, and Tan 2015). DA is the main molecule that has been well studied and investigated in addiction research as most drugs of abuse including alcohol, nicotine, and opioids were found to increase its synaptic levels by different mechanisms (reviewed by Ikemoto, Yang, and Tan 2015).

The activity of the dopaminergic system rises and peaks through adolescence and then stabilizes during adulthood (Tarazi & Baldessarini, 2000). For example, Tarazi and his colleagues demonstrated that DA subtype receptors density including D_1, D_2, and D_4 in frontal cortex and hippocampus increased by several fold in postnatal days 35-60 in the rat (Tarazi & Baldessarini, 2000). Using positron emission tomography (PET) in

humans to study DA receptor densities, and radioligand binding studies examining dopamine 1 receptor (D1R) densities, a reduction of 14–26% in the in the adult group compared to adolescents in the striatum and cerebral cortex were observed (Jucaite, Forssberg, Karlsson, Halldin, & Farde, 2010). In addition, DA transporter density was found to be increased by 6-7-fold in caudate-putamen (CPu, one of the structures that composes the basal ganglia) and NAc of rat brain in postnatal days 7-60 (Tarazi, Tomasini, & Baldessarini, 1998). Levels and activity of catechol-*o*-methyltransferase (COMT, one of the enzymes responsible for catecholamine degradation) are reported to increase two-fold from neonate to adulthood in primate PFC (Tunbridge et al., 2007). Also, immunohistochemistry studies showed that the level of TH, the rate-limiting enzyme involved in DA synthesis, peaks in PFC during the late-postnatal period and then declines in adulthood (Rosenberg & Lewis, 1994). In one study, haloperidol responsiveness and sensitivity was tested in different age groups to compare between younger and adult rats finding that the younger group was more sensitive to haloperidol in a manner associated with increased DA turnover in striatum and NAc - demonstrating that haloperidol was more potent in younger animals (Teicher et al., 1993). Progressive decreased expression of elements of the dopaminergic signaling system neurotransmission from adolescence to adulthood suggests that this period of life is critical in context of brain maturation, development and related psychopathology. Any external intervention to alter CNS activity during this critical period (adolescence) may have persistent consequences both negative (i.e. drug addiction) or positive (i.e. disease treatment).

1.2.2.2 The GABAergic (γ-aminobutyric acid) System

The GABAergic system, controlled by the major inhibitory neurotransmitter in the brain, is another system that is undergoes remarkable changes during adolescence and has a major role in cortical remodeling (reviewd by He & Hodge, 2007). In one study, amygdala fibers that form synaptic connections with the GABAergic interneurons in the PFC have been found to be largely increased during adolescence (Cunningham, Bhattacharyya, & Benes, 2008). The density of pre- and postsynaptic markers of GABA neurotransmission increased in the PFC of monkeys during adolescence (Lewis, Cuz, Eggan, & Erickson, 2004). In addition, Kellogg and his colleagues reported that the $GABA_A$ receptor density in adolescence was higher in the cortex compare to adults (Kellogg, Taylor, Rodriguez-Zafra, & Pleger, 1993). Also, it was reported that the GABA system may play a role in hippocampus formation when Hachiya and Takashima found that GABA transporter 1 (GAT-1) and glutamic acid decarboxylase (GAD) expression, a marker for GABAergic neurons, peaked in early infancy (Hachiya & Takashima, 2001). $GABA_A$ receptor α1 subunit, that mediates some of the pharmacological effects such as sedation and anticonvulsant actions, expression was found to be increased in the PFC in adolescents followed by a reduction during adulthood (Yu, Wang, Fritschy, Witte, & Redecker, 2006).

The maturation of GABAergic signaling from infancy to adolescence to adulthood is important as it is contributing to brain development and related behaviors including cognitive control, risk taking, and decision making (Silveri et al., 2013). Any intervention by alcohol and other drugs of addiction during adolescence may disrupt and remodel the

GABA system that may result in alcohol dependence and other drug addiction through adulthood.

1.2.2.3 Glutamate and NMDA Receptor Systems

In one of the *in vitro* receptor autoradiography studies in rat forebrain that was conducted by Insel and his colleagues, they found that the binding of glutamate to its N-methyl-D-aspartate (NMDA) receptors increased in adolescents compared to adults (Insel, Miller, & Gelhard, 1990). Moreover, NMDA-receptor dependent long-term potentiation (LTP), a measure of synapse strength, was more frequent in NAc in adolescent (3 weeks old) mice compared to adulthood (Schramm, Egli, & Winder, 2002). In addition, LTP and plasticity were also reported in other brain regions such as VTA (M. J. Thomas, Beurrier, Bonci, & Malenka, 2001), amygdala (Ungless, Whistler, Malenka, & Bonci, 2001), and hippocampus (Walter Adriani et al., 2004).

Addictive drugs are known to modulate the maturation of neurotransmission during adolescence (Crews, Rudolph, & Chandler, 2002). Thus, the impact of the environmental factors, such as abusing illicit drugs, is critical to understanding how adolescents are more vulnerable to drug addiction.

1.2.3 Learning New Skills During Late-Postnatal Development

Adolescence is a critical period of development where learning ability is active. At this critical period, learning new skills or traits are easier due to the physiological changes and development that occur in the brain. Examples of skills that are acquired and learned during this time are language, sports, music, communication and memory (He & Hodge, 2007; L.P. Spear, 2000). Evidence indicates that learning the first language starts at early

age of life and ends in puberty and demonstrates that language acquisition is unique to childhood (Grimshaw, Adelstein, Bryden, & MacKinnon, 1998). In addition, it was reported that children who grew up in isolation or had hearing impairment did not develop their first language normally because they miss sensory inputs during a critical window of learning in the first years of life (Friedmann & Rusou, 2015).

In terms of memory, one spatial working memory task study that had been conducted in a group of adolescents (9 to 20 years old) showed improvement in working memory abilities including developing the recall-guided action for single units of spatial information, maintaining and manipulating multiple spatial units, and strategic self-organization. PET studies showed the spatial working memory tasks activate ventrolateral PFC (Luciana, Conklin, Hooper, & Yarger, 2005). This development of working memory abilities was found to recruit the frontal lobe regions that continue to develop over childhood and adolescence (Conklin, Luciana, Hooper, & Yarger, 2007). A weight of prior evidence suggests the importance of this period of development.

1.3 Cannabis-related Phytocannabiniod

Cannabis is known to have been used by the ancient Chinese, Indian, Egyptian and Assyrian civilizations for both medical and recreational purposes (Mechoulam & Parker, 2013). Approximately 115 different cannabinoids have been isolated and identified from the Cannabis plant but Δ9-tetrahydrocannabidiol (THC), cannabidiol (CBD), and cannabinol (CBN) are the most well-studied constituents (Aizpurua-Olaizola et al., 2016). THC is the principal psychoactive constituent of Cannabis and was first isolated by Gaoni and his colleague in 1964 (Gaoni & Mechoulam, 1964). Cannabis users

experience mental and physical effects including euphoria, increased appetite and heart rate and impaired coordination (Anderson, 2018). When smoked, its effects begin within minutes of use and reaches peak levels shortly afterward in about 10 to 30 minutes. As THC is a lipid, it partitions to fat that may serve as a slow release depot, making the influence of THC last for a longer time than expected based upon blood levels (Anderson, 2018). It was formerly believed that THC exerted its effects by a general nonspecific mechanism by binding to and altering the structure of neuronal cell membranes. This view was disproven following discovery of the cannabinoid 1 receptor (CB1R) in 1988 by Devane & Howlett (Devane, Dysarz, Johnson, Melvin, & Howlett, 1988). THC is behaving like the endogenous chemical, anandamide, as partial agonist at CB1 receptors and displaying lower affinity at CB2 receptors (reviewed by Pertwee, 2008). In many studies, administration of THC was found to increase brain reward effects as it increases place preference and self-administration of a number of drugs of abuse such as heroin, methamphetamine, and alcohol (Panagis, Mackey, & Vlachou, 2014)

Cannabidiol (CBD) is a phytocannabiniod that was discovered in 1940. CBD is a non-psychoactive constitute that does not produce the similar psychoactive effects of THC. Depending on strain, CBD may account for up to 40% of the Cannabis plant's extract (Campos, Moreira, Gomes, Del Bel, & Guimaraes, 2012). CBD has been variously studied in clinical research for its effects and role in pain, anxiety, cognition, and movement disorders (Boggs, Nguyen, Morgenson, Taffe, & Ranganathan, 2018). CBD showed a little affinity for CB1 and CB2 receptors (Mechoulam, Peters, Murillo-Rodriguez, & Hanuš, 2007). However, CBD was found to work as an antagonist for the cannabinoid-receptor agonists (CP55940 and WIN55212) in CB1 mouse model and in human CB2

transfected cells (A. Thomas et al., 2007). Moreover, multiple evidence demonstrates that CBD effects are mediated by a number of receptors (Reviewed by Boggs et al., 2018). It was reported that CBD produces its effects such as antidepressant-like effect and anxiolytic effect by binding to the serotonin 1A (5-HT_{1A}) receptor in rodent models (Fogaça, Reis, Campos, & Guimarães, 2014; Sartim, Guimarães, & Joca, 2016). Also, other reports demonstrated interactions between CBD and G protein coupled receptor 55 (GPR55), μ and δ opioid receptors, and transient receptor potential vanilloid type-1 (TRPV1) cation channels (reviewed by Pertwee, 2008). Recently, the Food and Drug Administration (FDA) of the United States has approved marketing of the CBD formulation, Epidiolex, for the treatment of epilepsy (FDA, 2018).

Cannabinol (CBN) is another phytocannabiniod that is found in trace amounts in the Cannabis plant (Karniol, Shirakawa, Takahashi, Knobel, & Musty, 1975). Also, CBN is a metabolite of THC degradation (McCallum, Yagen, Levy, & Mechoulam, 1975). CBN is a mildly psychoactive constitute compared to THC and binds partially to CB1R and has high affinity at CB2R (Mahadevan et al., 2000).

1.4 Endocannabinoid System (eCB)

1.4.1 Endocannabinoid System Background

The endocannabinoid system is composed of endocannabinoid ligands, cannabinoid receptors and synthetic and degradative enzymes. This system is involved in a variety of physiological processes such as mood, memory, pain, and appetite (reviewed by Mechoulam & Parker, 2013). The CB1 receptor was first cloned in 1990 (Matsuda 1990). This was followed by discovering and identifying the CB2 receptor in

peripheral tissues, particularly spleen, soon after in 1993 (Munro, Thomas, & Abu-Shaar, 1993). Both CB1 and CB2 receptors are G protein-coupled receptors (GPCR) that have similarly in 48% of amino acid sequence and couples to adenylyl cyclase and mitogen-activated protein kinase (Howlett et al., 2002). CB1 receptors are mainly expressed in the central nervous system (CNS) and they are one of the most abundant GPCRs in brain (Howlett et al., 2002). As they are found on GABA and glutamatergic neurons, they are involved in GABA and glutamate neurotransmission (Howlett et al., 2002). Activation of CB1 receptors decreases cyclic adenosine monophosphate (cAMP) accumulation and this inhibits the cAMP-dependent protein kinase (PKA). Also, CB1R activation stimulates the mitogen-activated protein (MAP) kinase activity. In addition, CB1R are coupled through G proteins to many types of potassium and calcium channels. On the other hand, CB2 receptors are mainly present in the immune system and recently were found to be expressed at low levels in CNS (Ashton, Friberg, Darlington, & Smith, 2006; Onaivi et al., 2008; Van Sickle et al., 2005). Similar to CB1, activation of CB2R inhibits cAMP accumulation through $G_{i/o}$ proteins and hence decreases the MAP kinase activity (Felder et al., 1995; Kobayashi, Arai, Waku, & Sugiura, 2001). Moreover, CB2R activation results in activation of phospholipase C (PLC) and modulates the intracellular Ca^{2+} concentration (Zoratti, Kipmen-Korgun, Osibow, Malli, & Graier, 2003). In addition, there are also former orphan receptors that are now considered as cannabinoid receptors expressed in several peripheral systems and brain including G-protein coupled receptor 18 (GPR18), GPR55 and GPR119. These formerly orphan receptors play a role in various physiological and disease conditions such as melanoma, pain, diabetes, obesity, and osteoarthritis (A. Irving et al., 2017).

The endogenous cannabinoid ligands are lipids that bind to the cannabinoid receptors. These include most notably anandamide (AEA) and 2-arachidonoyl glycerol (2-AG, Figure 1.1). AEA was first identified by Mechoulam and his colleagues in 1992 (Devane et al., 1992) while the 2-AG was first described in 1995 (Mechoulam et al., 1995). Both of those ligands are retrograde synaptic messengers and synthesized on demand and are degraded by different enzymes. Multiple biosynthetic pathways play roles in the synthesis of AEA and this includes enzymes such as Ca^{++}-dependent *N*-acyltransferase (NAT) and N-acyl phosphatidylethanolamine phospholipase D (NAPE-PLD, (Wang & Ueda, 2009). AEA has a very short half-life (< 5 minutes) and is degraded by fatty acid amide hydrolase (FAAH) into arachidonic acid and ethanolamine (Cravatt et al., 2001). On the other hand, phospholipase C (PLC) and diacylglycerol lipase (DAGL) are involved in the 2-AG synthesis and formation while monoacylglycerol lipase (MAGL) is responsible for its degradation (Dinh et al., 2002). AEA has a low affinity for and is a partial agonist at both CB1 and CB2 receptors, however 2-AG has a high affinity (a full agonist) to CB1R and low affinity for CB2R (Gonsiorek et al., 2000; Luk et al., 2004). In addition, there are other ligands that may serve as endocannabinoids including N-dihomo-γ-linolenoylethanolamine, N-docosatetra enoylethanolamine, O-arachidonoylethanolamine, oleamide, N-arachidonoyl dopamine and N-oleoyl dopamine (Roger G. Pertwee, 2005).

1.4.2 Importance of Endocannabinoids (eCB) Signaling for Early CNS Development and Function

Many studies have found that eCB signaling emerges and is operating at an early age. Autoradiography experiments show that CB1 receptor levels peak during developmental and decrease in the adult rat (Bossong & Niesink, 2010). CB1 receptors are distributed to different extents in different brain regions, such as the cerebellum, the hippocampus, the basal ganglia, and the cortex, which suggests a role in a number of physiological processes including memory, mood, appetite, and pain-sensation (Bossong & Niesink, 2010). The use of cultured hippocampal neurons, it can be demonstrated that the activation of CB1 receptors located in presynaptic GABAergic neurons leads to the inhibition of GABA release (A. J. Irving et al., 2000). Axonal growth cones in GABAergic neurons are rich with CB1 receptors and endocannabinoids trigger the collapse of these cones (Watson, Chambers, Hobbs, Doherty, & Graham, 2008). These studies suggest that the eCB signaling has a critical role in early CNS development and function.

1.4.3 Adolescent but not Adult Cannabinoid Exposure Leads to Persistent Changes in Behavior and Neurophysiology

In one study, adolescent rats exposed chronically to THC showed a large deficit in their ability to learn. This effect was not seen when adults were exposed to the same treatments (Cha, Jones, Kuhn, Wilson, & Swartzwelder, 2007). Further experiments indicated that exposure to THC during development caused other persistent behavioral effects, such as affecting cognition and memory due to changes in hippocampal morphology and neuroplasticity (Moore et al., 2010; Rubino et al., 2009). These

experiments suggest that adolescents are more vulnerable to behavioral and neurophysiological changes than adults. The mechanisms underpinning these effects are still poorly understood. In our lab using the zebra finch songbird model, exposure to cannabinoid agents during a critical period of CNS development (adolescence) resulted in similar effects (Soderstrom & Tian, 2006). Also, exposure to a cannabinoid agonist during development leads to elevations of dendritic spine densities in the basal ganglia (Area X) and HVC regions (Gilbert & Soderstrom, 2011).This indicates that the exposure to cannabinoid agonist disrupts the normal decrease of dendritic spine densities over the course of development (Gilbert & Soderstrom, 2011).

1.5 Endocannabinoid System and Reward

CB1 receptors are the most abundant G protein-coupled receptors in the brain and they are expressed in brain regions that are important for reward and addiction including NAc, ventral tegmental area (VTA), lateral hypothalamus, PFC, and amygdala (Glass, Faull, & Dragunow, 1997; X. Wang, Dow-Edwards, Keller, & Hurd, 2003). Activation of pre-synaptic CB1R inhibits neurotransmitter (e.g. GABA & glutamate and acetylcholine) release that have many effects on neuronal signaling. There is a body of evidence demonstrating that both opioid and dopaminergic systems are influenced by eCB signaling in the context of reward and reinforcement behaviors (Wenzel & Cheer, 2018). For example, opioids are rewarding because of their effects on the mesolimbic DA system. Opioids exert their effects such as analgesia, respiratory depression, and euphoria by binding to opioid receptors GPRCs ($G_{i/o}$) including mu opioid receptor (MOPR), delta opioid receptor (DOPR), and the kappa opioid receptor (KOPR). Those receptors are widely expressed in the brain limbic system regions such as VTA and NAc.

Therefore, in one study it was found that administration of MOPR agonist (including morphine and endorphins) produced conditioned rewarding effects (reviewed by Shippenberg, LeFevour, & Chefer, 2008). Much evidence shows that there are interactions between the eCB and opioid systems. For example, it has been found that exogenous THC administration results in releasing endogenous opioids (enkephalins) in the NAc (Valverde et al., 2001). Additionally, heroin self-administration was found to increase the extracellular levels of endocannabinoids (particularly, 2-AG levels) in the NAc shell (Caillé, Alvarez-Jaimes, Polis, Stouffer, & Parsons, 2007). Moreover, cross tolerance can occur as a result of chronic administration of either THC or opioid drugs (Newman, Lutz, & Domino, 1974). Further, the administration of either WIN 55,2121-2 (a CB1 receptor full agonist) or heroin led to alterations of the levels and activity of μ-opioid (MOR) and CB1-cannabinoid receptors (CB1R) in the PFC, NAc , caudate-putamen (CP) and other brain areas that mediate rewarding effects (Fattore et al., 2007). The mechanism behind the interaction between the eCB and opioid systems is still unclear. One explanation could be that the co-localization of the two systems in the brain regions that are responsible for the rewarding effects might play a role (Pickel, Chan, Kash, Rodríguez, & MacKie, 2004). Moreover, one study reported that CB1 cannabinoid and μ-opioid (MOR) receptors in the NAc core may heterodimerize and synergistically inhibit GABA release (Schoffelmeer, Hogenboom, Wardeh, & De Vries, 2006).

Also, CB1 and μ-opioid (MOR) receptor interaction meditate reward and reinforcement effects. This evidence shows the rewarding effects of THC are abolished in μ-opioid (MOR) knockout mice (Ghozland et al., 2002). In addition, another study demonstrated that administration of naloxone (opioid receptor antagonist) blocked

cannabinoid-induced conditioned place preference, CPP (Braida, Pozzi, Cavallini, & Sala, 2001). On the other hand, the absence of CB1 receptor blocked the opiate-induced CPP (Ledent et al., 1999). These data suggest that the interaction between eCB and opioid system may enhance and facilitate the DA release the brain reward areas.

Moreover, exogenous AEA and 2-AG administration increases DA levels in the limbic system through a CB1R dependent mechanism (Solinas, Justinova, Goldberg, & Tanda, 2006). However, in most animal studies, neither a FAAH inhibitor nor a MAGL inhibitor administered alone increased the reward-related behaviors or drug reinforcement (Panlilio, Justinova, & Goldberg, 2013). However, concurrent administration of FAAH and MAGL inhibitors was found to increase self-administration, a result suggesting the need for a robust elevation of both AEA and 2-AG in order to result in a THC-like discrimination effects (Wiley et al., 2014; Wise et al., 2012).

Other drugs of abuse such as alcohol, nicotine, opiates were found to alter the level of endogenous cannabinoids (AEA and 2-AG) in different brain regions which indicates their important role in the brain reward effects and addiction of these drugs (González et al., 2002; Simonnet, Cador, & Caille, 2013; Viganò et al., 2003). For example, evidence from *in vivo* microdialysis in the NAc after ethanol administration demonstrated an increasing level of extracellular 2-AG and with no effect on the level of AEA (Simonnet et al., 2013). However, opioid exposure increased the content of AEA and slightly decreased 2-AG concentrations (Simonnet et al., 2013). In addition, chronic nicotine exposure resulted in a reduction in the levels of both AEA and 2-AG in midbrain while it increased their levels in brainstem, and AEA only in the limbic forebrain (González et al., 2002). Additionally, nicotine self-administration increased the level of both AEA and

2-AG in VTA (Buczynski, Polis, & Parsons, 2013). Moreover, reports of chronic opiate administration in rats demonstrated a reduction in the 2-AG levels without any alterations of AEA content in limbic brain regions and hypothalamus (Viganò et al., 2003). Moreover, a psychostimulant drug, such as methylphenidate, decreased the concentration of both AEA and 2-AG in the limbic forebrain of the mice (Patel, Rademacher, & Hillard, 2003). All of the previous examples indicate that many of the drugs of abuse alter the levels of endocannabinoids and sometimes produce similar or opposite effects on brain AEA and 2-AG contents. The differences in the effects on the endocannabinoid levels in those studies could be due experimental differences such as drug route of exposure, duration of treatment, or dose of drug.

1.6 Endocannabinoid System and Stress

Interaction between the endocannabinoid system and hypothalamic-pituitary-adrenal (HPA) axis is important in order to regulate stress responses. CB1R is highly expressed in brain regions that are important for HPA-axis regulation such as amygdala, hippocampus, hypothalamus and PFC (Herkenham et al., 1990, 1991). Activation of the HPA axis is known to be responsible for a stress response trigger. It starts when the activation of hypothalamus leads to release corticotropin-releasing factor (CRF). CRF binds and activates the anterior pituitary gland to release adrenocorticotropic hormone (ACTH). ACTH binds to adrenal receptors adrenal and stimulates release of cortisol (reviewed by Micale & Drago, 2018). CB1R is found to be expressed in hypothalamus, pituitary gland, and adrenal gland which suggests it may have a role in releasing CRF, ACTH and cortisol. Moreover, AEA and 2-AG are expressed in the pituitary gland too and

may have a potential role for eCB system in stress response (reviewed by Micale & Drago, 2018).

1.6.1 Acute Stress

Restraint stress was found to increase the level of FAAH, a degradative enzyme for AEA, and reduce the content of AEA in amygdala and hippocampus (Matthew N Hill et al., 2009; M. Wang et al., 2012) and this was in parallel with a higher level of corticosterone in blood (Matthew N Hill et al., 2009). In contrast, results from a foot shock paradigm demonstrated an increase in the level of AEA in mPFC, amygdala and hippocampus (Morena et al., 2014). These discrepancies could be explained by using different protocols (restraint stress *vs* foot shock paradigm) or by using different animal species (mice *vs* rats). Other studies showed an increase in the level of 2-AG in response to acute stress in different brain areas including the hypothalamus, hippocampus, and mPFC. In addition, higher levels of corticosterone were found to modulate 2-AG levels in the hypothalamus, hippocampus, and mPFC (Evanson, Tasker, Hill, Hillard, & Herman, 2010; Matthew N. Hill et al., 2011; M. Wang et al., 2012). No changes in CB1 and CB2 receptor expression or activity in brain were found following the restraint stress exposure (Matthew N Hill et al., 2009; MacDowell et al., 2017).

1.6.2 Chronic Stress

Chronic restraint stress was found to increase FAHH activity and reduce the level of AEA in brain areas such as hypothalamus, hippocampus, and amygdala (M N Hill et al., 2013). Also, it was found that corticosterone treatment decreases AEA content via corticotropin releasing hormone receptor 1 (CRHR1), a regulator of the HPA-axis-

mediated mechanism (Bowles et al., 2012). In contrast, using another model of chronic unpredictable stress (CUS) did not show any changes in the level of AEA (Bowles et al., 2012; Gray et al., 2016). On the other hand, prolonged restraint stress increased the level of 2-AG in hypothalamus, hippocampus, amygdala and mPFC (Dubreucq et al., 2012; Patel, Roelke, Rademacher, & Hillard, 2005). In the context of the CB1R, chronic stress exposure reduced its expression and activity in striatum, hypothalamus, midbrain, hippocampus (Bortolato et al., 2007; Matthew N. Hill et al., 2008).

1.7 Zebra Finch Songbirds as a Developmental Learning and Drug Abuse Model

Zebra finch songbirds are naturally vocal learners, like humans: these birds provide advantages for studying drug effects during critical periods of learning. Zebra finch brain contains specialized song regions that are involved in song learning and production. There are two critical pathways for song learning and production. The song learning pathway known as the anterior forebrain pathway (AFP) is comprised of at least three regions: lateral magnocellular nucleus of the anterior neostriatum (lMAN); the basal ganglia (Area X) and the thalamic region DLM. These interconnected regions form a circuit that is important during the sensorimotor period of vocal learning. The other pathway is the song production pathway that contains most notably HVC and the robust nucleus of the archistriatum (RA). In addition, an auditory pathway comprised of CMM and NCM, interface with the motor circuit, that in turn influences the learning circuit (Alvarez-Buylla, Theelen, & Nottebohm, 1990; Mooney, 2009).

1.8 Goal of Research and Statement of Hypothesis

As described above, adolescence is a distinct and critical period of learning new skills such as language, motor behaviors, and social interaction. To the best of our knowledge, chronic Cannabis abuse during adolescence and its persistent effects though adulthood are not adequately investigated. Our model, zebra finch songbirds, because of their specific features and characteristics as discussed above compared to other animal models, were used in this project to address our questions that are related to chronic cannabinoid exposure. Previously in our lab, reported that chronic cannabinoid treatments during adolescence, not adulthood, causes: 1) alteration in the song learning ability (Soderstrom & Johnson, 2003) and; 2) persistently altered axonal and dendritic spine densities in zebra finch brain regions that are important for song (Gilbert & Soderstrom, 2011, 2014; Soderstrom, Poklis, & Lichtman, 2011). Also, acute cannabinoid treatments inhibited zenk and Arc/Arg3.1 expression in NCM, one of the auditory perception regions, which in turn affects song recognition (Gilbert & Soderstrom, 2013). However, it is still unclear whether endocannabinoid signaling is important during the critical period of drug reinforcement and song learning or not. Zebra finch song brain regions contain high level of CB1 receptors and 2-arachidonoyl glycerol (2-AG), and their levels change over the course of development (Soderstrom & Wilson, 2013). Preliminary evidence that CB1 receptor antagonism alters song in a manner similar to agonists suggests that this is the case, but it has not yet been experimentally confirmed.

The goal of this research was to evaluate the chronic exposure of THC, the psychoactive constitute of Cannabis, during adolescence and how that can affect both vocal learning and drug addiction processes through adulthood. It is important to evaluate

the behavioral effects of Cannabis, the most widely abused drug by teenagers, and whether if it becomes a gateway drug for more toxic substances such as cocaine later in life. Moreover, the importance of endocannabinoid signaling to critical period-dependent learning will be investigated.

This project will provide information to better understand the effects of developmental Cannabis exposure and the role of the endogenous cannabinoid signaling during a natural sensorimotor learning period. Effects on vocal learning will be compared to changes in cocaine sensitivity later in life as a model of learning related to addiction. We suspect that similar neuronal circuits control development-dependent learning of both vocal communication and drug abuse.

Specific Aims

We investigated how developmental THC exposure (relevant to Cannabis abuse) and manipulation of the principal CNS endocannabinoid (2-AG) cause both persistently altered vocal learning and learning related to reinforcement. Both of these behavior effects may arise from similarly-altered neurophysiology involving the same neural circuits. To test this hypothesis, three aims were developed as follows:

Aim 1: Determine if THC exposure during zebra finch sensorimotor vocal learning alters cocaine reinforcement through adulthood

This aim evaluated chronic THC daily treatment for developmental and adult zebra finches prior to cocaine challenge tests. A conditioned place preference (CPP) paradigm similar to that used in rodents was used to evaluate drug reinforcement (Riters & Stevenson, 2012). First, the appropriate cocaine dose to be used for a songbird model in the CPP experiment was determined by doing an acute dose response curve and then

immunohistochemistry (IHC) for the expression of c-Fos as an indicator of neuronal activity in song brain regions was conducted in order to investigate their involvement in response to different cocaine doses. Second, the delayed time to introduce animal to CPP chambers was determined for cocaine preference or aversion behaviors. Third, persistent effects of chronic THC and nicotine treatments was evaluated.

Aim 2: Determine if brain regions involved in endocannabinoid-altered drug reinforcement and vocal learning overlap

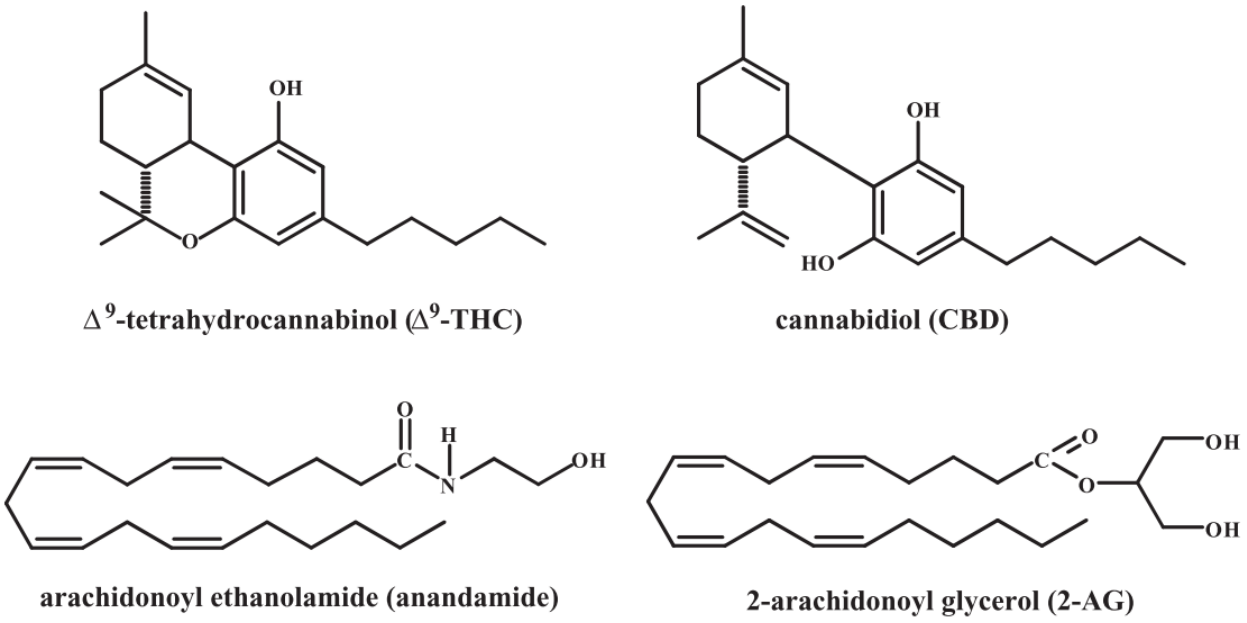
This aim evaluated the role of 2-Arachidonoyl glycerol (2-AG), one of the endogenous cannabinoids, in drug reinforcement. First, developmental and adult animals were chronically treated with JZL-184 (selective MAGL inhibitor) in order to systemically increase the level of 2-AG prior to cocaine challenge using CPP. Moreover, different methods such as HPLC, mass spectrometry, IHC (for what protein?) were used to test the involvement of song brain regions in drug reinforcement.

Aim 3: Does chronic activation of endocannabinoid signaling with an indirect agonist (JZL-184) mimic the effects of exogenous agonist (THC) on vocal learning?

This aim focused on the effects of chronic THC and JZL-184 on song alteration in both developmental and adult zebra finches. Different software for song analysis was used after the chronic treatment and maturation. SAP2011 software helped us to characterize syllables with measures of 14 acoustic parameters and with song recording. In addition, Kullback-Leibler (KL) distances is a statistical method to compare the 2D probability distributions between different groups after chronic treatments was used. Other software programs such as Avisoft SASLab, Bulk Rename Utility, Spectrogram16 were also used to help us to rename the song files and remove extraneous non-song

recordings. The song activity was quantified and the variability in phonology was evaluated.

Figure 1.1. Structures of the phytocannabinoids delta-9-tetrahydrocannabinol and cannabidiol and of the endogenous cannabinoids anandamide and 2-arachidonoyl glycerol



(Adapted from Mechoulam and Parker 2013)

CHAPTER 2: DELTA-9-THC EXPOSURE DURING ZEBRA FINCH SENSORIMOTOR VOCAL LEARNING INCREASES COCAINE REINFORCEMENT IN ADULTHOOD

2.1 Introduction

Zebra finches learn vocal patterns during a critical period of development (Eales, 1985). Because of this they represent an animal model suitable for studying neurobiology underlying maturational stage-dependent learning ability (Mello, 2014).

We have previously found that the critical period for vocal learning in these animals is associated with distinct sensitivity to CNS-active drugs (Soderstrom & Johnson, 2003). For example, exposure to a cannabinoid agonist during distinct sub-periods of sensorimotor vocal learning have differing effects on the relative numbers of syllables improvised vs. learned from adult male tutors (Soderstrom & Tian, 2004). Thus, this songbird model is suitable for investigating effects of drugs to alter behavior (and underlying neural circuitry) dependent upon learning during distinct, sensitive periods of development. Ability to study naturally learned vocal behavior is a unique to songbirds and is not possible with more common rodent models.

A missing feature of the zebra finch model is ability to study drug reinforcement of behavior (Spealman & Goldberg, 1978; Tzschentke, 1998). This is important as developmental exposure to multiple classes of abused drugs are associated with increased self-administration and sensitivity to reinforcement in adulthood (Linda Patia Spear, 2016) As drug exposures during similar periods of development cause both persistently-altered vocal learning and learning related to reinforcement, both may arise from similarly-altered neurophysiology. Testing this hypothesis requires ability to study

both types of learning within the same animal model. To fill this gap, we have developed methods to measure cocaine-conditioned reinforcement of place preference in zebra finches. The method we have employed is adapted from that described previously to demonstrate rewarding effects of undirected singing (Riters & Stevenson, 2012).

We have used the resulting place preference method developed to test ability of both THC and nicotine administered during vocal learning periods to persistently alter cocaine reinforcement in adulthood.

2.2 Materials and Methods

2.2.1 Subjects

Subjects were adult zebra finches raised in our breeding aviary. Both male and female animals were used as indicated. Birds were housed under 14:10-hr light/dark cycles and provided ad libitum food (Sun Seed VitaFinch) and water.

2.2.2 Chemicals and Reagents

Except where noted all supplies, chemicals and reagents were purchased from Sigma or Fisher Scientific. The phytocannabinoid partial agonist THC was obtained from the NIDA drug supply program. Drugs were suspended in vehicle for injections from concentrated DMSO stocks.

2.2.3 Drug Treatments

Drugs were delivered in 50 µl via IM injection into pectoralis using 30 ga needles. For chronic exposures, injections were given once daily in the morning for a period of 25

days. For developmental exposures, treatments were given from 50 – 75 days of age during a period of sensorimotor vocal learning. Following developmental treatments, animals were allowed to mature to adulthood at 100 days of age. For adult exposures, mature animals (>=100 days of age) were given 25 daily injections and were unmanipulated for an additional 25 days to simulate the maturation period in the developmental group. Following the maturation or simulated maturation period, animals underwent cocaine-reinforced place preference testing as described below.

2.2.4 Place Preference Apparatus

The place preference chambers used were constructed from two standard wire 16 X 16 X 18” finch cages with right or left side panels removed. Cages were joined at the missing panels by steel cage rings. An opening for a removable panel was created between the two cages. This removable panel was constructed from stiff poster board with bright yellow construction paper glued to one side, and bright green construction paper glued to the opposing side. The same yellow or green construction paper was affixed with zip ties to the sides, roof and floor of respective cages. A standard wooden perch was used in one cage, and an aluminum foil-covered perch was used in the opposing cage. Thus, chambers were distinguished by color and perch texture.

2.2.5 Cocaine-Conditioned Place Preference

Experiments were conducted in the afternoon. During the first five days of experiments, the panel separating the two chambers of the place preference apparatus was removed and animals were allowed to freely explore both sides for a period of 15 min. Behavior was video recorded and time spent in each chamber documented. The

chamber that each animal spent the least amount of time in during these pre-conditioning tests was designated as the least-preferred chamber. For conditioning, the chamber-dividing panel was installed, and animals were randomly assigned to receive cocaine or vehicle as their first treatment. Vehicle or cocaine injections were given IM and unless specified otherwise, animals were confined in a chamber 5 min after injections for a period of 15 min. Also, unless otherwise specified, animals receiving cocaine were placed in least-preferred chambers. Vehicle and cocaine treatments were made on alternating days for a total of eight days. The day following this conditioning period, animals were given a preference test by removing the dividing panel and allowing free exploration of both sides of the apparatus. The amount of time spent in the cocaine-paired chamber was determined and used to calculate a preference score (post-conditioning minutes spent in the cocaine-paired chamber minus pre-conditioning minutes spent in the cocaine-paired chamber).

2.2.6 Cocaine Dose-Response Experiment

To determine an appropriate dosage of cocaine to use in chronic treatment studies, a dose response experiment was done to evaluate efficacy of 0, 1.25, 2.5, 5 and 10 mg/kg dosages. Each group consisted of n = 6 animals. In an effort to ensure that some level of place preference was observed, half of the animals in each dosage group were assigned to receive cocaine in their least-preferred chamber (determined as described above) and the other half in their preferred chamber. Cocaine-conditioned place preference experiments were done as described above. For conditioning, animals were placed in the appropriate chamber five minutes after injections.

2.2.7 Anti-c-Fos Immunohistochemistry

The day following post-cocaine conditioning preference tests, animals in the dose-response experiment were killed by Equithesin overdose and PBS (pH = 7.4) followed by phosphate-buffered 4% paraformaldehyde, pH = 7.0 transcardially perfused. After brains were removed and immersed overnight in buffered 4% paraformaldehyde, they were blocked down the midline, and hemispheres were sectioned parasagittally (lateral to medial) on a vibrating microtome. Immunohistochemistry was performed using a standard protocol reported in (Whitney, Soderstrom, & Johnson, 2000) except that anti-c-Fos primary antibody was employed. For immunohistochemistry experiments, 30-µm sections of zebra finch brain were reacted with a 1:3,000 dilution of polyclonal anti-c-Fos antibody raised in rabbit (Santa Cruz Biotechnology, cat #sc-253). Tissue sections were rinsed in 0.1% H2O2 for 30 min, blocked with 5% goat serum for 30 min, and incubated overnight in blocking solution containing anti-c-Fos antibody (1:3,000). After antibody exposure, sections were rinsed in PBS (pH = 7.4), incubated in blocking solution containing biotinylated anti-rabbit antiserum (1:500) for 1 h, rinsed with PBS again, and then submerged in avidin–biotin–peroxidase complex solution (purchased as a kit from Vector Laboratories) for 1 h. Antibody labeling was visualized with DAB (diaminobenzidine) solution. Control sections that were not incubated in primary antibody were not immunoreactive.

Staining was examined in various brain regions at 200X using an Olympus BX51 microscope with Nomarski DIC optics. Images were captured using a Spot Insight QE digital camera and Image-Pro Plus software (MediaCybernetics, Silver Spring, MD, USA) under identical, calibrated exposure conditions. These images were background-

corrected, converted to grey scale, and borders of brain regions traced manually. Two-dimensional counts of labeled nuclei from images and areas enclosed within traced areas were determined without knowledge of treatment condition for each brain region of interest from five separate sections per animal using Image-Pro Plus software. Mean densities (within region counts of stained nuclei/area of the region) were compared across treatment group and brain region with a two-way ANOVA as described below.

2.2.8 Timing of Opposing Cocaine Responses

When studied in rodents, cocaine initially produces positive reinforcement shortly after administration that is later followed by a negative affective state (Ettenberg, 2004). Therefore, a complete characterization of cocaine reinforcement in our songbird model system depended upon understanding the timing of these responses in zebra finches. Zebra finches (n = 6) were randomly assigned to timing groups to receive 1, 5, 10, 15 and 30-minute delays after cocaine injections before introduction to the least preferred chambers. As this study was not related to singing behavior that is only produced by male zebra finches, these experiments provided an opportunity to employ adult females (>= 100 days of age).

2.2.9 Chronic Treatment with THC and Nicotine

Developing (50 +/- 3 days of age) and adult (> 100 days of age) animals were randomly assigned to subgroups (n = 7 - 8) to receive either vehicle, THC (3 mg/kg suspended in vehicle) or nicotine (0.4 mg/kg suspended in vehicle). The vehicle for THC was DMSO:Alkamuls EL-620 [Rhodia, Cranberry, NJ]:PBS, 18:1:1. The vehicle for nicotine was (ethanol:Alkamuls EL-620 [Rhodia, Cranberry, NJ]:PBS, 18:1:1).

Treatments were given by IM injection of 50 µl into pectoralis. Injections were made once daily, in the morning, over a period of 25 days. Following chronic exposures, developing animals were allowed to mature to adulthood (100 +/- 3 days of age). Adult animals underwent the same 25 days treatment regimen followed by 25 days of no manipulation to simulate the maturation period in developing birds. After the maturation period or its simulation, animals were evaluated for cocaine-reinforced place preference as described above.

2.2.10 Statistics

Results of the dose-response experiment were evaluated by 2-way ANOVA with cocaine dosage and chamber preference as main effects. Results of chamber placement delay experiments were evaluated by 1-way ANOVA with delay time as the main effect. Results of chronic treatment studies that compared vehicle and drug-treated groups were evaluated using two-tailed t-tests. Statistics were calculated using SigmaStat 3.1 software running on a Windows XP emulation. Means +/- standard error or 95 % confidence interval are reported as indicated.

2.3 Results

2.3.1 Cocaine Dose-Response

A dose-response experiment was done to determine an appropriate cocaine dosage to employ in place preference experiments. Groups of six adult males were assigned to receive 0, 1.25, 2.5 or 5 mg/kg cocaine. Twelve animals received 10 mg/kg cocaine. For this initial experiment, half of each group were given cocaine and placed in

their preferred chamber, and in the other half cocaine was paired in their least preferred chamber. In pre-conditioning preference tests 65 % of animals preferred green chambers with standard wooden perches, 35 % preferred yellow with foil-covered perches. Birds demonstrated strong chamber preferences and spent an average of 82.2 +/-2.9 % of the total pre-conditioning exploration time in their preferred chamber. Results of the dose-response experiment are summarized in figure 2.1. Two-way ANOVA indicated that both dosage and chamber preference had significant effects on preference scores without an interaction (dosage $F [4,26] = 5.00$, $p = 0.004$; chamber preference $F [1,26] = 8.28$, $p = 0.008$; interaction $F [4,26] = 2.22$, $p = 0.094$). In the case of animals receiving cocaine in their preferred chambers, all cocaine dosages resulted in 99.3 +/-5.4 % of preference test time in preferred chambers (Figure 2.1A). In contrast, a clear dose-response relationship (variable slope EC50 = 2.55 mg/kg) was observed in animals that received cocaine in their least preferred chamber (Figure 2.1B). As the goal of chronic exposure experiments was to determine potential persistent changes in sensitivity to cocaine reinforcement, a dosage of cocaine that produces a partial response, liable to either increases or decreases following chronic treatments with THC and nicotine was necessary. Thus, the 2.5 mg/kg cocaine dosage, paired with least preferred chambers, was employed in the following experiments.

2.3.2 Timing of Opposing Cocaine Responses

Similar to what is observed following IV administration of 0.75 mg/kg cocaine to rats (Ettenberg, 2004) we found that 2.5 mg/kg cocaine delivered IM resulted in place preferences when introduction to chambers was delayed 1, 5 or 10 min following injections (Figure 2.2). Place aversion was observed after 15 or 30 min delays. One-way

ANOVA confirmed a significant effect of chamber placement delay on preference scores ($F [4,25] = 3.61$, $p = 0.019$). As our dose-response study employed a five minutes chamber placement delay following cocaine injections, for consistency we chose to continue to use this time in the subsequent experiments.

2.3.3 Persistent Effects of Chronic THC and Nicotine Treatments

In rats, nicotine administered during periadolescence, but not adulthood, persistently increases both nicotine self-administration (W Adriani, Macrì, Pacifici, & Laviola, 2002) and sensitivity to cocaine or diazepam reinforcement (McMillen et al. 2005; James-Walke et al., 2007) following maturation. Persistently-altered rodent behavior following adolescent exposure to multiple abused drug classes is established (reveiwed by Spear, 2016). To test the hypothesis that a similar phenomenon occurs in songbirds, we evaluated effects of periadolescent THC or nicotine treatments to persistently alter sensitivity to cocaine reinforcement.

Animals (n=8) were treated once daily for 25 days with either vehicle, the cannabinoid partial agonist THC (3 mg/kg, in a DMSO-containing vehicle) or nicotine (0.4 mg/kg, in an ethanol-containing vehicle). Groups of both developing (50 days of age) and adult (> 100 days of age) animals were compared. Following the 25-day treatment period, animals were allowed to mature without further treatment for an additional 25 days. Thus, at the time of cocaine conditioning, all animal were adults (>= 100 days of age) and any drug effects observed must have persisted for at least 25 days. Results of these chronic exposure experiments are summarized in figure 2.3.

When administered during development, 3 mg/kg THC significantly increased preference scores for the cocaine-paired chamber compared to vehicle-treated controls

(Figure 2.3A, difference between mean preference scores = 457.9, 95 % CI = 104.5 – 810.5, p = 0.015, two-tailed t-test). No significant preference score differences were observed between vehicle- and THC-treated adults (Figure 2.3B, mean difference = 162.1, 95 % CI =-292.6 – 616.8, p = 0.457, two-tailed t-test). These results suggest that developmental, but not adult THC treatments increases the sensitivity to the reinforcing effects of cocaine. This increased sensitivity is persistent, lasting at least 25 days.

Seven adult animals were treated with nicotine, in all other groups n = 8. Although there was a trend for 0.4 mg/kg nicotine treatments delivered during development to increase preference scores for the cocaine-paired chamber, the difference from the vehicle group was not significant (Figure 2.3C, mean difference = 208.4, 95 % CI = -245.3 – 662.2, p = 0.313, two-tailed t-test). Daily nicotine treatment of adults did not result in preference scores different from the vehicle-treated group (Figure 2.3D, mean difference = 27.5, 95 % CI = -462.4 – 517.8, p = 0.905, two tailed t-test). Although direct comparisons cannot be made across different experiments, preference scores in animals treated with the ethanol-containing vehicle used in the nicotine experiments (Vehicle, Figure 2.3C and D) appeared higher than those treated with the DMSO-containing vehicle in THC experiments (Vehicle, Figure 2.3A and B). These apparent differences may be due to aversive effects of the DMSO-containing vehicle or positive reinforcing effects of the ethanol-containing vehicle. Thus, the apparent lack of expected developmental nicotine efficacy to increase sensitivity to cocaine reinforcement may be related to elevated preference scores in animals treated with the ethanol-containing vehicle.

2.3.4 c-Fos Expression in Song Regions Following Preference Tests

To begin to identify brain regions involved in learning cocaine reinforcement in songbirds, and to test the hypothesis that these regions may include those required for vocal learning (Figure 2.4), we assessed c-Fos expression as a function of cocaine dosage used for conditioning. Expression of c-Fos was induced by placement of animals back into their cocaine-conditioned chambers on the day following final preference tests. Neither drug nor vehicle were injected so that any response was a reaction to placement in the environment. As expected, we observed high-level expression in striatum that receives prominent midbrain dopaminergic input. Less expected were the lowest expression levels in the striatal sub-region, Area X, and relatively low activity in a second nucleus of the learning-essential anterior forebrain pathway, lMAN. The vocal motor-related cortical-like areas HVC and RA also showed clear dose-dependent sensitivity to cocaine. Statistics summarizing cocaine effects on c-Fos expression, and post-hoc comparisons of dosage groups to vehicle controls by brain region are presented in Table 2.1.

2.4 Discussion

Consistent with effects observed in other vertebrates, cocaine dose-dependently conditioned a place preference in zebra finches, with a potency similar to what is characteristically-produced in rodents (Jones and McMillen, 1995; Mueller and Stewart 2000). In addition, initial reward followed later by an opposing aversive response was observed over a time frame similar to that reported in rats (Ettenberg, Fomenko, Kaganovsky, Shelton, & Wenzel, 2015; Su, Santoostaroam, Wenzel, & Ettenberg, 2013).

Taken together, these results support the validity of the procedure we have developed to study cocaine reinforcement of songbird behavior.

2.4.1 Persistent Effects of Developmental Drug Exposure in Songbirds

We previously employed zebra finches as a vocal learning model to study effects of CNS-active drugs administered during late post-natal development. These songbirds have the advantage of natural learning vocal patterns during a sensitive period of late-postnatal development that approximates mammalian adolescence. Repeated cannabinoid agonist exposure during the sensorimotor stage of vocal learning reduces the stereotypy of song motifs and the number of distinct note types produced in adulthood (Soderstrom and Johnson, 2003). Agonist exposure during this learning period also resulted in persistent changes to increase dendritic spine densities and expression of synaptic markers - physiological effects similar to those reported following developmental drug exposure in mammals (Gilbert & Soderstrom, 2014). Our current results contribute to this prior work by the demonstration of developmental cannabinoid exposure that not only alters vocal learning, but also persistently increases sensitivity to cocaine reinforcement. This is important as the same neural circuitry may control both.

For example, the activation of dopaminergic projections from mammalian VTA to dorsal striatum is well known to be important to drug reward (Krebs, Boehler, Roberts, Song, & Woldorff, 2012). This circuit shares features with songbird midbrain projections to Area X (a region of basal ganglia). Positive reinforcement of singing behavior that is dependent upon this circuit is important to the process of vocal learning (Hoffmann, Saravanan, Wood, He, & Sober, 2016). Optogenetic activation of this songbird dopaminergic pathway promotes, and inactivation inhibits, vocal learning (Xiao et al.,

2018). Thus, the same neurodevelopmental changes responsible for altered vocal learning may also increase sensitivity to cocaine reinforcement. The songbird model represents a uniquely useful system with which to study such potential convergent developmental effects on a naturally learned, reward-dependent behavior.

An unexpected result of this study was the inability to clearly document an effect of developmental nicotine exposure to increase cocaine sensitivity. Similar nicotine treatment during rat development clearly increases cocaine reinforcement in adulthood (McMillen et al., 2005). A toward such an effect in zebra finches may indicate that increasing group sizes will confirm an effect. However, it may be the case that songbirds are less sensitive to cholinergic modulation than are rodent species. The apparently higher sensitivity to effects of THC during development may be related to distinctly high level CB1 receptor expression in relevant brain regions that notably peak during sensorimotor vocal learning (Soderstrom & Tian, 2006).

2.4.2 Persistent Developmental Effects of Reinforcing Drugs

The songbird drug reinforcement procedure we employed here is based on that developed by (Riters & Stevenson, 2012), that was used to demonstrate that singing is rewarding to these animals. We are not the first to have used an avian species to study drug reinforcement. Pigeons have long been used in operant conditioning experiments, and reinforcing effects of both THC (Dykstra, McMillan, & Harris, 1975; Henriksson, Johansson, & Järbe, 1975) and cocaine (Evans & Wenger, 1990) were evaluated in these semi-terrestrial birds. To the best of our knowledge, however, the current report documents the first use of avian reinforcement to evaluate persistent effects of chronic, developmental drug exposures over periods of vocal learning. In humans early initiation

of drug abuse is associated with increased probability of addiction and dependence throughout life (Hingson, Heeren, & Winter, 2006; Mechoulam et al., 1995). In rodent models, adolescent exposures to several classes of CNS active drugs are associated with persistent increases in reinforcement and self-administration (reviewed by Spear, 2016). For example, in the case of nicotine, repeated injections for 10 days during adolescence increase both nicotine self-administration (W Adriani et al., 2002) and cocaine reinforcement (McMillen et al., 2005). These drug abuse-relevant behavioral changes are only produced following exposures during distinct, sensitive periods of development and persist to adulthood – similar to the sensitive period of zebra finch vocal learning.

2.4.3 Brain Regions Involved

Comparative studies employing non-mammalian species hold promise in revealing the extent to which persistent effects of developmental drug exposures have been conserved over the course of evolution. To date, few such studies have been done. Our results demonstrating songbirds can be used to study drug-reinforced place-preference represent one of the first steps toward addressing this gap. Distinct changes in neuronal activity as indicated by changes in c-Fos expression suggest that brain regions important to vocal learning (Area X and lMAN) and motor production (HVC and RA) are also involved in cocaine-induced place preference. Evidence for cocaine-related increases in neuronal activity within songbird striatum is consistent with effects observed in rats (Graybiel, Moratalla, & Robertson, 1990). Higher sensitivity to these c-Fos expression effects within vocal motor regions (HVC and RA) relative to that within the learning-essential regions (Area X and lMAN) is interesting and perhaps involves a direct

dopaminergic projection from midbrain A11-type neurons to drive activity in HVC (Hamaguchi & Mooney, 2012).

Because chronic, developmental exposure to THC persistently increased sensitivity to cocaine reward in adulthood, increased activity of dopaminergic midbrain to striatal projections important to reward are likely involved. This is a hypothesis that should be tested and suggests that cocaine sensitivity involves altered development of the VTA to Area X/basal ganglia projection responsible for vocal-learning-related reinforcement of singing behavior. Thus, the songbird model promises to allow determination of the extent to which development altered by CNS drug exposure may generally impact all learning that is dependent upon intrinsic reinforcement.

2.5 Conclusion

We have developed a songbird model to study drug-reinforced place preference. This model has allowed us to demonstrate that songbird behavior is reinforced by cocaine in a manner consistent with rodent species. Use of this model has also allowed us to determine that exposure to THC during periods of sensorimotor vocal learning persistently increases sensitivity to cocaine in adulthood. Because vocal learning and drug reinforcement involve similar dopaminergic projections from midbrain to ventral striatum, the songbird model provides an opportunity to study how developmental drug exposure may generally alter processes of sensorimotor learning in a persistent manner.

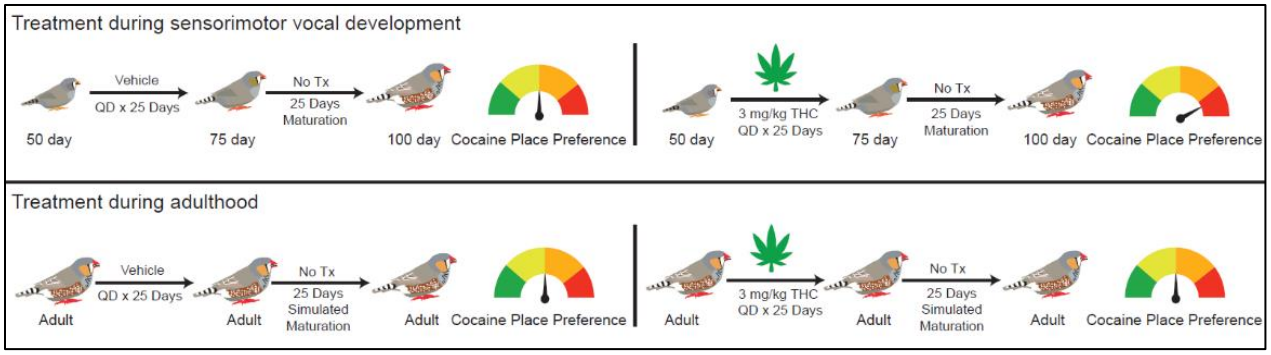

Treatment during sensorimotor vocal development
Vehicle
QD x 25 Days
No Tx
25 Days
Maturation
50 day
75 day
100 day
Cocaine Place Preference
3 mg/kg THC
QD x 25 Days
Treatment during adulthood
Adult
Simulated

Table 2.1. Dosage Group Differences in c-Fos Expression (c-Fos Nuclei/mm2)

Table 1: Dosage Group Differences in c-Fos Expression (c-Fos Nuclei/mm²)

Brain Region	ANOVA	Cocaine Dosage (mg/kg)	Vehicle - Dosage Mean c-Fos Nuclei/mm²	95% CI	q	p
HVC	F (4, 137) = 9.29, p<0.001	1.25	-4649	-8111 to -1186	3.317	0.004
		2.5	-3222	-6346 to -98.46	2.549	0.04
		5	-6361	-9345 to -3377	5.266	<0.001
		10	-6524	-9676 to -3372	5.114	<0.001
lMAN	F (4, 135) = 10.59, p<0.001	1.25	-330.8	-1847 to 1185	0.5393	0.95
		2.5	-157.7	-1611 to 1295	0.2681	>0.99
		5	-2828	-4117 to -1539	5.42	<0.001
		10	-1945	-3320 to -570.9	3.498	0.002
Area X	F (4, 137) = 10.60, p<0.001	1.25	-42.3	-837.6 to 753	0.1314	>0.99
		2.5	-182	-912.9 to 548.8	0.6154	0.93
		5	-141.6	-818.5 to 535.3	0.5169	0.96
		10	-1619	-2343 to -894.8	5.525	<0.001
RA	F (4, 134) = 7.27, p<0.001	1.25	-1206	-4083 to 1670	1.036	0.69
		2.5	-1468	-4138 to 1202	1.358	0.46
		5	-4471	-6950 to -1991	4.456	<0.001
		10	-4280	-6898 to -1661	4.039	<0.001
Striatum	F (4, 121) = 47.68, p<0.001	1.25	-3118	-4297 to -1940	6.549	<0.001
		2.5	-2994	-4057 to -1931	6.97	<0.001
		5	-2359	-3598 to -1120	4.711	<0.001
		10	-5846	-6900 to -4792	13.73	<0.001

Figure 2.1. Cocaine dose-dependently reinforces place preference in zebra finches

Groups were assigned to receive 0, 1.25, 2.5, 5 or 10 mg/kg cocaine (n = 6 - 12). Significant effects of both cocaine dosage and pre-treatment chamber preference were confirmed by 2-way ANOVA (p = 0.004 and 0.008, respectively). A, animals that received cocaine within most preferred chambers spent greater than 99% of post-conditioning preference test time in those chambers. B, animals administered cocaine in least preferred chambers dose-dependently changed preferences with EC50 = 2.55 mg/kg.

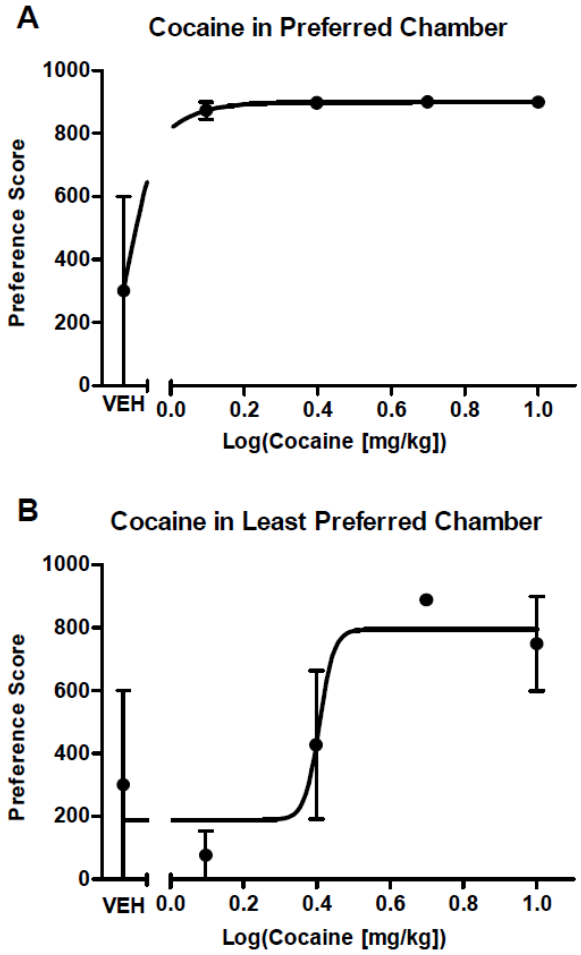

A
Cocaine in Preferred Chamber
Preference Score
1000
800
600
400
200
0
VEH
0.0
0.2
0.4
0.6
0.8
1.0
Log(Cocaine [mg/kg])
B
Cocaine in Least Preferred Chamber
Preference Score
1000
800
600
400
200
0
VEH
0.0
0.2
0.4
0.6
0.8
1.0
Log(Cocaine [mg/kg])

Figure 2.2. Cocaine produces both positive- and negative reinforcement of place preference

The valence of opposing responses depends upon the duration of intervals between cocaine injection and placement into least-preferred chambers (1-way ANOVA, $p = 0.019$).

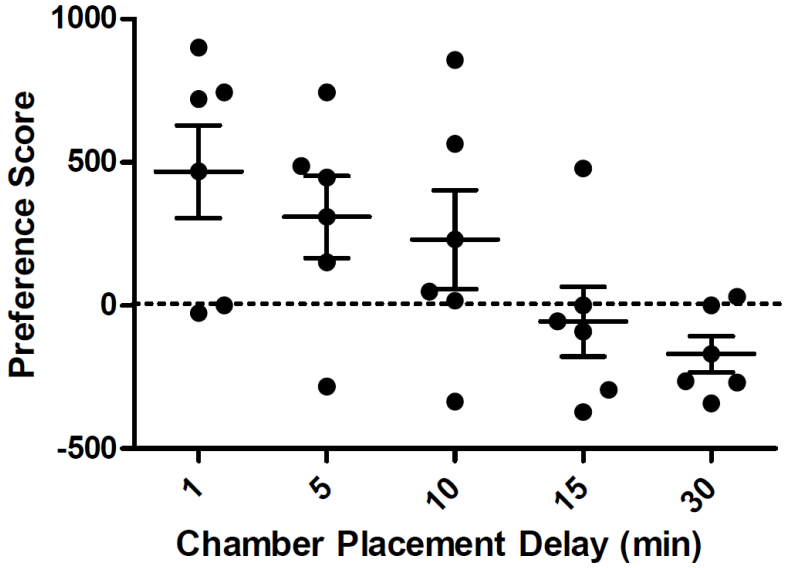

1000
500
0
-500
Preference Score
1
5
10
15
30
Chamber Placement Delay (min)

Figure 2.3. THC (3 mg/kg, A and B) but not nicotine (0.4 mg/kg, C and D) increases sensitivity to cocaine when delivered during a sensitive period of vocal learning, but not in adulthood

Daily treatments were administered for 25 days from 50-75 days in developing animals (A and C) or to adults (>= 100 days, B and D). Animals were allowed to mature an additional 25 days following treatments to adulthood. Animals in the adult treatment group were not manipulated for 25 days post-treatment to simulate the maturation period of the developmental group. Different vehicles were used for THC and nicotine (DMSO- and ETOH-containing, respectively). A potential increased cocaine sensitivity in the control animals treated with the ETOH-containing vehicle during development may have reduced the difference from nicotine-treated animals (panel C).

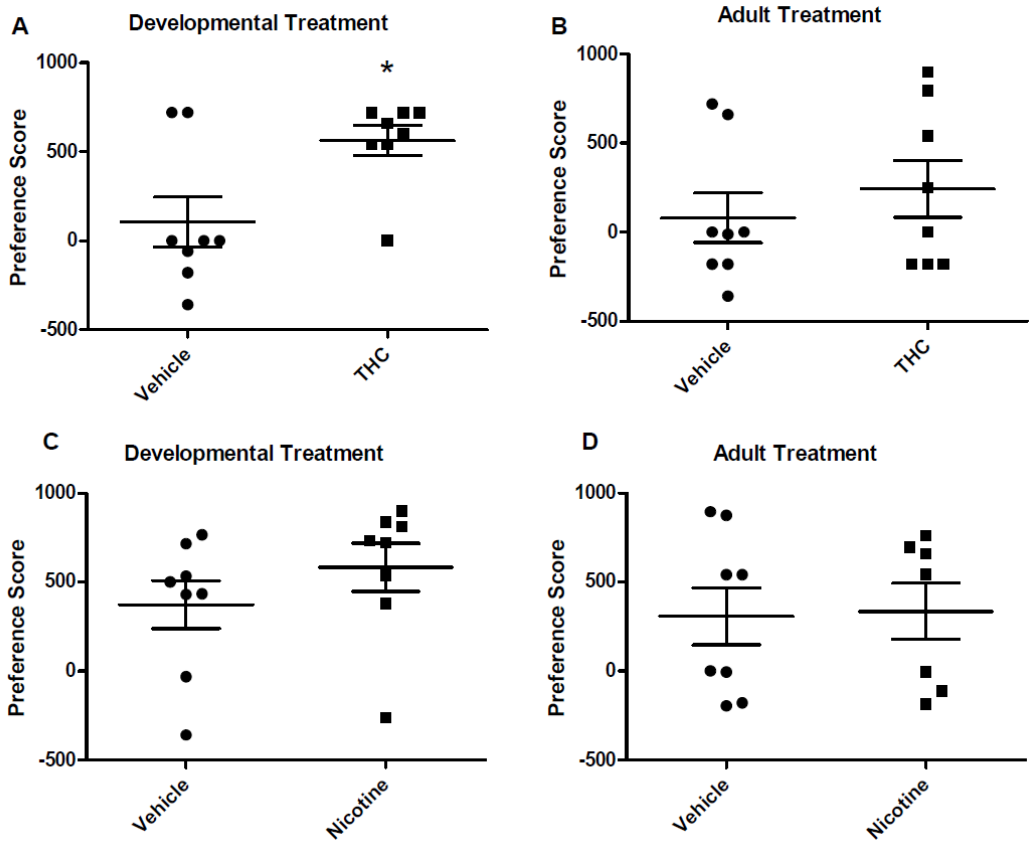
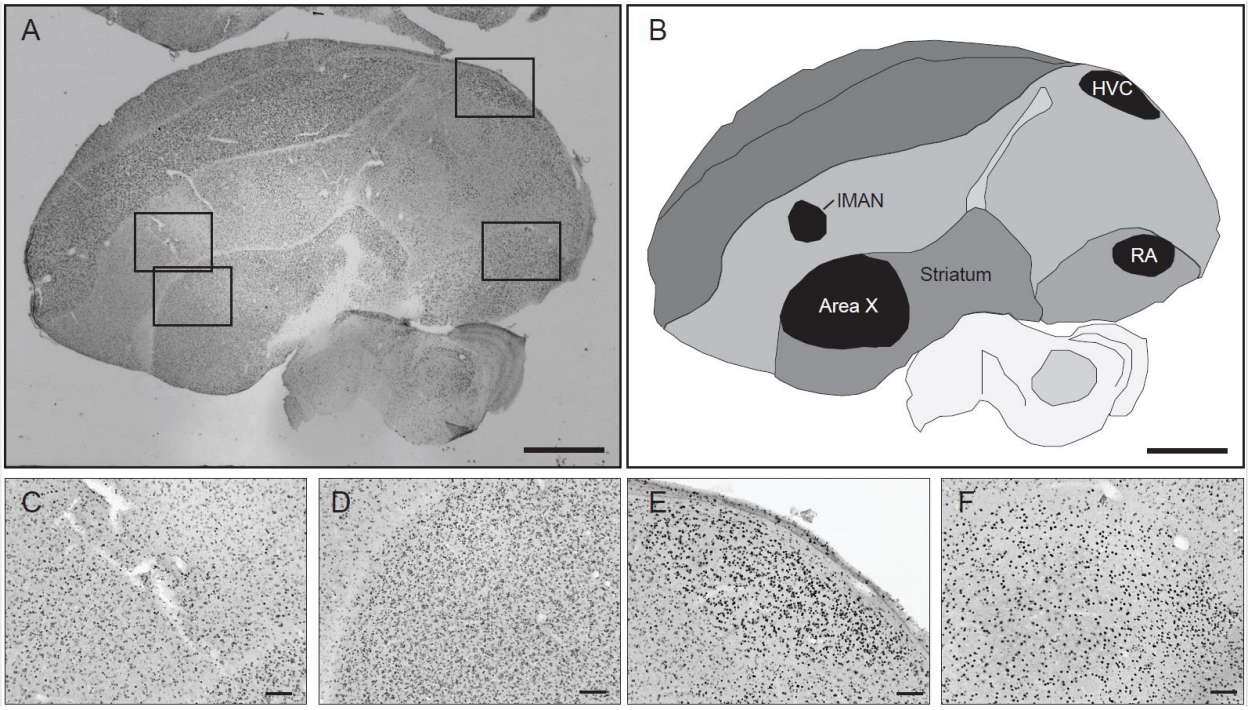

A
Developmental Treatment
Preference Score
1000
500
0
-500
*
Vehicle
THC
B
Adult Treatment
Preference Score
1000
500
0
-500
Vehicle
THC
C
Developmental Treatment
Preference Score
1000
500
0
-500
Vehicle
Nicotine
D
Adult Treatment
Preference Score
1000
500
0
-500
Vehicle
Nicotine

Figure 2.4. Examples of anti-c-Fos immunohistochemistry staining quality and spatial relationships between song regions of interest

A, 12.5 X image of a 30 µm parasagittal section from about 2 mm lateral of the midline. Rostral is left, dorsal is up. Bar = 1 mm. Insets correspond to 100 X images: upper left, lMAN in panel C; lower left, Area X in panel D, upper right, HVC in panel E; lower right RA in panel F. 100 X bars = 100 µm. B, neurolucida-type tracing of 12.5 X image presented in panel A illustrating spatial relationships of song regions studied (black shading). Stratum is the dark grey region surrounding Area X.


A
B
HVC
IMAN
RA
Striatum
Area X
C
D
E
F

CHAPTER 3: CONTRASTING REWARDING AND AVERSIVE RESPONSES TO COCAINE REINFORCEMENT FOLLOWING DEVELOPMENTAL EXPOSURE TO DELTA-9-THC (THC) AND THE MAGL-INHIBITOR (JZL-184)

3.1 Introduction

We previously developed a CPP paradigm using zebra finches to study drug reinforcement (Chapter 2). Songbirds, like the zebra finch, are notable in that they learn a form of vocal communication during sensitive periods of development (Eales, 1985). Early developmental exposure to cannabinoid agonists persistently alter song patterns produced in adulthood (Soderstrom & Johnson, 2003). In addition, exposure to a cannabinoid agonist during development led to elevation of dendritic spine densities in the basal ganglia (Area X) and HVC regions (Gilbert & Soderstrom, 2011). This indicated that the exposure to cannabinoid agonist disrupts the normal decrease of dendritic spine densities over the course of development (Gilbert & Soderstrom, 2011). In a study where a rat model was used, Schneider and her colleagues found that enhancing the CB1R, by induction with a missense mutation when a single nucleotide is replaced by another one in the DNA sequence of the rat Cnr1 gene during adolescence, resulted in an adolescent-like phenotype during adulthood (Schneider et al., 2015). This behavior was characterized by enhanced impulsivity, high risk seeking, increasing the reward sensitivity for drug and non-drug needs: all indicating that the eCB activity plays an important role in mediating the adolescence behaviors (Schneider et al., 2015). More recently we have found that early exposure to THC increases cocaine sensitivity in a persistent manner that lasts through adulthood. This effect was only produced following developmental exposures and

was not seen in adults treated for the same period of time. This suggested that the brain regions responsible for vocal learning were also involved in long-lasting changes in drug reinforcement, supporting the hypothesis that early drug exposure may generally alter learned behaviors in a persistent manner that lasts throughout life.

The current study extends results obtained with the exogenous cannabinoid agonist (THC) to establish effects of augmenting endocannabinoid signaling with the MAGL inhibitor (JZL-184) that acts as an indirect agonist by enhancing the level of the endogenous cannabinoids, 2-AG, that binds and activates the CB receptors. The hypothesis was that enhancing endocannabinoid signaling with JZL-184 would produce cocaine sensitization similar to that observed with THC. Interestingly, this hypothesis was not confirmed, and developmental JZL-184 treatments produced a persistent aversive response to cocaine in adulthood. These results suggest a more selective regional activity of endocannabinoid-related activity relative to the global cannabinoid receptor activation caused by exogenous agonist. In other words, JZL-184 may exert its effects in brain regions where there is tonic eCB signaling activity whereas THC will affect the entire brain. Comparisons of the patterns of neuronal activity associated with THC and JZL-184 treatments will provide insight to brain regions responsible for distinct efficacy.

The zebra finch model is well established for studying neurobiological changes underlying a naturally learned behavior that is dependent upon successful progress through a sensitive period of development. This learning requires activity of a basal ganglia-thalamocortical circuit termed the anterior forebrain pathway (AFP, that includes Area X → Dlm → lMAN → RA, Figure 3.10). Notably, Area X receives prominent dopaminergic input from VTA in a manner similar to the dopaminergic reward projection

from mammalian VTA to ventral striatum (Person, Gale, Farries, & Perkel, 2008). This is evident from effects of 6-OHDA lesions of dopaminergic terminals within Area X that greatly impair vocal learning, but have no detectable effect on vocal production (Hoffmann et al., 2016). Moreover, direct optogenetic activation of dopaminergic VTA projections to Area X improve vocal learning while inhibition impairs it (Xiao et al., 2018). Importantly, singing behavior itself is rewarding, as is evident in from the fact males singing undirected song develop a place preference to where singing was done consistent with striatal DA release (Riters & Stevenson, 2012). Singing-induced reward is evidence that both singing behavior and drugs produce reinforcement through similar mechanisms and suggests that all forms of incentive learning may involve the same basal ganglia-thalamocortical circuitry.

Thus, the mechanism that is responsible for the drug reinforcement for either reward or negative emotions in zebra finch model after chronic developmental cannabinoid exposure is not known. It is important to study the neurobiological changes in the brain regions, that are responsible for song learning and production, if they are involved in drug reinforcement. If not, we will investigate the involvement of other brain areas such as TnA of amygdala, which is known for its role in negative emotions, and striatum for its association with the reward effects.

Here, we tested the effect of chronic developmental THC and JZL-184 on drug reinforcement later in life using a CPP paradigm. Moreover, we measured the c-Fos expression, DA & DOPAC levels, and the content of AEA and 2-AG in brain regions that are important for vocal learning and production.

3.2 Materials and Methods

3.2.1 Subjects

Subjects were zebra finch songbirds that were bred in our aviary at East Carolina University. Juvenile zebra finches were removed to separate cages that contained adult tutors who already have characterized songs. The juvenile birds were in visual and auditory contact with the adult tutors from the age of 33-50 days. After that, they were removed from the tutoring cages and assigned to different treatment groups. During experiments, juveniles were housed singly in visual isolation with free access to grit, water, mixed seeds (Sunseed Vita Finch), and cuttlebone, and provided multiple perches. Animals were maintained on a 14:10 light/dark cycle, and ambient temperature was maintained at 78 F. All experiments were conducted based on protocols approved by East Carolina University's Animal Care and Use Committee.

3.2.2 Chemicals and Reagents

Cocaine hydrochloride and paraformaldehyde were purchased from Sigma-Aldrich (St. Louis, MO). The phytocannabinoid partial agonist THC was obtained from the NIDA drug supply program. Equithesin was prepared from reagents (40% propylene glycol, 10% ETOH, 5% chloral hydrate, 1% pentobarbital). Immunochemicals were purchased from Vector Laboratories (Burligame, CA). The primary c-Fos polyclonal antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, California). MAG Lipase inhibitor (JZL-184) was purchased Cayman Chemical (Cyman, Michigan). Alkamuls EL-620 was a generous gift from Rhodia (Cranberry, NJ). DMSO and sucrose were purchased from Fisher Scientific (Pittsburgh, PA). Prism data analysis software was purchased from GraphPad (San Diego, CA).

3.2.3 Drug Treatments

Different treatments, such as THC 3 mg/kg, JZL-184 4 mg/kg, and cocaine 2.5 mg/kg were used. THC and JZL-184 were diluted from concentrated DMSO or ethanol stocks and suspended in a vehicle to produce saline: DMSO/ethanol: Alkamuls EL-620, 18:1:1. Cocaine was dissolved in saline [pH = 7]. 50 µl of drug solutions were injected intramuscularly in the pectoralis muscle. For chronic exposures, injections were given once daily in the morning for a period of 25 days. For developmental exposures, treatments were given from 50 – 75 days of age during a period of sensorimotor vocal learning. Following developmental treatments, animals were allowed to mature to adulthood at 100 days of age. Following the maturation or simulated maturation period, animals underwent cocaine-reinforced place preference testing as described below.

3.2.4 Place Preference Apparatus

The place preference chambers used were constructed from two standard wire 16 x 16 x 18-inch finch cages with right or left side panels removed. Cages were joined at the missing panels by steel cage rings. An opening for a removable panel was created between the two cages. This removable panel was constructed from stiff poster board with bright yellow construction paper glued to one side, and bright green construction paper glued to the opposing side. The same yellow or green construction paper was affixed with zip ties to the sides, roof and floor of respective cages. A standard wooden perch was used in one cage, and an aluminum foil-covered perch was used in the opposing cage. Thus, chambers were distinguished by color and perch texture.

3.2.5 Conditioned Place Preference (CPP) Test

Experiments were conducted in the afternoon. During the first five days of experiments, the panel separating the two chambers of the place preference apparatus was removed and animals were allowed to freely explore both sides for a period of 15 min. Behavior was video recorded and time spent in each chamber documented. The chamber that animals spent the least amount of time in during these pre-conditioning tests was designated as the least-preferred chamber. For conditioning, the chamber-dividing panel was installed, and animals were randomly assigned to receive cocaine or vehicle as their first treatment. Vehicle or cocaine injections were given IM and unless specified otherwise, animals were confined in a chamber 5 min after injections for a period of 15 min. Also, unless otherwise specified, animals receiving cocaine were placed in least-preferred chambers. Vehicle and cocaine treatments were made on alternating days for a total of eight days. The day following this conditioning period, animals were given a preference test by removing the dividing panel and allowing free exploration of both sides of the apparatus. The amount of time spent in the cocaine-paired chamber was determined and used to calculate a preference score (post-conditioning minutes spent in the cocaine-paired chamber minus pre-conditioning minutes spent in the cocaine-paired chamber).

3.2.6 Immunohistochemistry Experiments

The day following post-cocaine conditioning preference tests animals were killed by Equithesin overdose and PBS (pH = 7.4) followed by phosphate-buffered 4% paraformaldehyde, pH = 7.0 were transcardially perfused. After brains were removed and

immersed overnight in buffered 4% paraformaldehyde, they were blocked down the midline, and hemispheres were sectioned parasagittally (lateral to medial) on a vibrating microtome. Immunohistochemistry was performed using a standard protocol reported in (Whitney et al., 2000) except that anti-c-Fos primary antibody was employed. For immunohistochemistry experiments, 30-µm sections of zebra finch brain were reacted with a 1: 3,000 dilutions of polyclonal anti-c-Fos antibody raised in rabbit (Santa Cruz Biotechnology, cat #sc-253). Tissue sections were rinsed in 0.1% H_2O_2 for 30 min, blocked with 5% goat serum for 30 min, and incubated overnight in blocking solution containing anti-c-Fos antibody (1: 3000). After antibody exposure, sections were rinsed in PBS (pH = 7.4), incubated in blocking solution containing biotinylated anti-rabbit antiserum (1:500) for 1 h, rinsed with PBS again, and then submerged in avidin–biotin–peroxidase complex solution (purchased as a kit from Vector Laboratories) for 1 h. Antibody labeling was visualized with DAB solution. Control sections that were not incubated in primary antibody were not immunoreactive.

Staining was examined in various brain regions at 200X using an Olympus BX51 microscope with Nomarski DIC optics. Images were captured using a Spot Insight QE digital camera and Image-Pro Plus software (MediaCybernetics, Silver Spring, MD, USA) under identical, calibrated exposure conditions. These images were background-corrected, converted to grey scale, and borders of brain regions traced manually. Two-dimensional counts of labeled nuclei from images and areas enclosed within traced areas were determined without knowledge of treatment condition for each brain region of interest from five separate sections per animal using Image-Pro Plus software. Mean

densities (within region counts of stained nuclei/area of the region) were compared across treatment group and brain region using one-way ANOVA as described below.

3.2.7 HPLC Analysis

The day following post-cocaine conditioning preference tests animals were killed by Equithesin overdose. Brains were removed and placed on a cold glass plate. Microdissection of the VTA and Area X was done using the Palkovits punch technique as described in Charlier, 2010 and Vockel, Balthazart, 1990. Collected tissues were frozen over dry ice and stored at -80 for high performance liquid chromatography (HPLC) analysis. HPLC was used to determine the levels of DA and its metabolites, DOPAC. The internal standard utilized was 3,4-dihydroxybenzylamine (DHBA). Tissue was thawed on ice, weighed, and placed into homogenizing tubes containing 0.8 ml of 0.4 N perchloric acid + 50 ng DHBA. Tissue was then homogenized (Eberbach Con-Torque Homogenizer, Ann Arbor, MI, USA) and transferred to clean, labeled microcentrifuge tubes. An alumina extraction was performed, and extracted samples and standards were injected into a high-performance liquid chromatography system equipped with a Coulochem 5100A carbon electrode detector and a Brownlee Velosep Column (ESA, Bedford, MA, USA and RP-18, 3 mm, Shelton, CT, USA, respectively). The mobile phase for high performance liquid chromatography consisted of a 0.1 M potassium phosphate buffer, containing 0.10 mM disodium-EDTA, 2.10 mM sodium octyl sulfate (SOS), 9% methanol, pH established at 3.9, and run at a flow rate of 0.5 ml/min.

3.2.8 Determination of Anandamide (AEA) and 2-Arachidonylglycerol (2-AG) Content

Following developmental exposure, birds were allowed to mature to adulthood (100 days of age) prior the cocaine challenge experiments and then euthanized. Brains were removed rapidly to a glass plate on dry ice and dissected to punch the areas of interest including Area X of striatum, Nucleus Taenea of amygdala (TnA), and the VTA. Tissues were frozen and stored at -80°C until extracted. On extraction day, tissues were homogenized in and extracted with chloroform:methanol:Tris-HCl 50 mM, pH = 7.5, 2:1:1, (v/v) containing internal standards (10 pmol of anandamide-d8, and 100 pmol of 2-arachidonyl glycerol-d8, obtained from Cayman Chemicals). The lipid-containing organic phase was collected and dried under nitrogen. Samples were reconstituted in 100 µL of 10:90 (v/v) water:methanol and placed in autosampler vials for analysis. The electrospray ionization-mass spectrometry-mass spectrometry (LC-ESI-MS-MS) method was used to detect and quantitate AEA and 2-AG.

3.2.9 Statistics

One-way analysis of variance (ANOVA) was used for experiments employing three or more treatment groups. Post-hoc analysis of ANOVA results was made with Newman-Keuls post-tests, where appropriate. Student's t-test was used for experiments employing two groups. Statistical analyses were done using GraphPad Prism software.

3.4 Results

3.4.1 JZL-184 Increases the 2-AG Level

Adult animals (n = 3) were treated IM with either vehicle or indirect endogenous agonist JZL-184 (4 mg/kg, in a DMSO-containing vehicle). A trend for an increased concentration of the level of 2-AG was observed in JZL-184-treated animals compared to vehicle-treated group. A t-test was conducted to compare between groups. (Figure 3.1. mean difference = 200.3, 95 % CI =-74.68 - 475.7, p = 0.11)

3.4.2 Persistent Effects of Chronic THC and JZL-184 Treatments

Animals (n = 8 - 24) were treated once daily for 25 days with either vehicle, the cannabinoid partial agonist THC (3 mg/kg, in a DMSO-containing vehicle) or indirect endogenous agonist JZL-184 (4 mg/kg, in a DMSO-containing vehicle), SR (6 mg/kg). Groups of developing (50 days of age) and adults (>= 100 days of age) were recruited. Following the 25-day treatment period, animals were allowed to mature without further treatment for an additional 25 days. Thus, at the time of cocaine-induced conditioning, all animal were adults (100 days of age) and any drug effects observed must have persisted for at least 25 days. Results of these chronic exposure experiments are summarized in figure 3.2. When drugs were administered during development, one-way ANOVA confirmed a significant effect of treatments on preference scores ($F [5,73] = 10.34$, $p < 0.0001$). Newman-Keuls post-tests revealed significant increases in mean preference in THC-treated animals (from 248.4 ± 182.2 sec to 672 ± 34.47 sec, $p < 0.05$) and decreases in mean preference in JZL-184 treated animals (from 248.4 ± 182.2 sec to -138.1 ± 54.88 sec, $p < 0.05$).

3.4.3 No Effect of Chronic JZL-184 Treatment in Adults

No significant preference score differences were observed between vehicle- and JZL-184 treated adults (Figure 3.3, mean difference = 177, 95 % CI =-325.6 – 697.6, p = 0.462, two-tailed t-test). These results suggest that developmental, but not adult, JZL-184 treatments cause aversion to the reinforcing effects of cocaine. This aversion is persistent, lasting at least 25 days. Earlier work showed that chronic THC treatment in adult has no effect compared to vehicle-treated group.

3.4.4 Dopamine and Its Metabolites Levels Following Preference Tests

DA levels were found to be elevated in the NAc in rats that developed conditioned place preference (Duvauchelle et al., 2000). To test the hypothesis that the mesolimbic DA system is involved in the cocaine preference effect after chronic developmental treatment, we assessed DA and DOPAC levels as a function of treatments (see Figure 3.4 & 3.5). The mesolimbic DA system in zebra finches consists of DA pathways that originate in the VTA and terminate in Area X and HVC. In THC-treated animals, both DA and DOPAC levels were induced by placement of animals back into their cocaine-conditioned chambers on the day following the final preference tests in Area X, and only DA levels in VTA. Although the concentration of DOPAC was higher in the VTA, the greater variability did not allow for significance. This increase in DA and DOPAC levels was not seen in JZL-184 treated animals. As expected, we observed high-levels of DA and DOPAC content in the striatum that receives prominent midbrain dopaminergic input in THC-treated animals. These results suggest that chronic developmental THC treatment might increase the synthesis of DA in response to cocaine later in life.

3.4.5 c-Fos Expression in Song Regions Following Preference Tests

To begin to identify brain regions that are involved in learning cocaine reinforcement and aversion in songbirds, and to test the hypothesis that these regions may include those required for vocal learning, we assessed c-Fos expression as a function of treatments used for conditioning. Expression of c-Fos was induced by placement of animals back into their cocaine-conditioned chambers or vehicle-conditioned chambers on the day following final preference tests. As expected, we observed high-level expression in striatum (Area X) that receives prominent midbrain dopaminergic input in THC treated animals. While we observed high-level expression in TnA (avian analog of amygdala) in JZL184-treated animals. The amygdala is a part of the limbic structure that plays a major role in a variety of physiological processes such as emotions (fear and anxiety), memory, and decision making (Amunts et al., 2005) . These results might explain the cocaine reinforcement in THC-treated animals and cocaine aversion in JZL-184 treated animals.

In THC-treated animals, one-way ANOVA confirmed a significant effect of treatments on expression of c-Fos in the Area X region ($F [3,56] = 18.51$, $p < 0.0001$) followed by Newman-Keuls post-tests. Results of experiments in THC-treated animals are summarized in figure 3.5. In JZL-184 treated animals, one-way ANOVA confirmed a significant effect of treatments on expression of c-Fos in TnA of the amygdala region ($F [3,56] = 11.45$, $p < 0.0001$ followed by Newman-Keuls post-tests). Results of experiments in JZL184-treated animals are summarized in figure 3.6.

3.5 Discussion

3.5.1 JZL-184 Increased the Level of 2-AG

one goal of this study was to investigate whether increasing the level of endogenous cannabinoid, particularly 2-AG because it is found in higher concentration compared to other endogenous cannabinoids in brain (Soderstrom et al., 2011), would result in effects similar to that of the exogenous cannabinoid THC. Thus, we injected animals with the indirect acting cannabinoid agonist JZL-184 in order to increase the 2-AG content in brain. JZL-184 is a selective blocker of monoacylglycerol lipase (MAGL), the enzyme that is responsible for 85 % of 2-AG degradation (Long et al., 2009). Animals were euthanized 20 minutes after injections and the concentration of 2-AG was measured by mass spectrometry. We found that the concentration of 2-AG was elevated to double amount compared to the vehicle group. This result is consistent with what Long and his colleagues found when using 4 mg/kg of JZL-184: increased levels of 2-AG 30 minutes following injections (Long et al., 2009).

3.5.2 Persistent Effects of Chronic THC and JZL-184 Treatments

In harmony with our previous work, developmental THC treatment increased cocaine sensitivity later in life, during adulthood. To further understand the role of endocannabinoids, we manipulated the endogenous cannabinoid by treating animals with MAGL inhibitor JZL-184. Treating developing animals with JZL-184 during the sensitive period of vocal learning (50-75 days) resulted in conditioned place aversion to cocaine weeks later in adulthood. This persistent effect was not seen following similar treatment of adults. The aversive effect of JZL-184 treatment in the current study is consistent with

what was found in other studies. For example, in mice, an increased level of 2-AG in the basolateral nucleus of the amygdala (BLA) induced by systemic JZL-184 treatment promoted conditioned-fear (Llorente-Berzal et al., 2015). Another study showed that the impairment in fear extinction after JZL-184 administration was mediated by CB1 receptors expressed on GABAergic neurons (Llorente-Berzal et al., 2015). Thus, these results suggested that THC may enhance the DA signaling in striatum (Area X) which is associated with the reward emotions similar to the other drug of abuse while JZL-184 exposure may lead to the augmentation of the tonically active endocannabinoid signaling in brain regions that are associated with negative emotions. Moreover, pre-treatment with SR before administration of THC and JZL-184 blocked their effects and suggested that the preference and aversion effects caused by THC and JZL-184 were both mediated by the CB1 receptor.

3.5.3 Mesolimbic Pathway and DA Levels

Most CNS active drugs that induce conditioned place preference exert their actions by increasing the synaptic level of DA in the limbic system structures, including the nucleus accumbens. In one study, repeated administration of cocaine to male Sprague–Dawley rats in a distinctive environment had led to place preference for the cocaine paired compartment and increased the level of DA in NAc (Duvauchelle et al., 2000). Acute THC administration was found to increase the level of DA in the human striatum (van Hell et al., 2012), PFC (Pistis et al., 2002), and NAc (Chen et al., 1990). Moreover, repeated THC administration resulted in increase in the level of DA in the shell of the NAc (Tanda, Pontieri, & Di Chiara, 1997). To the best to our knowledge, there is a lack of studies that investigate the relationship between the chronic developmental THC exposure and the

DA system. In our current study, we found that developmental THC exposure increased cocaine sensitivity, and this was associated with the increase in the DA and DOPAC levels in both VTA and Area X. An increase in both the transmitter and metabolite suggests that an increase in synthesis and turnover of DA occurred as a result of the exposure to the cocaine-paired environment by the THC-exposed birds. The dopaminergic neuron transmission from midbrain to ventral striatum has been found to be important for drug reward (Krebs et al., 2012), and vocal learning in zebra finches (Hoffmann et al., 2016). Therefore, the hypothesis that the same circuit is likely to be involved in drug reinforcement is involved was tested. Thus, the songbird model is a promising model that allows the dertemination of the effects of the CNS active drugs during CNS development and how this exposure may generally impact all learning that is dependent upon intrinsic reinforcement. Moreover, the finding of the current study suggests that chronic developmental THC administration could be a gateway for increasing the sensitivity of drug of abuse by involving a persistent changing to DA system. On the other hand, to what extent that the eCB system is involved in the drug reward was first tested by Solianas and his group when they intravenously injected AEA and methanamide to rats. This group found an increase in the level of extracellular DA in the shell of the NAc that suggests the involvement of AEA in the reward processes (Solinas et al., 2006). However, in our study we revealed that chronic developmental JZL-184 exposure did not alter the DA and DOPAC levels in VTA and Area X in response to cocaine later in life in adulthood. This could be explained by increased levels of 2-AG alone not mimicking the discriminative stimulus effects of THC (Hruba et al., 2015). Though in a recent *in vitro* study 2-AG was found to enhance the DA neuron excitation

(Gantz & Bean, 2017). Thus, further studies are needed to characterize the interaction and relationship between the eCB, particularly 2-AG, and DA systems when the 2-AG levels are manipulated during late-postnatal development.

3.5.4 Developmental THC and JZL-184 Exposure Persistently Altered Neuronal Activity Measured Through c-Fos Expression

Area X receives DA input from VTA/Sn in a manner similar to the dopaminergic reward circuit of mammalian ventral striatum (Person et al., 2008). In this study we found that chronic THC exposure increased the c-Fos level in Area X. Our results are consistent with most of the previous work that has reported an increase in neuronal activity in the limbic brain regions in response to drugs of abuse such as cocaine, amphetamine, (Graybiel et al., 1990) and morphine (Chang, Squinto, & Harlan, 1988). A focus of drug abuse research has been to investigate neuronal activity in brain regions believed to be involved in addiction. These regions notably include striatum and the NAc (reviewed by Spear, 2016). Also, involvement of the neurotransmitters glutamate and DA are considered to be associated with drug abuse and addiction (reviewed by Spear, 2016). Glutamatergic excitatory neurons (Vanhoutte et al., 1999) and dopaminergic neurons (Badiani et al., 1998) induced c-Fos levels in the striatum and cocaine abuse is associated with elevated expression of c-Fos in CNS. For example, in rats, cocaine administration was found to induce c-Fos expression in striatum and this was inhibited by NMDA glutamate receptor antagonists (Torres & Rivier, 1993). Moreover, acute administration of THC increases c-Fos expression in limbic brain regions including striatum, and to a lesser degree the NAc (Erdtmann-Vourliotis, Mayer, Riechert, & Höllt, 1999). To the best to our knowledge, our work may be the first to study the effect of chronic and

developmental THC exposure and how that could be involved to increase the cocaine reinforcement in adulthood. We report here that THC exposure during development may increase neuronal activity that was indicated by the increased basal level of c-Fos expression in response to cocaine later in life. On the other hand, chronic development exposure to JZL-184 resulted in conditioned place aversion in response to cocaine challenge. This effect was accompanied with the increase of neuronal activity in TnA of amygdala as c-Fos level was higher in JZL-184 treated group compared to the control. The amygdala is known as a brain structure that is responsible for emotional responses, such as fear. In addition, the amygdala is connected to other brain regions by sending and receiving projections. These other regions include hypothalamus, VTA, NAc and olfactory cortex (Swanson, 2006). In one study, it was reported that both unconditioned and conditioned fear elevated the level of c-*fos* mRNA in the amygdala (Campeau et al., 1991). Moreover, activation of c-Fos expression and overlap between some brain regions including amygdala, PFC, hippocampus, amygdala, hypothalamus were reported following shock-induced conditioned fear (Tulogdi et al., 2012). These previous studies support our finding that c-Fos activation in TnA of amygdala may be associated with the aversive effects following chronic and developmental JZL-184 exposure. Taken together, our c-Fos finding in this study demonstrated that the song brain regions may be involved in drug reward and aversion. These results suggest the positive and negative reinforcement effects that result from chronic and developmental THC and JZL-184 respectively, which is indicated by the increased neuronal activity in brain regions that are important for reward or aversion, may increase spine densities that normally wane during development in some specific brain regions. THC exposure might increase the spine

densities in Area X while JZL-184 may elevate the spine densities in TnA of amygdala. For future work, it is important to test this hypothesis, knowing that early cannabinoid agonists exposure causes elevation in spine densities in song brain regions (Gilbert & Soderstrom, 2011).

3.5.5 The Levels of Endocannabinoids (2-AG & AEA) in Brain Areas

Following the 25 days of chronic injections with THC and JZL-184 and after waiting until maturation, animals were euthanized and the brain areas of interest such as Area X of striatum, VTA, and TnA of the amygdala were punched to determine the concentrations of 2-AG and AEA. We found that both chronic developmental THC and JZL-184 administration decreased 2-AG level in Area X of striatum, TnA, and VTA while increased AEA in in Area X of striatum, and VTA levels. Interestingly, only chronic JZL-184 exposure was found to increase AEA content in TnA. Given that amygdala has a principal role in emotion, particularly fear, elevation the level of AEA in TnA of amygdala might be associated with JZL-184 aversion effect. In general, the evidence showing a relationship between stress and endocannabinoid levels are solid (Morena, Patel, Bains, & Hill, 2016). One of the main roles of the endocannabinoid system is to regulate the stress response. Stress exposure activates the HPA axis. This starts when the hypothalamus releases CRF in response to stress. CRF activates anterior pituitary gland to release ACTH. ACTH binds to its receptors in adrenal gland causing cortisol releasing (reviewed by Micale & Drago, 2018). The elevation of the level of cortisol in the body triggers endocannabinoid biosynthesis in brain in order to terminate and adapt the HPA axis. Moreover, FAAH levels increased in response to stress which ultimately led to decrease AEA content in amygdala, a result that suggests its involvement and contribution to the

stress (M N Hill et al., 2013). In the current study, we found that AEA concentrations were increased in TnA of amygdala. The increasing AEA level may have a role in reducing the aversion effect that is seen after developmental JZL-184 treatment. This might occur after AEA binds to and activates pre-synaptic CB1Rs on glutaminergic neuron terminals in TnA. Inhibition of these excitatory synapses can result in reduction of neuronal activity, possibly contributing to reducing the aversion affects. In one study, glutaminergic neuronal activity has been found to be reduced in day one after stress in a model of post-traumatic stress disorder (Fang et al., 2018). Future studies should investigate the excitatory/inhibition balance in amygdala by studying the activity and expression of glutaminergic and GABAergic neurons to understand how the endogenous cannabinoids (AEA and 2-AG) are involved in fear behavior.

3.6 Conclusion

In conclusion, here we found that early developmental exposure to THC and JZL-184 resulted in conditioned-place preference and aversion respectively that persist through life. These effects are associated with increased neuronal activity in distinct limbic brain regions. This suggests that these regions are persistently altered by developmental, but not adult exposures to the exogenous cannabinoid, THC and also by enhancing signaling of the endocannabinoid 2-AG. The findings of the current study suggest that adolescents who abuse Cannabis may be more sensitive to the reinforcing effects of other drugs. This increased sensitivity may be due to persistently altered neuronal activity following early exposure. Moreover, another finding of the current work is that zebra finches are useful for studying drug reinforcement in addition to their well-established role in investigating vocal learning. The dopaminergic neuron transmissional from midbrain to

ventral striatum is important for drug reward (Krebs et al., 2012), and vocal learning in zebra finches (Hoffmann et al., 2016). Moreover, Area X receives prominent dopaminergic input from ventral tegmentum (VTA) in a manner similar to the dopaminergic reward projection from mammalian VTA to ventral striatum (Person et al., 2008). This suggests similar circuits that are responsible for vocal learning and production may also participate in drug reinforcement. More studies are needed to find the effects of early exposure of THC and JZL-184 on vocal learning and how this persistent change in brain may alter a learned behavior. Moreover, changes in the DA signaling in VTA or NAc may be accompanied with alteration of signaling by other neurotransmitters, thus, research into the role of other neurotransmitters such as glutamate may provide more information about how the reward and aversion occurred.

Figure 3.1. JZL-184 (4 mg/kg) increases 2-AG levels

JZL-184 (4 mg/kg) increases 2-AG levels to almost double that of vehicle-treated animals after 20 minutes. Treatments were delivered via IM injection once to adult zebra finches. Two-tailed t-test was done with $p = 0.11$. Error bars = standard error.

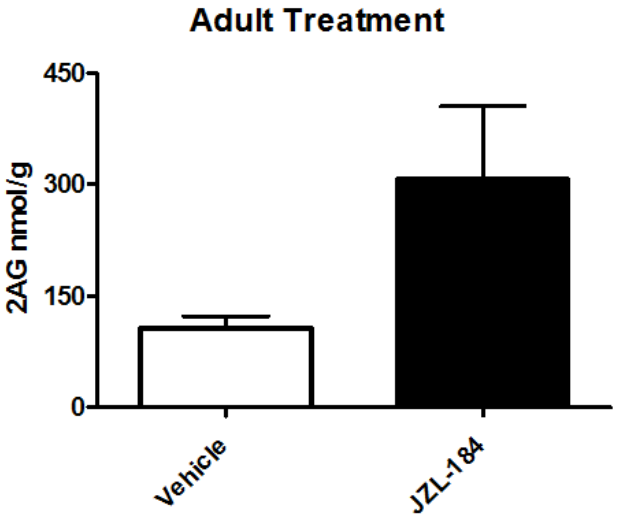

Adult Treatment
2AG nmol/g
450
300
150
0
Vehicle
JZL-184

Figure 3.2. Developmental JZL-184 (4 mg/kg) exposure caused aversion while THC (3 mg/kg) increases the preference in response to cocaine challenge test

THC (3 mg/kg) increases sensitivity of the adult songbird to cocaine when delivered during a sensitive period of vocal learning, while JZL184 (4 mg/kg) causes aversion. Those effects were reversed by pretreatment with the CB1-selective antagonist SR and demonstrates CB1 receptor mediation of the preference and aversion behaviors. Daily treatments were administered for 25 days from 50-75 days of age in developing animals. Animals were allowed to mature an additional 25 days following treatments to adulthood. Differences were determined using one-way ANOVA followed by Newman-Keuls post-tests. *$p < 0.05$ vs. Vehicle. Error bars = standard error.

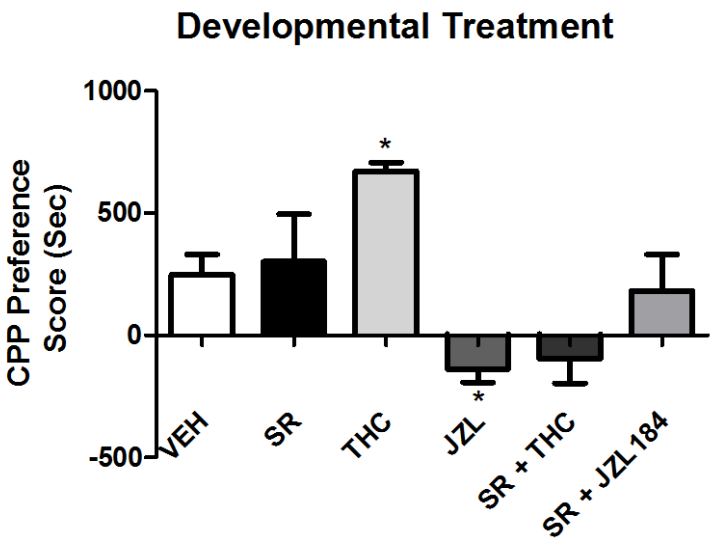

Developmental Treatment
CPP Preference Score (Sec)
1000
500
0
-500
*
*
VEH
SR
THC
JZL
SR + THC
SR + JZL 184

Figure 3.3. Adult JZL-184 exposure has no clear effect on drug reinforcement

Daily treatments were administered for 25 days to adults (>= 100 days). Animals in the adult treatment group were not manipulated for 25 days post-treatment to simulate the maturation period of the developmental group (see Figure 3.2). Differences were determined using a two-tailed t-test.

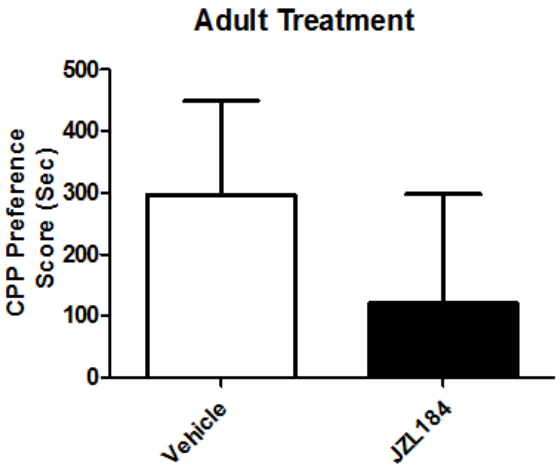

Adult Treatment
CPP Preference Score (Sec)
500
400
300
200
100
0
Vehicle
JZL184

Figure 3.4. Striatal and midbrain DA & DOPAC levels increased following developmental THC, but not JZL-184 treatments

Developing animals (50-75 days old) were treated with vehicle, the partial agonist THC (3 mg/kg) or the MAG lipase inhibitor JZL-184 (4 mg/kg). Treatments were followed by 25 days of no treatment in order to allow developing animals to mature. Animals were placed back into their cocaine-paired chambers on the day following final preference tests and euthanized 15 min later. Significant increases in DA (A) and DOPAC (B) levels were observed following developmental THC treatments. Differences were determined using one-way ANOVA analysis followed by Newman-Keuls post-tests. $^{*}p < 0.05$ vs. Vehicle. Error bars = standard error.

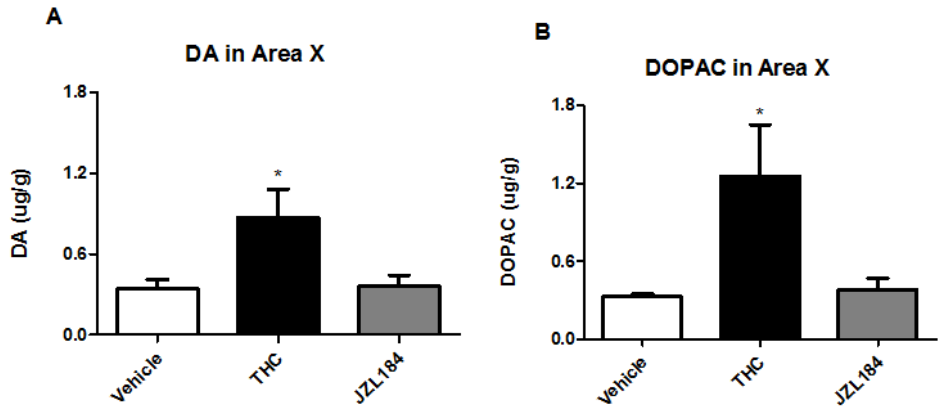

A
DA in Area X
DA (ug/g)
1.8
1.2
0.6
0.0
*
Vehicle
THC
JZL184
B
DOPAC in Area X
DOPAC (ug/g)
1.8
1.2
0.6
0.0
*
Vehicle
THC
JZL184

Figure 3.5. DA & DOPAC levels increased following developmental THC and JZL-184 treatments

Songbirds during adolescent development (50-75 days old) were treated with vehicle, partial agonist THC (3 mg/kg) or MAG Lipase inhibitor JZL184 (4 mg/kg). Treatments were followed by 25 days of no treatment in order to allow developing animals to mature. Animals were placed back into their cocaine-conditioned chambers on the day following final preference tests and euthanized 15 mins later. A, a significant increase in DA levels was observed following developmental THC treatments. B, there was not different in DOAPC levels among groups. Differences were determined using one-way ANOVA analysis ($p = 0.06$) followed by Newman-Keuls post-tests. *$p < 0.05$ vs. Vehicle. Error bars = standard error.

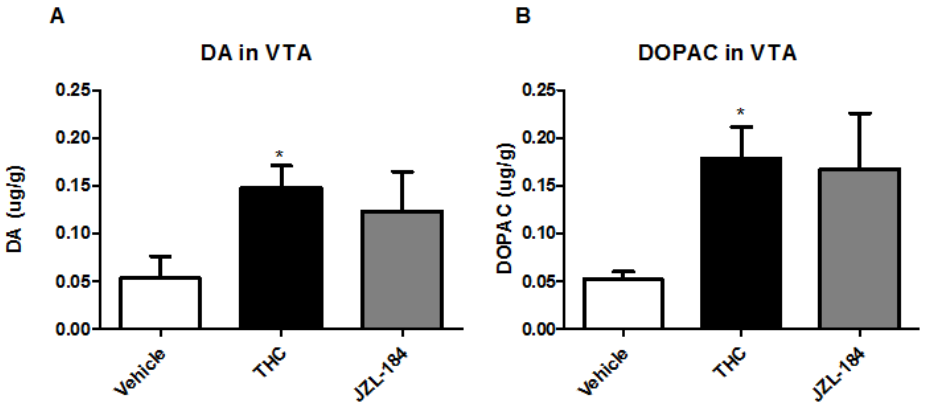

A
DA in VTA
DA (ug/g)
0.25
0.20
0.15
0.10
0.05
0.00
*
Vehicle
THC
JZL-184
B
DOPAC in VTA
DOPAC (ug/g)
0.25
0.20
0.15
0.10
0.05
0.00
*
Vehicle
THC
JZL-184

Figure 3.6. Developmental THC increases striatal c-Fos expression following placement in cocaine-paired chambers

Developmental THC exposure increases basal densities of c-Fos reactive nuclei within both Area X and medial striatum. Initial daily treatments over 25 days are indicated by first designations (VEH-, THC-). Later, animals were placed back in CPP apparatus to stimulate c-Fos expression in adulthood are indicated second (-VEH, -COC). Treatments were delivered during sensorimotor song learning (from 50 to 75 days) and measured in adulthood (>110 days). In F, levels of c-Fos expression are elevated following repeated THC exposure during development in Area X (compare VEH-VEH vs. THC-COC and THC-VEH vs. THC-COC). Asterisks indicate differences from VEH-VEH treatment groups ($p < 0.05$, one-way ANOVA followed by Tukey post-tests). Pound indicates a difference from THC-VEH group ($p < 0.05$, one-way ANOVA followed by Newman-Keuls post-tests). COC = cocaine-paired chamber.

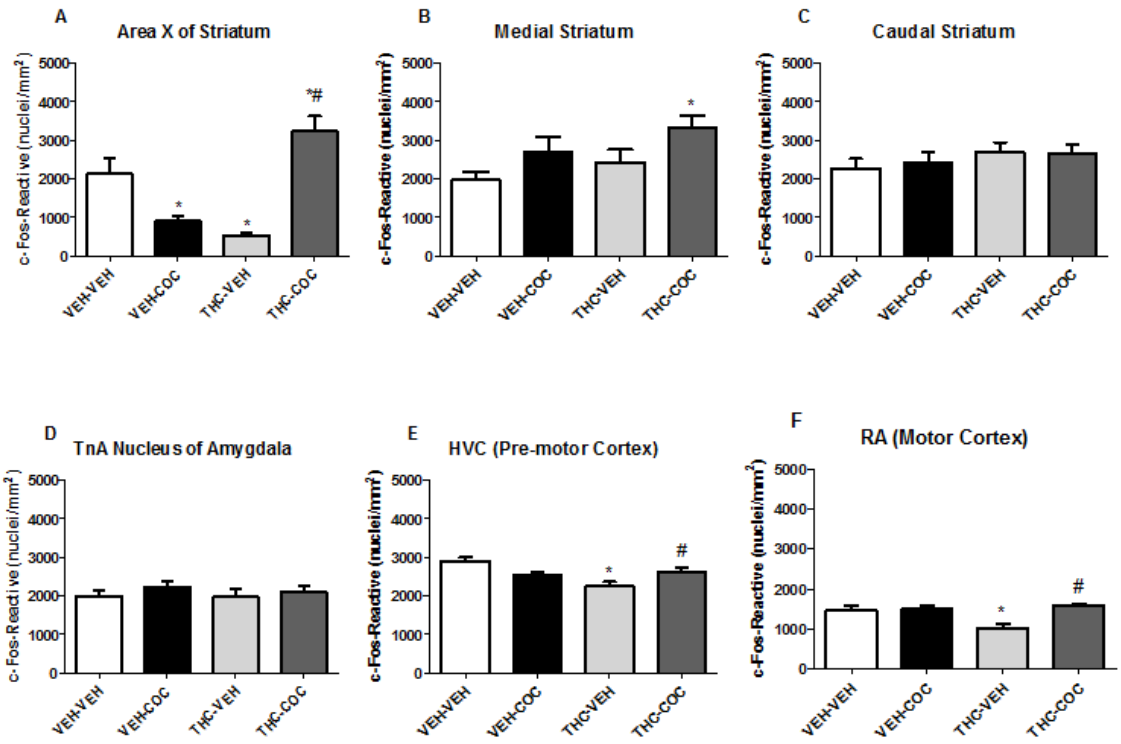

A
Area X of Striatum
c-Fos-Reactive (nuclei/mm²)
5000
4000
3000
2000
1000
0
*
*
*#
VEH-VEH
VEH-COC
THC-VEH
THC-COC
B
Medial Striatum
c-Fos-Reactive (nuclei/mm²)
*
C
Caudal Striatum
c-Fos-Reactive (nuclei/mm²)
D
TnA Nucleus of Amygdala
c-Fos-Reactive (nuclei/mm²)
E
HVC (Pre-motor Cortex)
c-Fos-Reactive (nuclei/mm²)
*
#
F
RA (Motor Cortex)
c-Fos-Reactive (nuclei/mm²)
*
#

Figure 3.7. Developmental JZL-184 decreases striatal c-Fos expression following placement in cocaine-paired chambers

Developmental JZL-184 exposure increases basal densities of c-Fos reactive nuclei within TnA. Initial daily treatments over 25 days are indicated by first designations (VEH-, JZL-). Later, animals were placed back in CPP apparatus to stimulate c-Fos expression in adulthood are indicated second (-VEH, -COC). Treatments were delivered during sensorimotor song learning (from 50 to 75 days) and measured in adulthood (>110 days). In C, levels of c-Fos expression are elevated following repeated JZL184 exposure during development in TnA (compare VEH-VEH vs. JZL-COC and JZL-VEH vs. JZL-COC). Asterisks indicate differences from VEH-VEH treatment groups ($p < 0.05$, one-way ANOVA followed by Tukey post-tests). Pound indicates a difference from JZL-VEH group ($p < 0.05$, one-way ANOVA followed by Newman-Keuls post-tests). JZL = JZL-184 and COC = cocaine-paired chamber.

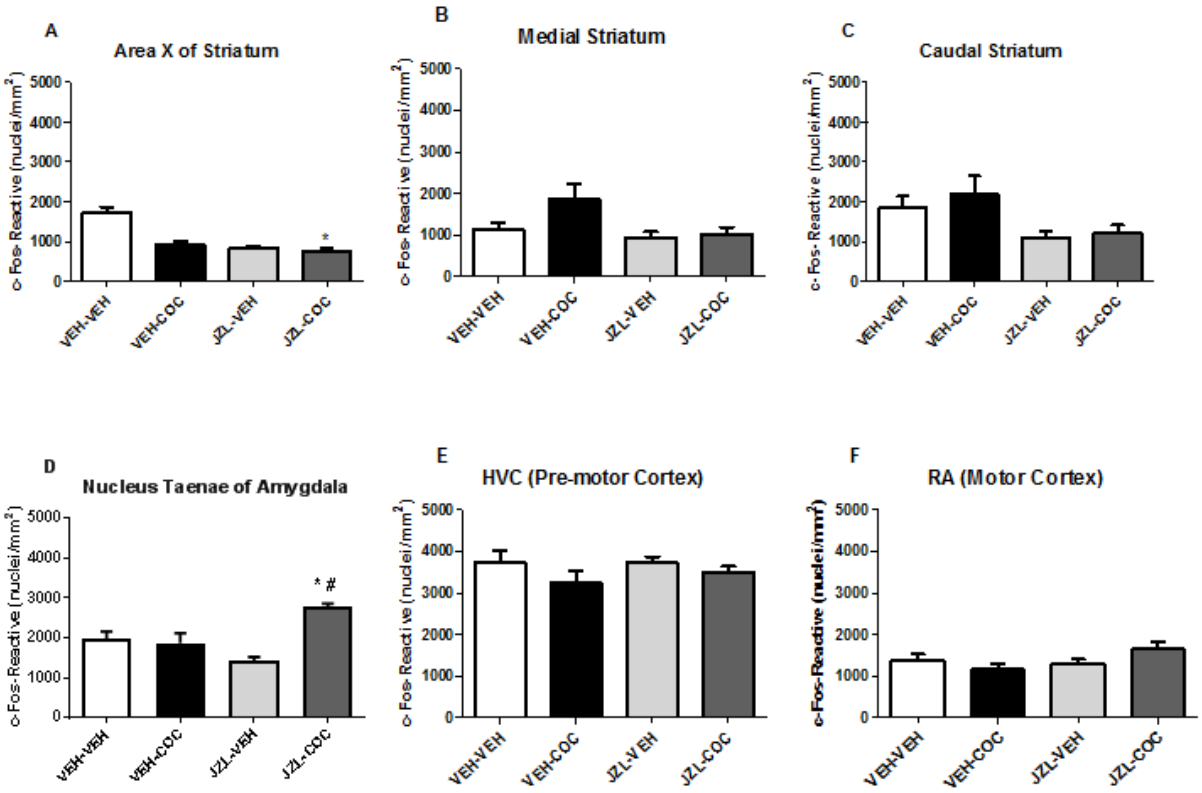

A
Area X of Striatum
c-Fos-Reactive (nuclei/mm2)
5000
4000
3000
2000
1000
0
*
VEH-VEH
VEH-COC
JZL-VEH
JZL-COC
B
Medial Striatum
c-Fos-Reactive (nuclei/mm2)
VEH-VEH
VEH-COC
JZL-VEH
JZL-COC
C
Caudal Striatum
c-Fos-Reactive (nuclei/mm2)
VEH-VEH
VEH-COC
JZL-VEH
JZL-COC
D
Nucleus Taenae of Amygdala
c-Fos-Reactive (nuclei/mm2)
* #
VEH-VEH
VEH-COC
JZL-VEH
JZL-COC
E
HVC (Pre-motor Cortex)
c-Fos-Reactive (nuclei/mm2)
VEH-VEH
VEH-COC
JZL-VEH
JZL-COC
F
RA (Motor Cortex)
c-Fos-Reactive (nuclei/mm2)
VEH-VEH
VEH-COC
JZL-VEH
JZL-COC

Figure 3.8. Developmental THC & JZL-184 decreases 2-AG level in TnA, and VTA

Developmental animals (50-75 days old) treated with vehicle, partial agonist THC (3 mg/kg) or MAG Lipase inhibitor JZL-184 (4 mg/kg). Treatments followed by 25 days of no treatment in order to allow developing animals to mature. Animals were placed back into their cocaine-conditioned chambers on the day following final preference tests and euthanized 15 mins later. Significant decreases in 2-AG levels was observed following developmental THC and JZL-184 treatments in TnA and VTA. Differences were determined using one-way ANOVA analysis followed by Newman-Keuls post-tests. $^{*}p < 0.05$ vs. Vehicle. Error bars = standard error.

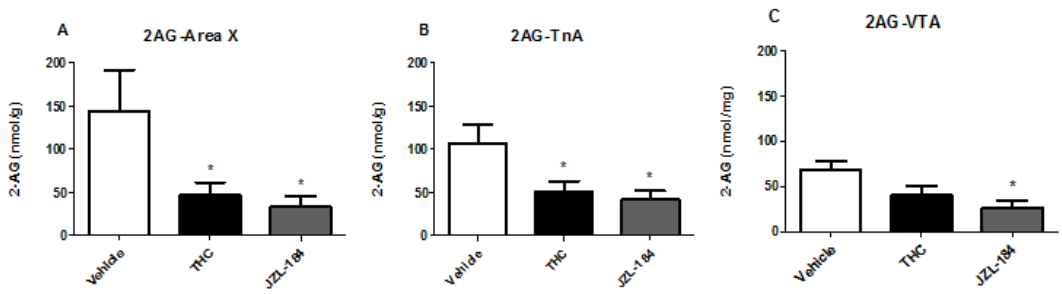

A
2AG -Area X
2-AG (nmol/g)
200
150
100
50
0
*
*
Vehicle
THC
JZL-184
B
2AG -TnA
2-AG (nmol/g)
200
150
100
50
0
*
*
Vehicle
THC
JZL-184
C
2AG -VTA
2-AG (nmol/mg)
200
150
100
50
0
*
Vehicle
THC
JZL-184

Figure 3.9. Developmental THC & JZL-184 persistently increases AEA levels in the Area X of striatum, TnA of amygdala, and VTA

Developmental animals (50-75 days old) treated with vehicle, partial agonist THC (3 mg/kg) or MAG Lipase inhibitor JZL-184 (4 mg/kg). Treatments followed by 25 days of no treatment in order to allow developing animals to mature. Animals were placed back into their cocaine-conditioned chambers on the day following final preference tests and euthanized 15 mins later. Significant increases in AEA level was observed following developmental THC and JZL-184 treatments in Area X, TnA and VTA. Differences were determined using one-way ANOVA analysis followed by Newman-Keuls post-tests. $^{*}p < 0.05$ vs. Vehicle. Error bars = standard error.

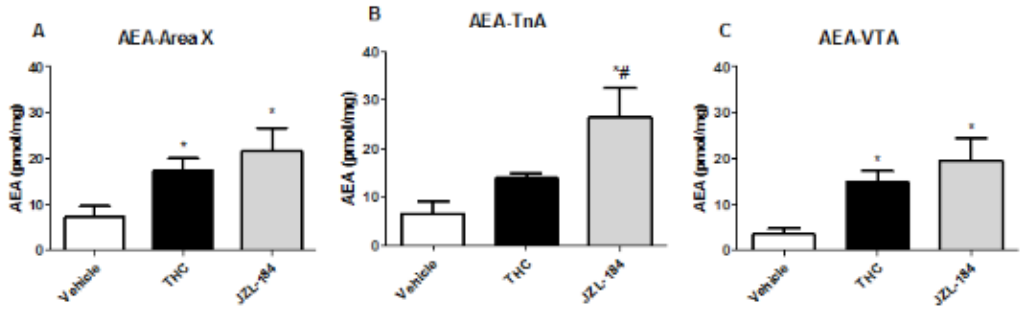
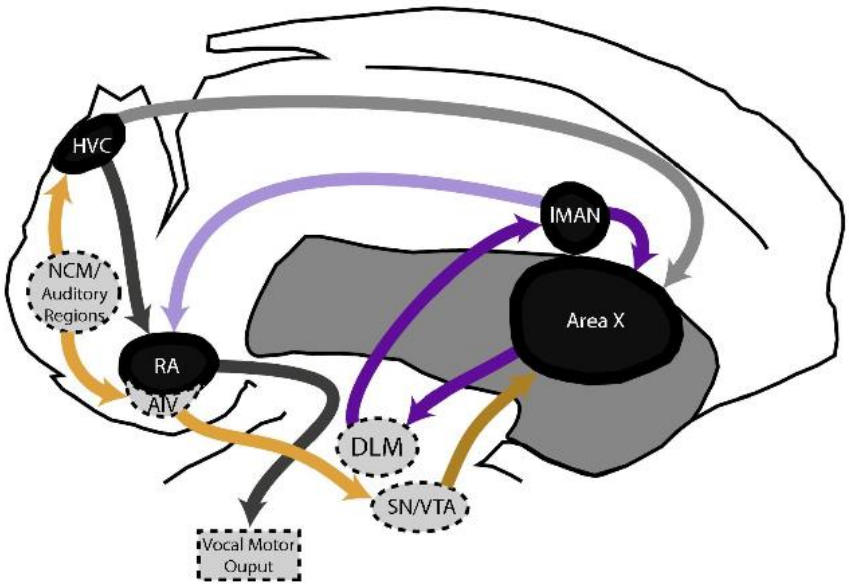

A
AEA-Area X
AEA (pmol/mg)
40
30
20
10
0
*
*
Vehicle
THC
JZL-184
B
AEA-TnA
AEA (pmol/mg)
40
30
20
10
0
*#
Vehicle
THC
JZL-184
C
AEA-VTA
AEA (pmol/mg)
40
30
20
10
0
*
*
Vehicle
THC
JZL-184

Figure 3.10. Illustration of zebra finch song brain regions.

Dark purple arrows indicate connections of the anterior forebrain pathway (AFP), a cortico-basal ganglia-thalamic loop critical for sensorimotor vocal learning. Light purple arrow illustrates AFP output from lMAN to the vocal motor output region, RA. Dark grey indicates vocal motor pathways, light grey illustrates the output from pre-motor HVC to the basal ganglia region, Area X. Light gold arrows indicate relevant auditory input to the motor system and from the ventral portion of the intermediate arcopallium (AIV) to dopaminergic neurons within substantia nigra (SN)/VTA. Dark gold indicates SN/VTA dopaminergic projections to spiny interneurons within Area X. Abbreviations: DLM (nucleus dorsolateralis anterior, pars medialis), HVC (proper name), lMAN (lateral magnocellular nucleus of the anterior nidopallium), NCM (caudal medial nidopallium), RA (robust nucleus of the arcopallium).



(Adapted from Holland and Soderstrom 2017)

CHAPTER 4: DEVELOPMENTAL THC AND JZL-184 EXPOSURE PERSISTENTLY ALTER SONG PHONOLOGY THROUGH ADULTHOOD

4.1 Introduction

In our previous work, we showed that both chronic THC and JZL-184 exposure during adolescence increased zebra finch sensitivity to cocaine into adulthood (chapters 2 and 3). We found that 3 mg/kg THC acts as a positive reinforcer while 4 mg/kg JZL-184 acts as a negative reinforcer. Interestingly, increasing the sensitivity to cocaine later in life following both THC and JZL-184 was accompanied with many physiological changes including the DA & DOPAC level, c-Fos expression, and the levels of the endocannabinoids (AEA & 2-AG) in brain areas including Area X of striatum and TnA of amygdala.

Previously in our lab, it has been found that chronic WIN55212-2, a full agonist cannabinoid**,** treatments during adolescence, not adulthood, causes: 1) altered song learning (Soderstrom & Johnson, 2003) and; 2) persistently altered axonal and dendritic spine densities in zebra finch brain regions that are important for song (Gilbert & Soderstrom, 2011, 2014; Soderstrom, Poklis, & Lichtman, 2011). Also, it has been found that acute cannabinoid treatments inhibit expression of the immediate-early genes zenk and Arc/Arg3.1 expression in NCM, one of the auditory perception regions, which in turn affects song recognition (Gilbert & Soderstrom, 2013).

However, as THC is the main psychoactive constitute of Cannabis, to best of our knowledge, there is limited data about the effects of THC on vocal learning behavior. Moreover, it is still unclear whether endocannabinoid signaling is important during the

critical period of song learning or not. As mentioned above in chapter 1, the zebra finch song brain regions contain high levels of CB1 receptors and 2-AG, and their levels change over the course of development (Soderstrom & Wilson, 2013). Preliminary evidence that CB1 receptor antagonism alters song in a manner similar to agonists suggests that this is the case, but it has not yet been experimentally-confirmed.

Therefore, we tested the hypothesis that both chronic developmental THC and JZL-184 exposure increase the variability of songs in a manner similar to increasing the sensitivity to cocaine in the zebra finch (see chapters 2 and 3).

4.2 Method & Materials

4.2.1 Materials

The phytocannabinoid partial agonist THC was obtained from the NIDA drug supply program. Equithesin was prepared from reagents (40% propylene glycol, 10% ETOH, 5% chloral hydrate, 1% pentobarbital). Immunochemicals were purchased from Vector Laboratories (Burligame, CA). MAG Lipase inhibitor (JZL-184) and rimonabant, also known as SR141716 were purchased from Santa Cruz Biotechnology (Santa Cruz, California). Alkamuls EL-620 was purchased from (Rhodia, Cranberry, NJ). DMSO and sucrose were purchased from Fisher Scientific (Pittsburgh,PA) . Prism data analysis software was purchased from GraphPad (San Diego, CA).

4.2.2 Drug Treatment

Drugs were delivered in 50 µl via IM injection into pectoralis muscle using 30 ga needles. For chronic exposures, injections were given once daily in the morning for a

period of 25 days. For developmental exposures, treatments were given from 50 – 75 days of age during a period of sensorimotor vocal learning. Following developmental treatments, animals were allowed to mature to adulthood at 100 days of age. For adult exposures, mature animals (>=100 days of age) were given 25 daily injections and were unmanipulated for an additional 25 days to simulate the maturation period in the developmental group. Following the maturation or simulated maturation period, animals underwent vocalizations recorded testing as described below.

4.2.3 Animals and Audio Recording Environment

Following treatments, at age 110 days, the male adult songs were recorded. The recording took place in a recording room (14/10-hour light/dark cycle) that is equipped with chambers with lights and microphones. The birds had free access to water and food. Prior to recording, habituation took place for 3 days. The birds were visually isolated during the experiments and were not female-directed. Songs were recorded by using Sound Analysis Recorder software stored in Waveform Audio File (WAV) format. All animal procedures were approved by the East Carolina University Animal Care and Use Committee.

4.2.4 KL Distance Measures of Phonology

Songs were segmented into syllables using Sound Analysis Pro 2011 (SAP 2011) software (Tchernichovski, Nottebohm, Ho, Pesaran, & Mitra, 2000). Segmentation using SAP was done by thresholding based upon entropy, syllable and syllable gap durations. SAP characterizes syllables with measures of 14 acoustic parameters (e.g. syllable duration, mean amplitude, mean pitch, mean FM, mean AM^2, mean entropy, mean

goodness of pitch, mean frequency, pitch variance, FM variance, entropy variance, goodness of pitch variance, mean frequency variance, AM variance). Kullback-Leibler (KL) distances, a statistical method to compare two 2D probability distributions that developed by (Daou, Johnson, Wu, & Bertram, 2012; Elliott, Wu, Bertram, & Johnson, 2014), was used to assess the phonology using the acoustic features measures. Greater KL distance means greater divergence of patterns. We used acoustic measures from vehicle-treated animal recordings as “template” distributions, and recordings from each group of treatments as individual “targets”. KL distances between template and target distributions were calculated using software we developed (KLFromRecordingDays) available for download as described in Soderstrom and Alalawi, 2017).

4.2.5 Statistical Analyses

To assess differences in phonology and vocal production across treatment groups, we used one-way ANOVA followed by Newman-Keuls post-hoc tests. Statistical analyses were done using GraphPad Prism software. Probabilities less than 0.05 were considered significant. Means +/- standard error or 95 % confidence interval are reported as indicated.

4.3 Results

4.3.1 Developmental THC and JZL-184 Increased KL Distance Measures

KL distances, a measure of phonological variance, differed across treatments after developmental exposures (Figure 4.1). Developmental treatments (n = 8) altered the KL distance measures (F[5, 42], p=0.005, 1-way ANOVA) in figure 4.1A. Following

developmental treatments, THC and JZL-184-treated animals showed significantly higher KL distance measures (including all the acoustic features) than vehicle treated animals (Figure 4.1A). The 3 mg/kg THC dosage significantly increased KL distance by 2.1 ([2.1-4.2], p=0.023). The 4 mg/kg JZL-184 dosage significantly increased KL distance by 2.5 ([2.1-4.6], p=0.0048). Treatment with the SR antagonist (6 mg/kg) prior to administration of both 3 mg/kg THC and 4 mg/kg lowered KL distances by 0.5 ([3.6-4.2], p=0.4 and; 2 [4.6-2.6], p=0.007, respectively). Adult treatments showed no clear effects on KL distance measures (F [5, 42], p=0.156, 1-way ANOVA) in figure 4.1B. Specifically, developmental THC and JZL-184-treated animals had higher KL distance at amplitude (by 5.2 [3.9-9.1], p=0.003, 3.4 [3.9-7.3], p=0.03, respectively) and entropy (by 3 [4.0-7.0], p=0.03, 3 [4.0-7.0], p=0.017, respectively) of songs compared to vehicle-treated animals (Figure 4.2).

4.3.2 THC and JZL-184 Have No Effects on Motif Syllable Numbers

Number of motif syllables, a stereotyped sequence of syllables (figure 4.5), was counted based on the KL distance outputs (Figure 4.4). There were no significant differences in the number of motif syllables across treatment groups following both developmental and adult exposure. 1-way ANOVA was done separately for both the developmental and adult groups, and their results were p=0.96 and p= 0.063, respectively.

4.4 Discussion & Conclusion

This study demonstrated that the chronic developmental THC and JZL-184 exposure persistently increases song variability in adulthood. As we expected, the cannabinoid exposure in adults had no effects on the song phonology. These results

demonstrate that the effects of both THC and JZL-184 on song phonology were specific to the learning and development of songs during a critical period of learning (adolescence). Alterations of the song phonology during adolescence could be the result of THC and JZL-184 effects on neuronal development. In zebra finches, the circuit of basal ganglia-thalamocortical, anterior forebrain pathway (Area X >> Dlm >> lMAN >> Area X) is required for songbird vocal learning until the maturation of stereotyped song that leads to “hand off” of control to a motor cortical circuit (HVC >> RA) (Bottjer, Miesner, and Arnold 1984; Herrmann and Arnold 1991). This was demonstrated by quantitative electron microscopic studies (EM) that measured the motor cortical synaptic densities (within RA) derived from afferents from the learning circuit output, lMAN and the motor circuit output, HVC (Herrmann & Arnold, 1991). The quantitative EM had been done using three different groups of ages, 25-day, 53 day, and adulthood. They found that the density and total number of RA synapses derived from the learning circuit output lMAN decreased significantly between days 25 and 53 and did not change thereafter. In contrast, the density and total number of synapses in RA derived from HVC increased significantly between days 25 and 53 but did not change significantly thereafter. This result suggested the importance of lMAN in learning processes at early age, as well as the importance of HVC for song production in adulthood (Herrmann & Arnold, 1991). The anterior forebrain pathway and its lMAN projection neurons to RA seems to be responsible for vocal variations after HVC microlesion (Kao, Doupe, & Brainard, 2005; Thompson & Johnson, 2007; Vu, Mazurek, & Kuo, 1994). This was confirmed after lMAN ablation prior the HVC microlesion prevented vocal disruption (Thompson & Johnson, 2007). Moreover, some preliminary data in our lab has shown that lesions of lMAN improved cannabinoid-altered

songs that indicates a potential mechanism of how cannabinoid agents affect vocalization by interfering and preventing the normal “hand-off” of RA control by lMAN to HVC. Thus, developmental THC- and JZL-184-altered song phonology may involve inappropriate involvement of learning circuit control over vocal motor programs. The role of lMAN output in the cannabinoid-altered vocal patterns is a hypothesis to be tested.

Figure 4.1. KL distance measures of phonology for different treatment following developmental and adult exposure

Developmental (50 – 75 days of age, panel A) and adult (>=100 days of age, panel B) animals were given once daily IM injections for 25 days period as described above in the method section. Following the maturation or simulated maturation period, animals underwent vocalization recording. We used acoustic measures from vehicle-treated animals' recordings as template distributions, and recordings from each group of treatments as individual targets. KL distances between template and target distributions were calculated using KL distance software. Greater KL distance means compared to vehicle, greater divergence of patterns. In developmental animals, KL distances are clearly increased in THC and JZL-184-treated animals indicating significant song variability compared to the vehicle-treated group. Pre-treatment animals with SR reduced the KL distances measures. In adult group, both THC and JZL-184 has no clear effects on KL distance measures compare to vehicle-treated animals. Differences were determined using one-way ANOVA ($p = 0.005$) followed by Newman-Keuls post-tests. *$p < 0.05$ vs. Vehicle. Error bars = standard error.

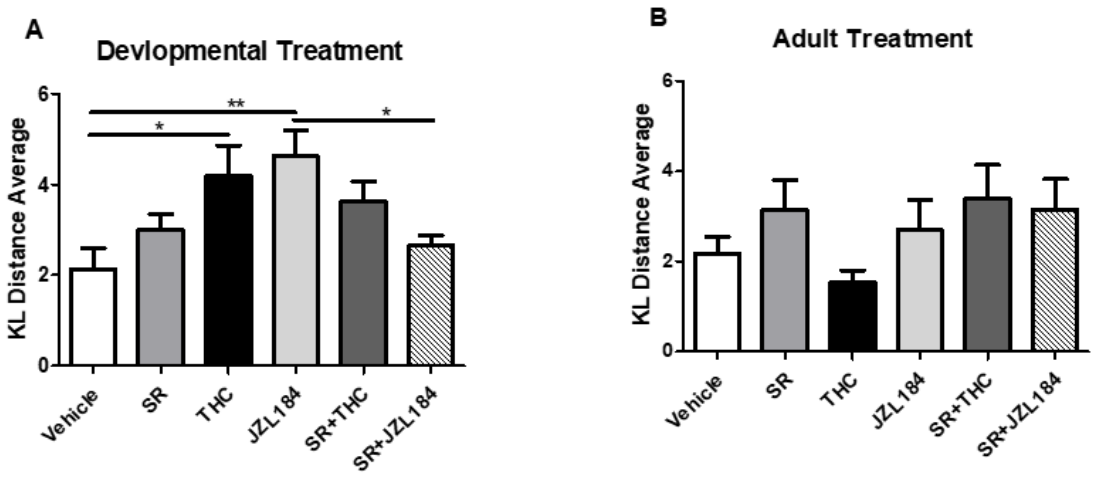

A
Devlopmental Treatment
KL Distance Average
0
2
4
6
*
**
*
Vehicle
SR
THC
JZL184
SR+THC
SR+JZL184
B
Adult Treatment
KL Distance Average
0
2
4
6
Vehicle
SR
THC
JZL184
SR+THC
SR+JZL184

Figure 4.2. Developmental THC and JZL-184 exposure increase amplitude and entropy vocal variance through adulthood

The THC and JZL-184-treated animals have higher mean KL distance at amplitude (by 5.2 [3.9-9.1], p=0.003, 3.4 [3.9-7.3], p=0.03, respectively) and entropy (by 3 [4.0-7.0], p=0.03, 3 [4.0-7.0], p=0.017, respectively) of songs compared to vehicle-treated animals. Differences were determined using one-way ANOVA followed by Newman-Keuls post-tests. *p < 0.05 vs. Vehicle. Error bars = standard error.

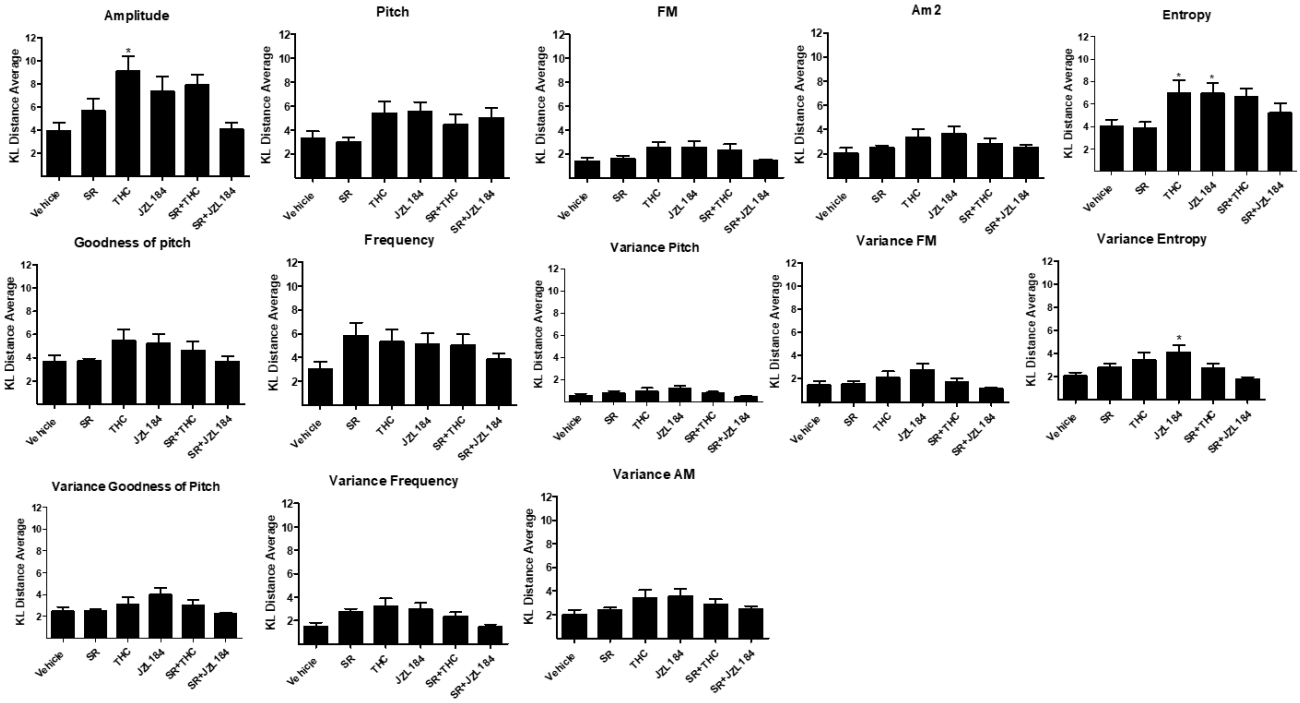

Amplitude
Pitch
FM
Am2
Entropy
Goodness of pitch
Frequency
Variance Pitch
Variance FM
Variance Entropy
Variance Goodness of Pitch
Variance Frequency
Variance AM
KL Distance Average
Vehicle
SR
THC
JZL184
SR+THC
SR+JZL184

Figure 4.3. Adult THC and JZL-184 exposure have no clear effects on the KL distance at any acoustic parameters

KL distance at each acoustic parameter did not show any song variability when THC or JZL-184 were given alone but THC + SR significantly altered entropy.

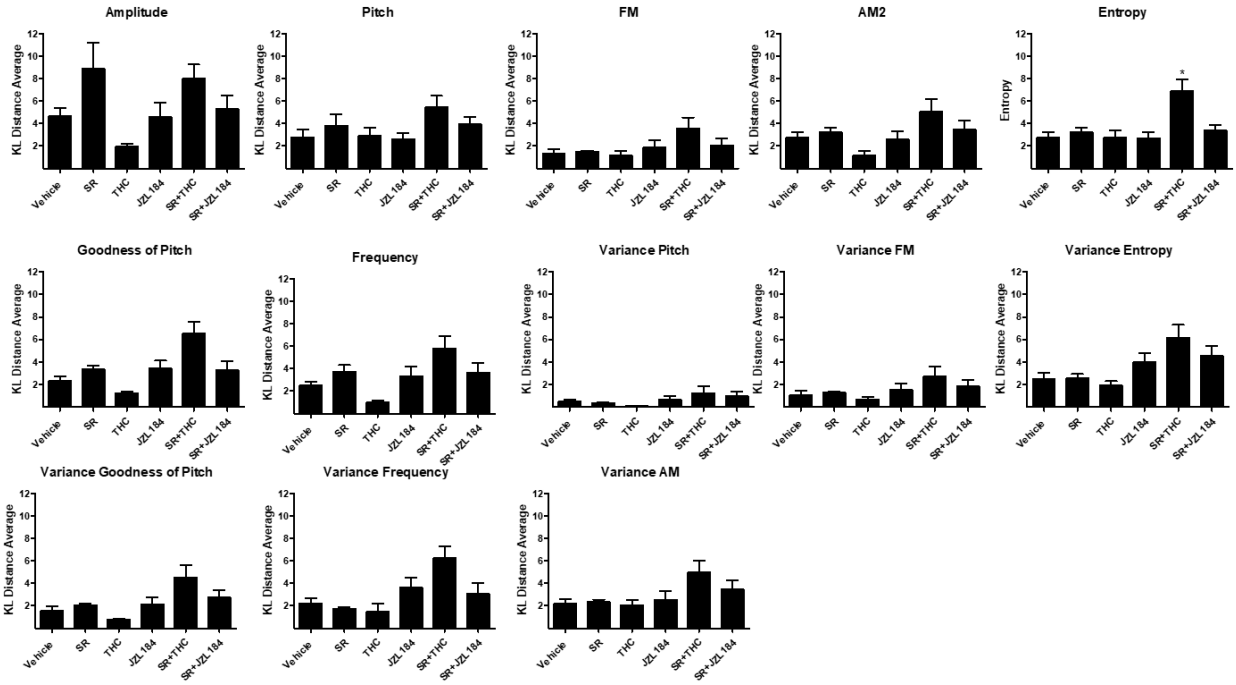

Amplitude
Pitch
FM
AM2
Entropy
Goodness of Pitch
Frequency
Variance Pitch
Variance FM
Variance Entropy
Variance Goodness of Pitch
Variance Frequency
Variance AM
KL Distance Average
Entropy
*
Vehicle
SR
THC
JZL184
SR+THC
SR+JZL184

Figure 4.4. THC and JZL-184 had no effect on song production measured as motif syllables uttered per hour

No treatment group sang significantly more- or less complicated song patterns when compared to other groups following both chronic developmental and adult exposure ($p=0.96$ and $p= 0.063$, respectively, 1-way ANOVA done separately for each age group)

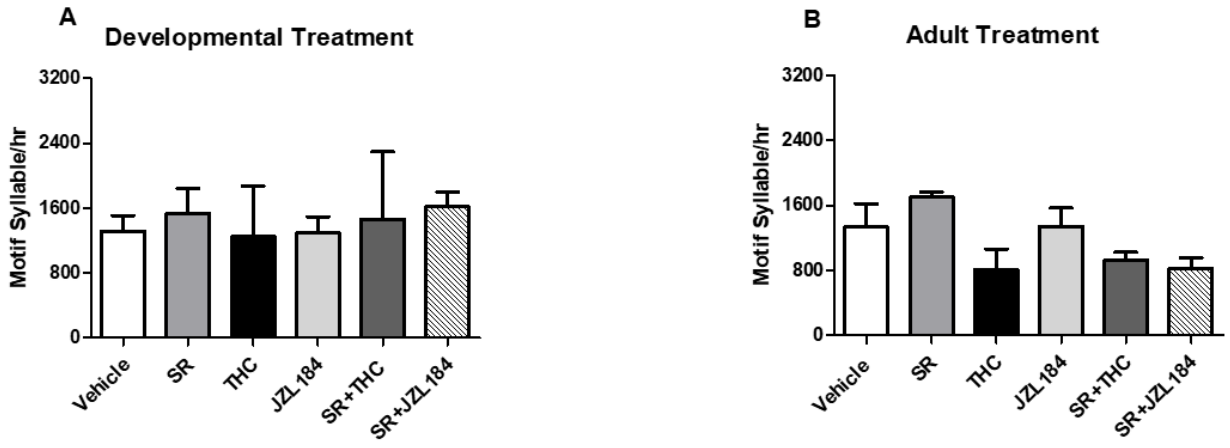
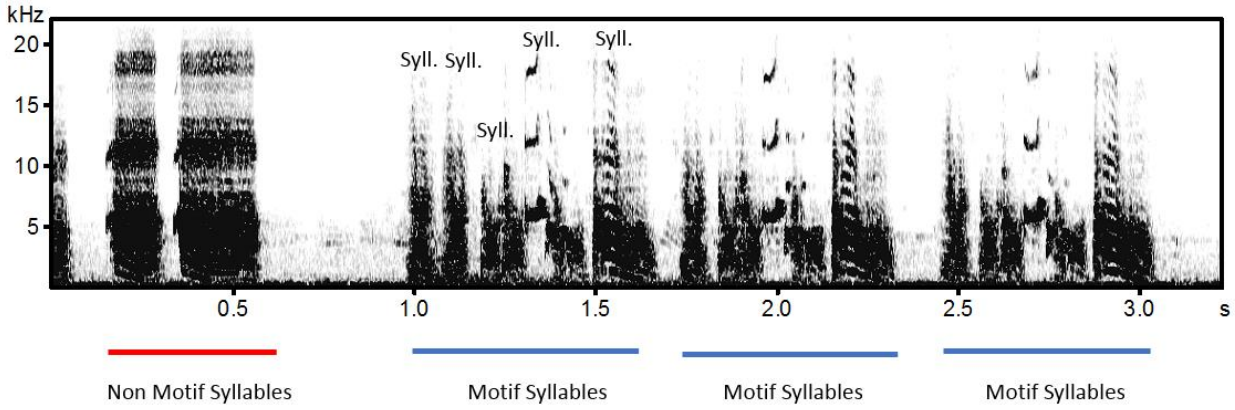

A
Developmental Treatment
Motif Syllable/hr
3200
2400
1600
800
0
Vehicle
SR
THC
JZL184
SR+THC
SR+JZL184
B
Adult Treatment
Motif Syllable/hr
3200
2400
1600
800
0
Vehicle
SR
THC
JZL184
SR+THC
SR+JZL184

Figure 4.5. A sonogram example demonstrating the difference between the motif syllables and non-motif syllables. A motif is composed of a stereotyped sequence of syllables.


kHz
20
15
10
5
Syll.
Syll.
Syll.
Syll.
Syll.
0.5
1.0
1.5
2.0
2.5
3.0
s
Non Motif Syllables
Motif Syllables
Motif Syllables
Motif Syllables

CHAPTER 5: CONCLUSION AND FUTURE DIRECTIONS

5.1 General Discussion

The present study was conducted to evaluate the effects of chronic developmental THC exposure on a learned behavior such as drug reinforcement and vocal learning that persist through adulthood, to further understand the role of endocannabinoid signaling during a critical period of development (adolescence), and the degree to which similar neuronal circuits control both of these behaviors. The zebra finch was used as the model for drug reward and vocal learning

In Aim 1, we developed a new animal model, through the use of zebra finches to study drug reinforcement of behavior in adulthood after drug exposure during a critical period of development. Zebra finch songbirds are natural vocal learners that learn how to communicate and sing during a sensitive and critical period of life (Eales, 1985). Because of this, they represent an animal model suitable for studying neurobiology underlying maturational stage-dependent learning ability (Mello, 2014). Exposure to cannabinoid drugs during this sensitive period of development results in alterations of vocal learning (Soderstrom & Johnson, 2003).

As this model is useful to study drug effects during development, the missing gap is to use this model study the drug reinforcement of behaviors. In this study, a modified conditioned place preference (CPP) paradigm was used to evaluate the rewarding and aversive effects of a drug. CPP paradigm involves the Pavlovian association of a particular environment with a drug treatment or some other stimulus. Adult zebra finch song birds were exposed to different dosages of cocaine hydrochloride, 0, 1, 2.5, 5, 10

mg/kg in the least preferred chamber in order to find the appropriate cocaine dosage that can be used in the following experiments. The dose response curve showed the EC50 was 2.5 mg/kg. At this dosage, we found that half of the animals preferred the cocaine-associated compartment while the other half preferred the vehicle-associated compartment. Consistent with other vertebrate studies, cocaine dose-dependently produced place preference in our model with a potency similar to rodents (Mueller & Stewart, 2000). In addition, cocaine was found to initially induce place preference that followed by aversion in rats (Su et al., 2013). This finding was consistent with our results for the chamber placement delay experiment. Animals who were placed back into the CPP apparatus after 1, 5, or 10 minutes after injection of 2.5 mg/kg cocaine developed preference for the cocaine-paired side, while animals who were placed back after 15 or 30 minutes developed aversion. As our dose-response study employed a five-minute chamber placement delay following injections of cocaine, for consistency we chose to continue to use this time in the following experiments.

The effects of different drugs of abuse in rodents after chronic adolescence exposure has been evaluated (Linda Patia Spear, 2016). In rats, it was found that periadolescent exposure to nicotine increased self-administration (W Adriani et al., 2002). Moreover, adolescent exposure to nicotine was reported to increase sensitivity to cocaine or diazepam following maturation (McMillen et al. 2005; James-Walke et al., 2007). In our study, we found that chronic developmental THC exposure, but not adult THC treatment, increased sensitivity to cocaine through the adulthood. However, the developmental nicotine exposure showed a trend toward a cocaine-induced place preference, but the

result was not significant compared to the vehicle group and this could be due to the positive reinforcing effects of using an ethanol-containing vehicle.

Previous studies have reported that the administration of cocaine increased neuronal activity in the striatum in rats (Graybiel et al., 1990). In our study, distinct changes in neuronal activity as indicated by changes in c-Fos expression suggest that brain regions known to be important for vocal learning (Area X and lMAN) and motor production (HVC and RA) are also involved in drug-induced reinforcement of behavior.

Taken together, these finding suggests that zebra finch model is a potential model to study the effects of developmental drug exposure in drug reinforcement.

The focus of Aim 2 was to compare the developmental influence of elevated levels of the endogenous cannabinoid 2-AG to effects of the exposure to exogenous THC on the drug reinforcement. These results improve our understanding of the involvement and the role of the endocannabinoid signaling in learning related to both drug abuse and vocal communication. 2-AG is one of the principal endogenous cannabinoids along with AEA in the brain (Mechoulam et al. 1995). JZL-184 is a MAGL inhibitor that increases 2-AG levels in brain (Long et al., 2009). Availability of a selective pharmacological tool to block the degradation of 2-AG helps to understand the role of eCB system in some pathological conditions such as pain, cancer and addiction. Moreover, the eCB system was found to be involved in addiction of many drugs of abuse such as alcohol, opiates, and nicotine (González et al., 2002; Simonnet et al., 2013; Viganò et al., 2003). Limited data are available, however, from the evaluaition of the chronic elevation of 2-AG levels in a learned behavior such as drug reinforcement or vocal learning during development.

Our current study investigated the persistent behavioral effect of chronic and developmental JZL-184 (indirect cannabinoid agonist) exposure through adulthood. A conditioned place preference (CPP) was used to evaluate cocaine reinforcement after developmental and adult treatments. While exogenous THC exposure caused a positive cocaine reinforcement, developmental JZL-184 exposure caused a negative cocaine reinforcement (Figure 3.2). Even though the adult JZL-184 treatments decreased preference scores compared to the vehicle group, it did not result in an aversive effect (Figure 3.3). Similar to what was found in a study that used a mouse model, a systemic JZL-184 treatment was found to promote conditioning-fear in mice (Llorente-Berzal et al., 2015). Moreover, JZL-184 administration resulted in impairment in fear extinction (Llorente-Berzal et al., 2015). These findings demonstrate that THC increases sensitivity to the positive reinforcing effects of cocaine, while the JZL-184 sensitizes negative cocaine reinforcement. This suggests that each drug modulates activity within distinct brain regions.

The limbic system mediates the brain reward of most CNS active drugs (Duvauchelle et al., 2000). In our zebra finch model, Area X receives DA input from VTA/Sn in a manner similar to the dopaminergic reward circuit of mammalian ventral striatum (Person et al., 2008). In our current study, we found that the concentrations of DA and DOAPC increased in Area X and VTA following the developmental chronic THC treatments (Figure 3.4 & 3.5). DA and DOPAC level did not increase after the JZL-184 exposure. These results concur with other reports in which acute and repeated THC administration were found to be associated with the increased levels of DA in NAc, striatum, and PFC (Chen et al., 1990; Pistis et al., 2002; Tanda et al., 1997). These

findings suggest that elevating the DA and DOPAC contents in Area X and VTA might be involved in cocaine reinforcement after developmental THC exposure.

c-Fos is a proto-oncogene that expressed in neurons and used as an indirect marker of neuronal activity (VanElzakker, Fevurly, Breindel, & Spencer, 2008). In addition, high c-Fos expression indicates a recent neuronal activity (Dragunow & Faull, 1989). In our current study, the neuronal activity in brain regions that are important for song learning and production were investigated following chronic THC and JZL-184 treatment and cocaine challenge. Interestingly, THC exposure was found to increase the striatal c-Fos level (Figure 3.6) while the JZL-184 exposure decreased striatal c-Fos expression and increased it in TnA of amygdala (a brain region that is responsible for emotions including fear, Figure 3.7). These findings are consistent with what was reported by Graybiel and his collogues when they found that neuronal activity (indicated by increasing c-Fos levels) increases within striatum after the administration of cocaine (Graybiel et al., 1990). Moreover, both unconditioned and conditioned fear were found to elevate levels of *c-fos* mRNA in amygdala (Campeau et al., 1991). These findings suggest that these different brain regions may be responsible for the behavioral differences following chronic and developmental THC and JZL-184 treatments.

Many studies have reported that the eCB system is involved in abuse of alcohol, nicotine and opiates. Those drugs were found to alter the levels of the endogenous cannabinoids including AEA and 2-AG (González et al., 2002; Simonnet et al., 2013; Viganò et al., 2003). In our current study, there was a pattern of reduction in the 2-AG levels in the Area X, TnA of amygdala, and VTA after both developmental THC and JZL-184 exposure (Figure 3.8). However, a pattern of elevating AEA levels in Area X, TnA of

amygdala, and VTA was found (Figure 3.9). Interestingly, high level of AEA in TnA of amygdala after JZL-184 exposure compared to the vehicle and THC groups was notable. Other reports found that neither FAAH inhibition (the enzyme responsible for AEA degradation) nor MAGL inhibition (2-AG degradative enzyme) alone increased reward behaviors or drug reinforcement (Panlilio et al., 2013). However, concurrent administration of FAAH with MAGL inhibitors lead to an increase in self-administration of cocaine, a result that suggests a need to robustly elevate of both AEA and 2-AG in order to give produce THC-like discrimination effects (Wiley et al., 2014; Wise et al., 2012). Interestingly, our results suggest that JZL-184 may exert its effects by acting in brain regions where normal tonic eCB signaling takes place, (regions such as VTA, striatum, TnA of amygdala, hippocampus, hypothalamus) and thus indicate their importance roles in regulating behaviors such as rewarding, stress, memory, and appetite, whereas the THC will affect the entire brain. Moreover, in the zebra finch model, the normal tone of eCB signaling in song brain regions (e.g. Area X of striatum) is important particularly during the sensorimotor period of learning where higher level of eCB activity is occurring. Thus, any perturbation or dysregulation to eCB neurotransmission may affect learned behaviors.

The focus of Aim 3 was to evaluate the effects of THC as well as the role of the endogenous cannabinoids on song learning during critical period of development. In addition to that, THC and JZL-184 caused positive and negative cocaine reinforcement effects, both of these treatments increased the mean KL distance measures which indicates high song variability within drug treated groups compared to the vehicle control groups. These behavioral effects (drug reinforcement and altered vocal phonology) may

be due to altered activity within similar brain regions. Our c-Fos results indicate changes in neuronal activity within specific brain regions that includes those regions important for song learning and production. For example, we found that chronic developmental THC treatments increased the c-Fos levels in Area X, while JZL-184 decreased compared to the vehicle groups. Area X is known to be important for song learning that is normally active at an early age when song learning process occur, However, during the development and maturation, the pathway that is responsible for song production becomes more active. Therefore, THC and JZL-184 exposure might lead to a selective upregulation of neuronal activity in one of these regions, this will provide important indication of learning- and motor-circuit regions that are persistently altered, and provide novel insight to circuits influenced by Cannabis abuse. Moreover, given distinct neurochemistry in the regions studied (e.g. basal ganglia is enriched with GABAergic and dopaminergic signaling) results will inform potential therapeutic approaches to address problems associated with cannabinoid-altered development. GABAergic and dopaminergic system drugs could be tested to find whether it improves song or not.

5.2 Future Directions

Research targeting enzymes is one of main focus of drug research in order to discover new treatments for diseases. JZL-184 was developed in 2009 as the first selective blocker of 2-AG degradation. Recently, much research is focusing on using JLZ-184 as an analgesic in pain models. One of the main findings of our current study is that the JZL-184 treatment did not mimic THC effects. We found that the chronic developmental JZL-184 exposure reduced the preference score in response to cocaine treatment. This raises a question about whether pretreatment with JZL-184 prior to the

administration of cocaine could be a potential approach to treat cocaine addiction. The administration of amperozide, a 5HT2A receptor and FAAH inhibitor prior to cocaine prevented a cocaine-induced place preference (Jones and McMillen, 1995). A proposed experiment may involve giving a systemic JZL-184 treatment before cocaine injections and then measure the neuronal activity in brain regions in which cocaine induced c-Fos expression (e.g. brain regions such as striatum, VTA and amygdala, see chapter 2 and 3 above). The results of this experiment will help us to understand if JZL-184 will acutely reduce the preference score prior to the administration of cocaine and to find the brain regions that are involved in drug reinforcement in the songbird model.

The study of the neurophysiological changes that occur after chronic developmental THC and JZL-184 exposure that are associated with the persistent increase in cocaine sensitivity and alteration of vocal behaviors are important to understand what areas in the brain are responsible for both the negative and positive effects following the chronic developmental cannabinoid exposure. Generally, late-postnatal CNS development is associated with a reduction in dendritic spine densities across the CNS (Changeux, 1997; Changeux & Dehaene, 1989). This is true of our songbird model and includes Area X and lMAN, which indicates both activity- and experience-driven synaptic changes (Gilbert & Soderstrom, 2011, 2014; Soderstrom et al., 2011). Previously, our lab reported that chronic cannabinoid treatments during adolescence, not adulthood, causes persistently altered axonal and dendritic spine densities in zebra finch brain regions that are important for song (Gilbert & Soderstrom, 2011, 2014; Soderstrom et al., 2011). Therefore, chronic THC or JZL-184 may affect the spine densities and neuron size within brain regions that are responsible for drug

reinforcement and altered vocal behaviors. Golgi-Cox staining can be used to study the morphological changes of neurons in several brain regions. This will help us to study and to more understand the behavioral-morphological relationships.

Additionally, the hypothesis that whether the cannabinoid-altered development is attributable to inappropriate involvement of learning circuit control over vocal motor activity is worthy to be tested as some of reports showed that lMAN projections to RA are responsible for song variations - and this was prevented after lMAN ablation (Thompson & Johnson, 2007). Songs will be recorded following reversible lMAN inactivation by a sodium channel blocker, tetrodotoxin. We will test the hypothesis that song patterns altered by daily exposure to THC (3 mg/kg) or JZL-184 (4 mg/kg) will improve under lMAN inactivation conditions and degrade as the effect of the sodium channel block wanes. Dye-conjugated dextrans will be added to the microinfusion solutions for histological confirmation of infusion site. If this expected result is not confirmed, we will repeat experiments except that lMAN will be lesioned as in preliminary experiment (Elliott et al., 2014). Quantitative EM experiments will be done to measure the number of synapses in motor cortical region RA derived from pre-motor HVC, and the learning circuit output lMAN. The ratio of lMAN:HVC derived synapses in RA decreases during the course of normal development. We will test the hypothesis that exposure to cannabinoid treatments during development, but not in adults, alters learning by preventing this normal developmental change, resulting in a high ratio of lMAN: HVC synapses and inappropriate lMAN control over RA activity. Therefore, the fraction of total synapses derived in RA from lMAN or HVC after lesion or treatments will be determined by using TEM methods as described in (Herrmann & Arnold, 1991). We will measure the ratio of lMAN:HVC derived

synapses in RA as a function of developmental cannabinoid treatments and following ablation of each region. If our hypothesis is confirmed, this will indicate that exposure to cannabinoid treatment during development that altered learning inhibits the normal “hand off” of the learning circuit to the motor control of learned behavior. This is consistent with our hypothesis that cannabinoids act to maintain stasis, preserving immature phenotypes following developmental exposure (Soderstrom & Gilbert, 2013). This finding will suggest that the now well-established persistent behavioral effects following early onset Cannabis abuse likely involve a similar interference with maturation of neural circuits for learning that involve a similar transition from ventral to dorsal striatal circuits (Volkow et al., 2006).

Even though we measured c-Fos levels as an indirect marker for recent neuronal activity, doing electrophysiology studies can help to record the electrical activity in specific brain regions such as Area X and TnA of amygdala following chronic THC and JZL-184 treatments. The electrophysiology experiments will help us to find out exactly which brain regions ae responsible for the preference and aversion effects.

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APPENDIX

ANIMAL CARE AND USE COMMITTEE APPROVAL LETTER

East Carolina University

Animal Care and
Use Commitee
212 Ed Warren Life
Sciences Building
East Carolina University
Greenville, NC 27834

252-744-2436 office
252-744-2355 fax

January 30, 2017

Ken Soderstrom, Ph.D.
Department of Pharmacology
Brody 6S-10
East Carolina University

Dear Dr. Soderstrom:

Your Animal Use Protocol entitled, "Cannabinoid-Altered Vocal Development" (AUP #W190e) was reviewed by this institution's Animal Care and Use Committee on January 30, 2017. The following action was taken by the Committee:

"Approved as submitted"

Please contact Aaron Hinkle at 744-2997 prior to hazard use

A copy is enclosed for your laboratory files. Please be reminded that all animal procedures must be conducted as described in the approved Animal Use Protocol. Modifications of these procedures cannot be performed without prior approval of the ACUC. The Animal Welfare Act and Public Health Service Guidelines require the ACUC to suspend activities not in accordance with approved procedures and report such activities to the responsible University Official (Vice Chancellor for Health Sciences or Vice Chancellor for Academic Affairs) and appropriate federal Agencies. **Please ensure that all personnel associated with this protocol have access to this approved copy of the AUP and are familiar with its contents.**

Sincerely yours,

Susan McRae, Ph.D.
Chair, Animal Care and Use Committee

SM/jd

Enclosure